University of East Anglia

The Role of Matrix Metalloproteinase-8 in Anti-Cancer Immunity

Kate Alice Makin

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Abstract

The existing literature suggests that MMP-8 is protective in the context of several cancers. In breast cancer (BC) patients, MMP-8 expression correlates with increased relapse free survival. *In vivo*, absence of *Mmp8* increases tumour burden and lung metastasis in the MMTV-PyMT spontaneous mouse mammary cancer model. Data imply that the putative host-protective role of MMP-8 may be *via* its ability to orchestrate the immune system. Therefore, *in vivo* studies were required to establish the mechanistic link between MMP-8 and immunity. Using *Mmp8* null mice orthotopically injected with MMTV-PyMT-derived mammary tumours, we have found conflicting data in this alternative model. There was no impact on tumour volume in the absence of *Mmp8* and there were no consistent changes to intra-tumoural immune infiltrates by flow cytometry or cytokine gene expression in comparison to wild-type controls. As a caveat to these findings, upon sequencing of the *Casp11* gene in *Mmp8* null mice, a 5 base-pair deletion was discovered, rendering caspase-11 non-functional. This finding prevented definitive conclusions to be made on the impact of MMP-8 in our existing mice, therefore investigations were carried out to ascertain whether the passenger mutation contributed to any results by using the *Mmp8* KO mouse without the *Casp11* mutation. However, using these animals there was still a lack of tumour or immune phenotype. This leads to two conclusions: firstly, our data suggests that the passenger mutation did not contribute to any phenotype or lack thereof. Moreover, MMP-8 did not suppress primary growth of orthotopically implanted BC tumours via co-ordination of the immune system. The discrepancy with previous findings advocates for further exploration of the differences between the spontaneous and orthotopic implant model in *Mmp8* KO mice to pinpoint the role of MMP-8 in tumourigenesis.

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Acknowledgments

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List of Abbreviations

ADAM	A Disintegrin and Metalloproteinase
ADAMTS	A Disintegrin and Metalloproteinase with thrombospondin repeats
Ab	Antibody
APC	Antigen-presenting cell
ApoE	Apolipoprotein-E
Arg1	Arginase 1
BLBC	Basal like breast cancer
bp	Base pair
BCR	B-cell receptor
BMM	Bone marrow-derived macrophages
BC	Breast Cancer
BRCA	Breast cancer type 1 susceptibility protein
CCR-	C-C chemokine receptor-
C/EBP	CCAAT/enhancer-binding protein element
CCL-	Chemokine C-C ligand-
CD	Cluster of differentiation
CFU	Colony forming unit
CFU-GEMM	Colony forming unit- granulocyte, eosinophil, monocyte, megakaryocyte
cMoP	Common monocyte progenitor
CNA	Copy number alterations
CXCR-	CX_3C-chemokine receptor-
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DCs	Dendritic Cells
DCIS	Ductal carcinoma in situ
DARC	Duffy Antigen Receptor for Chemokines
E-	Embryonic day
ESC	Embryonic stem cell
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal-Transition
EPO	Erythropoietin
ER	Estrogen Receptor
ECM	Extracellular Matrix
FMO	Fluorescence Minus One
FACS	Fluorescence-Activated Cell Sorting
G-CSF	Granulocyte Colony Stimulating Factor
GMP	Granulocyte-Monocyte Progenitor
HSC	Haemopoietic stem cells
HSPG	Heparan Sulphate Proteoglycan
HRT	Hormone Replacement Therapy
HER2	Human Epidermal Growth Factor Receptor 2
HIF-1α	Hypoxia-inducible factor 1-α

ICI	Immune Checkpoint Inhibitor
Ig	Immunoglobulin
INDEL	Insertion or Deletion of Bases
IGF-1	Insulin-like Growth Factor-1
IFN	Interferon-
IL-	Interleukin-
LAP	Latent Activating Protein
LPMP	Lymphoid-primed Pluripotent Progenitor
MDP	Macrophage and Dendritic Precursor
MCSF	Macrophage Colony-Stimulating Factor
Mϕ	Macrophages
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase-
MT-MMP	Membrane-type Matrix Metalloproteinase
miRNA	micro RNA
MMTV	Mouse Mammary Tumour Virus
MPO	Myeloperoxidase
NICE	National Institute for Clinical Excellence
NK	Natural Killer
NET	Neutrophil Extracellular Trap
PDGF	Platelet Derived Growth Factor
PECAM	Platelet Endothelial Cell Adhesion Molecule
PMN	Polymorphonuclear Neutrophil
PyMT	Polyoma Middle T antigen
PR	Progesterone receptor
PDCD-4	Programmed Cell Death-4
PD-L1	Programmed Death-Ligand 1
PGP	Proline-Glycine-Proline
ROS	Reactive Oxygen Species
RAG-2	Recombination-activating Gene-2
RBC	Red Blood Cell
SNP	Single Nucleotide Polymorphism
SCF	Stem Cell Factor
TCR	T Cell Receptor
T_C	T Cytotoxic cell
T_H	T Helper
TREG	T Regulatory cell
TEB	Terminal end buds
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll-like Receptor
TGFβ	Transforming Growth Factor β
TNBC	Triple Negative Breast Cancer
TME	Tumour Microenvironment

TNF	Tumour Necrosis Factor
TNM	Tumour, Node, Metastasis
TAA	Tumour-associated Antigens
TAM	Tumour-associated Macrophage
TAN	Tumour-associated Neutrophil
US	Unstained
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell

1. Introduction

1.1. Breast Cancer

1.1.1. Overview

Breast cancer (BC) is the most common cancer in women worldwide [1] with women in the UK facing a 1 in 8 lifetime risk of developing the disease [2]. Many predict the incidence of BC to rise in the next twenty years due to societal and lifestyle changes such as abstaining from breastfeeding, having children later in life and the impact of the environment, with increases in obesity and hormone replacement therapy prescribed during the menopause all contributing risk factors [3].

Considerable progress has been made in increasing 5-year survival rates in recent years through improved prevention and treatment programmes but despite this success, the prognosis for patients with metastatic BC remains poor due to limited treatment options. Currently, the 5-year survival rate stands at a disappointing 15% for patients diagnosed with metastatic BC [4]. As is stands, there is no cure for metastatic BC. Therefore, there exists a need to develop therapies that target the primary tumour before metastasis can occur.

1.1.2. Anatomy of the Breast

It is necessary to discuss the anatomy of the breast before considering the pathology of BC. The taxonomic class Mammalia is itself named after the Latin for breast: '*mamma'*, which speaks volumes of the significance of the breast in evolution and survival. The breast is a highly complex organ that undergoes dramatic changes throughout life including puberty, pregnancy, lactation and the menopause. The term 'breast' strictly refers to the organ housing the exocrine mammary gland in humans as well as primates. Mice do not have breasts but have 5 pairs of mammary-glands that develop in a similar fashion to humans, particularly during embryogenesis.

Mammary gland development begins prenatally at embryonic day 10 (E10) whereby bilateral milk lines arise from the ectoderm, forming five pairs of placodes located at

the site of each future nipple [5]. Each placode expands to form a mass of cells that transcends into the underlying mesenchyme, the precursor to the mammary fat pad, and this leads to formation of the mammary anlage, or primitive organ. Once at the fat pad, the epithelial 'sprout' branches to produce 10-15 rudimentary ductal branches. This structure remains quiescent until puberty.

Interestingly, unlike mice, human prenatal breast development is identical between genders, and does not significantly differ until hormonal differences occur during puberty [6]. The process of branching morphogenesis occurs at the onset of puberty under influence of hormones and growth factors such as oestrogen and insulin-like growth factor-1 (IGF-1) [7]. Highly-proliferative bulb-shaped structures called terminal end buds (TEBs) form to direct arborized growth throughout the remaining area of the fat pad to create an extensive system of branched ducts.

Post-development, the mammary gland is fully formed and ready for further changes to occur during pregnancy, lactation and the menopause. The overall structure of the adult human breast can be found in Figure 1.1, which also illustrates lobules. Lobules are grape-like structures formed of clusters of alveoli: the site of milk production during lactation. In mice, these structures do not appear until the onset of pregnancy [8].

In BC, it is the epithelial cells lining the ducts and lobules that cancer most frequently arises from; termed ductal and lobular cancers respectively [9]. Polarised cells lining the lumen of the ducts are termed 'luminal' and the outer layer of myoepithelial cells are referred to as 'basal'. These classifications are used to subtype BCs and will be discussed further in 0 .

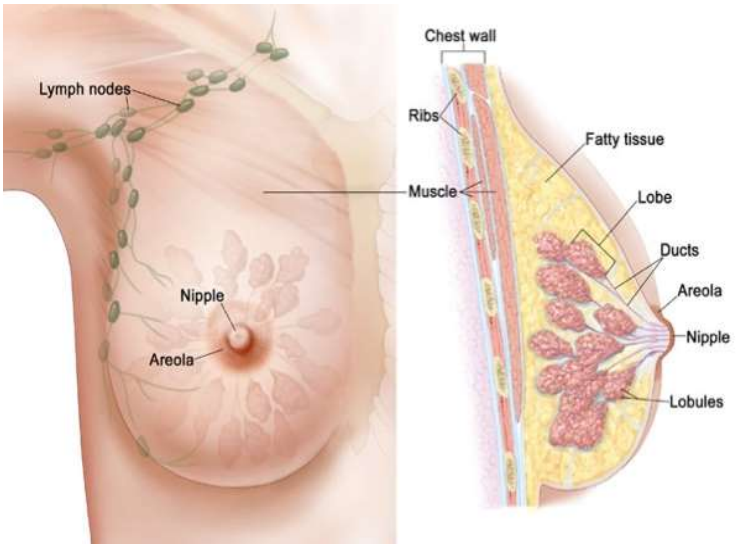


Figure 1.1. Gross anatomy of the human breast from an external and internal view [449].

1.1.3. Aetiology

Several known risk factors exist for the development of neoplastic diseases of the breast, however the single biggest risk factor is increasing age. Figures from the US in 2016 attribute 99.3% deaths from BC to women over 40 years old [10]. The underlying reasons for this are not entirely clear, but a lifetime exposure to environmental and chemical triggers may increase the likelihood of acquiring genetic mutations [11]. Further research points to the impact of reproductive factors such as the menopause. The average age to begin the menopause is 51 years old in the UK and each 1-year delay increases BC risk by 3% due to longer oestrogen exposure [12,13]. Similarly, an early menarche (before 11 years of age) increases the risk [14]. There is a speculated relationship between lifetime number of ovulatory cycles and BC risk. Post-childbirth, a woman has a short-term increased risk of BC however this disappears in the long term and is instead replaced with a reduced overall lifetime risk of developing BC. Similarly, more pregnancies correlate with reduced long-term risk [15]. Pregnancy results in mammary stem cells that are less likely to multiply – influenced by variations in hormonal signalling. However, this reduction in risk is not reflected in women that become pregnant later in life. In fact, the opposite is true. This may be due to longer lifetime exposure to oestrogen and greater accumulation

of genetic changes – where it is then too late to counteract any damage [16]. Furthermore, BC risk is diminished in breastfeeding women. Meta-analyses have shown that 12 months of breastfeeding reduces risk by 14% compared to parous women who have never breast-fed [17]. Some studies even estimate that current rates of breastfeeding prevent 20,000 deaths from BC annually [18].

Another contributing risk factor is familial history of BC. A recent study of over 113,000 women indicated a 1.75-fold increase in risk if a first degree relative had BC and a 2.5 fold increase with more than one affected relative [19] compared to individuals with no direct family history. Often, familial risk is linked to inherited mutations in key tumour-suppressor genes, the most well-known examples are the *BRCA1* and *BRCA2* mutations. Both are tumour-suppressor genes that facilitate essential DNA double-strand repair mechanisms. Germline mutations in these genes lead to an extremely high risk of developing both breast and ovarian cancer compared to the general population [20]. Overall, approximately 5% of BCs are attributed to genetic mutations [21].

Many other risks of developing BC are related to lifestyle choices. The oral contraceptive pill and hormone replacement therapy (HRT) have both been associated with increased BC risk [22], but the data remain controversial and discontinuing oral contraceptive use for 10 years negates the risk encountered during usage [23,24]. There is scientific basis for these risks relating to hormone sensitivity in the breast. Epithelial cell growth and division in the breast tissue is responsive to oestrogen and progesterone. These hormones peak during the menstrual cycle whilst HRT supplies these hormones once the body has naturally slowed down production. Oestrogen has been shown to play a causal role in the aetiology of BC and has a known carcinogenic effect in both malignant and normal breast epithelial cells [25,26].

And finally, as with the majority of cancers, high consumption of alcohol, a diet rich in fat and smoking are also risk factors for BC development [27].

1.1.4. Breast Cancer Subtypes

If a BC diagnosis is suspected, a biopsy will be performed to obtain a sample of cells from the breast tissue for analysis. Classification of BC is essential for an accurate prognosis and in designing treatment regimes. Broadly, BC is categorised into non-invasive (*in situ)* or invasive. The former represents neoplasms with cells that are not invading the surrounding tissue, whilst the latter pertains to cells that have begun to invade local connective tissues. BC *in situ* generally has a good prognosis. Some but not all *in situ* BC's will become invasive. Invasive BC accounts for around 80% of all BC diagnoses of which most are invasive ductal carcinomas [28].

Treatment of BC is dictated by the type that a patient presents with; factoring in stage, histological grade of the breast tumour and specific gene expression signatures. Staging refers to how extensive the cancer is at time of diagnosis and is based on the TNM (tumour, node, metastasis) system. 'T' represents the size of the primary tumour, 'N' refers to the number of lymph nodes nearby with detectable cancer present and 'M' is the presence or absence of metastases. However, for simplicity the stage is commonly referred to in a numerical system: Stage 0 is representative of the presence of abnormal cells that have not yet become cancerous. Stage I, II and III involves spread to nearby tissues with a higher number representing more severe cases and finally stage IV is confirmed when metastasis has occurred [29]. Furthermore, a measure is made of how abnormal the cancer cells have become in comparison to the tissue they have originated from. This is referred to as being 'well-differentiated' for a low grade and 'poorly-differentiated' for a high grade [30] – referring to how easy it is to distinguish their tissue of origin. Grading and staging is key in developing a prognosis for the patient and can indicate likelihood of recurrence.

BC is not merely a single disease, but a multifaceted one with distinct but sometimes overlapping subtypes. Traditionally, subtyping has been based on immunohistochemical validation of three markers: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2).

Differing combinations of receptor expression can exist and the status influences the biological properties of BC, guides therapy and affects patient outcome.

Due to the heterogeneous nature of tumours, classification has gone further and through genome-wide gene microarrays such as PAM50 there now broadly exists four intrinsic subtypes: luminal A, luminal B, $HER2^+$, and basal-like, that are detailed in Table 1.1 [31]. Alongside ER, PR and HER2, this system also considers expression of Ki67, a marker of cell proliferation.

Table 1.1. Molecular classification of BC subtypes and percentage of cases

Subtype	Molecular Profile	Percentage Cases in UK
Luminal A	ER^+, $HER2^-$, $Ki67^{low}$	71% [32]
Luminal B	ER^+, $HER2^-$, $Ki67^{high}$	12% [33]
$HER2^+$	$HER2^+$	5% [34]
Basal-like	ER^-, PR^-, $HER2^-$	12% [33]

Some consider normal-like breast cancer as an additional category. These tumours are difficult to characterise, often expressing adipocyte-related genes but test negative for basal-like markers such as cytokeratins and epidermal growth factor receptor (EGFR). There are several lines of evidence to suggest this subtype actually arises from normal breast cell contamination, obtained during sampling [35].

A complete lack of receptor expression is termed triple negative breast cancer (TNBC) and is associated with the worst prognosis in part due to lack of targeted therapies [36]. It is categorised under 'basal-like' BC, has a particularly high grade and low differentiation, characteristically expressing high levels of basal myoepithelial markers and frequent mutations in the *tumour protein 53* (*TP53)* gene and inactivation of the *retinoblastoma (Rb)* pathway [37]. Almost 40% of patients will relapse within 5 years of diagnosis [38].

Whilst these microarray-based molecular stratifications are indeed very useful for prognosis and treatment, there exists a need to further delve into the heterogeneity

that exists in tumours. The technology used to carry out these analyses has been further refined to also look at genome-wide copy number. In the METABRIC study, Curtis *et al.* integrated both the copy number alterations (CNAs) and gene expression data to create ten novel integrative clusters that BCs can be grouped into [39]. These clusters divide up some of the intrinsic subtypes, however some subtypes can still be captured in distinct clusters. Stratification using integrative clustering is well-reviewed by Russnes *et al* [40].

In the UK, this in-depth analysis is not carried out during diagnosis and treatment planning as it is time-consuming and not deemed cost-effective by the National Institute for Clinical Excellence (NICE) [41]. In fact, for diagnosis, intrinsic subtyping is not carried out and instead focusses on ER, PR and HER2 expression. Slowly, the NHS is beginning to use gene expression profiling to identify recurrence risk and guide treatment decision. Recently, the use of the molecular test Oncotype DX, changed clinical practice for women diagnosed with lymph node negative, ER^+, $HER2^-$ BC. Results from the TAILORx trial revealed that patients can avoid chemotherapy if they receive a mid-range recurrence score in the Oncotype DX test [42]. There appears to be a metastasis signature indicative of likelihood of metastasis in primary tumours, that these molecular tests have begun to capitalise on [43]. Another example is MammaPrint. This assay was developed using samples from 78 young BC patients, half of whom experienced metastasis within 5 years [44]. Looking at over 5000 genes, researchers looked for gene signatures that could predict good *vs* poor prognosis, and found that 70 of those genes could be correlated with prognosis [45]. With time and reduced cost, the UK may adapt their guidelines to include such tests, but for now these detailed arrays are not used in everyday clinical practice.

1.1.5. Treatment

Attempts to treat cancer have been made for centuries; Egyptian artefacts exist from 1600 B.C. containing treatment plans [46]. Modern treatment as we know it has undergone many changes even in the last 100 or so years. In the early 20th century, heavily mutilating surgery was carried out by surgeons such as William Halstead;

removing the entire breast, large areas of chest muscle and lymph nodes from the armpits. These radical mastectomies attempted to remove absolutely all cancerous tissue however the consequences for the woman were often life-changing and debilitating. It was not until the early 1980s with research to suggest that cancer was a systemic disease, that several radical surgeons began to change things. Surgery was transformed from radical mastectomies to segmented mastectomies known as lumpectomies, which facilitated targeted, local removal of cancerous tissue [47]. Often this was followed up with radiotherapy and is still carried out in this way today.

Mid-way through the 20th century there was a new era in cancer treatment. Generous funding from the NIH aided in the development of new chemotherapeutics such as cyclophosphamide, methotrexate and fluorouracil. These drugs came in to use after surgical removal of the primary tumour, where it was recognised that adjuvant therapy could be used to help prevent metastasis. However, cancers are not one and the same. The same chemotherapeutics were administered for many different types of cancers. Recognition of specific cancers as their own entities was an important step for improved treatment.

In BC, there exists a relationship between sex hormones and tumour progression. This was first realised by the surgeon Thomas Beatson. In animals who received an ovariectomy, it was observed that their breast tumours regressed [48]. Meanwhile, the discovery of the oestrogen receptor in 1967 had paved the way for oestrogen-modulating drugs such as tamoxifen. Tamoxifen is an anti-oestrogen that has been in the clinic for over 40 years, used in adjuvant therapy and more recently in chemoprevention. BC patients with ER+ tumours are generally prescribed tamoxifen for 5 years post-surgery. However, this mechanism of blocking ovarian oestrogen is ineffective in post-menopausal women considering the menopause represents a cessation in ovarian function. Post-menopause, the enzyme aromatase found at high levels in adipose tissue is responsible for converting estrogen from androgens such as testosterone [49]. Therefore, a class of drugs called aromatase inhibitors are prescribed to post-menopausal women [50].

More recently, the use of ‘biologics’ has become commonplace in BC treatment. These therapies target proteins overexpressed on tumour cells and often harness the

immune system or via the use of antibodies work in a similar way. In patients overexpressing HER2, administration of the monoclonal antibody trastuzumab (Herceptin) is particularly effective, and has been heralded as a major advance in BC treatment [51]. Other similar drugs have been developed including pertuzumab and lapatinib that also target the HER2 pathway, as well as bevacizumab that targets VEGF, approved for use in patients with metastatic HER2- BC [52,53] .

Nowadays, treatment for most cancers, BC included, is based on three pillars: chemotherapy, surgery and radiotherapy. Some patients will receive all three, some will receive only one and some receive a combination. Often the full extent of the cancer is not known until investigatory surgery is performed. After local surgical removal, adjuvant chemotherapy and radiotherapy can be given. In contrast, for some cancers, patients receive neoadjuvant chemotherapy and/or radiotherapy which aims to shrink a tumour before surgical removal. Each treatment option is made on a case by case basis. There is also a fourth pillar of treatment that has come into the light in the past decade or so: immunotherapy. This is harnessing and improving the ability of the immune system to eliminate cancerous cells. In several cancers, notably liquid cancers such as leukaemia and lymphomas, immunotherapy has become a *bona fide* treatment option, particularly those that do not respond to conventional therapies.

It must be noted here that whilst these hormonal and biological therapies are very efficacious, they are not without their disadvantages. Treatments such as trastuzumab that work on a single oncoprotein are more prone to the emergence of resistance mechanisms [54]. Cancerous cells are evolutionarily inventive and are very good at treatment evasion; perhaps utilising a different but similar biological pathway to aid their growth and proliferation. From a societal and economic perspective, these innovative new medications come at quite a cost. Figures from 2014 state it costs on average around $135,000 a year for an orally administered cancer medicine per year in the US [55] and the newer cancer drugs in development such as immunotherapies that will be discussed in further detail in 1.3.3 are significantly more expensive. These factors will have huge implications in the healthcare system's use of these costly pharmaceuticals in modern treatment of BC.

1.2. The Immune System

1.2.1. General Overview

Humans would not have evolved into the species they are today without the evolution of their intelligence-guided defence system: otherwise known as the immune system. From the Latin word '*immunis*' meaning exemption from military service or tax, the etymology of 'immunity' has changed somewhat over time to 'the balanced state of multicellular organisms having adequate biological defences to fight infection, disease, or other unwanted biological invasion, while having adequate tolerance to avoid allergy, and autoimmune diseases. A plethora of species hoping to themselves flourish and evolve have been attempting to invade humans for millions of years and ways have been devised to prevent this. For many years, primitive species had basic defence systems, most likely involving release of toxic chemicals. However, as life became more complex with the generation of entire organ systems and a dedicated control centre: the cerebral cortex, we had to make our defence systems more sophisticated and elaborate. This multifaceted network of cells, messenger molecules and proteins as we now know as our immune system can be broadly categorised into innate; as in encoded in the germline, and adaptive – built over time. However, our first line of defence does not rely on the complex but rather a simple physical barrier. Several organs including the skin, lung and the gut are lined with epithelial cells packed tightly together to provide a border between the outside world and inside the human body. The exterior of these cells is coated with a thin mucus layer containing chemicals that aid in protection from microbial, mechanical and chemical trauma. These chemicals include mucin and defensins – the latter of which are broad-spectrum antimicrobials.

If a microorganism has managed to cajole its way past the epithelial barrier, it is time for the innate and adaptive immune systems to step up – recognise it as foreign and destroy it without host damage – a process that has been refined over millions of years. From the origin of immune cells to the intricate details that underlie their function, this chapter will attempt to cover what is one of the most highly-tuned systems in the human body.

1.2.2. Haematopoiesis

At any one time, under normal disease-free conditions, our blood is teeming with the soldiers of the immune system: white blood cells (WBCs). In a complete blood count, the reference range for WBC's is 4500-11,000 cells per mm^3 of blood [56]. To scale that up to a human body: that equates to on average 7.5 x 10^9 cells / L and at any one time there is around 4.5 – 5L of blood circulating resulting in an impressive 37.5 x 10^9 WBCs, with constant renewal ongoing. However, WBCs do not develop in isolation. Our entire collection of blood cells originates from early progenitor cells that undergo differentiation to become distinct populations that perform specific functions. This process is termed haematopoiesis and begins early in embryonic development. In vertebrates, haematopoiesis is divided into two waves: primitive and definitive [57]. Briefly, the primitive wave exists to facilitate rapid production of red blood cells (RBCs). This occurs in the extra-embryonic yolk sac and its main purpose is to provide sufficient tissue oxygenation for foetus growth [58]. This phase is transitory, and the resulting erythroid progenitors have no renewal capacity. For a self-renewing population of haematopoietic cells to exist, development switches to the definitive wave, occurring slightly later in development. Haematopoiesis moves from the yolk sac, briefly to the liver and then finds permanent residence in both the bone marrow and the thymus. [59]

Formation of the entire adult haematopoietic system is reliant upon the emergence of haematopoietic stem cells (HSCs) during development (Figure 1.2). The mechanisms driving this process are evolutionarily-conserved however whilst much research has delineated many of the stages, the entire process is yet to be recapitulated *in vitro*, limiting some of our understanding. HSCs are a population of self-renewing pluripotent stem cells that under precise conditions in the HSC niche produce a continuous supply of mature haematopoietic cells. Upon asymmetrical division, two daughter progeny arise from HSC division: a progenitor cell and a replacement stem cell. These progenitor cells become committed myeloid and lymphoid progenitor cells that upon further differentiation can subsequently become the cells that make up our immune system.

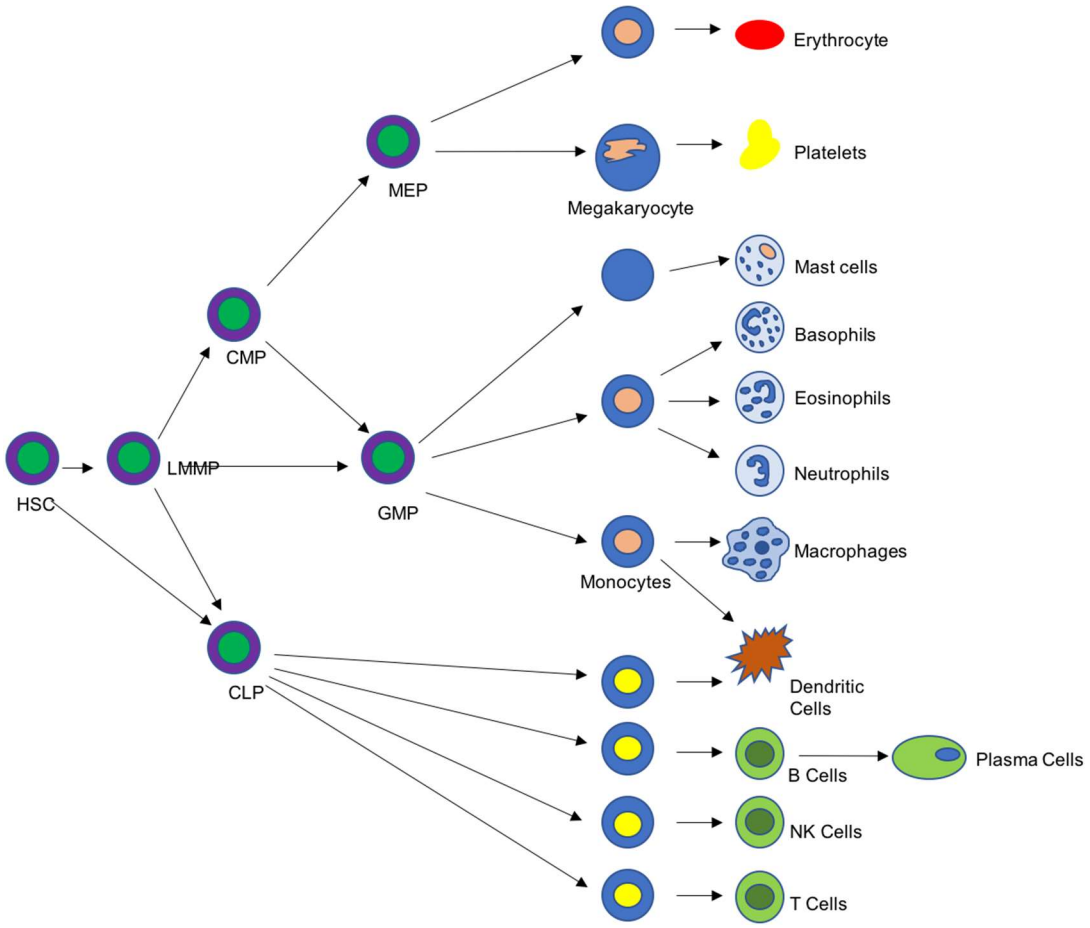


Figure 1.2. Hierarchical overview of haematopoiesis. The haemopoietic stem cell (HSC) is the origin of the entire haemopoietic lineage – a multipotent cell able to self-renew and differentiate into the common lymphoid progenitor (CLP) or lymphoid-primed multipotent progenitors (LMPP). The entire lymphoid lineage is derived from the CLP that can differentiate to become small lymphocytes – the origin of T and B cells or can become dendritic cells or NK cells. The myeloid lineage originates from the common myeloid progenitor (CMP) that can differentiate into either megakaryocyte-erythrocyte progenitor (MEP) or granulocyte-monocyte progenitor (GMP) to produce erythrocytes and platelets or granulocytes, monocytes, mast cells and dendritic cells respectively.

Specific spatiotemporal expression of cytokines and stem cell factors governs progenitor cell lineage fate. For the myeloid lineage, erythropoietin (EPO) stimulation alongside interleukin-3, -9 (IL-3, -9) and stem cell factor (SCF) generates proerythroblasts: precursors to erythrocytes [60]. All granulocytes and monocytes arise from a common progenitor termed colony forming unit- granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), that upon a distinct set of

signals including SCF, IL-3, flt3 ligand and granulocyte-macrophage colony-stimulating factor (GM-CSF) becomes CFU-GM [61]. This population can then give rise to monocytes and thus macrophages (Mϕ), dendritic cells (DCs), neutrophils and eosinophils. The three remaining populations that originate from the CFU-GEMM are thrombocytes, basophils and mast cells; the latter two of which are heavily influenced by IL-4 signalling [62].

From the common lymphoid progenitor arises every variation of lymphocyte: B-cells, plasma cells, T-cells and dendritic cells. As with the myeloid lineage, specific cytokine patterns govern differentiation. However, the development and maturation of T and B cells requires other cytokine-independent processes including antigen-driven selection and will be discussed in 1.2.4.

Discovering which factors drive each population has been largely carried out *in vitro* by colony-forming assays. In this assay, precursors are cultured in soft agar with cytokines to allow colonies to form that can be identified visually [63] .

The clinic has benefitted from the knowledge gained in regulation of haematopoiesis. For example, for patients undergoing radiotherapy or receiving immunosuppressive chemotherapeutics, both the myeloid and lymphoid progenitors are highly proliferative and therefore very susceptible. However, pluripotent stem cells are resistant to cytotoxic drugs because they reside in G0 phase – quiescent and non-cycling [64]. In stem cell transplantation, prior to the collection of stem cells/ bone marrow from the donor, injections of growth factors are given to stimulate production of higher quantities of haematopoietic cells. This is most often granulocyte-colony stimulating factor (G-CSF), which aids in mobilisation of HSCs from the bone marrow into the bloodstream as well as direct influences on neutrophil differentiation via downstream JAK/STAT signalling [65].

1.2.3. Innate Immunity

1.2.3.1. Phagocytic cells

At the heart of the innate immune system is the ability of cells to engulf pathogens and cell debris, scientifically referred to as phagocytosis. The process of phagocytosis is not unique to complex life; it is even used by simple protists for feeding [66]. The word 'phagocytosis' is derived from Greek and translates as 'the process of cells devouring'. Élie Metchnikoff, the Nobel Prize for Physiology and Medicine winner in 1908, was responsible for the discovery and naming of these professional devourers, of which all are WBCs that will be discussed in detail in this thesis. Many cells have phagocytic capabilities, however there exist professional phagocytes that carry out much of the work including neutrophils, monocytes and dendritic cells.

1.2.3.2. Monocytes

The first mononuclear phagocytic cell to introduce is the monocyte – more than just a humble precursor to the Mϕ. Monocytes make up an impressive 10% of all WBCs in humans and 4% in mice [67]. Whilst most monocytes circulate in the bloodstream, many also reside in the spleen and the lungs; ready for mobilisation [68]. As previously described, all WBCs originate from a common progenitor cell: the haemopoietic stem cell. All monocytes are derived from a common myeloid progenitor with further commitment steps occurring in the bone marrow. In mice, the myeloid committed precursor can differentiate into the Mϕ and dendritic precursor (MDP) and studies have shown that this precursor has two possibilities for differentiation: a common monocyte progenitor (cMoP) – responsible for the more classical Ly6C expressing monocytes, or common DC precursors – that can produce classical DCs and plasmacytoid DCs [69,70].

Upon completion of the necessary development in the bone marrow, monocytes circulate for around three days before migrating into peripheral tissues [71]. There are two main circumstances for the mobilisation of monocytes into tissues: homeostasis and inflammation. Once in a tissue, monocytes receive local stimulatory

signals that encourage differentiation into one of several subsets of Mϕ and dendritic cells.

Monocytes can be categorised into three subpopulations that are based on expression of various markers including cluster of differentiation (CD) markers. These expression markers vary between human and mouse. Mouse monocytes are subdivided into three populations: classical, non-classical and intermediate. The predominant way to distinguish between these populations is via their expression of Ly6C and CD43. Classical and intermediate monocytes both have a $Ly6C^{hi}$ phenotype but differ on their CD43 levels with $Ly6C^{hi}CD43^{lo}$ cells representative of classical monocytes and $Ly6C^{hi}CD43^{hi}$ cells characteristic of the intermediate phenotype [72]. Around 85-90% of monocytes are classical circulating monocytes [73]. Non-classical monocytes are $Ly6C^{lo}$ and represent a sizeable amount of the circulating monocytes – estimated to be around 40% [74]. Some characterisations also use expression of C-C chemokine receptor type 2 (CCR2) and CX_3C-chemokine receptor 1 (CX_3CR1) to delineate different populations [75]. Since of these subsets appear to possess functional differences, the traditional view that monocytes are merely developmental intermediates for Mϕ is clearly outdated.

Monocytes themselves possess a strong phagocytic ability, their surfaces littered with toll-like receptors and phagocytic receptors. During steady state, monocytes do not proliferate and are in fact inefficient at differentiating into DCs and Mϕ [76]. However, during inflammation and upon appropriate inflammatory stimulation they can carry out their differentiation program into descendant cells. There is some controversy as to the theory of monocyte differentiation, largely due to conflicting results from experiments *in vitro vs in vivo.* What is debated is whether monocytes pre-exist as subsets with very specific fates or whether they exhibit plasticity and their differentiation is in response to local factors in the immune milieu. Some experiments *in vitro* have demonstrated that the original monocyte subset is irrelevant: GM-CSF and IL-4 are able to stimulate all monocytes into DCs [77]. If monocytes are stimulated with macrophage-colony stimulating factor (M-CSF) they exhibit an M1 pro-inflammatory phenotype, and M-CSF with the addition of IL-4 results in an anti-inflammatory M2 phenotype [78,79].

It is worth noting at this stage that there are several issues regarding terminology and experimental standards in describing activation [80]. Macrophage polarisation/ activation upon stimulation is not linear, and exists more as a continuum, whereby cells are extremely plastic to their environmental cues. The concept of M1 and M2 phenotypes will be elaborated on in 1.3.2.1.

Our understanding on the role of classical monocytes has developed over the last decade. It is now clear that there are several fates for monocytes in various homeostatic and inflammatory processes. Broadly speaking, classical monocytes can migrate into tissues to either generate tissue resident Mϕ or accumulate undifferentiated in a monocyte reservoir such as the spleen [68]. Furthermore, monocytes can differentiate into non-classical monocytes that appear to have roles in maintenance of vascular structures or simply change phenotype once in tissues to take on roles such as antigen-presentation and tissue repair [81,82].

To reach their target tissue in the first instance, monocytes must possess the ability to respond to a signal and traffic to the correct site – often traveling long distances. Their surfaces are packed with receptors that recognise and bind circulating chemokines released from tissues to trigger downstream signalling pathways that facilitate migration to target tissues. One example of which is the chemokine (C-C motif) ligand 2/7 (CCL2/CCL7) and CD192 signalling axis. CCL2 otherwise known as monocyte chemoattractant protein 1 and CCL7 are produced during inflammation and bind to CD192 on monocytes for recruitment [83]. CCL2/CCL7$^{-/-}$ mice display a 40-50% reduction in monocyte recruitment during *Listeria monocytogenes* infection demonstrating the importance of the interaction between chemokine receptors and their respective cytokines [84]. There are other chemokine signalling axes that exist to aid in attraction of monocytes towards tissues however the role of adhesion molecules is also essential in monocyte recruitment. Moving monocytes into tissues is a form of diapedesis, or extravasation, and briefly, is the process whereby monocytes adhere to an activated endothelium via integrins and adhesion molecules such as platelet endothelial cell adhesion molecule (PECAM) and L-selectin to then extravasate between epithelial cell junctions to finally reach tissues [85]. This mobilisation must occur rapidly during times of inflammation when there is need for

high levels of differentiation into Mϕ. This process is not exclusive to classical monocytes, however the genetic program for tissue migration does not exist in non-classical monocytes [86].

Once monocytes have reached their target tissue, they very rapidly differentiate into Mϕ. Monocyte-derived Mϕ share very similar genetic programs to resident tissue Mϕ even though the majority of tissue-resident Mϕ are derived embryonically rather than via bone marrow precursors whilst there becomes a clear distinction from the counterpart circulating monocytes [87–89]. Nevertheless, as previously mentioned not all monocytes become Mϕ. Ly6C^{hi} monocyte populations are maintained in some tissues and maintain a monocyte reservoir or become Ly6C^{lo} non-classical monocytes [68]. How this fate is decided in the tissue is still the subject of research.

Something important to discuss at this stage and will be discussed further in 1.3, is that not all monocytic activities are beneficial. Relative levels of monocytes exist in homeostasis and any variations to that number can cause disease. Monocytosis – the overproduction of monocytes is characteristic of autoimmune diseases, tuberculosis infection and cancer, and can be detrimental [90]. Conversely, monocytopenia – reduced absolute levels of monocytes can be a risk for infection [91]. Furthermore, the populations that arise from differentiated monocytes may be detrimental to the host, as is the case for M2 Mϕ in cancer for example [92]. However, paradoxically, M2 Mϕ are also important in restoring physiology such as in wound healing [93]. Therefore, there is need for a fine-tuned balance of monocyte levels and their differentiated counterparts.

1.2.3.3. Macrophages

1.2.3.3.1. Overview

It is logical to follow on from monocytes with their descendants: macrophages (Mϕ). The immune cell that precedes all others in development: the Mϕ has become one of the most well-studied cells in immunology and subsequently in disease. Mϕ exist in all adult tissues in vertebrates and have a plethora of roles in both physiology and pathology. Whilst many assume all Mϕ are derived from monocytes, it turns out that this is not the case. Several experiments have shown that Mϕ have two origins during ontogeny. Firstly, the classical paradigm: HSCs differentiate into monocytes in the blood before migrating into tissues upon stimulation and differentiating into Mϕ. And secondly, what has become evident in recent years is the existence of a population of tissue-resident Mϕ that arise from the yolk sac during embryonic development at around embryonic day 8.5 (E8.5) (Figure 1.3). Furthermore, in the period post-yolk sac development and pre-bone marrow development there is a transitory wave of production of cells from the foetal-liver (E10.5) [94,95]. These haemopoietic progenitor populations arise from two sources: the hematogenic endothelium of the aorta-gonad-mesonephros as well as the yolk-sac and become foetal liver monocytes before infiltrating into peripheral tissues and differentiating into tissue-resident Mϕ capable of self-renewal [59]. The yolk sac-derived and foetal liver-derived tissue-resident Mϕ coexist demonstrating chimerism in the tissue.

As it turns out, tissue-resident Mϕ are predominately embryonic-derived and maintain themselves during adult life with little contribution from circulating monocytes. Elegant experiments have solidified this theory including a complex parabiosis study that involved anatomically joining two mice expressing two allelic variants of CD45: CD45.1+ and CD45.2+ via their circulation. Using fate mapping and lineage tracing it was shown that after around 2-5 months, between 15-40% non-host (donor) monocytes can be detected in the host circulation. However, this percentage was not reflected in tissue compartments for the number of host vs non-host Mϕ where the percentage was less than 1% [96]. This indicates that at steady-

state, monocyte populations do not differentiate into tissue-resident Mϕ and therefore most must be embryonically derived.

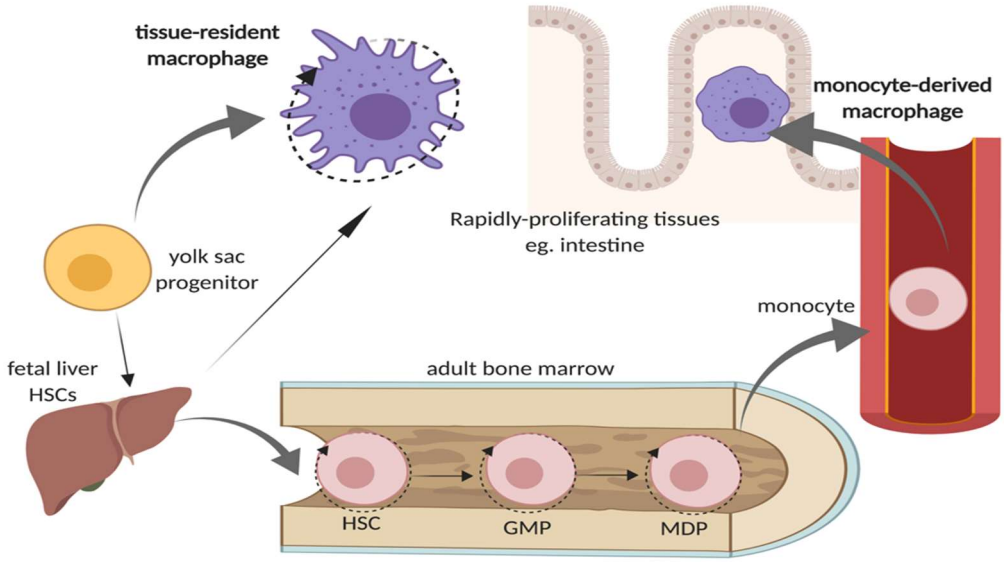


Figure 1.3. Ontogeny of tissue-resident and monocyte-derived macrophages. Macrophages are derived from two main sources dependent on development stage. During embryogenesis, haemopoietic stem cells (HSCs) originating from the yolk sac can either differentiate to produce tissue-resident macrophages or can translocate to the fetal liver where they become monocytes and infiltrate into peripheral tissues. During adult life, HSCs in the bone marrow differentiate into granulocyte-monocyte progenitors (GMP), then macrophage/dendritic cell progenitors (MDP) before becoming monocytes in the blood stream and finally differentiating into macrophages in tissues.

In terms of monocyte-derived Mϕ, the classical Ly6C^{hi} monocyte population is responsible for the generation of Mϕ resident to organs that are subject to continued inflammation such as the intestine, as well as organs undergoing vast amounts of remodelling such as the mammary gland and myometrium [97–99]. These Mϕ are short-lived and non-self-renewing in comparison to tissue-resident Mϕ which self-renew and survive for much longer periods [100]. The exact reason for this is unclear but if there is a continuous supply of circulating monocytes available, there seems little use in maintaining a long-term resident population.

1.2.3.3.2. Non-Immune Functions of ϕ

The classical view of a Mϕ is that it functions as a pathogen-eating rubbish bin in infection and immunity. However, many other non-immune responses rely on Mϕ including development, steady state haematopoiesis and wound healing.

In the bone marrow, Mϕ are key in maintaining steady-state levels of haematopoiesis. During erythropoiesis, when RBCs are reaching maturity, Mϕ gather to remove the nuclei discarded during RBC enucleation. As well as RBCs, Mϕ clear neutrophils in the spleen and any cells in the bone marrow that do not express CD47. Interestingly, this is one mechanism leukemic cells utilise to avoid phagocytosis through upregulation of CD47 [101].

Most knowledge on Mϕ in development has come from studies using the *M-CSF*$^{-/-}$ mouse model [102]. *M-CSF*$^{-/-}$ mice exhibit a plethora of abnormalities including a lack of teeth, fertility issues in both males and females and skeletal defects [103]. Osteoclasts are differentiated macrophages that form from Mϕ fusion events in the bone. Osteogenesis: the development and formation of bone is heavily reliant on the ability of osteoclasts to resorb bone during remodelling [104].

Mϕ are also critical in wound healing - several parallels can be drawn between this process and cancer immunity. Mϕ are involved in all three stages in the wound healing response: inflammation, proliferation and remodelling [93]. Of note, the production of several MMPs by Mϕ is a feature of each stage in this process. At each stage, Mϕ exhibit very different phenotypes; they are extremely plastic cells and can sense and respond to their environment. Mϕ can be broadly classified into two phenotypes: M1 pro-inflammatory and M2 anti-inflammatory. This classification is in fact a spectrum as mentioned in 1.2.3.2 however there are some functional differences that arise from different stimuli. M1 ϕ are essential for debris and bacterial clearance in the early wound however this population must be replaced with M2 ϕ to resolve inflammation and promote re-epithelialisation [93][105]. This M1/M2 paradigm will be discussed in greater detail in 1.3.

1.2.3.3.3. Macrophages in the Mammary Gland

To detail how Mϕ function in each specific organ within the body is beyond the scope of this thesis. However, the reliance on Mϕ by the mammary gland is of interest for this body of work. In BC, up to 50% of a tumour is comprised of Mϕ and generally speaking they have a deleterious effect on survival [106]. How this population encourages BC growth is discussed in 1.3.2.1 but it is worth noting that the mammary gland is not devoid of Mϕ throughout health with their first exposure occurring during disease. It is reported that within just 2 weeks of birth, bone-marrow derived Mϕ begin homing to the mammary gland [107]. When this migration is interrupted, such as in *M-CSF* null mice, branching morphogenesis, stem cell activity and alveolar budding are all interrupted. Therefore, Mϕ play a key role in the development and maintenance of the highly dynamic mammary gland. Recently, the dogma that mammary gland Mϕ are derived from bone marrow-derived monocytes has been challenged by a group that determined it is actually fetal-derived Mϕ that dominate adult mammary glands [108]. Delineating the ontogeny and role of Mϕ in both development of the mammary gland and during each stage of breast disease will be essential in designing novel therapeutics targeting monocytes or Mϕ in BC.

1.2.3.3.4. Macrophages in Immunity

The original role of Mϕ as professional phagocytes is arguably of most relevance to the innate immune response. However, their ability to recognise stimuli, become appropriately activated and trigger downstream events to mount an adaptive and thus more complex immune response with memory is also essential. Mϕ must recognise a plethora of molecules from different domains, let alone species. To do this, they express an abundance of receptors including toll-like receptors (TLRs), mannose-receptor and scavenger-receptor that recognise constituents of pathogens such as bacterial and fungal cell wall components. Recognition of these components leads to activation of Mϕ and often phagocytosis through production of a phagolysosome – a fusion of hydrolytic enzymes contained in the lysosome with a phagosome containing the foreign object to promote its destruction. However, not

all interactions lead to phagocytosis and instead some lead to the production of inflammatory cytokines or other cell death programmes such as pyroptosis [109].

Post-digestion, Mϕ process the pathogen and form pieces that become peptide antigens. These antigens are processed and packaged into a complex presented with major histocompatibility complex II (MHCII) on their surface. In this respect, Mϕ can function as antigen-presenting cells (APCs) presenting antigens to helper T-cells [71]. T-cells can thus directly induce an immune response or communicate with B-cells to stimulate antibody secretion. To come full circle, the formation of antibody-antigen complexes on the surfaces of target cells stimulates further phagocytosis via mechanisms such as agglutination and opsonisation [110].

Additionally, Mϕ can produce chemical messengers: cytokines that act locally and in some cases upon long distances to either promote or downregulate inflammatory responses. If Mϕ are stimulated to produce pro-inflammatory cytokines, they will release messengers such as tumour necrosis factor (TNF), IL-1, IL-6, IL-8 and IL-12. And conversely, their production of anti-inflammatory cytokines includes transforming growth factor β (TGF-β) and IL-10 [111]. Some of these cytokines will be discussed in greater detail in the context of cancer immunity, particularly those that relate to the topic of this thesis.

To summarise, Mϕ are first-line phagocytic cells that play an important role in mounting both a general and specific immune response. Furthermore, they function in several other processes in the body including early life during development and maintaining homeostasis. Although not discussed here, they also participate in many diseases and their presence is prognostic in many conditions. This includes cancer which will be discussed in 1.3.2.1.

1.2.3.4. Neutrophils

The most abundant yet shortest-living immune cell is the humble neutrophil; a member of the polymorphonuclear neutrophil (PMN) family, otherwise known as granulocytes. The discovery of neutrophils was made by Paul Ehrlich, observing distinct granular structures and a lobulated nucleus under the application of certain

stains. This property of neutrophils is responsible for their naming since neutrophils take up both acidic and basic dyes to become overall neutral. Of all the leukocytes in the body, neutrophils make up around 70%. However, at any one time only 1-2% are present in the circulation; the blood merely acts as a transport system for neutrophils to reach their target tissue [112].

In contrast to the M-CSF that drives Mϕ differentiation, neutrophil lineage commitment is driven by granulocyte colony-stimulating factor (G-CSF). From the early lymphoid-primed pluripotent progenitor (LPMP) comes the granulocyte-monocyte progenitor (GMP). It is these latter cells that under stimulation of G-CSF commit to the neutrophil lineage, first becoming a myeloblast before a maturation program that produces a mature neutrophil [113]. During these maturation phases, the granules inside the cell themselves develop from early azurophil granules to secretory vesicles in the cell's final form. These secretory vesicles are responsible for much of the anti-microbial function of neutrophils including production of elastase, myeloperoxidase (MPO) and MMPs – including MMP-8 (Figure 1.4) [114].

In any one day, 10^{11} neutrophils are produced in the bone marrow for homeostatic maintenance of granulopoiesis, where they linger for 4-6 days forming a reserve pool [115]. During infection, neutrophils are mobilised, and the pool becomes depleted leading to neutrophilia – driving further production. Neutrophil trafficking from the bone marrow is driven via an axis involving G-CSF, chemokine C-X-C motif ligand-12 (CXCL12) and CXCR4. Under homeostatic conditions, neutrophils are retained in the bone marrow via VLA-4/VCAM-1 signalling – enhanced by CXCL12 binding to its sole receptor: CXCR4 present on the neutrophils themselves [116]. During mobilisation, CXCR4 is downregulated, in part by G-CSF that inhibits CXCL12 production from bone marrow stromal cells [117]. Neutrophils also express CXCR2, which upon chemotactic CXCL1, -2, -5 and -6 ligand binding mediates their egression into sinusoidal blood vessels to travel to the site of infection [118].

In contrast to Mϕ, neutrophils have a more limited arsenal and participate in far fewer processes. This is probably related to their short half-life of around 6-12 hours in circulation and up to 2 days in tissues [119]. Generally, neutrophil functions can be divided into 3 categories: phagocytosis, degranulation and netosis - the release of

neutrophil extracellular traps (NETs) Figure 1.4). These functions will be discussed in the context of cancer in 1.3.

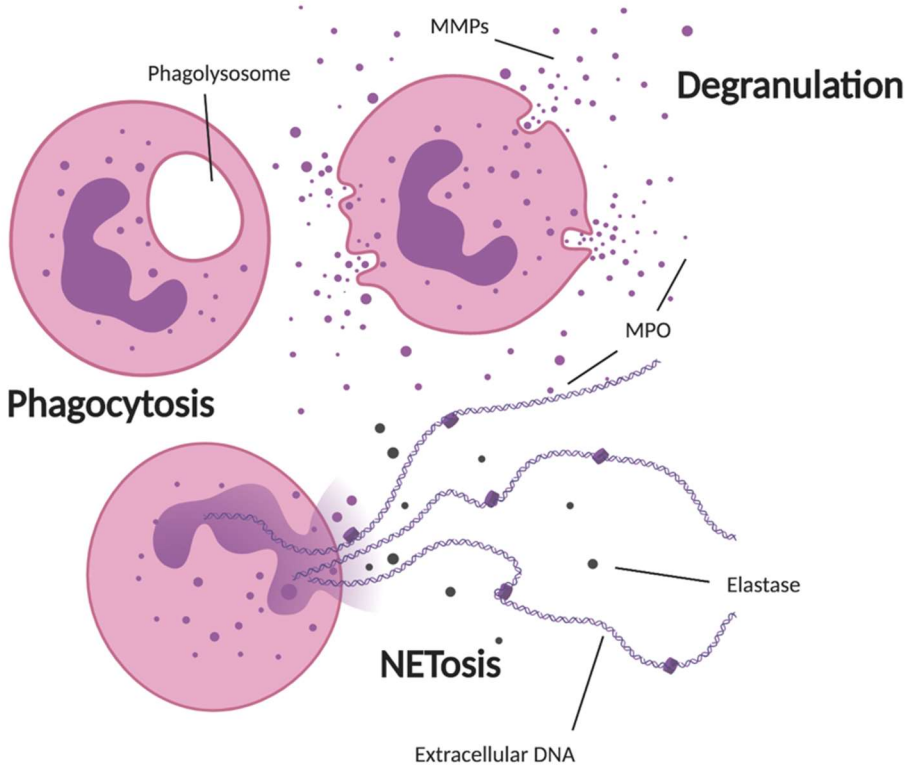


Figure 1.4. The main neutrophil processes in immunity. Neutrophils utilise three major mechanisms: phagocytosis, degranulation and NETosis to function against pathogens. Phagocytosis is an evolutionarily-conserved process of ingesting foreign cells or particles for their destruction. Degranulation involves releasing their cytosolic contents including MMPs and myeloperoxidase (MPO). NETosis is the release of a neutrophil extracellular trap (NET) that can stick onto foreign matter and enhance its removal.

Neutrophil clearance is as important as activation due to their highly cytotoxic contents. When neutrophils are not removed, their intracellular cargo can be released upon their death via necrosis or netosis [120]. Accumulation of neutrophils can be extremely detrimental leading to prolonged inflammation and tissue damage. Clearance of neutrophils is less well understood than their influx into tissues. Efferocytosis is one process of removal whereby Mϕ remove neutrophils 'in the battlefield'. However, an emerging mechanism for aged neutrophil removal is homing back to the bone marrow via re-expression of CXCR4, where they become destroyed by bone-marrow Mϕ Figure 1.5 [121].

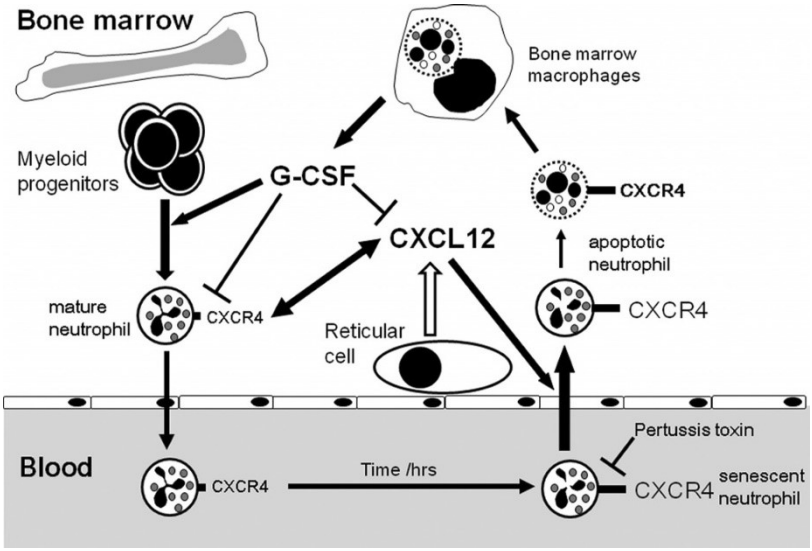


Figure 1.5. Neutrophil homing to the bone marrow. Senescent neutrophils re-express CXCR4 to return to the blood where they are engulfed by resident bone marrow macrophages [121].

1.2.3.5. Other cells in innate immunity

Neutrophils and Mϕ are of the most importance to this thesis, however for completeness the remainder of the granulocyte family will be briefly discussed. Representing a much smaller percentage of total leukocytes are: the eosinophils, basophils and mast cells. The most abundant of this group are the eosinophils, making up around 1-3% of total leukocytes [122]. Predominantly, eosinophils function as anti-parasitic immune cells, capable of protection against helminth infection [123]. During helminth infection eosinophil levels rise, with aggregation and degranulation occurring in the damaged area [124].

The rarest of all granulocytes are the basophils, representing a mere 0.5% of all leukocytes in peripheral blood [125]. This cell type is functionally similar to the last of our granulocytes: the mast cell and therefore they will be collectively discussed. The primary role of mast cells is in propagation of type 2 immune responses associated with helminth infection and allergy. When CD4+ Th2 cells activate naïve B-cells, antibody production commences with immunoglobulin M (IgM) and IgD classes [126]. Importantly in the context of mast cells, if these antibody-producing B-

cells interact with IL-4, class switching occurs to instigate IgE antibody production: commonly associated with allergic responses. IgE is able to bind to the FcεR1 present on mast cells (as well as basophils) and upon interaction with an antigen, FcεR1 crosslinking occurs leading to the release of mast cell granular contents; including histamine, a potent vasodilator [127].

Briefly, one more important leukocyte population is natural killer cells (NK cells). Originally discovered through their potent anti-tumour activity, NK cells are very effective at killing infected or cancerous cells without prior-stimulation. Whether they belong to the myeloid or lymphocyte lineage has been debated, but their cytotoxic ability is unquestionable. Their general role in immunity is beyond the scope of this thesis but their role in cancer is discussed in 1.3.

1.2.4. Adaptive Immunity

Cells of the myeloid lineage are of the most relevance to this thesis; however, it would not be a proper description of the immune system without mention to the more sophisticated arm: adaptive immunity. 'Adaptive' refers to how this system has developed over time to cope with such a wide range of pathogens in the first instance, and then variations of those pathogens that have mutated in attempt to subvert immune defences; a co-evolution arms race. The finely-tuned adaptive immune system has four defining characteristics: self vs non-self-recognition, antigenic specificity, diversity and immunological memory. This is in stark contrast to the innate immune system. For a system so complex, there are only two main types of cells comprising it: T-lymphocytes and B-lymphocytes. Broadly, the two can be categorised into cells involved in adaptive cellular immunity and adaptive humoral immunity respectively. However, there are many subsets within each category that perform their own functions

T and B lymphocytes perform very different functions; however, both rely on generation of a diverse repertoire of receptor specificities. This occurs during a process termed VDJ recombination whereby gene segments are recombined to produce antigen receptors that can recognise as many parts of a pathogen as possible. This process is beyond the scope of this thesis but is well-reviewed by

Market and Papavasiliou [128]. Nevertheless, this process is ultimately responsible for the diversity of the adaptive immune system.

T cells are classified according to their expression of two CD molecules: CD4 and CD8 that divide T cells into T helper (T_H) and T cytotoxic cells (T_C) respectively. T_H cells otherwise known as effector cells are further subcategorised according to their functions. T_H cells aid in the activity of other immune cells via production of cytokines. T_C cells are effective at killing infected and damaged cells [129].

The B cell lineage is less extensive than T cells. Their main responsibility is the production of antibodies – proteins that bind to and neutralise components of pathogens on the surfaces of infected cells and antigen-presenting cells. Mature B cells bind cell-surface antigens via their B-cell receptor (BCR). This begins a complex activation and differentiation programme that results in the generation of plasma cells from B-cells that themselves produce the antibodies. After antibody production, B-cells can undergo differentiation into memory B cells that are responsible for the immunological memory feature of the adaptive immune system. This means that upon a second exposure, a stronger more rapid response can be initiated [130].

B and T cells cooperate with one another, and cross-over occurs with the innate immune system. Much of this communication occurs via specialised APCs called dendritic cells (DCs) that facilitate many of the process described above [131]. Acting at the interface between the innate and adaptive immune system, DC's can function as pattern recognition receptors, process antigens, and migrate to draining lymph nodes to present antigen complexes to T cells [132,133].

1.3. Cancer and The Immune System

It is not a new concept that inflammation and cancer are intertwined; the presence of leukocytes in tumours was seen as early as the 1800s by Rudolph Virchow [134]. Today, we know that the tumour microenvironment (TME) is teeming with immune cells, that are not merely bystanders but interact with cancer cells to influence tumour progression (Figure 1.6).

However, it has only recently been accepted that inflammation plays a role in tumourigenesis, with some of the underlying molecular mechanisms having been elucidated. As previously discussed, the immune system has evolved to recognise self from non-self. It makes logical sense to categorise tumours as 'self' since they are composed of host cells. Because of this, it was a difficult notion to accept that the immune system could detect, let alone eradicate cancer. Evidence that the immune system could see these 'invisible' cancers came from both the clinic and experimental mouse models. Famous surgeon William Coley observed that his sarcoma patient who exhibited complete regression had a post-surgery *Streptococcus pyogenes* infection [135]. His theory was that this infection had prompted an anti-tumour immune reaction. Interestingly, to this day the cancer clinic benefits from his observation with the use of the infamous tuberculosis vaccine: Bacillus Calmette-Guérin (BCG) in treating bladder cancer patients, and the fact that Coley's toxin – a mixed bacterial vaccine - is still used as an immunotherapy by some clinicians. [136,137]

Further support of the cancer-immune system connection came from Burnet and Thomas in the 1950s who suggested the 'cancer immunological surveillance' theory. Their hypothesis suggested that emerging tumour cells expressed neoantigens that provoked an immune response [138,139]. Experimental research using hybridoma studies supported this notion. Mice were immunised with human tumours and large-scale screenings were carried out on the antibodies (Abs) produced. Many that were obtained were exclusive to the tumour antigen and not the tissue antigen – confirming the existence of neoantigens on tumour cells [140].

Nevertheless, some opposition to the immunosurveillance hypothesis arose when novel genetic tools created mice that lacked an effective immune system. In nude mice that lack a thymus and thus T-cells, there was no increased incidence in spontaneous or chemically-induced tumour development compared to wild-type controls [141]. Of note, nude mice still have functioning NK cells – the accepted hypothesis for the lack of observed phenotype.

Some years later, Robert Schreiber's group used alternative knockout mouse models that shaped a newer immuno-oncology paradigm. Mice deficient in recombination-activating gene-2 ($RAG2^{-/-}$) lack both T and B lymphocytes. When $RAG2^{-/-}$ mice were subjected to chemical carcinogenesis, they developed sarcomas earlier and with greater frequency than their wild-type counterparts. A similar story was seen in interferon γ ($IFN\gamma)^{-/-}$ mice and susceptibility increased further in double $RAG2^{-/-}$ $IFN\gamma^{-/-}$ mice, confirming the immune system is indeed essential in tumour prevention. However, additional data conflicted with the original immunosurveillance hypothesis. It was observed that tumours from immunodeficient mice were more immunogenic [142]. Immunogenicity is a term used to describe how well a tumour can stimulate an immune response – highly immunogenic tumours stimulate the most. This suggests a paradoxical role for the immune system in shaping the immune landscape to promote tumour outgrowth and coined the seminal use of the term 'immunoediting' to describe the dynamic process whereby the host immune system shapes the fate of a tumour. Immunoediting is the current accepted theory in cancer progression and will be discussed in greater detail.

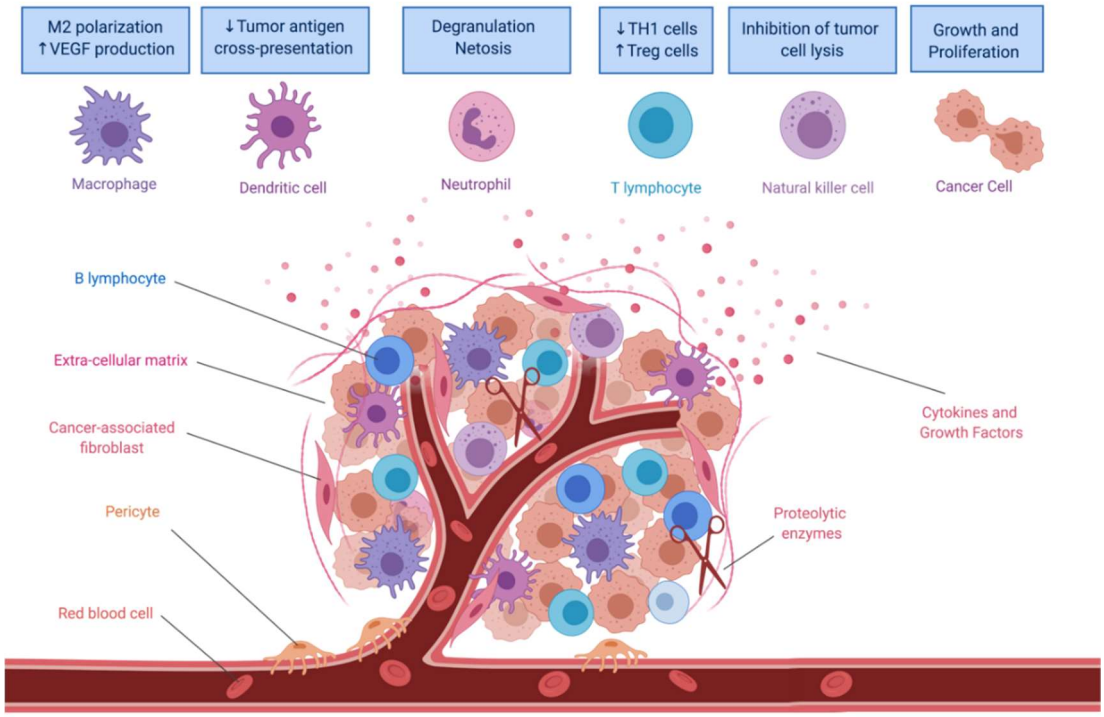


Figure 1.6. Contribution of immune cells and their products in the TME. In the TME, Mϕs are known to polarise to the pro-tumourigenic M2 phenotype and increase angiogenesis via upregulation of VEGF. Dendritic cells in the TME become less involved in presenting antigens to T-cells. The number of Treg cells is high in tumours, and other anti-tumourigenic T cells become less abundant. Neutrophils carry out their effector functions, natural killer cells prevent tumour cells being destroyed and due to all of this, the cancer cells themselves can continue to grow and proliferate.

1.3.1. Immunoediting

The process of immunoediting revolves around the idea that the immune system has a dual role in tumour development. It can either function to detect and eradicate cancers or it can promote the selection of tumours that are unable to promote a strong immune response and thus proliferate and thrive. The current theory proposes three stages of immunoediting: the elimination phase, also known as immunosurveillance, the equilibrium phase and escape [143].

During the elimination phase transformed cells are detected and eradicated by the immune system: tumours remain contained. The immune cascade commences with a mass of transformed cells that are recognised by the immune system via several mechanisms. Firstly, the mass of cells has most likely caused local tissue damage

whilst attempting to grow larger in size through neovascularisation for example [144]. This causes release of inflammatory cytokines from surrounding stromal cells. Secondly, the presence of tumour neoantigens on the surface of transformed cells prompt immune recognition. These tumour antigens come in several forms including mutational antigens and overexpressed proteins amongst others [145]. Similarly, tumours can produce so-called 'danger signals' such as uric acid that ultimately leads to their detection [146].

The common step after initial recognition seems to be via cytokine activation of immune cells such as NK cells and gamma-delta (γδ) T cells [147]. These cells produce IFNγ which has a cornucopia of effects in the TME. Two instances of this are the direct cytolytic effect of IFNγ on tumour cells themselves as well as stimulation of chemokine production (CXCL-9, -10 and -11) from tumour cells that results in recruitment of further immune cells such as Mϕ [148,149]. Tumour cell debris that arises in the TME can be ingested by local DCs that then home to draining lymph nodes where they are able to induce CD4+ T_H cell differentiation and eventually aid in the production of CD8+ T cells that are specific to the tumour. This then comes full circle when both T cell populations reach the tumour and remove the highly immunogenic cells [142].

The next stage of immunoediting relies on some tumour cells having escaped the elimination phase, either because of intrinsic or acquired heterogeneity. Whilst the elimination phase is still ongoing and highly immunogenic cells are eradicated, tumour cells evolve and due to genetic instability acquire new mutations such as the loss of major histocompatibility complex class I and II antigens or mutations in the IFNγ pathway that confer immune resistance. [143]. This dynamic equilibrium phase is essentially a long period of time, potentially years, where Darwinian selection occurs and results in a population of negligibly immunogenic tumour cells that can enter the final stage of immunoediting.

This final stage is known as the escape phase - the tumour becomes impassive to the attempts of the immune system and can divide uncontrollably. A generally immunosuppressive tumour microenvironment is encouraged, with high levels of cytokines such as VEGF, TGF-β and expression of immunoregulatory molecules such

as programmed death-ligand 1 (PD-L1) [150]. The tumour becomes clinically apparent and the host immune system obsolete in controlling the tumour.

1.3.2. Contributions of Specific Cell Types

1.3.2.1. Macrophages

Most immune subtypes function in some shape or form in the process of immunoediting: from the early NK cells to the later CD8+ T cells. However, the most abundant of all cell types present in the TME are tumour-associated macrophages (TAMs). The initial hypothesis of TAM ontogeny proposed that differentiation of circulating bone-marrow derived monocytes was responsible however in the past few years this dogma has been challenged - suggesting embryonic-derived tissue-resident Mϕ such as those discussed in 1.2.3.3 can also provide a pool of TAMs. Tumours therefore exhibit chimerism with respect to their Mϕ origins.

In human cancers, TAMs can constitute up to 50% of a tumour's mass [151]. TAM infiltration is synonymous with increased tumour growth – via their ability to influence pro-tumourigenic processes such as angiogenesis and immune-suppression. Meta-analyses have shown that in 80% of cases a high density of TAMs is associated with a poor prognosis in solid tumours including BC [106]. Tumour-derived chemotactic factors such as CCL2, VEGF and M-CSF all encourage the migration of Mϕ into the TME. Genetic depletion of Mϕ using transgenic MMTV-PyMT mice crossed with *M-CSF* null mice resulted in a delay in progression to malignancy as well as metastasis. The reverse experiment - overexpression of M-CSF, resulted in an acceleration of tumour progression and metastasis [152]. These data combined has led many to believe that TAM presence promotes tumour growth and progression.

However, as alluded to in 1.2.3.3, Mϕ exhibit a great deal of plasticity - essential to respond to a diverse range of signals and carry out multiple functions. Activation of Mϕ by different sets of signals leads to 'polarisation' – a narrow way of describing the different phenotypes that arise in this process (Figure 1.7). These subsets of Mϕ

are commonly referred to as M1 and M2; with M1 representing the classically activated Mϕ population and M2 the alternatively activated Mϕ [153]. The 'classically activated M1' phenotype is largely pro-inflammatory. Upon activation by bacterial lipopolysaccharide (LPS), IFNγ and (GM-CSF), M1 Mϕ secrete a wide variety of pro-inflammatory cytokines including TNF, IL-1β, IL-6 and IL-12, leading to the downstream activation of the adaptive immune system [92,154].

In comparison, 'M2 Mϕ', of a decidedly more heterogeneous nature than their M1 counterparts are associated with an anti-inflammatory phenotype. Activation does not occur via LPS or IFNγ but by numerous other cells and molecules. [78]. Fungal cells, parasites, complement proteins, IL-3, IL-4 and TGF-β can all induce the M2 phenotype [80]. Characteristically, M2 Mϕ express anti-inflammatory IL-10 as well as IL-1Ra and secrete angiogenic factors such as VEGF [155]. M2 Mϕ are further subdivided into three categories: M2a, b and c. M2a Mϕ are activated through exposure to IL-4 and IL-13 and participate in Th2 responses. Immune complexes and TLRs induce M2b Mϕ, which are involved in immuno-regulation. And finally, M2c Mϕ inducible via IL-10 aid in general suppression of the immune system and tissue remodelling through changes in matrix deposition [156].

The concept of M1/M2 ϕ existing on a continuum was discussed in 1.2.3.2 and 1.2.3.3.2. There is much debate in the field regarding the concept of M1/M2 ϕ polarisation, which many consider outdated [157]. For example, delineating ϕ subtypes through the use of one or two markers is flawed considering it is often changes in levels of hundreds of genes that define the phenotype of a ϕ [158]. Furthermore, there are variations in experimental conditions between labs such as tissue culture surfaces, activation conditions and even the origin mouse strain that can lead to changes to ϕ behaviour. These observations have led to new guidelines that attempt to generate consistency in the field [80].

TAMs are often considered as polarised M2 Mϕ, but this is likely an oversimplification. Some evidence exists that TAMs can be of the M1 phenotype. NK cells and APCs that produce IFNγ and TNFα can stimulate macrophage effector function leading to tumour cell killing and in melanoma, reprogramming TAMs from an M2 to an M1 phenotype led to changes in the vasculature, improving

chemotherapy delivery and tumour growth regression [159,160]. Perhaps M1 is the early Mϕ phenotype, that either metamorphose into M2 Mϕ or are outcompeted during tumour growth and immunoediting. But mostly, TAMs in established tumours do function as M2 Mϕ. Upon activation, TAMs produce a plethora of cytokines and growth factors that drive cancer initiation, angiogenesis, immune suppression and formation of metastatic niches.

Abnormally-sustained inflammation is a hallmark of cancer [161]. Sufferers of chronic inflammatory disorders such as inflammatory bowel disease are at an increased risk of developing cancer [162]. Mϕ are potent inflammatory cells and their secreted substances can create a microenvironment that promotes genetic instability and increases the likelihood of mutations. In colorectal cancer, microbial products that penetrate through a disrupted epithelial barrier are thought to upregulate TAM-derived IL-23 and IL-17 that drives cancer progression [163]. In hepatocellular carcinoma, TAM-derived IL-6 promotes tumour initiation via STAT3 signalling [164].

Secondly, TAMs drive angiogenesis. Solid tumours have areas lacking a blood supply that subsequently become hypoxic. Cells respond to lack of oxygen by upregulating expression of hypoxia-inducible factor 1-α (HIF-1α) – a transcription factor that drives the expression of pro-angiogenic genes to promote vascularisation [165]. TAMs present in hypoxic regions of tumours use this mechanism to form blood vessels that provide nutrition for tumour growth and prevent necrosis [151,166].

Another pro-tumourigenic process mediated by TAMs is Immune-suppression. Through release of immuno-suppressive cytokines such as IL-12, TGF-β and prostaglandin $E_{2,}$ activation of T regulatory cells (Tregs) occurs that suppress CD8+ T cell proliferation and function [167]. Similarly, high expression of arginase 1 (Arg1) in TAMs inhibits T-cell functions; specifically, antigen-specific responses [168].

And finally, TAMs can contribute to formation of secondary tumours – which is the most comprehensively described function of TAMs. TAMs secrete factors that enhance migration and invasion within the primary tumour as well as influencing sites of colonisation. TAM-derived CCL18 enhances BC metastasis by organising integrin clustering to facilitate enhanced adhesion to the extracellular matrix (ECM)

[169]. To become motile and invade the bloodstream, cells must first undergo epithelial-mesenchymal transition (EMT). Then, they must use the ECM as a conduit to travel. TAMs can alter cell adhesiveness, or produce proteases that reorganise the ECM to create this path [170]. Furthermore, TAMs can be recruited to distant sites such as the lung to form an environment suitable for colonisation. Tumour and stromal cells can release VEGF and TGF-β into the bloodstream to destination organs which encourage tissue-resident Mϕ to produce S100A8 and serum amyloid A3 that recruits tumour cells and Mϕ [171].

Because of these pro-tumourigenic functions, Mϕ have become a novel target in cancer treatment. There are several approaches to do this including preventing monocyte recruitment, reprogramming M2 TAMs to M1, or targeting molecules upregulated upon activation such as CD206 on M2 Mϕ [172].

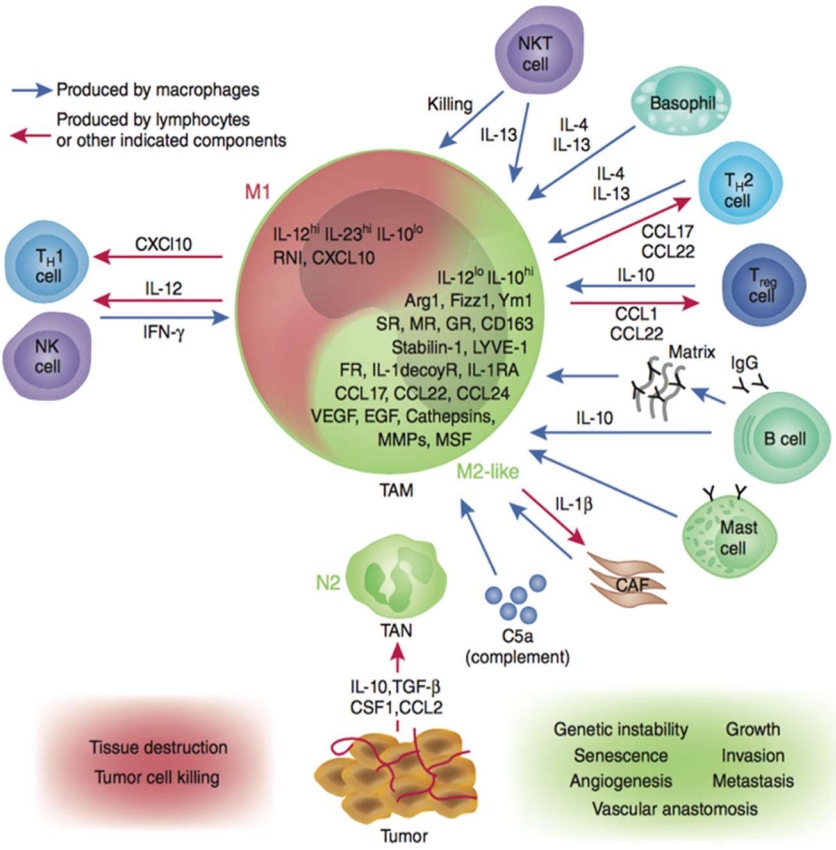


Figure 1.7. A comparison between M1 and M2 macrophages. Processes in red and green represent anti-tumourigenic and pro-tumourigenic functions respectively [450].

1.3.2.2. Neutrophils

It is perhaps counter-intuitive that short-lived neutrophils contribute to a chronic disease such as cancer, but more and more evidence is emerging that this is the case. Meta-analyses looking at peripheral blood samples and tumour biopsies have concluded that presence of neutrophils correlates with poor prognosis [173,174]. Systemic neutrophils are increased in cancer patients, perhaps unsurprisingly considering the inflammatory nature of cancers – though it is known that tumour cells produce G-CSF to mobilise neutrophils from the bone marrow [175,176]. Researchers have begun to correlate patient outcome with neutrophil-to-lymphocyte ratio. A ratio greater than 4 correlated with worse overall survival in every type of solid cancer at all stages [173]. However, changes in neutrophil numbers in peripheral blood is not necessarily representative of how tumour-associated neutrophils (TANs) behave. A meta-analysis on tumour specimens from many types of cancers revealed that presence of intra-tumoural neutrophils was also a prognostic factor [174]. So, if tumours actively recruit neutrophils, what is their role in tumourigenesis? Sustained inflammation is a known contributor to cancer initiation, growth and metastasis. Neutrophil activity in the TME can support each of these processes. TANs generally exhibit a pro-tumourigenic phenotype, and in mouse studies, just like Mϕ, neutrophils can also undergo polarisation. Using the Mϕ nomenclature, the N1 phenotype is anti-tumourigenic whereas the pro-tumourigenic phenotype is N2 – the phenotype of most TANs [177]. Like M1/M2 Mϕ polarisation, N1 and N2 is an oversimplification, representative of a spectrum, and notably has not been seen in humans. Polarisation towards the N2 phenotype is driven by TGF-β and stimulates the expression of Arg1 – known to suppress T-cell cytotoxicity [168]. It was elegantly shown through blockade of TGF-β that TANs can also be polarised to the N1 phenotype, driven instead by IFNβ [178]. N1 TANs possessed anti-tumourigenic activity including increased expression of pro-inflammatory cytokines such as TNFα and are more able to adhere to ICAM-1 that is upregulated on endothelial cells [177].

Products released from TANs such as reactive oxygen species (ROS) and proteases contribute to angiogenesis, cell proliferation, matrix remodelling and invasion [179]. For example, neutrophils produce MMP-9 which facilitates degradation of the ECM

and releases VEGF to enhance angiogenesis [180]. Additionally, NET release can have a two-fold impact in tumourigenesis: stimulating endothelial cells to release pro-angiogenic growth factors and at distant sites, can sequester circulating tumour cells contributing to metastatic niche formation [181,182]. Additionally, there is evidence that G-CSF promotes metastasis by driving neutrophils into distant sites to create a pre-metastatic niche that enhances the metastatic ability of several tumours [183].

Therefore, neutrophils represent a population that could be targeted to improve outcome. Currently there are three inhibitors in Phase I/II clinical trials targeting CXCR1 and 2 [184]. G-CSF inhibitors are also being developed and tested in pre-clinical models, although their effects on cancer patients with vulnerable immune systems may limit their use in the clinic [183].

1.3.2.3. B and T Lymphocytes

The adaptive immune system is critical to the eradication of transformed cells – implicating both B and T cells. Lymphocytes display much diversity in immunity, and the same is true in cancer. As with neutrophils and Mϕ, infiltration of lymphocytes into tumours can be correlated with clinical outcome; but the picture is less clear than with myeloid infiltrates. In the case of T cells, which subtypes dominate the tumour influences patient outcome. The presence of CD4+ and CD8+ T cells is associated with improved survival in many cancers [185–187]. Whereas, the opposite is true if there are high numbers of Tregs in the tumour [188–190]. Why is there a differential response between these populations? T cell differentiation is guided by cues in the local microenvironment such as cytokines and the presence of other immune cells. In cancer, aberrant cells express tumour-associated antigens (TAAs). These are presented as fragments in complex with MHC I on the cell surface recognised by the T-cell receptor (TCR) on T cells. If binding occurs, this leads to T cell activation dependent upon concomitant co-stimulation [191]. This then drives clonal expansion of T cells and brings in other effector cells to the site. Ultimately, the activation of CD8+ T cells either directly by MHC-antigen complexes or via stimulation by CD4+ helper T cells directs their cytotoxic activity. This can occur directly via granule exocytosis such as release of perforin and granzymes or via the FasL death

receptor pathway. Alternatively, cytotoxic activity can be indirect – using antibody/ complement-mediated mechanisms [192] .

How cancer cells resist this killing was detailed in 1.3.1 but in brief, T cell mediated cytotoxicity cannot occur if MHC has been downregulated, tumours are in an immune-privileged site not accessible to the immune system or they have upregulated immune checkpoints [193]. Immune checkpoints exist on cells to mediate immune tolerance. It is both unnecessary and detrimental to the host to maintain constant activation and so mechanisms are in place to dampen the response. Tregs exist in the T cell repertoire to do this very role. They do this via production of anti-inflammatory cytokines such as IL-10 and TGF-β which dampen effector functions of both B and T lymphocytes [194]. This considered, it is evident why an increase in Tregs is detrimental to cancer outcome. T cells are the biggest target in immunotherapy, and intervention methods for cancer are discussed in 1.3.3

Much less is known about B cells in cancer. However, what is known is that B cells often work in synchrony with T cells. Some tumours are comprised of up to one quarter B cells and in some BCs, 40% of the infiltrating lymphocytes are B cells [195,196]. The primary B cell function is production of antibodies, and in cancer there are TAAs that require antibody-neutralisation. Antibodies have manifold functions in cancer. Firstly, they coat tumour cells in a process termed opsonisation. This coating leads to the death of the cell via phagocytosis, antibody-dependent-cell-mediated cytotoxicity mediated by NK cells or activation of the complement pathway. Opsonisation can also lead to cross-presentation of tumour antigens by DCs [197].

It is important to note that whilst not discussed, other immune cells are present in the TME and contribute to immunoediting. This includes NK cells, DCs and a recently discovered subset of monocytes and neutrophils: the myeloid-derived suppressor cells [198].

1.3.3. Immunotherapy

The twenty-first century has seen the emergence of a fourth pillar of cancer treatment: immunotherapy. Immunotherapy harnesses the body's own immune

system to destroy tumours by stimulating anti-tumourigenic processes or countering desensitisation. The contribution of the immune system to tumour growth and metastasis is significant and has been discussed in detail in 1.3. Immunotherapies reverse the latter stages of the immunoediting process: primarily the equilibrium phase but also the escape phase – when cells look for ways to avoid immune detection. Tumour cells express neoantigens that stimulate an immune response and subsequently create an inflammatory TME. However, it is now appreciated that tumour cells manipulate the immune system for their own survival. The most-characterised example of this is inhibition of T cells. The surface of T cells is littered with ligands and receptors that facilitate the plethora of roles carried out by T cells– including stimulatory and inhibitory molecules. For antigen-mediated activation of T cells to occur, as well as TCR-MHC interaction, CD28 binds its receptors: CD80 or CD86 expressed on APCs for co-stimulation [199]. If this interaction is disturbed, TCR signalling is abrogated and T-cell activity is supressed. Sometimes, this is appropriate – for tolerance or resolution of inflammation, activity must be diminished and so pathways exist to regulate T-cell function. Tregs are the masters of this: they express inhibitory molecules on their surface that can interact with CD4+ and CD8+ T cells to inhibit their function. One example is cytotoxic T-lymphocyte antigen-4 (CTLA-4) which competes with CD28 for interaction with CD80/86 to antagonise TCR signalling [200]. However, CTLA-4 can become upregulated on activated T cells to suppress their cytotoxic activity thus shielding a tumour from immune destruction.

Therapeutic inhibition of CTLA-4 thus became a feasible option for treating cancer and ipilimumab, a monoclonal Ab against CTLA-4 was the first FDA-approved immunotherapy for treatment of melanoma in 2011 [201]. Several other immune checkpoint inhibitors (ICIs) have been developed against targets such as programmed death-1 (PD-1).

PD-1 is a receptor expressed on T and B cells and is important in regulating peripheral tolerance to prevent autoimmunity [202]. PD-L1 ligands are constitutively expressed on both haematopoietic cells and non-haematopoietic cells such as mesenchymal and endothelial cells [203,204]. PD-1: PD-L1 interaction leads to T cell inhibition. Tumour cells have exploited this mechanism to repress the immune response by

expressing PD-L1 on their surface [205]. Hence, PD-1 and PD-L1 also became therapeutic targets and several years after approval of ipilimumab, two more mAbs were approved that targeted this pathway [206].

In terms of success, ICIs have relatively low efficacies and some severe side effects not to mention a huge price tag. Ipilimumab treatment led to response rates of around 30-40% in monotherapy, increasing to 50% in combination therapy [207]. Billions of dollars have been channelled into developing immunotherapies and they have been miraculous for some patients. Yet, only around one third of patients have a durable and meaningful response to ICI's; many relapsing months to years later. Melanoma has been the biggest success, but the results are less convincing in other cancers, particularly solid cancers. This means that most patients simply do not respond to immunotherapies [208]. The explanation for this is not entirely clear. Current focus on improvements to ICIs is searching for biomarkers that predict response to treatment. Currently in the clinic, patients are stratified according to expression of checkpoint ligands, mutational burden and presence of tumour-infiltrating lymphocytes [209]. This latter marker is hypothesised to be key - tumours are referred to 'hot' with high levels of infiltration or 'cold' with low immune infiltration which can be due to immune exclusion or a total lack of response [210]. Cold tumours represent the biggest challenge for immunotherapy – since absence of immune cells or inactivity does not lend itself well to checkpoint inhibition. Most BCs are cold tumours [211].

The non-cellular component of tumours: the ECM, is often overlooked in immunotherapy, but it is a constant in an ever-changing landscape. However, it is remodelled during tumourigenesis and immunoediting. Bioactive fragments released from the ECM can influence immune cell trafficking, angiogenesis, blood vessel permeability and cell death; mostly mediated by proteolytic enzymes such as the MMPs [212]. The contribution of matrix composition on shaping immunogenicity is a new concept but should be studied in more depth to aid in the design and improvement of novel and existing immunotherapies respectively.

Whilst it is clear immunotherapy is a breakthrough field, it will be important in the coming years to address its limitations including cost, side effects and relapse.

1.4. Matrix Metalloproteinases

1.4.1. Overview

All cells exist surrounded by an ECM that acts not merely as a scaffold but also as a reservoir for signalling and growth molecules – rendering it a hub for cell-cell and cell-ECM communication. Fundamentally the ECM is composed of water, protein and polysaccharides with amount and distribution varying significantly between tissue types. By far the most abundant component is collagen – a triple helical fibrillar protein that impressively makes up over a quarter of the total protein present in the human body [213]. The ECM is not static but rather a dynamic environment that requires constant remodelling to facilitate changes in cell behaviour. This remodelling of constituents is carried out primarily by a group of proteolytic enzymes aptly named the matrix metalloproteases (MMPs).

Matrix metalloproteinases (MMPs) are a family of 23 zinc-dependent endopeptidases belonging to a wider metzincin superfamily that also includes a disintegrin and metalloproteinase (ADAMs) and a disintegrin and metalloproteinase with thrombospondin repeats (ADAMTSs). The founding member: MMP-1 was discovered in tadpoles from *Rane catesbiana*: a frog native to North America [214]. Collagen in tadpole tails requires remodelling during metamorphosis and subsequent transformation into an adult body – a process reliant on collagenases.

Collagen is not the exclusive substrate of MMPs – they are able to degrade absolutely all protein components of the ECM [215]. Because of this power, MMPs must be kept in check by various mechanisms including by the tissue inhibitors of metalloproteinases (TIMPs) which is discussed in greater detail in 1.4.2.

MMP classification is based on their substrate specificity forming 5 subgroups: collagenases, gelatinases, stromelysins, matrilysins and membrane-type (MT-MMPs) [216]. The members represented by each group can be found in Table 1.2**.**

Despite differences between subfamilies, all MMPs are multi-domain proteins that possess structural similarity. MMPs consist of a pre-domain, pro-domain, catalytic domain, hinge region and hemopexin domain– each with their own function (Figure

1.8). The pre-domain – an N-terminal signal sequence targets MMPs to the endoplasmic reticulum for secretion.

Table 1.2. Classification of the matrix metalloprotease family

Subfamily	Members
Collagenases	MMP-1, -8, -13
Stromelysins	MMP-3, -10,
Gelatinases	MMP-2, -9
Matrilysins	MMP-7, -11, -26
MT-MMPs	MMP-14, -15, -16, -17, -24, -25
Others	MMP-12, -19, -20, -21, -23, -27, -28

Post-secretion, the pro-domain which is just 80 amino acids in length holds the key to activation. A conserved PRCGVPDV sequence contains a cysteine residue that connects to the Zn^{2+} ion in the catalytic domain. This intramolecular complex formed functions to prevent catalysis. All methods of activation such as furin cleavage lead to a disturbance in the cysteine-zinc interaction to permit exposure of the Zn ion – essential as a cofactor for enzymatic activity - known as the cysteine-switch mechanism [217]. Lastly, the 170-amino acid hemopexin domain is a C-terminal four bladed β-propeller structure important in substrate and TIMP recognition [218]. This domain features a highly conserved histidine sequence that functions in this recognition as well as in zinc chelation. There are some exceptions including MMP-7, -23 and-26 that lack the hemopexin domain, and MMP-23 that features a further cysteine-rich and immunoglobulin domain [219]. MT-MMPs are an exception to convention and have an additional domain that enables anchoring in the cell membrane. This is either in the form of a transmembrane domain (MMP-14, -15, -16, ,-24 and -27) or a glycophosphatidylinositol anchor (MMP-17 and -25) [220].

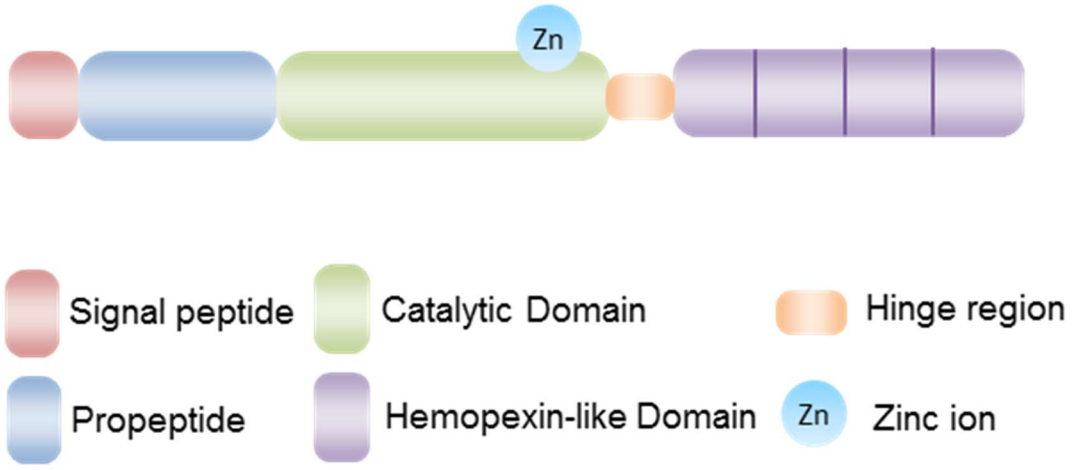


Figure 1.8. Basic domain organisation of the matrix metalloproteinase enzyme family.

It has been hypothesised that the diversity that exists between MMPs is due to duplication events that occurred during evolution in the tetrapod lineage [221]. Therefore, some members are derivatives of a single gene and this is evident in their location on the chromosome as clusters of MMPs. For example, on human chromosome 11: *MMP1, -3, -7, -8, -10, -12, -13, -20*, and *-27* all cluster closely together and this is reflected in mouse chromosome 9 [222]. Whilst this may explain some of the overlapping functions of MMPs, it has important consequences for genetic manipulation. The creation of mouse models specifically lacking one specific MMP may be difficult due to linkage between nearby genes. This issue is discussed in further detail in 1.5.

1.4.2. Regulation of MMPs

MMPs are responsible for maintaining homeostasis *via* a plethora of physiological processes and thus their activity requires stringent regulation. There are four main mechanisms of regulation: transcriptional control, proenzyme activation, compartmentalisation, and specific inhibition.

Constitutive transcription of MMPs is generally low under steady-state conditions but a transient increase occurs upon stimuli including cell-cell and cell-ECM

interactions that result in production of cytokines such as IL-1, IL-6, TNFα, TGF-β, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) [223,224]. Many of these molecules also regulate transcription via influencing mRNA stability. In recent years, micro-RNAs (miRNAs) have also been shown to regulate post-transcriptional control of MMPs – an emerging area in the field [225].

The second mechanism of regulation is the existence of MMPs as pro-enzymes that require activation. There are several ways to initiate proenzyme activation: endoproteinase cleavage, allosteric conformational change and chemical modification [226]. Membrane-bound MMPs are intracellularly activated by pro-protein convertases such as furins and therefore are active immediately upon appearance on the cell membrane [227]. In contrast, secreted MMPs are activated pericellularly. Upon exit from the cell, MMPs often undergo an initial conformational change that disturbs the zinc-cysteine interaction and leads to cleavage within the pro-domain by other proteases such as plasmin, trypsin and elastase as well as other MMPs that can lead to pro-domain cleavage and thus activation through autoproteolysis (Figure 1.9) [217,228]. Autoproteolysis also appears to be triggered by other non-proteolytic means such as the release of reactive oxygen species (ROS) from phagocytic immune cells and *in vitro,* the addition of compounds such as sodium dodecyl sulphate and 4-aminophenylmercuric acetate [229,230].

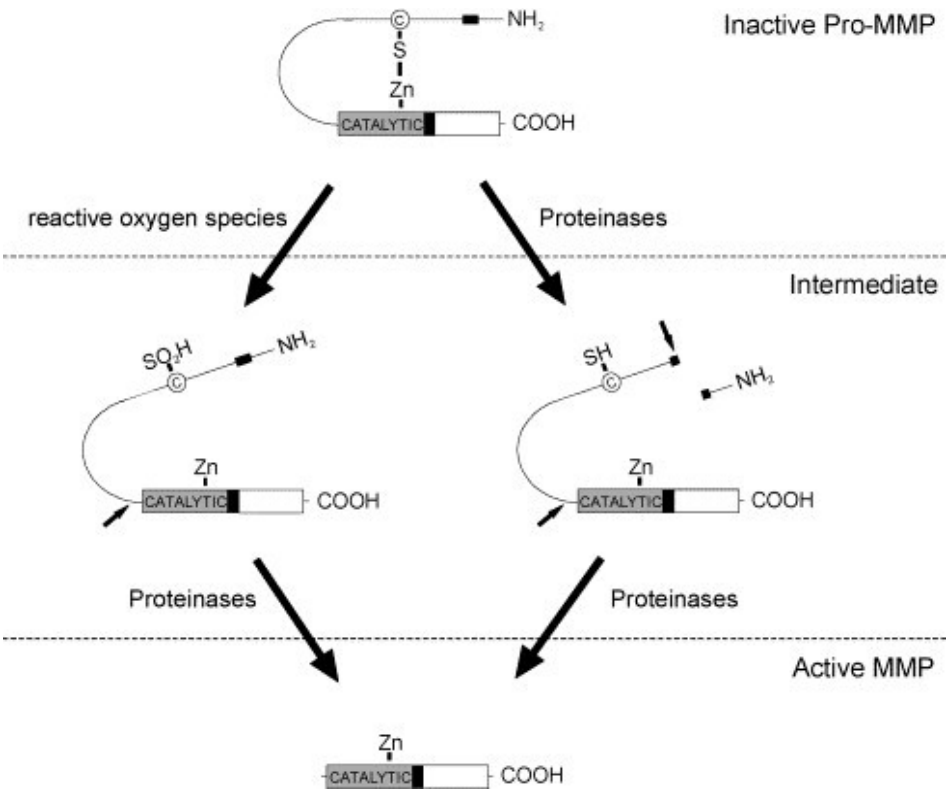


Figure 1.9. Schematic detailing the cysteine switch mechanism of MMP activation. Upon stimulation, the interaction between the catalytic zinc and a thiol group is disturbed and the catalytic region becomes exposed. [289].

The location of MMPs is perhaps the simplest method of regulation however it is very effective in ensuring appropriate substrate interaction. The cell surface and surrounding ECM is an exceptionally complex mesh of thousands of receptors, molecules and enzymes that require compartmentalisation to ensure a cell responds correctly to its environment. Secreted MMPs preferentially locate to the pericellular space and associate with cell-membrane proteins as is the case for MMP-1 binding to $\alpha 2\beta 1$ integrin [231,232]

And finally, there exists a brake on activity in the form of inhibitors. MMP activity is controlled by both a specific family of inhibitors: TIMPs and non-specific protease inhibitors including $\alpha 2$-macroglobulin.

The TIMPs are an ancient family of four inhibitors – a modest 190 amino acids in length comprising a simple structure of an N-terminal and a C-terminal domain [233].

The N-terminal domain is responsible for chelating the zinc atom necessary for proteolytic activation and sustained activity. The four TIMPs have overlapping functions but each have specific qualities. TIMP-1 is principally expressed in reproductive organs and the central nervous system [234]. Its limited expression perhaps relates to it being the only TIMP member not able to potently inhibit all MMPs – it is a poor inhibitor of MT1-MMP (MMP-14), MT3-MMP (MMP-16), MT5-MMP(MMP-24) and MMP-19 [235]. TIMP-3 is often considered the most important – able not only to inhibit all MMPs but also several members of the ADAM and ADAMTS families including ADAMTS-4, -5 and ADAM17 [236,237]. The ability to inhibit this latter member: ADAM17, has important consequences in inflammation acting as a sheddase, releasing ligands such as TNFα and epidermal growth factor (EGF) from the cell surface [238].

It is conceivably partly due to these interactions that the TIMP3 knockout mouse exhibits the most profound phenotype without challenge – lung damage and accelerated apoptosis in the mammary gland [239,240] . And finally, there is TIMP-4, that is restricted primarily to the heart [241].

Aside from the TIMPs, α2-macroglobulin is another well-studied MMP inhibitor. Present in tissue fluids and the blood, it is a homotetrameric protein of 725KDa that physically traps enzymes for subsequent endocytic removal [242]. And finally, non-enzymatic inhibitory methods exist including production of ROS from phagocytic immune cells during inflammation that potently inhibit MMP activity [231].

The need for such extensive regulation exemplifies the importance of a balance between activation and inhibition of these multi-functional metalloproteases in homeostasis to prevent disease.

1.4.3. MMPs in Physiology and Pathophysiology

MMPs are essential in both physiology and pathology: participating in a plethora of processes such as embryogenesis, wound healing and angiogenesis [243]. The list of MMP substrates is extensive and presented in the online database MEROPS [244].

However, many of their functions involve common substrates such as collagens and other large molecular weight proteins such as laminins, fibronectin and aggrecan. These latter proteins are cleavable by almost all known MMPs. The fibrillar collagens: Type I, II and III are cleaved by collagenases and MT1-MMP at a specific site three-quarters from the N-terminal [220]. Collagen IV, found in basement membranes is cleaved by gelatinases, matrilysins and stromelysins. Furthermore, MMPs can cleave cell-adhesion molecules such as E-cadherin (matrilysins and stromelysins) and integrin $\alpha v\beta 3$ (MT1-MMP) to influence cell migration and adhesion to particular substrata [245].

It is not simply that MMPs cleave large molecules for subsequent degradation. The cleavage of substrates often reveals cryptic epitopes that influence multiple cell behaviours. For example, cleavage of latent activating protein (LAP) by MMP-2, -9, -13 and -14 increases the bioavailability of TGF-β, since LAP complexes with TGF-β to keep it inactive. TGF-β is a potent growth-promoting cytokine which has a plethora of effects in both physiology and pathophysiology and is discussed in more detail in the context of MMP-8 in 1.4.5.2 [246].

Furthermore, MMPs can also liberate peptides that impact vascular development and homeostasis. MMP-2 and -9 can cleave collagen IV to reveal an angiogenesis-promoting neo-epitope subsequently termed tumstatin [247]. Similarly, MMP-9 can cleave VEGF in complex with heparan-sulphate proteoglycans (HSPGs) such as syndecans to again promote angiogenesis [248]. Conversely, MMP associated products can also inhibit angiogenesis. Cleavage of VEGF by MMP-3 and in some instances MMP-9, can lead to its inability to bind HSPGs leading to irregular vessel sprouting [249]. Thus, with such wide-ranging biological impacts, the spatiotemporal distribution of MMPs and their substrates are crucial to physiological function [250].

Much of our knowledge on function is derived from the phenotypes of MMP knockout mouse models. Somewhat surprisingly, absence of any single MMP is not embryonic lethal nor are there severe consequences on postnatal development except for in the case of *Mt1mmp* null mice which die prematurely after a few weeks [251]. It has been hypothesised that due their overlapping functions, other MMPs

compensate in these models – making interpretation more difficult. However, many of the phenotypes are defects in vascular development and bone growth/remodelling. Endochondral and intramembranous ossification – the formation of long and flat bones respectively both appear to be reliant on MMPs. *Mmp2*, *-9, -13, -14, and -16* null mice all exhibit defects in some aspect of bone biology. In MMP-13 this appears to be a general remodelling defect but the phenotype is better characterised in *Mmp2* and *Mmp9* null mice [252]. *Mmp2* null mice have decreased bone mineralisation and defects in osteoblast and osteoclast growth [253]. *Mmp9* null mice exhibit delayed repair upon bone fracture [254]. These phenotypes illustrate how important matrix remodelling is to skeletal development and maintenance.

Lastly, and of most relevance to this thesis is the importance of MMPs in immunity. A wealth of evidence has emerged over the past few decades that MMPs participate in both physiological immunity and immunopathology. There are three main mechanisms of MMP-mediated immunity: modulation of chemotactic gradients, physical breakdown of the ECM to facilitate immune cell migration and cleavage of anti-microbial peptides. The intricacy of the innate immune system has been dealt with in 1.2.3 but here much of the focus is on how leukocytes are recruited to sites of injury and inflammation.

Cytokines and chemokines are released from the site of injury to recruit circulating immune cells. Once at their target tissue, leukocytes must transmigrate across the endothelial barrier before degrading the surrounding basement membrane and migrating through the dense interstitial matrix [255]. The components of these structures are substrates for proteolytic enzymes such as the MMPs and so it is unsurprising that leukocyte migration involves MMPs [256]. Neutrophil migration is driven by cytokine and chemokine gradients; many of which are bound in some form to the ECM or cell-surface receptors. MMP-7, -8 and -9 all facilitate neutrophil migration to some extent. MMP-7 does this through cleavage of syndecan-1: releasing sequestered chemotactic cytokines such as CXCL1 – to guide neutrophils to damaged lung epithelia [257,258]. In an autoimmune skin blistering disease: bullous pemphigoid, absence of MMP-9 renders mice resistant due to an inability to recruit

neutrophils [259]. There also exists a feed-forward mechanism between neutrophil infiltration and release of MMP-8 that will be discussed in detail in 1.4.5 [260].

Modulation of chemotactic gradients is not specific to neutrophil recruitment however, and at least six MMPs are able to release TNFα from its precursor on the cell surface (MMP-1, -2, -3, -7, -9, and -12). Similarly proIL-1β is a target of MMP proteolysis [261,262]. Both cytokines can hugely influence promotion of systemic inflammation.

Aside from the creation of cytokine gradients, some MMPs aid in immune cell transmigration and extravasation. For example, MMP-2 and -9 are key in leukocyte transmigration across the blood brain barrier [263]. MMP-9 also mediates T cell and dendritic cell migration through the basement membrane [264]. Furthermore, mice treated with MMP inhibitors had impaired lymphocytes influx into lymph nodes – impacting on antigen-presentation to the systemic immune system [265].

The final mechanism whereby MMPs influence immunity is through direct cleavage of endogenous antimicrobial peptides. MMP-7 is key in innate defence against intestinal pathogens. MMP-7 null mice are more susceptible to bacterial infection in the gut due to an inability to release α-defensin from epithelial cells [266].

However, MMPs do not have uniquely positive effects on host immunity. Excess or inappropriate activity can lead to malignant transformation, chronic inflammation and ultimately disease; including HIV, meningitis, tuberculosis and periodontal disease [267,268]. This has led to ideas that modulation of MMP activity may be of benefit therapeutically. However, there are two principal issues: difficulty in targeting specific MMPs and the existence of so-called ‘anti-targets’. As previously discussed, MMPs function in homeostasis and many have positive effects that would be eliminated under inhibition. MMPs as anti-targets will be discussed in more detail in 1.4.4 in the context of cancer.

Numerous pathologies implicate MMPs however there are fundamentally three general mechanisms of action: tissue fibrosis, weakening of the ECM and tissue destruction. The fibrotic disease atherosclerosis is the primary cause of mortality worldwide and whilst the series of events that lead to eventual myocardial infarction

are complex, MMPs have an undisputed role in the progression of the disease and subsequent plaque rupture [269]. Less well understood is the role of MMPs in disorders involving reduced matrix stability such as the skin condition epidermolysis bullosa; nevertheless, upregulation of MMPs is seen and they have been considered as therapeutic targets [270]. Lastly, and of the most relevance to this thesis is the involvement of MMPs in diseases with characteristic tissue destruction such as osteoarthritis, inflammatory-related diseases (rheumatoid arthritis, periodontal disease, neuroinflammatory diseases) and cancer.

Turnover of the ECM is a precise event, stringently regulated to prevent excessive destruction as discussed in 1.4.1. When this process becomes dysregulated it has enormous consequences for the immediate tissue microenvironment as well as distant tissues as is the case in cancer invasion and metastasis. Cells that experience genomic instability and uncontrolled cell division require new territory and resources urgently and upregulate proteolytic enzymes that permit this; such as the MMPs. This happens both within tumour cells but most remarkably and predominately through tumour-host interactions – coercing nearby stromal cells.

1.4.4. Matrix Metalloproteinases in Cancer

In a cancer field constantly improving but devoid of targeted therapies, MMP inhibitors (MMPIs) were a logical venture. Through their ability to cleave matrix components and liberate growth factors, MMPs participate in many stages of the tumourigenesis cascade. Many years ago, the seminal paper by Hanahan and Weinberg proposed the six hallmarks of cancer: sustained proliferative signalling, evading growth suppressors, activating invasion and metastasis, replicative immortality, inducing angiogenesis and resisting cell death [161]. The list became more exhaustive in recent years to include: avoiding immune destruction, genome instability and mutation, tumour-promoting inflammation and deregulating cellular energetics [271]. Common to most of these characteristics is the activity of MMPs. There is evidence for enhanced angiogenesis through liberation of pro-angiogenic molecules, physical breakdown of barriers such as the basement membrane facilitating invasion and metastasis, cleavage of death receptors to prevent

apoptosis, liberation of growth factors to encourage cell growth and proliferation and changes in chemokine gradients to promote detrimental inflammation or avoid detection by the immune system. Furthermore, almost all MMPs are upregulated in virtually every type of solid cancer; expression generally correlative with tumour aggressiveness, stage and prognosis [272,273].

Substantial investment from pharmaceutical companies saw the development of broad-spectrum MMPI's such as marimastat and batimastat, designed to bind within the enzymes' catalytic domain thus preventing their activity [274]. Unfortunately, pan-MMPI's such as marimastat failed phase III clinical trials, failing to improve progression-free survival in metastatic BC patients and inadvertently causing severe side effects such as debilitating musculoskeletal pain and inflammation [275]. The hypotheses for failure were: MMPs function in early stages of tumour progression and the patient cohort was those with advanced disease. Secondly, there was a lack of specificity that led to accidental targeting of other closely related families such as the ADAMs and inhibition of MMPs that appeared to be host-beneficial rather than tumour-promoting [276]. This challenged the notion that MMPs simply pave the way for cancer cell invasion *via* the ECM. Instead, it is now appreciated that they have multifaceted roles in cancer, dependent upon type, stage and cellular source of the MMP [277]. In fact, several MMPs have been demonstrated to inhibit tumour initiation, cell migration, invasion and metastasis [278,279].

Despite this, robust evidence exists that many MMPs are tumour-promoting in a range of malignancies. Expression in clinical specimens such as tumour tissue or serum often has prognostic value – with an overwhelmingly negative impact on survival [273]. Furthermore, samples from metastatic sites have been linked with high MMP levels such as MMP-1, -7, -9, -11 and -13 in BC [280].

Several studies have looked at the expression patterns of the MMP family in BC. Köhrmann *et al.* profiled MMP expression at the protein and RNA level in both normal breast tissue and BC tissue, finding significant differences for many members of the MMP family [281]. The best example was the increased expression of MMP-9 and MMP-11 in BC specimens, which has been shown consistently [282,283]. Both MMPs are assessed in the clinical setting. *MMP9* is one of 70 genes in the MammaPrint gene

signature and *MMP11* expression is measured in the Oncotype DX 21-gene array discussed in 1.1.5, used prognostically in the UK for guiding treatment for $HER2^-$ BC [284,285]. Interestingly, one of the fourteen members with high expression in BC tissue was *MMP8*; high expression also correlated with increased tumour grade [281]. However, there was no demonstrated correlation with survival whereas other studies have revealed a contrasting tumour-protective role for serum and tissue MMP-8 in BC [286,287].

1.4.5. Matrix Metalloproteinase-8

MMP-8 is a member of the collagenase subfamily; efficient at cleaving triple helical collagen fibrils but also cytokines, growth factors and proteases [260,288]. It is alternatively known as neutrophil collagenase owing to its secretion in specific granules from neutrophils, but is also produced by a diverse set of cell types including Mϕ, T cells, endothelial cells and fibroblasts – all cells in the TME [289]. MMP-8 is not essential for life, since *Mmp8* knockout mice are viable and healthy with no overt phenotype. This is most likely due to compensation from other members of the MMP family. It is involved in many aspects of physiology and pathology, however of the most relevance to this thesis is its role in tumourigenesis and immunity.

1.4.5.1. MMP-8 in Tumorigenesis

Post-MMPI failure, pharmaceutical companies abandoned MMPs but researchers were still curious as to why. MMP-8 was the first so-called 'anti-target' to be discovered. Evidence that MMP-8 was tumour inhibitory came from a screen of differentially expressed genes in two BC cell lines with opposing metastatic ability. Derived from MDA-MB-231 triple negative BC cells; both clones were tumourigenic but only one could metastasise to the lungs of mice. *Mmp8* expression was 10-fold higher in the non-metastatic cell line compared to the metastatic, and this difference persisted in breast tissue after their orthotopic injection into athymic mice. Furthermore, upon stable antisense knockdown of MMP-8 in the metastatic clone, there was a 2.5-fold increase in invasion into a Matrigel matrix [290]. Follow-up work

found that conversely, overexpression by retroviral transduction in the metastatic line reversed their metastatic ability [291].

Later that year, a more focused approach was taken using an *Mmp8* knockout mouse in carcinogen-induced skin cancer. Mice deficient in *Mmp8* exhibited a much higher incidence of carcinogen induced skin papillomas – but this was a sex-specific phenomenon as this did not occur in females [292]. However, the male phenotype could be observed in females after ovariectomy or oestrogen-blocking tamoxifen treatment indicative of ovarian oestrogen being protective in female *Mmp8* null mice.

A similar observation was made using the same knockout mouse in tongue cancer – where *Mmp8* deficient mice were more susceptible to developing tongue carcinoma than wild-type controls [293]. Once again, there were differences in phenotypes between males and females: the incidence was far greater in females than in males. To further study this, a tongue carcinoma cell line was utilised to examine the interplay between MMP-8 and oestrogen. Oestrogen upregulated the expression of MMP-8 and MMP-8 cleaved both forms of the oestrogen receptor (ERα and ERβ). The *MMP8* promoter does not contain an estrogen response element but does contain a C/EBP element known to associate with ERα [294]. Both studies implicate oestrogen in the observed phenotypes but how MMP-8 mediates oestrogen signalling *in vivo* is yet to be determined.

Aside from skin and tongue cancer, the anti-tumourigenic role of MMP-8 in *in vivo* tumourigenesis has extended to melanoma and BC. Overexpression of *Mmp8* in B16F10 melanoma cells led to reduced lung metastases compared to control cells upon injection into immunocompetent mice. However, this was dependent on the catalytic activity of MMP-8 since this phenotype was lost when the B16F10 cells were transfected with a catalytically-dead mutant. To demonstrate the impact of host-derived MMP-8 on tumour formation and metastasis, *Mmp8* null mice were injected with B16F10 cells that did not express *Mmp8*. Whilst primary tumour growth was comparable between genotypes, lung metastasis was significantly higher in mice

deficient in *Mmp8*. This work demonstrates that host and tumour-derived MMP-8 contributes to the spread of melanoma [287].

In recent years, the Edwards group demonstrated the tumour and metastasis-inhibitory effects of MMP-8 in a spontaneous mouse model of BC. The mouse mammary tumour virus-polyoma middle T antigen (MMTV-PyMT) model involves expression of a viral oncoprotein (PyMT) driven by a mammary-specific promoter (MMTV) [295]. Post-weaning, mice develop hyperplastic legions that progress in a similar fashion to human BC including predictable lung metastases. Crossing the MMTV-PyMT model onto *Mmp8* null females revealed that absence of *Mmp8* led to accelerated primary tumour growth and an eleven-fold increase in lung macrometastases [296]. In the primary tumour, reduced vascularity was seen in wild-type mice compared to *Mmp8* heterozygote and null mice at 8 weeks of age.

Despite demonstration of MMP-8-mediated inhibition in tumourigenesis in several cancers, there is evidence that MMP-8 is detrimental in at least three cancer types: ovarian, liver and colorectal cancer. Tissue specimens from ovarian cancer patients were used to examine the expression of several MMPs at the protein and RNA level. Strong MMP-8 expression was observed in most ovarian cancer subtypes and significantly correlated with tumour grade and stage – implicating it as a prognostic factor for ovarian cancer progression [297]. Interestingly, MMP-8 expression often coincided with MMP-9 staining – perhaps indicative of the contribution needed from collagenases able to degrade type I and type IV collagen respectively. A connection with MMP-9 has been observed before in wounds from *Mmp8* null mice: whereby loss of MMP-8 led to a significant upregulation of MMP-9 [298].

In both colorectal and liver cancer, high serum MMP-8 levels are associated with decreased survival, however the primary tissue was not studied and systemic levels of MMPs are not necessarily predictive of effects on the tumour itself [299,300].

1.4.5.2. MMP-8 and the Immune System

The primary aim of the above studies was to determine the role of MMP-8 in tumourigenesis – where it is clear MMP-8 plays cancer type-specific roles. However, a running thread between many of the studies was concomitant impacts on the immune system. This is unsurprising considering that several immune cells including Mϕ and neutrophils are producers of MMP8, it cleaves several inflammatory cytokines and it is implicated in numerous inflammatory disorders.

During inflammation, neutrophil activation leads to degranulation and subsequent release of antimicrobial molecules such as myeloperoxidase and elastase as well as ROS and several types of granules. MMP-8 is released in so-called 'specific' granules from neutrophils or can be membrane-bound. It has been suggested that up to 90% of MMP-8 is membrane-bound and interestingly resistant to TIMP-inhibition [301]. The involvement of MMP-8 in several inflammatory disorders will be discussed and has been summarised in Table 1.3 for clarity.

In several models of inflammation and disease, absence of neutrophil-derived MMP-8 impairs the early inflammatory response through a delayed influx in neutrophils – suggestive of MMP-8 participating in a feed forward mechanism for neutrophil recruitment [260,296]. This is certainly true in wound healing. Matrix remodelling and recruitment of inflammatory cells are key processes for successful wound healing. Neutrophils are the first immune cells at the site of injury and survive for between 24-36 hours before undergoing apoptosis [302]. The death of neutrophils is essential for healing to progress and is followed by recruitment and differentiation of monocytes into Mϕ [303].

Table 1.3. **Relevance of MMP-8 in inflammatory disorders**

Disorder	Phenotype	Reference
Inflammatory Arthritis	Expression is **protective** – increased neutrophils in synovial tissue in null mice	[304,305]
Periodontal Disease	Activity mediates **tissue destruction**. Beneficial during *P.gingivalis* infection - aids in **resolution of inflammation**	[[43,307]285]
Allergen-induced asthma	**Protective** against airway inflammation – drives **neutrophil apoptosis**	[308]
TNF-induced hepatitis	**Promotes liver failure** through apoptosis of hepatocytes and neutrophils	[309]
Bleomycin-induced lung fibrosis	**Promotes lung fibrosis** by modulating MMP-9 and IL-10 activity	[43,307]
Sepsis	**Protective** against LPS-induced endotoxemia	[310]

MMP-8 is the predominant collagenase in healing wounds but high expression is also observed in chronic ulcers [311]. In *Mmp8* null mice, neutrophil influx is impaired in wound healing, and thus wound closure is delayed. This phenotype was rescued with bone marrow transplantation from wild-type mice indicating that immune-derived MMP-8 is essential for this process. Despite a delay in neutrophil entry, whilst neutrophils were cleared from wounds in wild-type mice, they remained in wounds from *Mmp8* null mice [298]. Therefore, MMP-8 has a two-fold impact on neutrophils: driving their recruitment and resolving inflammation through their clearance.

One hypothesis for the sustained inflammation seen in the absence of MMP-8 is a delay in neutrophil apoptosis. This was found to be the case in the wound healing model but is a phenomenon seen in other inflammatory disorders. In allergen-induced asthma, *Mmp8* null mice have increased airway inflammation attributed to both an initial increase in neutrophil numbers in the lungs, but also a significant decrease in neutrophil apoptosis [308]. A similar observation is made in inflammatory arthritis. *Mmp8* deficiency exacerbates arthritis and neutrophils

accumulate in the synovial tissue [304,305]. In a cancer-context, neutrophils linger at later stages of BC in the spontaneous PyMT model [296]. Similarly, in bleomycin-induced lung fibrosis, neutrophil activity persists and does not disappear [312].

It has been hypothesised that neutrophils do not dissipate in *Mmp8* null mice due to incomplete collagen breakdown, retaining some chemotactic signals. In periodontal disease, MMP-8 mediates tissue destruction [313]. Gum tissue is primarily composed of type I collagen. In mouse models of periodontitis, reduction in MMP activity reduces disease progression, and in *Mmp8* null mice again there is impaired neutrophil infiltration perhaps related to the accidental trapping of neutrophil chemoattractants [43,307]. However, during *Porphyromonas gingivalis* infection, the complete absence of MMP-8 led to enhanced bone degradation suggesting that MMP-8 is essential for the resolution of inflammation [306]. Inhibition of MMP-8 using doxycycline is currently carried out for treatment of periodontal inflammation [314]. Of note it is the only FDA-approved treatment to target MMPs in human disease.

The predominant chemotactic signal mediated by MMP-8 is lipopolysaccharide-induced CXC chemokine (LIX) now referred to as CXCL5. CXCL5 is one of the three mouse homologues of human IL-8; CXCL1 and CXCL2 being the others. CXCL5 is the most potent neutrophil chemoattractant both *in vivo* and *in* vitro [315]. It regulates neutrophil trafficking in a CXCR2-dependent manner but also binds nonsignaling receptors such as Duffy Antigen Receptor for Chemokines (DARC): a sink for at least 16 CXC and CC chemokines. CXCL1 and CXCL2 are both associated with DARC and are also important neutrophil chemoattractants [316]. In the ECM, the establishment of haptotactic gradients occurs when negatively charged glycosaminoglycan chains on proteoglycans interact with chemokines and their immobilisation leads to modulation of leukocyte migration [317]. This immobilisation is often mediated by proteolytic cleavage.

In vitro, it has been shown that MMP-8 cleaves CXCL5 at two sites: N-terminally at Ser^{4} ~ Val^{4} and C-terminally at Lys^{79} ~ Arg^{80} leading to truncated forms of CXCL5 that are more chemotactic than the precursor [260,318]. Proving that cleavage of MMP substrates occurs *in vivo* is a big challenge for the field due to the physiological

redundancy that occurs in knockout mouse models. However, upon air pouch injection of full-length CXCL5 in *Mmp8* null mice, PMN infiltration was severely reduced compared to the wild-type control [260]. MMP-1, -2, -9 and -13 all efficiently cleave CXCL5 but in this scenario, were not able to fully compensate for the loss of MMP-8 – indicative of the essential role of MMP-8 in leukocyte trafficking. In further support of this, in TNF-induced lethal hepatitis, CXCL5 was not released from the ECM in *Mmp8* null mice, and concomitantly they displayed an impaired neutrophil influx [309]. Nevertheless, more recently it has been shown MMP-8 is not predominantly responsible for the cleavage of CXCL5, instead operating as part of a protease web. MMP-8 cleaves and inactivates α1-protease inhibitor (α1-PI), a known inhibitor of neutrophil elastase [319]. It was consequently demonstrated that neutrophil elastase activity increases and CXCL5 cleavage is ultimately neutrophil elastase-dependent.

The MMP-8/IL-8 relationship has also been probed in humans. Thirkettle *et al* have demonstrated that overexpression of catalytically-active *MMP*8 in BC cells induces the expression of *IL-8* and *IL-6* [320]. Additionally, IL-6 enhanced endogenous *MMP8* expression and IL-8 enhanced *IL-6* expression in what is evidently an immunomodulatory network. Whether MMP-8 directly cleaves IL-8 and its homologues or mediates downstream cleavage is still unclear and it is likely both could be true depending on substrate availability and presence or absence of enzymes with overlapping functions.

The final aspect of MMP-8 in innate immunity is the link with TGF-β – which is mostly in the context of cancer. TGF-β is a multifunctional cytokine that is at first tumour suppressive in the epithelium before it can instigate tumourigenesis via several mechanisms. This switch in the role of TGF-β is known as the 'TGF-β paradox' [321]. Firstly, it has been shown to induce EMT [322]. EMT is a developmental process recapitulated in tumour progression whereby epithelial cells lose their polarity and cell-cell adhesion, gaining migratory and invasive properties to invade distant sites [323]. Further actions of TGF-β include modification of the cellular milieu to encourage tumour progression via deposition of ECM substances and immunosuppression [178,324].

TGF-β is a potent regulator of inflammatory cells in the TME. T cell inflammatory and cytotoxic functions are suppressed, Tregs are potently activated by TGF-β to repress downstream effector cell function and in both neutrophils and Mϕ, polarisation towards the N2, M2 pro-tumourigenic phenotype is induced [177,325]. Thus, the bioavailability of TGF-β can have a dramatic effect on inflammatory cells, cancer cells and stromal cells in the TME.

TGF-β is often complexed with ECM molecules – which can be released through proteolytic cleavage. One example is fibromodulin: a proteoglycan in the ECM that binds TGF-β. Wen *et al.* took monocytes from *Mmp8* null mice and found they struggled to polarise to the M2 phenotype endogenously or upon IL-4 stimulation. They demonstrated that MMP-8 cleaved fibromodulin, increasing the bioavailability of TGF-β [326]. In this context, MMP-8 could modulate the immune system to facilitate tumour progression.

Nevertheless, contrasting data shows MMP-8 cleaves the matrix proteoglycan decorin facilitating sequestration of TGF-β. Decreased availability of extracellular TGF-β led to reduced expression of miR-21 in turn leading to a release on the suppression of programmed cell death-4 (PDCD4) resulting in an increase in tumour cell apoptosis (Figure 1.10) [327]. It is likely that both scenarios could be correct, with the action of MMP-8 in the tumour microenvironment likely to be context-dependent, based on the local tissue type and array of cytokines and other enzymes in the vicinity.

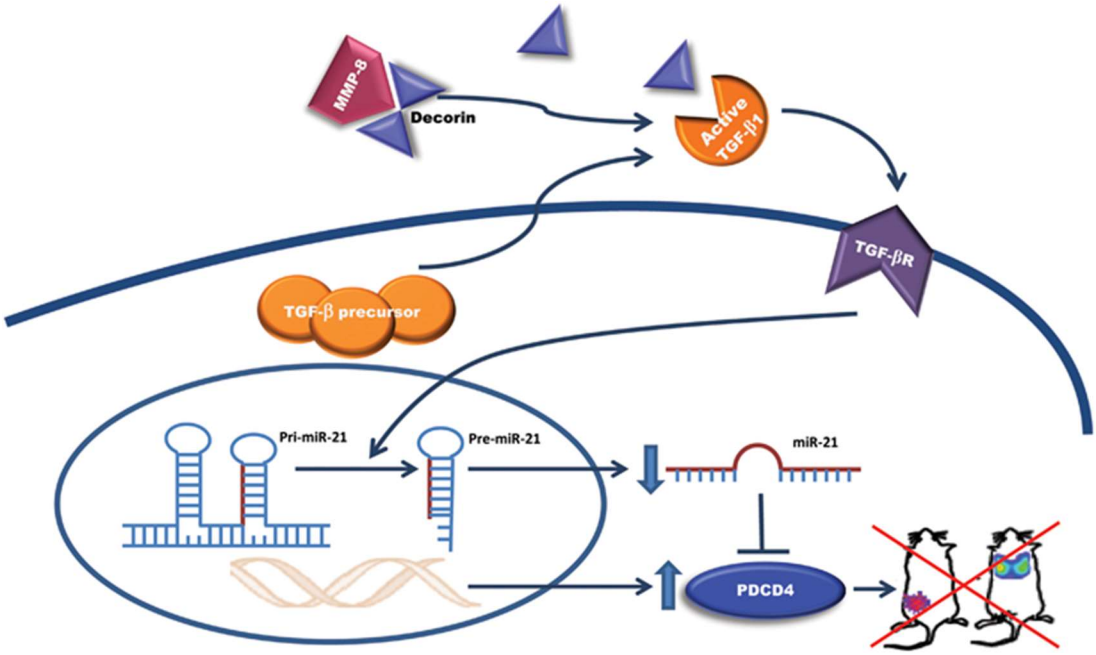


Figure 1.10. Proposed MMP-8 signalling pathway. MMP-8 cleaves decorin which is then able to sequester TGF-β. TGF-β is then unable to bind to its receptor which during tumourigenesis can increase the expression of miR-21. This micro RNA can suppress PDCD4 preventing tumour cell apoptosis [327].

1.5. Passenger Mutations

Before the advent of modern genetic engineering tools such as CRISPR-Cas9 and genetic manipulation of embryonic stem cells (ESCs), selective breeding approaches were used in mice to study the function of a gene *in vivo*. *Mus musculus* is an ideal model organism for genetic studies since 99% of genes are shared with *Homo sapiens,* their reproductive cycle is short and they contract many of the same diseases as humans [328].

Recently, more innovative techniques have been utilised to generate genetically-engineered knockout mouse models. Using ESCs, homologous recombination at the target locus is carried out to render the gene non-functional – often inserting a drug resistance cassette in its place before injecting the cells into blastocysts, which are then implanted into pseudo-pregnant female mice [329]. However, it was not always effective and ESC's from different mouse strains varied in their ability to undergo successful homologous recombination. Cells from the SV129 strain were particularly adept at colonizing and competing with cells from the inner cell mass of blastocysts [328]. Because of this, and the difficulty in obtaining manipulated ESCs from more favourable strains, thousands of gene knockout mice were produced on the 129 background. However, the 129 strain had breeding difficulties and researchers desired mice on the C57BL/6 (C57) or FVB backgrounds [330]. These strains are well characterised and have become the gold standard for genetic mouse models. To use C57 mice as example: in order to obtain a pure background, with the vast majority of the genome consisting of C57 DNA, a backcross approach is taken for at least 10 generations to be deemed a congenic mouse (Figure 1.11) [331]. The 129-transgenic donor mouse is bred to a C57 mouse, leading to the first generation of mice containing around 50% donor SV129 DNA and 50% C57 DNA. Further rounds of breeding with C57 mice are carried out to obtain a pure C57 mouse. If carried out effectively only 0.2% contamination from the 129 strain can be expected [332]. This 0.2% contamination exists in the region flanking the target gene due to genetic linkage and is referred to as passenger DNA. Difficulties arise when this passenger DNA harbours mutations: aptly known as passenger mutations. Backcrossing for 10 generations unfortunately does not always guarantee the loss of these passenger

mutations and in fact their presence is highly likely. The closer the passenger mutation exists in relation to the target gene, the greater the likelihood of allelic differences between the knockout mouse and its non-transgenic littermates. Furthermore, if the passenger mutation results in a functional consequence for that gene such as its inactivation or expression of an aberrant protein, this confounds interpretation of any results since it becomes difficult to separate which gene is responsible for a phenotype.

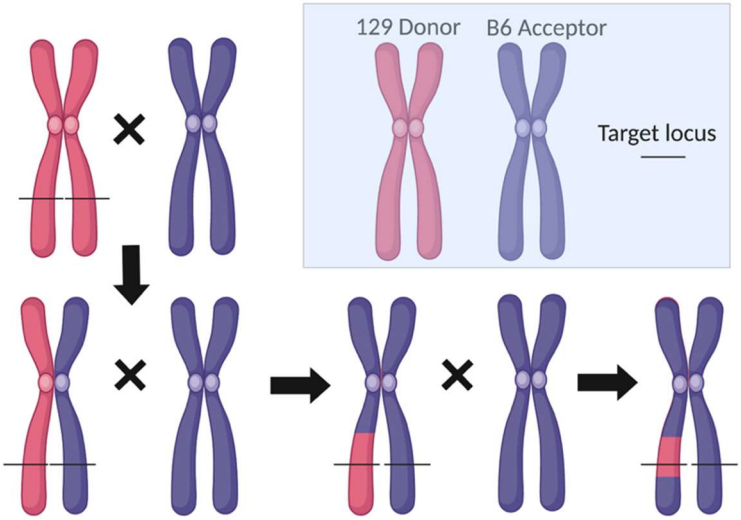


Figure 1.11. Persistence of passenger DNA despite backcrossing. DNA from the 129 donor persists around the target locus after each cross.

Whilst this concept of genetic variation in mouse strains and passenger mutations appears recent, it has been alluded to for some time. The existence of polymorphisms in the 129 genome were highlighted in a Nature paper in 1997 [333]. Ten years later, Lusis and Wang detailed complications with passenger mutations in the field of atherosclerosis but it is a problem that has been largely overlooked and ignored across many different fields in the last decade [334]. In 2015, Vanden Berghe *et al.* released a ground-breaking paper with the bold claim that interpretation of all genetically modified congenic mice may be impacted by passenger mutations [335]. By comparing the genomic sequence from the 129 strain to the C57BL/6J reference genome, an unprecedented number of insertion or deletion of bases (INDELS) and

single nucleotide polymorphisms (SNPs) were detected in 1,084 genes in total. Importantly, this paper also investigated the functional consequences of these genetic alterations with 13% resulting in a gained or lost STOP codon. The group also investigated the extent of passenger mutations that could be present in 129 ES cell-derived knockout mice. By looking at a small region of either 1, 5 or 10cM flanking the target gene, it was revealed that the percentage of mice carrying at least one passenger mutation was 70.7%, 96.7% and 99.5% respectively. This figure is exceptionally high and has implications for nearly all 129-derived knockout mouse models. Furthermore, a passenger mutation resulting in a new STOP codon and a potentially non-functional protein, affects 76% of all 129-derived congenic mice.

One example of the consequences of these mutations is the *Caspase11* passenger mutation. Seminal work in *Cell* and *Science* revealed IL-1β-converting enzyme (caspase-1) deficiency conferred resistance to endotoxic shock using C*asp1* knockout mice [336,337]. However, almost twenty years later Kayagaki *et al*. uncovered a phenotype common to both *Casp1* knockout mice and 129 mice (the origin strain of this model): a defect in IL-1β production and lack of caspase-11 expression. Upon investigation, a mutation in *Casp11* was detected in the 129 strain that was also present in the *Casp1* knockout mouse since the genes were too close on the chromosome to be segregated during recombination [338]. Subsequently, *Casp1* null mice were generated using CRISPR-Cas9 technology and already some functions attributed to *Casp1* have been shown to be *Casp11*-mediated [339].

Two concepts have unfortunately coalesced in *Mmp8* null mice. Firstly, smaller distance between genes increases the probability of linkage between those genes thus preventing recombination. Secondly, several members of the MMP family arose due to gene duplication events and thus many MMPs cluster tightly together on mouse chromosome 9. *Mmp8*, like the nine other MMPs that cluster nearby, is within 5cM of the *Casp11* gene. Almost all mouse studies were carried out in the *Mmp8* null model created by Carlos Lopez Otin, which in the hands of Vanden Berghe *et al.* almost 10 years later had the *Casp11* passenger mutation as described by Kayagaki [338]. However, that is not say that upon extensive breeding in other labs, the passenger mutation may have been eradicated. At this point, it is difficult to say

which phenotypes were solely due to the loss of MMP-8 and which were confounded by a caspase-11 deficiency. Therefore, much of the work on MMP-8 described in this introduction, particularly in immunity is to be interpreted with this caveat in mind.

1.6. Research Aims and Objectives

Most evidence indicates a tumour-suppressive function for MMP-8 in BC. This project intended to use the orthotopic model of BC, alternative to the spontaneous model used previously, to further expand the literature on MMP-8 in tumourigenesis. The main objective was to determine whether MMP-8 inhibited primary BC tumour growth through co-ordinating anti-tumour immunity by dissecting tumour growth metrics, immune populations and gene expression changes. The working hypothesis for this project was that MMP-8 orchestrated the innate immune system to suppress primary breast cancer progression.

To verify this hypothesis, the aims were as follows:

1. Determine whether absence of *Mmp8* contribute to primary tumour growth in an orthotopic model of BC
2. Characterise the intra-tumoural immune compartment in *Mmp8* null mice
3. Investigate intra-tumoural expression of lipid metabolism and cytokine genes in the absence of *Mmp8*
4. Delineate the role of specific cell types within orthotopic tumours using 2D cell culture models
5. Examine the impact of MMP-8 on immune organ populations to gain a wider understanding of the immune landscape in *Mmp8* null mice

2. Materials and Methods

2.1. Mouse Breeding

Mmp8 null mice were generated in the laboratory of Dr Carlos Lopez-Otin according to the protocol described in Balbín *et al.* [292]. Briefly, a targeting vector was engineered so that a PGK-neomycin resistance cassette replaced most of exon 2, the entirety of exon 3 and 4 and intron 2 and 3 of the *Mmp8* gene. Embryonic stem cells (129/SvJ-derived) were electroporated with the vector and successfully-transfected cells were injected into blastocysts of C57BL/6J mice before being transferred into pseudopregnant females. Chimeric offspring were mated and maintained on a C57BL/6J background. In our hands, animals were bred on a C57BL/6J background. All experiments were performed in accordance with UK Home Office regulations and the European Legal Framework for the Protection of Animals used for Scientific Purposes (European Directive 86/609/EEC) under Project Licence SR 70/8722 and Personal Licence #ICD0AEBB3.

2.2. Genotyping and PCR

Ear biopsies from mice were digested overnight (o/n) at 56°C in tissue lysis buffer (100 µl) (Tris-HCl (50 mM pH 8.5), EDTA (10 mM pH 8.0), NaCl (100 mM) and 0.2% SDS) supplemented with proteinase K (100 µg/mL). DNA was precipitated with isopropanol (100 µl), vortexing and centrifuged at 1400 x g for 30 mins. Isopropanol was removed, and the DNA pellet dried at 37°C for 2 hrs before being resuspended in TE buffer (200 µl) (Tris-HCl (10 mM pH 7.5), EDTA (1 mM)). PCR reactions were subsequently performed, as described below, in 96-well PCR plates (Corning, New York, USA) in the appropriate 96-well block thermal cycler PCR machine (Bioer Technology, Binjiang, China).

PCR analysis of the MMP8 allele was performed by combining: DNA (0.8 µL), MegaMix- Blue (Clent Life Sciences, Stourbridge, UK) (10 µL) and WT, Anchor and Neo primers (at a final concentration of 0.8 µM, sequences listed below) under the following conditions: initialisation step at 94°C for 1 min; followed by 40 amplification cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension

at 72°C for 1 min; terminating with a final elongation step at 72°C for 10 min. The resulting PCR product was visualised using a 1.8% agarose gel, PCR products produced are 500-base pair (bp) (MMP8 WT) and 300-bp (MMP8 KO) in size.

The oligonucleotide primers used were as follows:

MMP8-Neo: 5' – GCCAGAGGCCACTTGTGTAG – 3'

MMP8-WT: 5' – TCGTCTCAAGAGGTAGGCTCA – 3'

MMP8-Anchor: 5' – AGCCCTTAAACCGCTAAGGA – 3'

2.3. DNA Sequencing

PCR analysis of the caspase-11 allele was performed by combining: 1.4 µL genomic mouse DNA (processed as described in genotyping section), Accuprime Pfx SuperMix (Invitrogen, Paisley, UK) and forward and reverse caspase-11 primers with the following sequences:

Caspase-11 Forward: 5' - GGTTACTGTACAAAACGAGGCA – 3'
Caspase-11 Reverse 5'-AGCAAGCATGTTTCCGGAAG-3'

The PCR reaction proceeded under the following conditions:

95°C for 5 minutes, 3 cycles of: (95°C for 15 seconds, 60°C for 30 seconds, 68°C for 30 seconds) and maintained at 4°C.

PCR product clean-up was carried out using the PureLink™ Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The resulting PCR product was run on a 3% agarose gel to confirm amplification before sequencing. The capsase-11 PCR products were sequenced using the Mix2Seq Kit (Eurofins Genomics, Ebersberg, Germany) and subsequent analysis was carried out using DNADynamo (Blue Tractor Software Ltd, North Wales, UK).

2.4. Cell Culture

B6BO1 cells were kindly obtained from Professor Katherine Weilbaecher at Washington University, St Louis. EO771 cells were obtained from Kairbaan Hodivala-Dilke at Barts Cancer Institute, London. CMT-19T cells were purchased from CR-UK. All cell lines were mycoplasma-free and cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Paisley, UK) and 100 units/ mL penicillin/streptomycin (P/S) (Invitrogen, Paisley, UK) at 37°C and at 5% CO_2. Cell lines were grown in flasks coated in 0.1% gelatin from porcine skin (Sigma Aldrich, St Louis, USA).

2.5. Mouse Tumour Models

B6BO1 or E0771 cells grown for at least two days, harvested at >80% confluency and injected at a density of 1 x 10^5 in 50 µl of 1:1 PBS and Matrigel (Corning, New York, USA: #354248) into the mammary fat pad of 8-10 week old female mice. Tumour size (mm^3) was calculated by measuring the longest (L) and shortest (S) distance of tumour tissue and the formula 0.51 x L x S^2. Once palpable, tumours were measured at least every 5 days. On the final day, tumour size was calculated *ex vivo* after the mice were sacrificed using the same formula.

2.6. Macrophage Isolation, Stimulation and Polarisation

Femurs and tibias were harvested from WT and $MMP8^{-/-}$ mice aged between 6-8 weeks old. Both epiphyses were removed, and the bones placed into a tube containing a hole made with a 19G needle prior to centrifugation at 3500 rpm for 4 minutes. Bone marrow collected was centrifuged at 300 x g for 5 minutes, and cells underwent red blood cell lysis incubation for 5 minutes (NH_4Cl, $NaCO_3$, 1 mM EDTA, pH 7.3). Bone marrow was seeded into 6-well plates at a density of 2 million per well. Macrophage complete medium (50:50 Ham's F12 Nutrient Mixture (Invitrogen, Paisley, UK) / DMEM with / 10% FBS and 1X P/S) was added on Day 1 with 10 ng/ml macrophage colony-stimulating factor (M-CSF) (Peprotech, Princeton, USA), topped up on Day 3 and replaced on Day 6. For polarisation experiments, on Day 7,

macrophage culture medium was replaced with fresh medium containing 10 ng/ml M-CSF and either 100ng/ml recombinant LPS (Sigma Aldrich, St Louis, USA) or 10 ng/ml recombinant IL-4 (Peprotech, Princeton, USA) as experimentally validated in [340] . RNA and protein were collected 48hrs post-stimulation.

2.7. Co-Culture Experiments

For direct co-culture experiments, on Day 7 of the isolation of bone marrow-derived Mϕs, 200,000 B6BO1 cells were added directly on top. Culture media and RNA was collected 48 hours post-addition. For transwell experiments, 200,000 B6BO1s were cultured overnight in a 0.4 μM transwell to adhere before being placed on top of Day 7 bone marrow- derived Mϕs. Culture media and RNA were collected from each compartment.

2.8. RNA extraction, reverse transcription-PCR and Quantitative Real-Time PCR

For tissue RNA isolation, 1mL of TRIzol (Thermo Fisher Scientific, Massachusetts, USA) was added to 50-100mg of tissue and homogenised with RNase-free glass beads (Thistle Scientific, Glasgow, UK) in a tissue-lyser (Qiagen, Sussex, UK) for 2 minutes at 50Hz. For cells grown in culture, 1mL of TRIzol was used for every 1-2 x 10^6 cells.

One fifth of the volume of TRIzol, of chloroform was added, the sample was mixed by inversion and total cell RNA was extracted using the SV Total RNA Isolation Kit (Promega, Wisconsin, USA) according to the manufacturer's instructions. RNA samples were quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and their A260/280nm and A260/230nm absorbance ratios assessed for purity. Samples were reverse-transcribed using MMLV-Superscript (Promega, Wisconsin, USA) resulting in a final concentration of 0.5 ng/μL. Quantitative real-time polymerase chain reaction (qRT-PCR) TaqMan was carried out using 5ng cDNA for genes of interest and 1ng cDNA for 18S rRNA. The cycle conditions in the 7500 Fast Real Time PCR System (Applied Biosciences) were: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. All primer/probe sets were obtained from Applied Biosciences

(USA) (Table 2.1).

Table 2.1. Primer/probe set details of all genes profiled for in qRT-PCR from Applied Biosciences

Gene Name	Catalogue Number
18S	Mm03928990_g1
IL-6	Mm00446190_m1
CXCL1	Mm04207460_m1
CXCL2	Mm00436450_m1
CXCL5	Mm00436451_g1
Leptin	Mm00434759_m1
Acsl1	Mm00484217_m1
Perilipin-1	Mm00558672_m1
Lipase-E	Mm00495359_m1
Mmp8	N/A custom probe

2.9. Western Blotting

Cells were lysed in RIPA buffer (500mM NaCl, 50 mM Tris pH 7.4, 0.1% SDS and 1% Triton-X100) supplemented with protease inhibitor (Merck Millipore, USA). Protein content in cell lysates was determined using the DC BioRad protein assay (BioRad, Hemel Hempstead, UK) and 20 µg was resolved using 8% SDS-PAGE gels and transferred using the wet method onto nitrocellulose membrane for 3 hours. Protein transfer was confirmed using Ponceau S staining before 1 hour blocking in buffer (phosphate buffered saline [PBS], 5% skimmed milk and 0.1% Tween-20). Membranes were incubated with primary antibodies (as in Table 2.2) for 16 hours overnight at 4°C in 5% Bovine Serum Albumin (w/v) in PBS with 0.01% (w/v) Tween (PBST). Membranes were washed three times for 5 minutes with washing buffer (PBST) before HRP- conjugated secondary antibodies (Dako, Agilent Technologies, USA) (Table 2.3) were applied and the blots were incubated for 2 hours. After a

further three PBST washes, enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, USA) was applied according to manufacturer's instructions. Protein bands were imaged using a Fuji Film (LAS-3000) system.

Table 2.2 List of primary antibodies utilised for Western blotting

Target	Species and Clonality	Cat No.	Manufacturer	Dilution
iNOS	**Mouse Monoclonal**	**MAB9502**	**R&D**	**1:1000**
Arginase-1	**Rabbit Monoclonal**	**93668T**	**CST**	**1:1000**
MMP-8	**Goat Polyclonal**	**AF3245-SP**	**R&D**	**1:1000**
Heat Shock Protein 70	**Mouse Monoclonal**	**sc7298**	**Santa Cruz**	**1:2500**
Estrogen Receptor	**Rabbit Monoclonal**	**ab32063**	**Abcam**	**1:1000**
Progesterone Receptor	**Rabbit Polyclonal**	**ab63605**	**Abcam**	**1:1000**

Table 2.3 List of secondary antibodies utilised for Western blotting

Secondary Antibody	Cat No.	Manufacturer	Dilution
Anti-Rabbit HRP	**P0448**	**Dako**	**1:2000**
Anti-Mouse HRP	**P0447**	**Dako**	**1:2000**

2.10. Immunocytochemistry

Cells were incubated at a density of 15,000 on 0.1% porcine gelatin-coated, acid-washed glass coverslips overnight, before ice cold methanol fixation for 10 minutes. Cells were permeabilised in 0.1% Triton X-100 for 10 minutes prior to blocking in 5% goat serum for 30 minutes. Primary antibodies were diluted in blocking buffer to the concentrations found in Table 2.4 and applied overnight at 4°C. Cells were then washed with PBST and incubated in secondary antibody for 2 hours (Table 2.5). After further PBST washes, cells were mounted in fluoromount G + DAPI (Invitrogen,

Paisley, UK) and imaged using the Axioplan epifluorescent microscope and AxioCam MRm camera (Zeiss, Cambridge). Images were processed using ImageJ™ software.

Table 2.4 List of antibodies used for immunocytochemistry

Target	Species and Clonality	Cat No.	Manufacturer	Dilution
Estrogen Receptor	Rabbit Monoclonal	ab32063	Abcam	1:200
Progesterone Receptor	Rabbit Polyclonal	ab63605	Abcam	1:200

Table 2.5 List of secondary conjugated-antibodies for immunocytochemistry

Secondary Antibody	Cat No.	Manufacturer	Dilution
Anti-Rabbit Alexa 594	A11012	Invitrogen	1:250
Anti-Rat Alexa 594	A21209	Invitrogen	1:250

2.11. Flow Cytometry

2.11.1. Sample Collection

2.11.1.1. Bone Marrow

Bone marrow was collected according to the protocol in 2.6 to obtain a single cell suspension.

2.11.1.2. Spleen

Spleens were harvested from WT and MMP8$^{-/-}$ mice aged between 6-8 weeks old. The spleen was homogenised through a 70 µM cell strainer with 2 mL PBS to create a single cell suspension.

2.11.1.3. Tumours

Tumours harvested from mice were homogenised and digested in collagenase solution (0.2% Type IV collagenase, 0.01% sheep hyaluronidase and 1X DNase I) at 37°C for 1 hour with agitation every 10 minutes. The collagenase solution was then passed through a 70 µM cell strainer to obtain a single cell suspension.

2.11.2. Cultured Cells

For flow cytometry from cultured cells: cells were removed from the culture dish using 0.05% trypsin-EDTA (Thermo Fisher Scientific, Massachusetts, USA), and subjected to the same procedure post-single cell suspension as above.

2.11.3. Staining and Sample Acquisition

After centrifugation for 5 minutes at 300 x g and a PBS wash, cells isolated from tissues were incubated at room temperature with red blood cell lysis solution for 5 minutes (NH_4Cl, $NaCO_3$, 1 mM EDTA, pH 7.3). Cells were counted and the concentration was adjusted to 1 million cells in 100µl in FACS buffer (1% BSA in PBS). Cells were incubated with FcR block (Miltenyi Biotec, Cologne, Germany) for 10 minutes before the addition of conjugated primary antibodies in FACS buffer (Table 2.6) .After incubation, two further FACS washes were carried out before fixation in 4% paraformaldehyde (PFA) for 20 minutes. PFA was removed and cells were left overnight in FACS buffer. The following day, cells were run on the BD LSR Fortessa II Flow Cytometer (BD Biosciences, New Jersey, USA) with standard filter sets and five lasers. In total, 100,000 (immune organs) or 250,000 (tumours) events were collected per sample. Quantification and analysis were carried out using FlowJo software.

Table 2.6 List of conjugated-antibodies used for flow cytometry

Marker	Fluorochrome	Dilution	Clone	Manufacturer
Live Dead	Alexa 488	1:200	N/A	ThermoFisher
CD45	Pac Blue	1:400	30-F11	Biolegend
CD11b	BV605	1:400	M1/70	BD
Ly6C	PE	1:300	HK1.4	Biolegend
Ly6G	APC-Cy7	1:200	1A8	BD
F480	PE-Cy5	1:200	BM8	eBioscience
CD3	APC	1:200	145-2C11	eBioscience
CD4	PE	1:200	GK1.5	eBioscience
CD8	BUV395	1:400	53-6.7	BD
B220	eVolve605	1:200	RA3-6B2	eBioscience
NK1.1	eFluor450	1:200	PK136	eBioscience

2.12.Enzyme-linked immunosorbent assay (ELISA)

Media was collected from cells grown in culture for at least 48 hours and centrifuged at 4°C at 1500 rpm for 10 minutes. The concentration of IL-6 was quantified using the Mouse IL-6 DuoSet ELISA Kit (R&D, UK, DY406-25) according to manufacturer's instructions.

2.13.Statistical Analysis

Where appropriate, statistical analysis was performed using a two-tailed, unpaired *t*-test. For data containing multiple comparisons – a multiple *t*-test was used. Where a correction was applied, it is stated in the figure legend. For data with greater than two groups, a two-way ANOVA was used. Data bars represent the mean value with error bars indicating standard error of the mean (SEM). All analysis was carried out using Prism and R software.

3. Characterisation of a breast cancer mouse model in *Mmp8* null mice

Consistently, MMP-8 has been shown to play a tumour-suppressive role in numerous types of cancer including BC [287,293,296]. The prognostic value of MMP-8 has been demonstrated in BC specimens where it was found that increased expression of *MMP8* correlates with lengthier relapse-free survival [287]. This led to further research aiming to elucidate the mechanism by which MMP-8 expression improves clinical outcome. Previous work in our lab sought to address this using a mouse model lacking the enzyme. To study BC progression, the *Mmp8* null mice were bred onto MMTV-PyMT mice: an established model for studying spontaneous BC. In this study, mice lacking *Mmp8* developed palpable tumours at an earlier time-point than their wild-type counterparts – indicating that MMP-8 is involved in inhibiting primary tumour initiation and/or growth. Upon analysis of the tumours, it was found that late in tumour progression, in the absence of *Mmp8* there was an abundance of intra-tumoural neutrophils that were not present in tumours from wild-type counterparts [296]. This delay in neutrophil clearance has also been seen in chronic wounds and inflammation in other models utilising *Mmp8* null mice [298,308].

Because of this observation, a logical next step was to delve further into changes in the immune compartment in BC tumours from *Mmp8* null mice. This project sought to do just that – however, the spontaneous model was not the most appropriate model to examine this. Whilst the spontaneous model excellently replicates the histological changes that occur in human BC, it does not represent humans in terms of number of tumour foci. Multiple foci develop in the spontaneous model whereas this occurs in only 5.2% of human BC cases [341]. Furthermore, these foci experience their own unique tumour progression – when mice are sacrificed at 6 weeks old, some foci will have arisen 6 weeks ago and grown slowly, whereas some may have grown after 4 weeks of age and still be in an early growth stage [342]. This creates a challenge for dissecting immune compartment changes reflective of the entire animal in tumours at different immunological time points. To address this limitation, an alternative mouse model was used to study the interaction between BC and the immune system: the orthotopic injection model.

In this chapter, the potential anti-tumourigenic role of MMP-8 was investigated in an orthotopic mouse model of BC. The phenotype of tumours from *Mmp8* null mice have been examined through tumour growth experiments, flow cytometric and RNA analysis. However, as a caveat to these findings, it was discovered that the genome of the entire *Mmp8* null mouse colony contained a 5bp deletion in the neighbouring *Casp11* gene – known to lead to non-functional *Casp11* mRNA. Therefore, these mice harbour an inactivating passenger mutation that confounds interpretation of the model. During this chapter, the *Mmp8* null mice are referred to as double knockout (DKO) mice to reflect this.

3.1. B6BO1 and EO771 mouse mammary cancer cell lines do not express *Mmp8 in vitro* but have different hormone receptor statuses

Orthotopic injection into immuno-competent mice requires the use of mouse-derived cancer cells. In this study, two mouse mammary cancer cell lines were used that were suitable for orthotopic injection into the mammary fat pad: B6BO1 and E0771 cells. To study the function of MMP-8 in BC tumourigenesis, an *Mmp8* null mouse was utilised. This allowed investigation into the host effects of MMP-8, however the use of the orthotopic model requires introduction of cells that may express the gene of interest: in this case *Mmp8*. To ascertain whether *Mmp8* was endogenously expressed, profiling of both cell lines was undertaken at the protein and RNA level. MMP-8 expression was not detected at the protein level by Western blot (Figure 3.1A) blot nor at the RNA level using qRT-PCR (data not shown).

In human BC, the phenotype of a tumour is determined to guide prognosis and treatment options. The expression levels of three hormone receptors are distinguished in the clinic using an immunohistochemical approach. Those receptors are ER, PR and HER2. Different combinations of these receptors expressed can influence patient outcome. However, as discussed in 1.1.4, there are also methods to 'genotype' tumours into more distinct categories. The B6BO1 and E0771 cell lines utilised in this study are well characterised in terms of their origin, but not their hormone receptor status. The B6BO1 cell line is derived from a spontaneous mammary tumour that arose in an MMTV-PyMT mouse on a C57BL/6 background (B6) and was subsequently propagated to create an invasive cell line with a high incidence of bone metastases (BO-1). This cell line represents a luminal B subtype of BC. The E0771 cell line was isolated from spontaneous tumours again in C57BL/6 animals, but instead represents a basal-like subtype of BC. Both cell lines were already known to be HER2-, but their ER and PR status was uncertain. The receptor status of both cell lines was characterised using cell lysates for Western blots and cells grown on coverslips for immunocytochemistry (Figure 3.1B&C). ER expression could be detected in both cell lines in cell lysates, and this was confirmed with

staining. PR was not detected in either cell line by Western blot, but could be seen visually in B6BO1 cells, and not in EO771 cells.

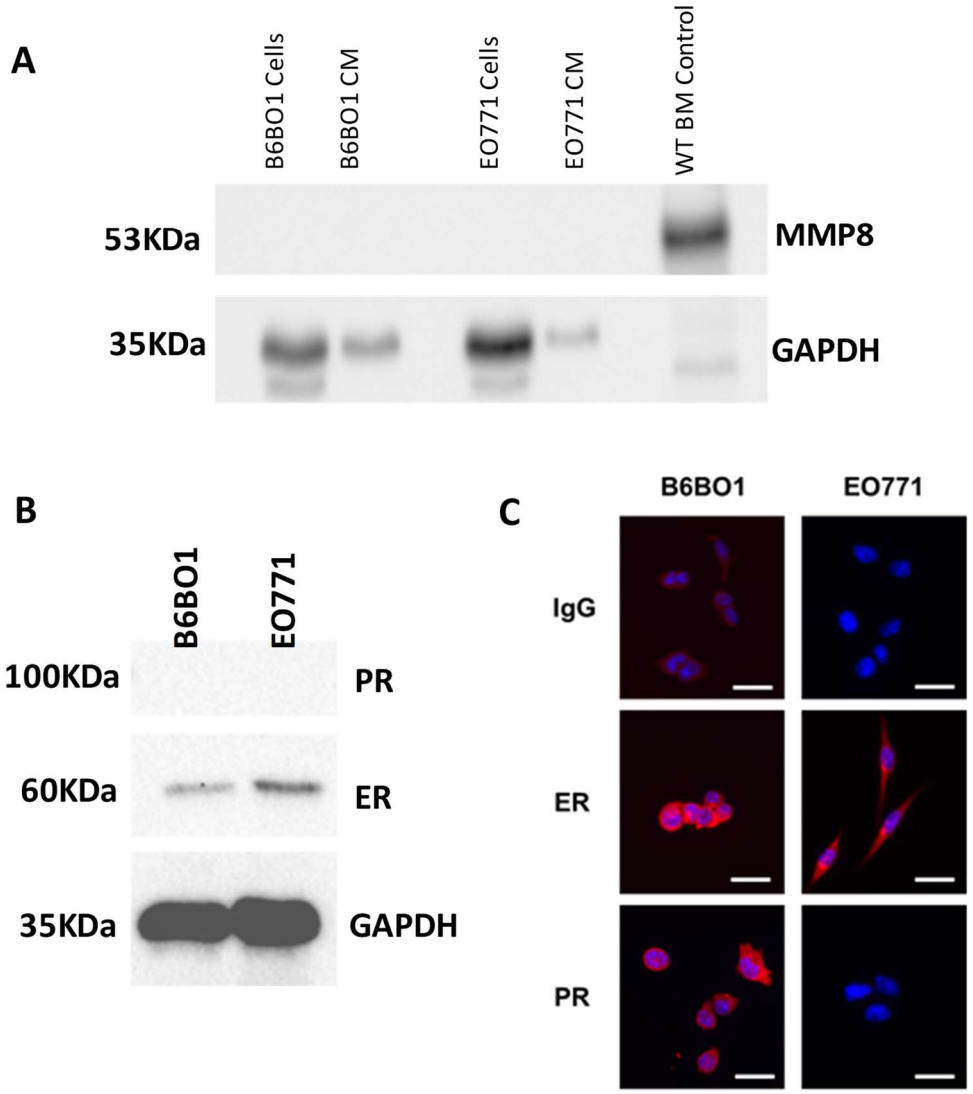


Figure 3.1. MMP-8, PR and ER expression in mouse mammary cancer cell lines. A) Lysates from both cell lines grown in vitro underwent SDS-PAGE electrophoresis and were Western blotted for MMP-8. Bone Marrow was used as a positive control and GAPDH was used as a loading control. B) Lysates from both cell lines grown in vitro were run on a Western blot and probed for progesterone receptor (PR) and estrogen receptor (ER). GAPDH was used as a loading control. C) Representative images of cell lines immuno-stained for ER and PR. IgG isotype control shown in top panel. Scale bar = 100 μM.

3.2. The absence of *Mmp8* does not impact tumour volume in an orthotopic model of BC

MMP-8 has been previously shown to inhibit tumour progression in a spontaneous model of BC. To investigate whether this phenotype was true in an alternative model of BC, the orthotopic injection model was utilised. Female mice 8-10 weeks of age on a C57BL/6 background were orthotopically injected with PyMT-derived B6BO1 cells into the inguinal mammary fat pad (Figure 3.2A). This was carried out in DKO mice and compared with wild-type controls. Tumours were excised at three distinct time points and measured *ex vivo* using callipers to give a metric for tumour growth (Figure 3.2B). At Day 13, upon excision, there were no differences in tumour volume between the DKO mice and control mice, with tumours on average 500 mm^3. This consistency was reflected at Day 15 with tumours between 800-1000 mm^3, but at Day 18 there appears to be a trend in decreased volume in tumours from DKO mice which are on average 1300 mm^3 in comparison to wild-type tumours reaching on average 1700 mm^3. However, this difference is not significant.

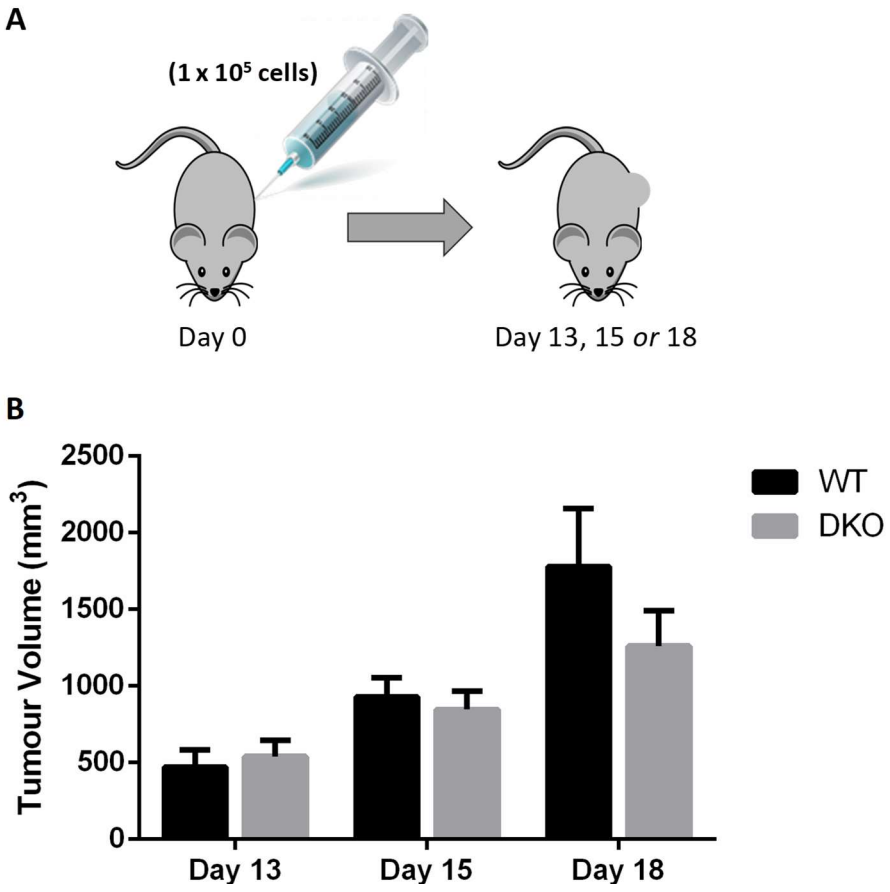


Figure 3.2. Orthotopically implanted B6BO1 volumes in DKO and wild-type mice. A) Schematic of experimental design: 1 x 10^5 PyMT-derived B6BO1 mammary carcinoma cells were injected into the mammary fat pad of female mice. After 13, 15 or 18 Days, tumours were excised and measured. B) Tumour volume measurements (mm^3) after excision on Days 13, 15 and 18. N ≥ 5, All data are presented as mean ±SEM.

3.3. Reduced numbers of intra-tumoural macrophages are found in DKO mice at Day 15

Leukocyte infiltration into tumours has a significant impact on prognosis, and the composition of the tumour microenvironment is known to shape tumour growth and progression. In order to look for changes in the populations of intra-tumoural immune cells in the absence of *Mmp8*, mammary tumours grown orthotopically were subjected to flow cytometric analysis. The tumour immune compartment was examined across three different time points: Day 13 (Figure 3.3A), Day 15 (Figure 3.3B) and Day 18 (Figure 3.3C) to pinpoint whether immune changes occur at specific stages in tumourigenesis.

Analysis focussed on a panel that could detect the most abundant myeloid populations in tumours: Mϕs and neutrophils using F4/80 and Ly6G as markers respectively. But to more precisely identify these specific cell populations, the antibody panel included markers of broader populations including the pan-leukocyte marker: CD45, to ascertain total numbers of immune cells and CD11b to delineate between myeloid cells and lymphocytes; CD11b a marker for cells of the myeloid lineage. For all samples, a gating strategy was devised to look at populations of immune cells and can be found in Supplementary Figure 7.1

Forward and side scatter was used as a parameter to exclude dead or grouped cells from the analysis. Mϕs were classified as $CD45^{+}CD11b^{+}Ly6G\&6C^{-}F4/80^{+}$ cells. Neutrophils were categorised as $CD45^{+}CD11b^{+}Ly6C^{-}Ly6G^{+}$ cells.

Overall, the B6BO1 tumours were composed of between 30-40% leukocytes, which was reflected in both genotypes and consistently across the three timepoints studied. At every time point, most leukocytes positively stained for CD11b; suggesting a large population of myeloid cells. At Days 13 and 18, around 95% of leukocytes were $CD11b^{+}$. However, at Day 15 this figure is much lower: approximately 80% of the cells were myeloid cells. Again, there were no statistically significant differences in the percentage of intra-tumoural myeloid cells between genotypes. The most abundant myeloid cell population in the B6BO1 tumours was TAMs: constituting on average 20% of all tumour cells. At Day 15, there was a

significant reduction in the number of F4/80$^+$ Mϕs in tumours from DKO mice. This was also reflected in a reduced number of myeloid cells in these mice, however this did not reach statistical significance.

One other population stained for was Ly6G$^+$ neutrophils. Very few to no neutrophils could be detected, independent of genotype.

In addition to appraising quantitative differences in immune cells, their qualitative characteristics were also investigated. Mϕs exhibit a large degree of plasticity and in tumours there exist populations that can either promote or inhibit tumour progression – representing M1 and M2 Mϕs respectively.

To investigate this, F4/80$^+$ Mϕs were further categorised according to their cell-surface expression of two polarisation markers: MHCII and CD206 – representative of M1 and M2 Mϕs respectively, via flow cytometry (Figure 3.3D). MHCII and CD206 expression exists as a spectrum and delineating between M1 and M2 Mϕs requires use of the scatter plot during flow cytometry analysis rather than a confirmatory staining approach.

At Day 13, the ratio of M1:M2 Mϕs was approaching 1:1. Approximately 40% of total Mϕs were M1 in phenotype and likewise for M2 – with around 20% of Mϕs not strongly expressing polarisation markers. At Day 15, the ratio was slightly more skewed in favour of M1 Mϕs (1.3:1), where they predominated over M2 Mϕs. This was similar between both genotypes. At Day 18, this effect was reversed, and M2 Mϕs were more abundant in a ratio of 1.3:1. However, this M2/M1 dominance was only statistically significant in tumours from wild-type mice. Overall, there were no differences in numbers of M1 or M2 Mϕs as a percentage of Mϕs at any time point between genotypes.

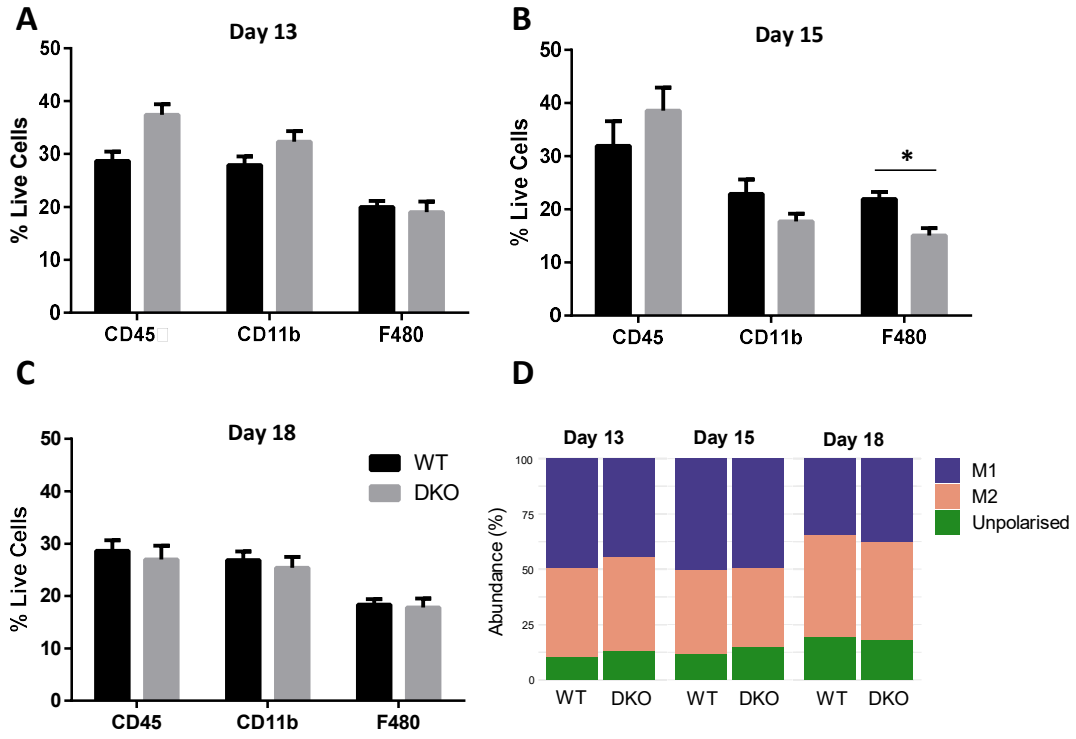


Figure 3.3. Flow cytometric analysis of intra-tumoural immune infiltrates from DKO and wild-type mice across three time points. A, B & C) Myeloid cell populations P.i. A) 13 Days, N =5,4. B) 15 Days, N = 9,8. C) 18 Days, N =5,5. Data are presented as a mean percentage of total live cells ± SEM. *Benjamini-Hochberg adjusted p value = 0.011. D) Tumour-associated macrophage polarisation across Day 13, 15 and 18. Data are presented as an abundance of total F4/80+ macrophages. N < 5. P.i = post-injection.

3.4. mRNA Expression of *IL-6* and *IL-8* homologs does not differ in in tumours from DKO mice and wild-type controls

MMP-8 is implicated in neutrophil chemotaxis. There are some data to suggest this is related to the proteolytic activity of MMP-8 on several chemokines including IL-6 and IL-8 [260,320]. If MMP-8 is responsible or involved in the cleavage and activation of these chemokines, in the absence of *Mmp8*, changes to their levels may be expected.

The same tumours that were subjected to flow cytometric analysis were analysed for their mRNA expression of *IL-6* and the three mouse homologs of human *IL-8*: *CXCL1, CXCL2* and *CXCL5* by qRT-PCR. Analogous to the flow cytometry, tumours were analysed at all three timepoints: Day 13 (Figure 3.4A&B), Day 15 (Figure 3.4C&D) and Day 18 (Figure 3.4E&F).

Across all time points, the expression of all four genes in the tumours remained consistent between genotypes and there were no statistically significant differences. One gene with a trend of increased expression in DKO mouse tumours was *CXCL5* at Days 13 and 15, that was expressed 3 times higher in tumours from DKO mice in comparison to tumours from wild-type mice. Contrastingly, at all-time points there was a trend for reduced *IL-6* expression in tumours from DKO mice.

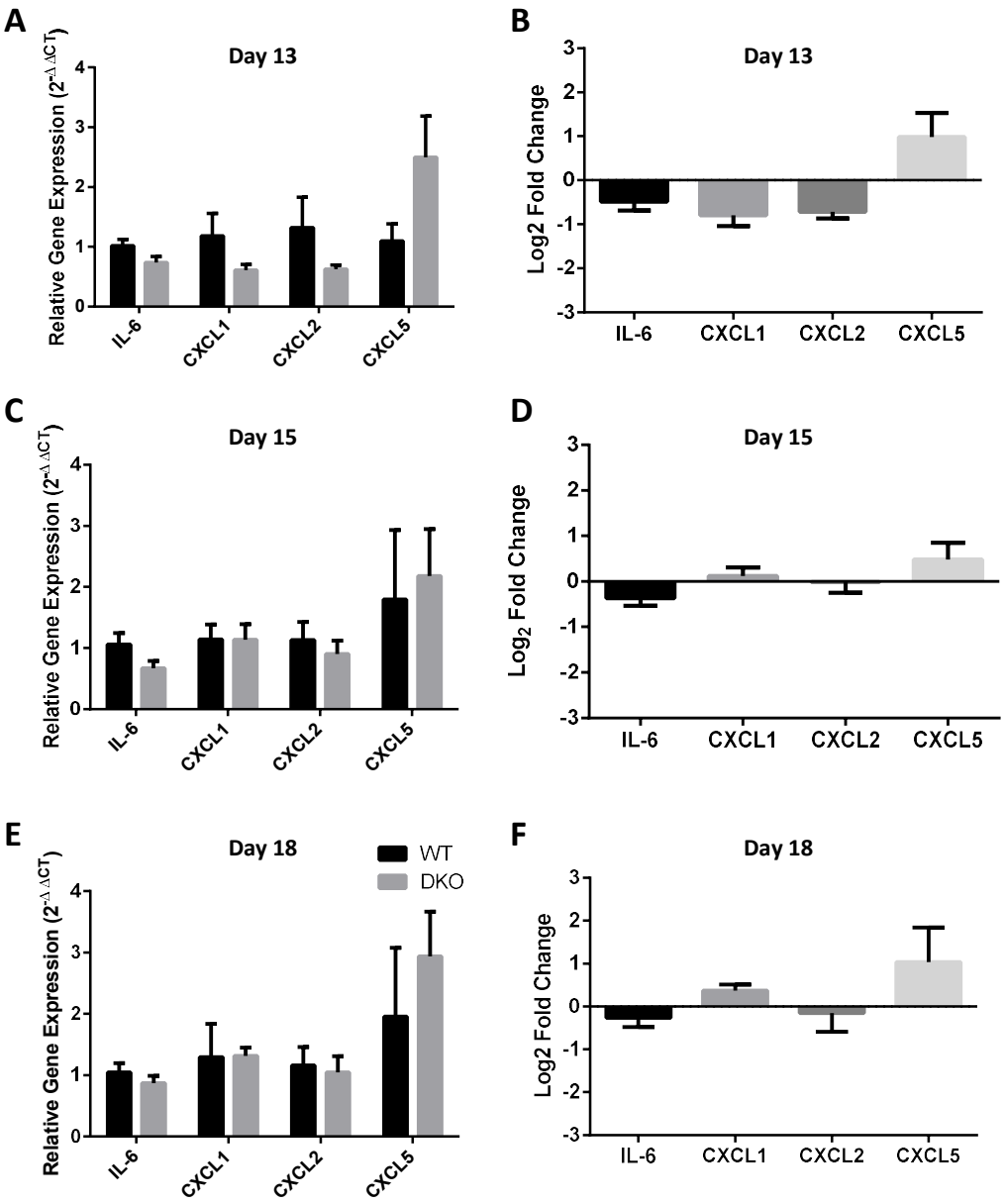


Figure 3.4. Cytokine gene expression in B6BO1 mammary tumours from DKO and wild-type controls across three time points. A, C & E) Relative mRNA levels of *IL-6, CXCL1, CXCL2 and CXCL5* at A) Day 13 N≥4. B) Day 15 N≥12 C) Day 18 N≥4. B, D & F) Data are displayed as a Log_2 fold change of ΔΔCT values (DKO/WT). in B) Day 13, D) Day 15 and F) Day 18. All data are displayed as the mean ±SEM.

3.5. *Perilipin-1* mRNA is reduced in B6BO1 tumours from DKO mice

Previous RNA-seq analysis of spontaneous tumours from *Mmp8* null mice revealed differential expression of several genes involved in lipid metabolism [343]. These genes were subsequently narrowed down to four, selecting those with most significant differences and biological relevance. The expression of each of these four genes in B6BO1 orthotopic mammary tumours was analysed using qRT-PCR.

RNA was isolated from the same tumours used for flow cytometric analysis. qRT-PCR was undertaken to measure the expression levels of four genes: *Lipase E, Acyl-CoA synthetase long chain family member 1 (Acsl1), Leptin* and *Perilipin-1* at Day 13 (Figure 3.5A&B), Day 15 (Figure 3.5C&D) and Day 18 (Figure 3.5E&F).

All genes were expressed at low levels in the earlier stages (Day 13 and 15) of tumour growth, however there was a significant reduction in *Plin1* mRNA in tumours from DKO mice compared to wild-type controls at Day 15, and an almost significant reduction at Day 13. At both Days, *Plin1* mRNA levels were 8 times lower in tumours from DKO mice compared to wild-type. By Day 18, this effect was lost.

Consistently at earlier time points, a trend existed for increased *LipE* and *Acsl1* mRNA in tumours from DKO mice. This trend had reversed by Day 18, but these genes were not significantly differentially expressed between genotypes at any timepoints. *Leptin* expression was low and did not differ much between genotypes at Day 13 and 15. By Day 18, *Leptin* expression was only detectable in two tumours.

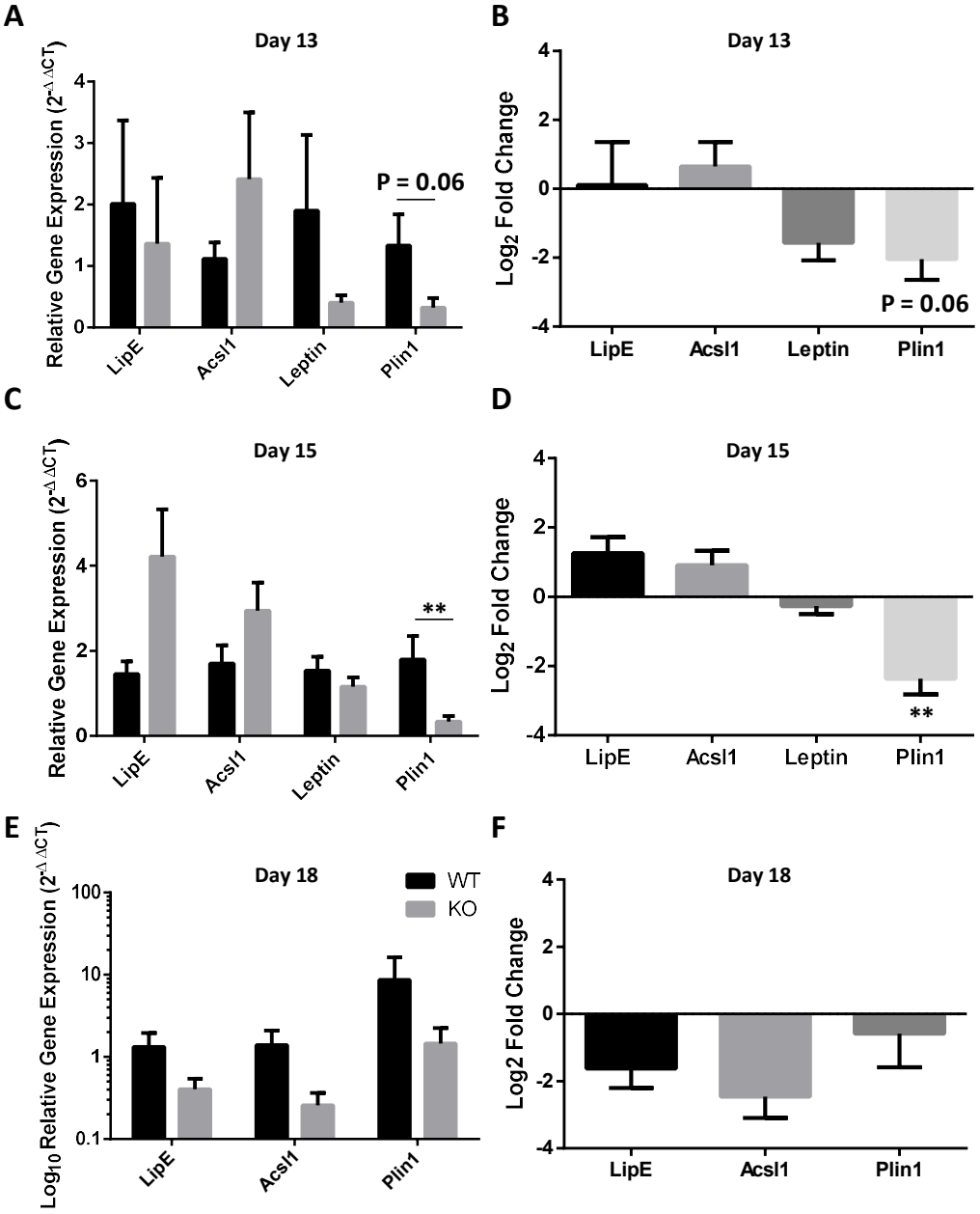


Figure 3.5. Lipid metabolism gene expression in B6BO1 mammary tumours from DKO and wild-type mice across three time points. A, C & E) Relative mRNA levels of *LipE, Acsl1, Leptin, Plin1* at A) Day 13 N≥3. C) Day 15 N≥12 E) Day 18 N≥5. B, D & F) Data are displayed as a Log_2 fold change of ΔΔCT values (DKO/WT) in B) Day 13, D) Day 15 and F) Day 18. All data are displayed as the mean ± SEM. N.B Leptin could not be detected at Day 18. FDR-adjusted p value **p<0.001.

3.6. Loss of *Mmp8* does not impact tumour growth or immune infiltrates in an orthotopic model of basal-like BC

There was no effect of loss of *Mmp8* on tumour growth or intra-tumoural immune populations in ER^+PR^+ B6BO1 tumours of a luminal B subtype. However, to exclude cell-type specific influences such as hormone receptor status or intrinsic subtype, tumourigenesis was studied in a different cell line: the E0771 basal-like ER^+PR^- cell line.

To mimic B6BO1 cell numbers, 100,000 E0771 cells were orthotopically injected into the inguinal mammary fat pad of 8-10-week-old female DKO mice and wild-type controls. These cells exhibit slower growth than B6BO1 tumours and were harvested at Day 19 (1 day after the latest time-point for B6B01 tumours). Upon excision, tumours were weighed and measured using callipers and on average, wild-type tumours were 200 mm^3, and tumours from DKO mice were 300 mm^3 (Figure 3.6A). Whilst there was a trend in increased tumour volume in DKO mice, there was no statistically significant difference between genotypes. To look at alternative metric of tumour growth, tumour weight was also measured. An increased trend was also apparent in DKO tumours, which were on average 0.35 g compared to 0.25 g in wild-type tumours. This difference was similarly not significant. The size of the tumours can be seen in (Figure 3.6B).

In order to be consistent with immunological profiling carried out in B6BO1 tumours, flow cytometric analysis was carried out using an identical myeloid panel to that previously to identify numbers of leukocytes, myeloid cells and Mϕs (Figure 3.6C). The total percentage of leukocytes ($CD45^+$) and myeloid cells ($CD45^+CD11b^+$cells) within the E0771 tumours was approximately between 45-50%, and 30% respectively. Concurrently, no significant differences between genotypes were found in Mϕ populations ($CD45^+CD11b^+Ly6C^-Ly6G^-F4/80^+$cells), which were between 5-10% of the tumour population. Overall, there were no statistically significant differences between genotypes in any of the myeloid cell populations analysed.

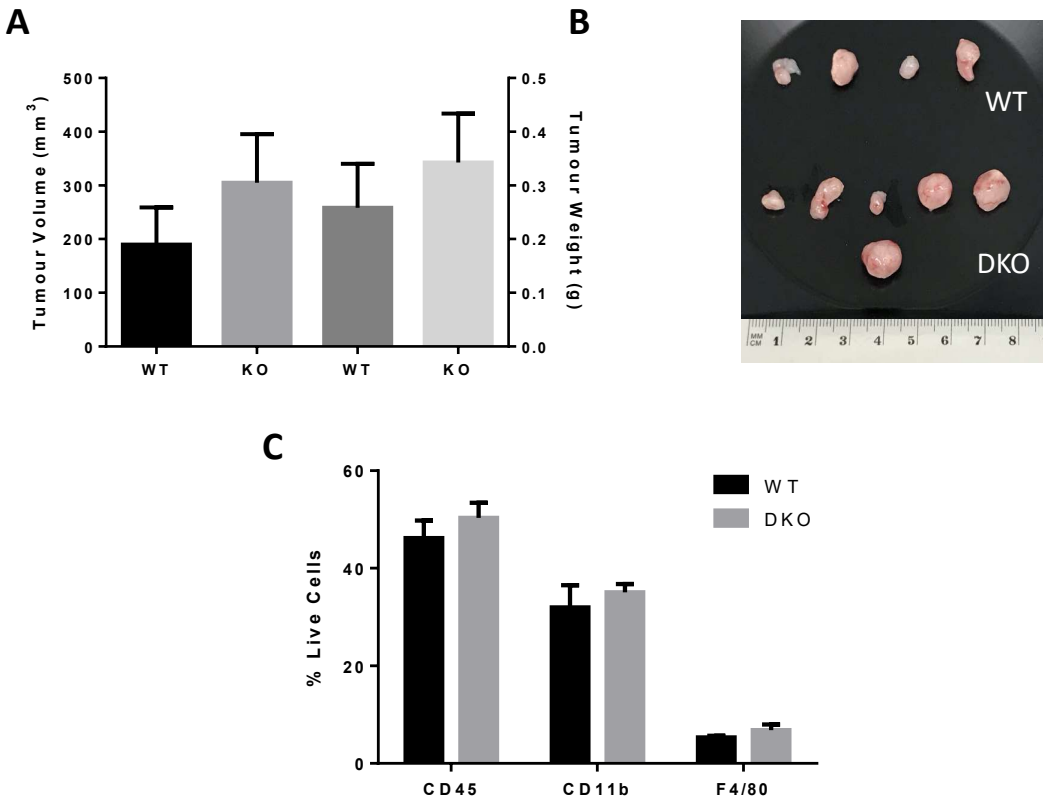


Figure 3.6. Tumour phenotype in DKO and wild-type mice orthotopically injected with E0771 mammary cancer cells. A) Tumour volume measurements (mm^3) performed using *in vivo* calliper measurements measured *ex vivo* and tumour weight (g) 19 days p.i. N = 4, 6. B) Representative photographs of E0771 mammary tumours from wild-type and DKO mice. Ruler shown for scale. Data are presented as mean ± SEM. C) Flow cytometric analysis of intra-tumoural immune infiltrates at Day 19. N = 4,5. Data are presented as a mean percentage of total live cells ± SEM. P.i = post-injection.

3.7. *Mmp8* is located in an MMP cluster with high probability of passenger mutations

The DKO mouse model utilised in this thesis was created on an SV129 background before extensive backcrossing onto the C57BL/6 strain that it was subsequently maintained on. It came to light recently that the SV129 genome contains many SNPs and INDELs. In the creation of a transgenic mouse model, if the target gene is located nearby to one of these mutations on the chromosome, there is a high chance it may exist in the model despite backcrossing. This is due to genetic linkage reducing recombination frequency. *Mmp8* clusters tightly with several other MMPs on mouse chromosome 9 and is also <5 cM from *Casp1* and *Casp4* (also known as *Casp11)* (Figure 3.7A). A database exists to check the likelihood of passenger mutations in the knockout mouse model of choice (me-PaMuFind-It) [335]. The output of this for *Mmp8* can be seen in (Figure 3.7B). The likely mutations affecting *Mmp8* null models are *Mmp1a*, *Olfr832, Fbxl12* and *Casp4* (otherwise known as *Casp11*). The SNPs most likely to be present in the *Mmp8* null mice are *Mmp1a* (91.35%) and *Casp11* (63.02%).

Figure 3.7. Location of *Mmp8* within the MMP cluster and likelihood of existence of passenger mutations. A) *Mus musculus* map of chromosome 9 containing the *Mmp* cluster downstream from the *Casp4* (*Casp11*) gene. Constructed using the NCBI Genome Data Browser [451]. B) Output from the me-PaMuFind-It passenger mutation finder on potential passenger mutations in *Mmp8* null mice and their probabilities after at least 10 backcrosses [335].

3.8. DKO mice harbour an inactivating passenger mutation in the *Casp11* gene but not in *Mmp1a*

In order to detect passenger mutations in the DKO mouse model, primers were designed that surrounded the known mutation-containing regions within *Casp11* and *Mmp1a*: utilising data from the me-PaMuFind-It tool [335].

DNA was isolated from a DKO mouse at random from a colony of 50 mice as well as a wild-type mouse as a control. The region of interest was amplified using PCR and the product was subsequently run on a 3% agarose gel. For the *Casp11* gene, there was a clear difference in the size of the band from the DKO mice compared to the C57 wild-type control – indicative of a difference in base pair number (Figure 3.8A). This was confirmed upon sequencing. The DNA sequence obtained from both genotypes was aligned with the mouse reference genome, and in the DKO mouse, but not the wild-type control, a 5bp deletion was detected in the *Casp11* gene (Figure 3.8B).

To confirm this was not a rare phenomenon, DNA was obtained from all DKO breeding pairs and run on a 3% agarose gel, confirming that all DKO mice harboured the *Casp11* passenger mutation (Figure 3.8C), thus authorising the DKO nomenclature.

For *Mmp1a*, the above process was repeated using primers specific for the region of interest in the *Mmp1a* gene. There was no difference in band size between the DNA from a DKO mouse, a C57 wild-type control, and an extra control: DNA from a wild-type mouse on a mixed C57/SV129 background (Ctrl) (Figure 3.8D). Upon sequencing of the PCR product, there was an identical sequence in all mice (Figure 3.8E).

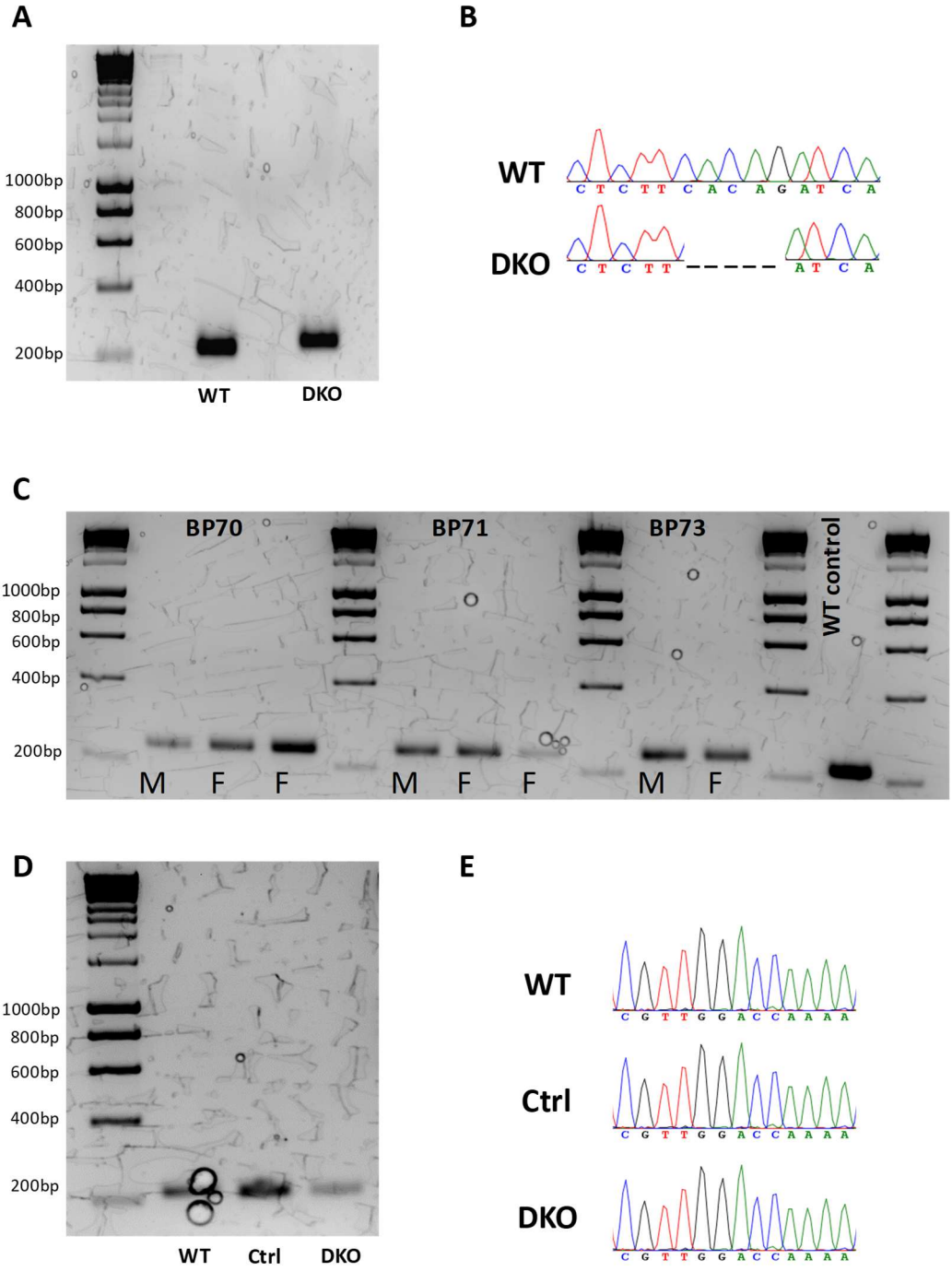


Figure 3.8. DNA agarose gel electrophoresis and sequencing of the *Casp11* and *Mmp1a* gene in the DKO mouse. A) The Casp11 PCR product from a DKO mouse was run on an agarose gel and compared to DNA from a wild-type C57 mouse and B) was sequenced and compared to a wild-type C57 mouse. C) Casp11 PCR products from all DKO breeding pairs. D) The MMP1a PCR product from a DKO mouse was run on an agarose gel and compared to DNA from a wild-type C57 mouse and a mouse on a mixed C57BL/6/SV129 background (Ctrl) and E) sequenced and compared to a wild-type C57 mouse and Ctrl mouse.

3.9. Discussion

The current literature on MMP-8 suggests a predominantly tumour-suppressive role in several cancers [287,292,293]. Of most relevance to this thesis is evidence obtained in our lab using the spontaneous MMTV-PyMT BC mouse model. It was shown that in the absence of *Mmp8*, mice develop tumours earlier and have greater burden of lung metastases compared to wild-type littermates [296]. Additional data linked this onset of tumour burden with changes to the immune compartment of tumours and therefore it was hypothesised that MMP-8 inhibited BC growth through orchestration of the immune system.

Firstly, it was necessary to profile the cell lines utilised in the injectable orthotopic model. One flaw of this model, which is discussed further on in greater detail, is that few mouse BC cell lines exist and there is a chance that the cell line of choice may express the knockout gene of interest: *Mmp8* in this instance. Expression of the gene of interest in the cells themselves could confound interpretation of the model since it would be difficult to delineate the impact of endogenous vs exogenous gene expression. Importantly, it has been shown both at the protein and RNA level, that MMP-8 is not endogenously expressed in either the B6BO1 or E0771 cell lines in 2D culture.

In tumours from wild-type mice, small amounts of MMP-8 have been occasionally detected at both the RNA and protein level (data not shown) indicative of host MMP-8 present in the TME. Many MMP-mediated effects upon tumours are stromal-derived, and this may be the case here. MMP-8 is expressed by many stromal cells including fibroblasts, endothelial cells and leukocytes; all cells present in the TME [344,345]. In the normal breast, MMP-8 expression is restricted to myoepithelial cells (MECs) where it is tumour-suppressive and subsequently lost during progression to ductal carcinoma *in situ* (DCIS) [346]. Therefore, the cellular origin of MMP-8 could be important in guiding its function during tumourigenesis. Occasionally, small amounts of *Mmp8* mRNA could be detected in tumours from DKO mice, the source of which could be from the tumour cells themselves. Although both cell lines do not express MMP-8 in 2D, they could be induced to express MMP-8 by locally-produced

factors in the 3D tumour microenvironment. This 3D-effect could be modelled using 3D spheroid culture, but it is explored in 2D later in Chapter 3. However, this raises the question of whether a phenotype could have been masked by inducible expression of *Mmp8*, albeit at a very low level in DKO mice.

Alongside profiling expression of the gene of interest, confirmation was carried out of the hormone receptor status of the injectable mouse BC cell lines: B6BO1 and EO771. Receptor status of BCs has a profound impact on tumour aggressiveness, growth and importantly, guiding treatment options in the clinic. It is important to regularly check the receptor status of BC cells since it is known that receptor status can drift over long periods of time. For example, there are reports that triple-negative variants exist in the established MCF-7 ER^+ BC cell line [347]. Hormone receptor status is also dynamic in human BC and can change over time [348].

Two techniques were attempted to confirm the ER and PR status of both cell lines. Both cell lines had already been confirmed as $HER2^-$ [349,350]. B6BO1 and EO771 cell lysates were Western blotted for ERα and PR. The presence of ER was detected in both cell lines. PR could not be detected in either cell line by Western blotting. However, by immunocytochemistry, PR staining was visible in the B6BO1 cell line but not in E0771 cells. To validate the positive ER finding by Western blot, immunocytochemistry was used. The luminal B B6BO1 cells were confirmed as $ER^+PR^+HER2^-$ and the basal-like E0771 cell line as $ER^+PR^-HER2^-$, thus representing two unique cell lines.

Several links have been made between MMP-8 and both sex hormone receptors. In human BC, MMP-8 expression is inversely correlated with levels of both ER and PR [351]. Whilst the *MMP8* promoter does not contain an oestrogen-receptor element to be directly influenced by oestrogen alone, it does contain a CCAAT/enhancer-binding protein element (C/EBP) that ERα can associate with to become a transcription factor complex that can regulate activity of the promoter [294]. Oestrogen has been shown to drive expression of MMP-8 at both the protein and RNA level, perhaps via this mechanism [293]. Previously, MMP-8 has been demonstrated to cleave ERα and ERβ in tongue carcinoma cells, and there are further

links with oestrogen since female *Mmp8* null mice exhibit a higher incidence of skin papillomas only when deficient in oestrogen [292]. Therefore, the cell line ER status could be of importance in any observed phenotype following ovariectomy.

Since previous data suggested a tumour-suppressive role for MMP-8 in BC, the aim here was to confirm whether this was also the case in an alternative model of BC: the orthotopic model. The experimental design was to inject mouse mammary cancer cells into the inguinal mammary fat pad of DKO mice and compare tumour progression with wild-type controls. No significant differences were observed in tumour volume between genotypes at Day 13, 15 or 18, when it would have been expected that tumour growth would increase if MMP-8 is tumour-inhibitory.

One disadvantage of the orthotopic model, and of stark contrast to the spontaneous MMTV-PyMT model is the short time frame of tumour growth. Eighteen days post-injection, tumour volume reaches on average 1500 mm^3, tumours begin to ulcerate, and the health of the mice deteriorates. Therefore, this is the latest time point that can be studied. Tumours are first palpable at around Day 7 and grow slowly until Day 13 where they begin to reach their exponential growth phase. Day 13, Day 15 and Day 18 were chosen for two reasons in this study. Firstly, to attempt to mimic the 6-, 8- and 10-week time points utilised in the MMTV-PyMT model and secondly to investigate whether immunological changes occur at specific time points during tumour growth. At Day 13, tumours were approximately 400-500 mm^3; this is the minimum tumour size necessary to look at intra-tumoural immune populations by flow cytometry based on the need to collect and stain 1 million cells. There is a possibility that even at the 'early' time point of Day 13 that the tumour is already well-established and the role of MMP-8 in tumour initiation has been missed. It is for these reasons that it is difficult to compare the two models side by side.

Despite no change in volume of tumours from DKO mice, more subtle changes to tumour phenotype were investigated. MMP-8 is involved in a signalling nexus that drives changes in inflammatory cytokine levels – all of which can increase influx of immune cells into tumours. This includes CXCL5 – a prominent neutrophil chemoattractant and IL-6 – a predominantly pro-tumourigenic cytokine

[260,320,352]. If MMP-8 is a key player in this network, it could be anticipated that immune cell populations within tumours would shift. For example, if MMP-8 increases levels of IL-6 and CXCL5, then in its absence, reduced IL-6 and CXCL5 may lead to lower numbers of infiltrating neutrophils and Mϕs.

Looking within the myeloid compartment of B6BO1 mammary tumours, relative percentages of total cells were consistent between time points and mirror the percentages seen in B6BO1 tumours by Kirkup *et al* [353]. Some changes in populations were observed between DKO and wild-type mice, however none of these changes were consistent throughout the three time points. At Day 13, there appeared to be an increase in leukocytes and myeloid cells, but this was not significant. The Mϕ population was consistent between both groups.

At Day 15, there was a significantly lower percentage of Mϕs in tumours from DKO mice, and this decreased population size was reflected in a reduced number of $CD11b^+$ myeloid cells. This effect is lost by Day 18 where the populations are consistent between both genotypes. It is unclear why there are less Mϕs in tumours from DKO mice at Day 15 but not at Day 13. However, by Day 18, with the tumours having reached over 1000 mm^3, it may be that subtle changes in immune cells are lost. According to the immunoediting theory, tumours that are large have successfully subverted the immune response and as a result, there is a generally immunosuppressive environment [354].

TAMs are generally considered to play a tumour-promoting role in BC and their presence is associated with a poor prognosis [355]. In DKO tumours, the reduced number of Mϕs observed could be indicative of a less immunosuppressive environment however it is important to remember that no changes in tumour volume occurred in DKO mice, so this is unlikely to have contributed to primary tumour growth.

However, it is important to remember that a spectrum of TAMs exist in tumours with differing effects on tumourigenesis. Therefore, it could be that immune populations do not differ in number but rather in function. Other groups have shown *in vitro* that

MMP-8 influences polarisation towards the M2 pro-tumourigenic phenotype [326]. If MMP-8 does impact Mϕ polarisation within tumours, changes would not necessarily be observed in population numbers, but instead Mϕ phenotype. The expression of two Mϕ markers was analysed: MHCII (M1 anti-tumourigenic) and CD206 (M2 pro-tumourigenic) within B6BO1 tumours and no significant differences between DKO and wild-type mice could be detected, which indicates that MMP-8 does not influence Mϕ polarisation within B6BO1 tumours.

The B6BO1 cells used in the orthotopic model preferentially metastasise to the bone and to the lung [349], however metastasis was not studied. The time constraint of this model makes long-term metastasis studies complicated. Primary tumours must be resected, and half of the mice will die during this procedure. Previously MMP-8 has been shown to be metastasis-suppressive in BC [296], but the mechanism remains elusive. It has been hypothesised that MMP-8 interacts with β1-integrin to regulate adhesion, thus preventing migration and invasion, and this could be one mechanism [356]. An alternative proposal is that cells within the tumour may abide by the 'seed and soil' hypothesis. Perhaps the transient decrease in Mϕs at Day 15 leads to changes in cytokine production that prevent dissemination of tumour cells, however this is speculation and would require more in-depth analysis on the profile of this Mϕ population within the tumour.

Since only small changes in immune populations were observed, it was proposed that absence of *Mmp8* may not lead to quantitative differences in immune cells but may impact the abundance of inflammatory mediators such as cytokines and chemokines. MMP-8 is a known driver of cytokine production including IL-6 and IL-8 [260]. In culture, overexpression of *Mmp8* leads to upregulation of *IL-6* and *IL-8* at the protein and RNA level. The catalytic domain of *Mmp8* was found to be critical for this upregulation, However, the upregulation at the RNA level is indicative of an effect on gene transcription rather than direct proteolytic activity [320]. In mice, IL-8 is found as 3 homologs: *CXCL1, -2* and *-5*, and CXCL5 has been validated as a downstream target of MMP-8. MMP-8 cleaves and inactivates α1-PI, to release

inhibition of neutrophil elastase which can subsequently cleave and increase activity of CXCL5 – a potent neutrophil chemoattractant [319].

The gene expression of *IL-6, CXCL1, -2* and *-5* in B6BO1 tumours was evaluated to ascertain whether MMP-8-dependent changes to intra-tumoural cytokines could be detected. At all three timepoints no significant up- or down- regulation of any of the genes was detected. However, there was a general trend for downregulation in *IL-6* mRNA in DKO tumours at all timepoints that was reaching statistical significance. It could be theorised that MMP-8 does induce the expression of IL-6 in B6BO1 tumours, since in the absence of MMP-8, *IL-6* transcripts are reduced. However, there may be a loss of resolution in a mass of cells and molecules as complex as a tumour. It may be that the tumour cells do produce less IL-6 in the absence of MMP-8 but this effect is diluted by the vast amounts of IL-6 produced from immune cell populations. This theory is plausible given that B6BO1 tumours are made up of between 30-40% leukocytes and monocytes, Mϕs and T cells are the major sources of IL-6 [357]. To confirm this theory, fluorescence-activated cell sorting (FACS) could be utilised to separate cancer cells from immune cells to study population-based transcriptomics.

Measurements of IL-6 protein levels were attempted in the tumours by ELISA but every sample was below the limit of detection (data not shown). Concentrations of IL-6 are low in tumour tissue, probably owing to it being a secreted molecule [358], therefore a high-sensitivity ELISA may solve this issue to reveal whether as in Thirkettle *et al*, MMP-8 induces the expression of IL-6 at both the protein and RNA level in mouse mammary tumours.

As consistent immunological changes in DKO tumours could not be found, other changes in tumour phenotype were investigated. It was observed by Otto Warburg over a century ago that cellular metabolism is altered in cancer cells [359]. It is now acknowledged that the ability of cells to reprogram their energy metabolism is a fundamental capability acquired by tumours [360]. In cancer, abnormalities occur in all aspects of energy metabolism including glucose, lipid and amino acid metabolism. Previous RNA-seq data generated in our lab on MMTV-PyMT tumours identified several biological pathways that were up- or down-regulated in *Mmp8* null mice

[343]. Many genes involved in the process of lipid metabolism were significantly downregulated at 8 weeks of age including fatty acid metabolism and fat cell differentiation. These genes were conversely upregulated at 10 weeks of age. Focus was placed on genes that were either highly significantly different or through a literature search, genes known to have links to cancer.

The mRNA expression of four lipid metabolism genes was studied: *LipE (*hormone-sensitive lipase), *Acsl1*, *Leptin,* and *Plin1,* in B6BO1 orthotopic mammary tumours from both DKO and wild-type mice at 3 different time points. At earlier time points: Day 13 and 15, consistent changes in lipid metabolism gene expression were observed. Despite not reaching statistical significance, an almost 3-fold increase in transcripts for *LipE* and *Acsl1* in tumours from DKO mice was detected. This contrasts with Day 18: *LipE* and *Acsl1* mRNA was downregulated by approximately 3-fold in DKO tumours. These concomitant increases and decreases contrast with the previously generated RNA-seq data. In tumours from MMTV-PyMT/*Mmp8* null mice, at 10 weeks of age, the latest time point studied, all four lipid metabolism genes were strongly upregulated, particularly *Leptin.* Whereas, at the latest time point of Day 18, *Leptin* was undetected, and *LipE and Acsl1* were downregulated in B6BO1 orthotopic tumours. However, lipid metabolism gene expression was downregulated in tumours from MMTV-PyMT/*Mmp8* null mice at 6 weeks of age, the earliest time point studied. This is in agreement with the downregulation of *Leptin* and *Plin1* expression at Day 13 and Day 15 in B6BO1 tumours from DKO mice. Although it is difficult to directly compare the two models at a molecular level as well as a temporal one, the sequencing was a useful unbiased approach to begin to further dissect gene expression changes.

What is clear from both the previous and current data is that lipid metabolism is dysregulated in mammary tumours lacking *Mmp8*. A consistent observation was made across earlier timepoints that *Plin1* mRNA is significantly downregulated in tumours from DKO mice. At Day 13 and Day 15, there is an 8-fold decrease in *Plin1* transcripts. By Day 18 this phenotype is absent. What is not certain from the use of

DKO mice is whether absence of *Casp11* influenced this finding. This has been considered in Chapter 4. and will be discussed further there.

Because BC is such a heterogenous disease, it is important to use BC cell lines that represent more than one subtype. Using the ER^+PR^+ B6BO1 cell line, representative of a luminal B subtype, it was shown that tumour growth was not inhibited by MMP-8, since in its absence there was no change to tumour volume. Furthermore, no consistent immunological changes were detected, suggesting that MMP-8 does not orchestrate the immune system to inhibit BC progression, as was first hypothesised. However, to look at whether this hypothesis held true in another subtype, the B6BO1 tumour experiment was repeated using the E0771 BC cell line, lacking the PR expression found in the B6BO1 cells. These cells are representative of a basal-like subtype, which is often characterised by an aggressive clinical outcome [361]. Using this alternative model, results consistent with those in the B6BO1 model were found, with absence of MMP-8 playing no role in primary BC tumour growth. There were also no changes to immune populations. These results both suggest that in the presence of estrogen receptor on BC cells, absence of *Mmp8* has no effect. This has been seen before in cancer, where female *Mmp8* null mice were protected from skin carcinoma in the presence of estrogen [292]. It would be interesting to use a triple negative cell line to ascertain whether estrogen receptor status plays a role in MMP-8-related tumour biology.

As a caveat to the findings thus far, potential pitfall was discovered in the mouse model utilised throughout this chapter. Detailed in a paper by Vanden Berghe *et al*, was the existence of passenger mutations in the SV129 mouse strain: the origin of many transgenic mouse models, including the one used here [335]. In brief, passenger mutations are SNPs and INDELs in the genome of 'donor' mice that due to genetic linkage persist near to genes of interest despite extensive backcrossing onto alternative strains. If these mutations change the gene sequence dramatically, they can lead to a non-functional protein. If this is the case, a mouse model can become a 'double knockout' and interpretation of the contribution from the gene of interest *vs* the passenger mutation becomes extremely difficult to interpret.

Using the me-PaMuFind-It web tool by Vanden Berghe *et al*, there was a strong possibility (over 70%) that the genome of the *Mmp8* null mice used thus far contained two passenger mutations – one in the *Mmp1a* gene and the other in the *Casp11* gene [335]. Upon DNA sequencing of the region containing the known mutation of both genes in these mice, the presence of the *Casp11* 5bp passenger mutation and the absence of mutations in the *Mmp1a* gene was confirmed. It has previously been shown that this 5bp deletion in *Casp11* leads to a non-functional protein rendering the mice *Mmp8*$^{-/-}$/*Casp11*$^{-/-}$ or double knockout (DKO) as they are referred to throughout this thesis [339].

Whilst there is little evidence to suggest caspase-11 plays a role in BC specifically, it has many functions within the immune system [339,362]. The lack of tumour and immune phenotype observed in this orthotopic BC model could have been obscured by the passenger mutation, and contrastingly, any observations made could have been the result of the passenger mutation. Thus, repeat investigations in *Mmp8* null mice with functional *Casp11* were warranted.

To summarise:

- The two BC cell lines utilised in the orthotopic injection model do not endogenously express *Mmp8*
- The E0771 cell line representing a basal-like subtype is ER^+PR^- and the B6BO1 cell line representing a luminal B subtype is ER^+PR^+
- Loss of *Mmp8* does not impact tumour growth in an orthotopic model of BC or alter the intra-tumoural myeloid immune compartment
- There is differential lipid metabolism gene expression in tumours from *Mmp8* null animals including a reduction in *Plin1* expression
- DKO mice are so-called due to the detection of a *Casp11* inactivating passenger mutation

4. Characterisation of the *Mmp8* KO breast cancer model in tumourigenesis

In the previous chapter, results indicated that MMP-8 does not inhibit primary BC tumour growth, and its absence only led to minor changes in tumour phenotype. However, the entire body of work was carried out in DKO mice that it was discovered harboured a passenger mutation in the *Casp11* gene.

The caspase-11 deficiency in the DKO mouse colony is of importance in the interpretation of the BC model for several reasons, but primarily due to its role within the immune system. Caspase-11 belongs to the family of caspases: cysteine-aspartic proteases with critical roles in several forms of programmed cell death including apoptosis, pyroptosis as well as inflammation [363]. Eleven caspases exist in humans whereas there are only 10 in mice. The caspase family is grouped into three categories: inflammatory caspases (-1, -4, -5, -12) initiator caspases (-8, -9, -10 and -2) and executioner caspases (-3, -6, -7) [364]. Caspase-11 is the mouse homolog of human caspase-4: an inflammatory caspase. As a cytosolic LPS-binding receptor, it plays a central role in pyroptosis: a form of programmed cell death often triggered upon infection with intracellular pathogens [365] [366]. It does this by forming a complex called the inflammasome with many other proteins that upon stimulation causes the release of IL-1β and IL-18 resulting in pyroptosis [365].

A plethora of defects have been associated with the absence of caspase-11 including abnormal immune cell apoptosis and decreased circulating cytokine levels [362,367]. Other research groups have acknowledged the presence of the *Casp11* passenger mutation in their models, and in some cases published data have been proven to be heavily influenced by its absence. In total, nine MMPs are within 5cM of the *Casp11* gene including *Mmp7, -8* and *-13*. *Mmp7, -8 and -13* null mice were found to be resistant to LPS-induced endotoxemia [310,368,369], notably as were *Casp11* null mice [370]. Subsequently, all three of the MMP- knockout mice were found to have the same 5bp mutation in the *Casp11* gene found in our model. Upon repeat studies in knockout mice *without* the passenger mutation, this phenotype was completely abrogated – suggestive that it was a result of the passenger mutation [335].

Here, the notion that the *Casp11* passenger mutation may have confounded the interpretation of the specific role of MMP-8 in tumour growth and immune orchestration has been acknowledged. To correct for this, extensive repeat experiments have been carried out on tumour volume, immune populations and gene expression analysis using *Mmp8* null mice without the passenger mutation, that will be referred to as *Mmp8* KO hereafter.

4.1. *Mmp8* KO mice no longer harbour a *Casp11* passenger mutation

The discovery of the *Casp11* passenger mutation in our DKO mouse colony meant that interpretation of the specific role of MMP-8 in this model would not be possible. Therefore, mice were sought that were *Mmp8* KO with a functional *Casp11* gene. Mice were obtained from Roosmarijn Vandenbroucke in Ghent and a new colony of *Mmp8* KO mice; free of passenger mutations was established.

Firstly, the MMP-8 status was confirmed in these mice. The region of interest surrounding the neomycin resistance cassette inserted into the *Mmp8* gene was amplified using PCR and the product was run on a 1.8% agarose gel (Figure 4.1A). Controls were run alongside for wild-type mice, *Mmp8* heterozygote mice and *Mmp8* null mice. The MMP-8 band from a wild-type mouse is found at ~500bp, as seen in the wild-type control DNA. The knockout status of the *Mmp8* KO mouse is confirmed by the band size at ~280bp, identical to that of the *Mmp8* null control DNA.

Next, the passenger mutation status was examined using DNA isolated from the newly acquired *Mmp8* KO mice, and compared to DNA from a wild-type mouse as a control. The region of interest in the *Casp11* gene was amplified using PCR and the product was subsequently run on a 3% agarose gel (Figure 4.1B) The PCR product was then sequenced (Figure 4.1C). For the Casp11 product, there was no longer a difference in the size of the band from the *Mmp8* KO mouse compared to the C57 wild-type control – indicative of no difference in base pair number. This was confirmed upon sequencing, where an identical sequence was obtained from both genotypes, unlike the 5bp deletion seen in DKO mice. To reiterate nomenclature: *Mmp8* null mice **with** the *Casp11* inactivating mutation will continue to be referred to as DKO and those from Ghent demonstrated to have functional *Casp11* will be referred to as *Mmp8* KO.

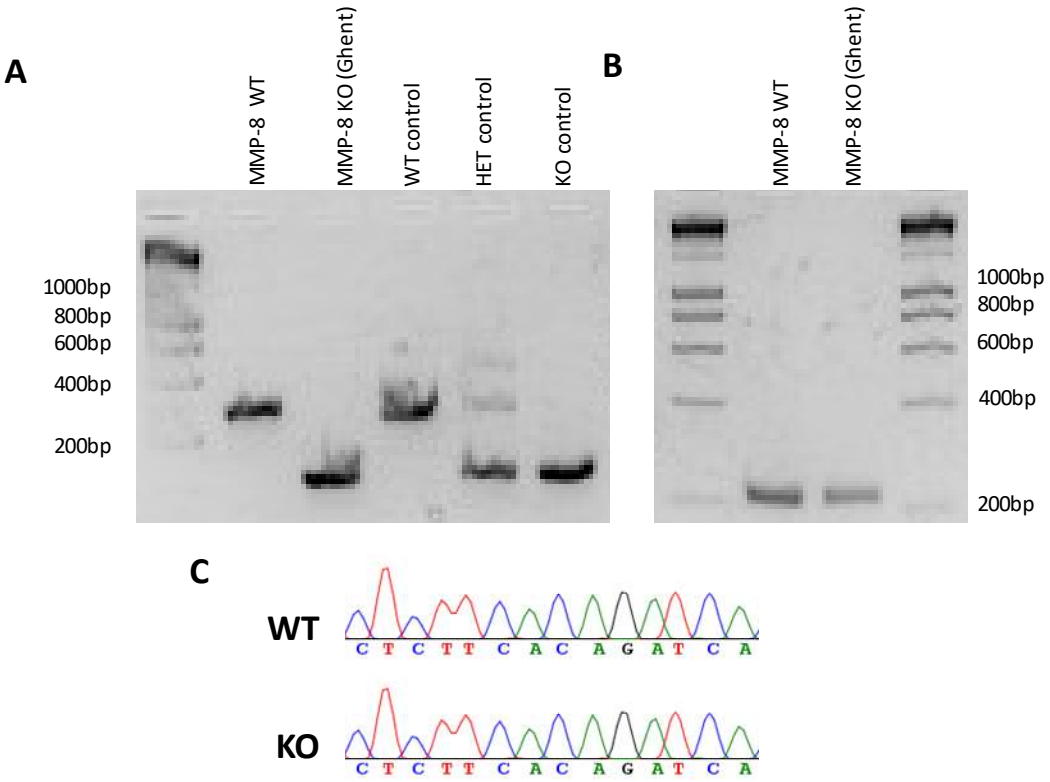


Figure 4.1. DNA agarose gel electrophoresis of the *Mmp8* and *Casp11* gene and sequencing of the *Casp11* gene in the *Mmp8* KO mouse. A) & B) The MMP-8 (A) and Casp11 (B) PCR products from an *Mmp8* KO mouse were run on an agarose gel and compared to DNA from a wild-type C57 mouse. Controls were run alongside from wild-type, *Mmp8*-heterozygous and *Mmp8*-null mice. C) The Casp11 PCR products were sequenced and compared to a wild-type C57 mouse.

4.2. The absence of *Mmp8* does not impact tumour volume in the B6BO1 orthotopic breast cancer model independent of the *Casp11* mutation

In DKO mice, the rate of primary breast tumour growth was not significantly different to that of tumours harboured by wild-type animals. However, as previously discussed, these results may be confounded by the *Casp11* passenger mutation. Therefore, to determine whether MMP-8 is important in primary BC tumour growth, the orthotopic injection model was again utilised, but this time in *Mmp8* KO mice without the *Casp11* passenger mutation. Female mice 8-10 weeks of age were orthotopically injected with B6BO1 cells into the inguinal mammary fat pad. To mimic the previous tumour stages, tumours were excised on Day 13, 15 and 18. The size of the tumours on each day can be seen in (Figure 1.2A-C) respectively. Tumours were measured with callipers *ex vivo* to calculate tumour volume (Figure 1.2D).

The tumour volume can be seen to increase consistently between genotypes from Day 13 to Day 18. At Day 13, tumours from both genotypes are on average 500-600 mm^3. At Day 15, wild-type tumours are 800 mm^3 and *Mmp8* KO tumours are larger, measuring on average 1200 mm^3. At Day 18, tumours from wild-type mice reached on average 2000 mm^3 whereas tumours from *Mmp8* KO mice were smaller at 1700 mm3. These averages are mostly consistent with those in Chapter 3 at each time point, for both genotypes. Previously, at Day 13, DKO and wild-type tumours were approximately 500 mm^3. At Day 15, they were between 800-1000 mm^3 and by Day 18 there was a slight decreasing trend in volume in the DKO tumours, which were on average 1300 mm^3 in comparison to 1700 mm^3 in wild-type tumours.

Overall, no differences in tumour volume between genotypes were observed at Day 13, 15 or 18 by tumour volume measurement.

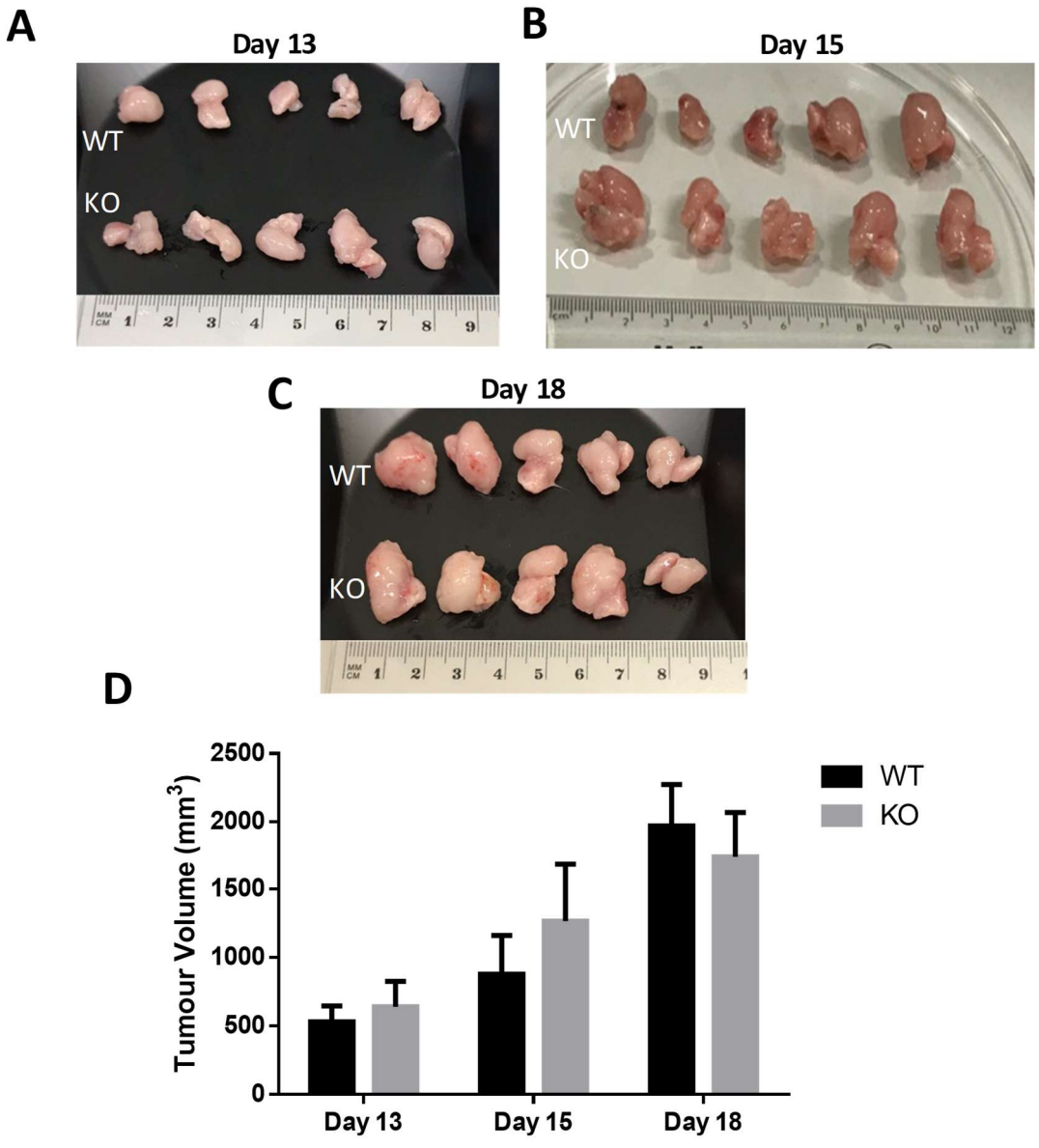


Figure 4.2. Orthotopically implanted B6BO1 tumour volumes in wild-type and *Mmp8* KO mice. A-C) Representative photographs of tumours from wild-type and *Mmp8* KO mice excised at A) Day 13. B) Day 15. C) Day 18 D) Tumour volume measurements (mm^3) after excision on day 13, 15 and 18. N = 5. Data are presented as mean ±SEM.

4.3. The absence of *Mmp8* has no effect on intra-tumoural myeloid immune populations in the B6BO1 orthotopic injection model

Despite no changes in tumour volume observed upon repeat experiments in *Mmp8* KO mice, an investigation was carried out to examine whether *Casp11* status had any influence on immunological processes within the tumour, that had not impacted tumour volume. Since caspase-11 plays a critical role in inflammation, it was reasonable that DKO and *Mmp8* KO mice may behave differently in the context of the immune system.

Myeloid cells made up the largest proportion of cells within the B6BO1 tumours and are of most relevance to MMP-8, considering its release from Mϕs and neutrophils. Therefore, again the antibody panel was designed to look at $CD45^+$ leukocyte populations, $CD45^+CD11b^+$ myeloid cells and $CD45^+CD11b^+Ly6C^-Ly6G^-F4/80^+$ macrophages.

Whilst re-establishing the *Mmp8* KO mouse colony, there was time to optimise and refine the flow cytometry panel. In the following experiments, the panel had the addition of a fixable Live/Dead marker to more definitively remove dead cells from analysis, and Ly6C, a marker to further delineate Mϕ/monocyte subsets. Previously, to identify neutrophils in tumours a gating strategy was used inclusive of $CD45^+CD11b^+Ly6G^+$ cells, whereby no neutrophils could be detected. In this panel, neutrophils could not be found, and the addition of Ly6C to more definitively label this population ($CD45^+CD11b^+Ly6C^-Ly6G^+$) also ruled out the contribution of $Ly6G^+$ cells such as myeloid-derived suppressor cells (MDSCs).

The number of immune cells present in tumours was analysed at Day 13 (Figure 4.3A), Day 15 (Figure 4.3B) and Day 18 (Figure 4.3C) as a percentage of total cells. In the DKO mice, significant reductions were seen in Mϕ number at the Day 15 time point, where the percentage of Mϕs within the tumours was 16% in comparison to 22% in wild-type animals. However, this reduction was not present in the *Mmp8* KO animals. Instead, at Day 15, tumours from both wild-type and *Mmp8* KO mice had approximately 15% Mϕs.

The relative proportions of all cells remained at levels consistent with those in Chapter 3, except for Day 18, whereby tumours from both wild-type and *Mmp8* KO mice contained up to 40% $CD45^+$ leukocytes. In tumours from DKO mice and wild-type mice at the same time point, this number was 30% of total cells.

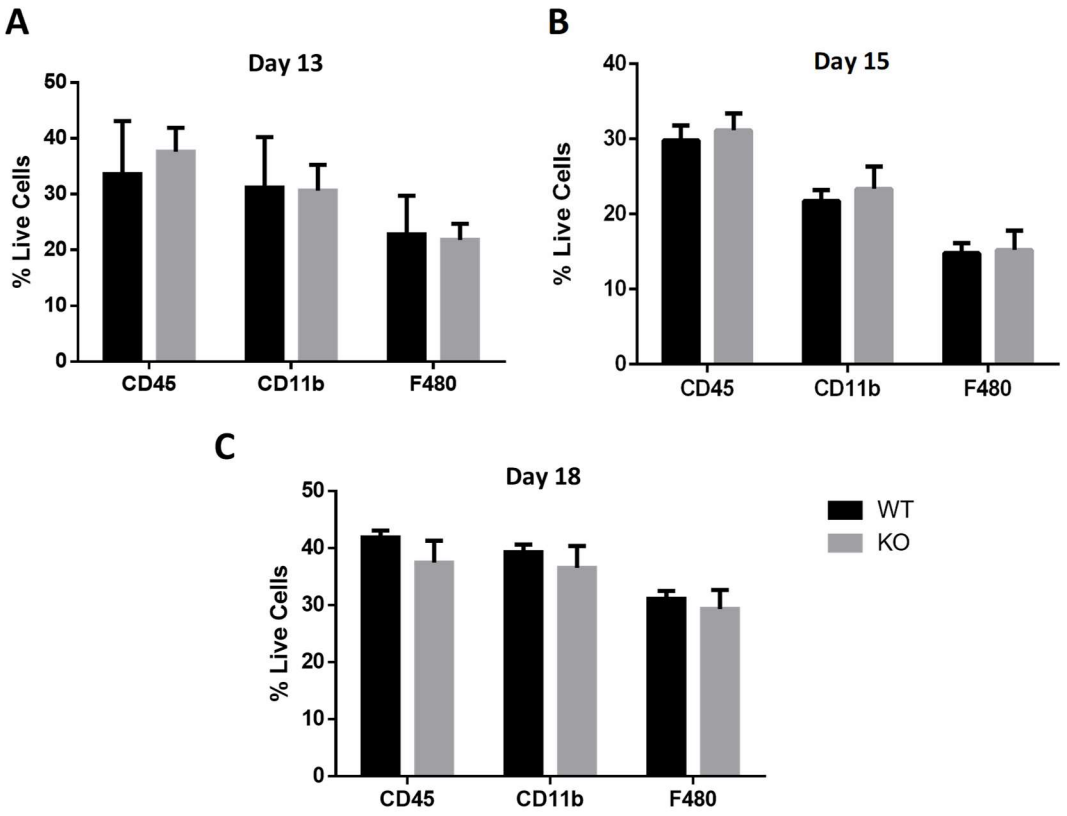


Figure 4.3. Flow cytometric analysis of intra-tumoural immune infiltrates across three time points in *Mmp8* KO and wild-type mice. A-C) Myeloid cell populations A) 13 days, B) 15 days and C) 18 days p.i. n≥5. Data are presented as a mean percentage of total live cells ± SEM. P.i = post-injection.

4.4. Intra-tumoural cytokine levels do not differ between *Mmp8* KO and wild-type mice

There is some evidence to suggest that MMP-8 modulates the expression of the cytokines IL-6 and IL-8 [260,320]. If MMP-8 is responsible or involved in the cleavage and activation of these chemokines, in the absence of *Mmp8*, changes to their levels could be expected. In Chapter 3. , no differences were found in levels of *IL-6* or homologs of *IL-8* at the RNA level. However, upon discovery of the *Casp11* passenger mutation, to ensure this had not affected previous findings, this analysis was repeated on tumours from *Mmp8* KO mice with functional *Casp11*.

Utilising the same tumours subjected to flow cytometric analysis in 4.3, mRNA expression of *IL-6* and the three mouse homologs of human IL-8: *CXCL1, CXCL2* and *CXCL5* was analysed by qRT-PCR. Analogous to the flow cytometry, tumours were analysed at all three time points: Day 13 (Figure 4.4A&B), Day 15 (Figure 4.4C&D) and Day 18 (Figure 4.4E&F).

Across all time points, all four transcripts could be detected. Expression of all four genes in the tumours remained consistent between genotypes and there were no statistically significant differences. However, there was a trend in reduced *CXCL2* expression at Day 18 and increased *CXCL5* expression at Day 15 in *Mmp8* KO tumours.

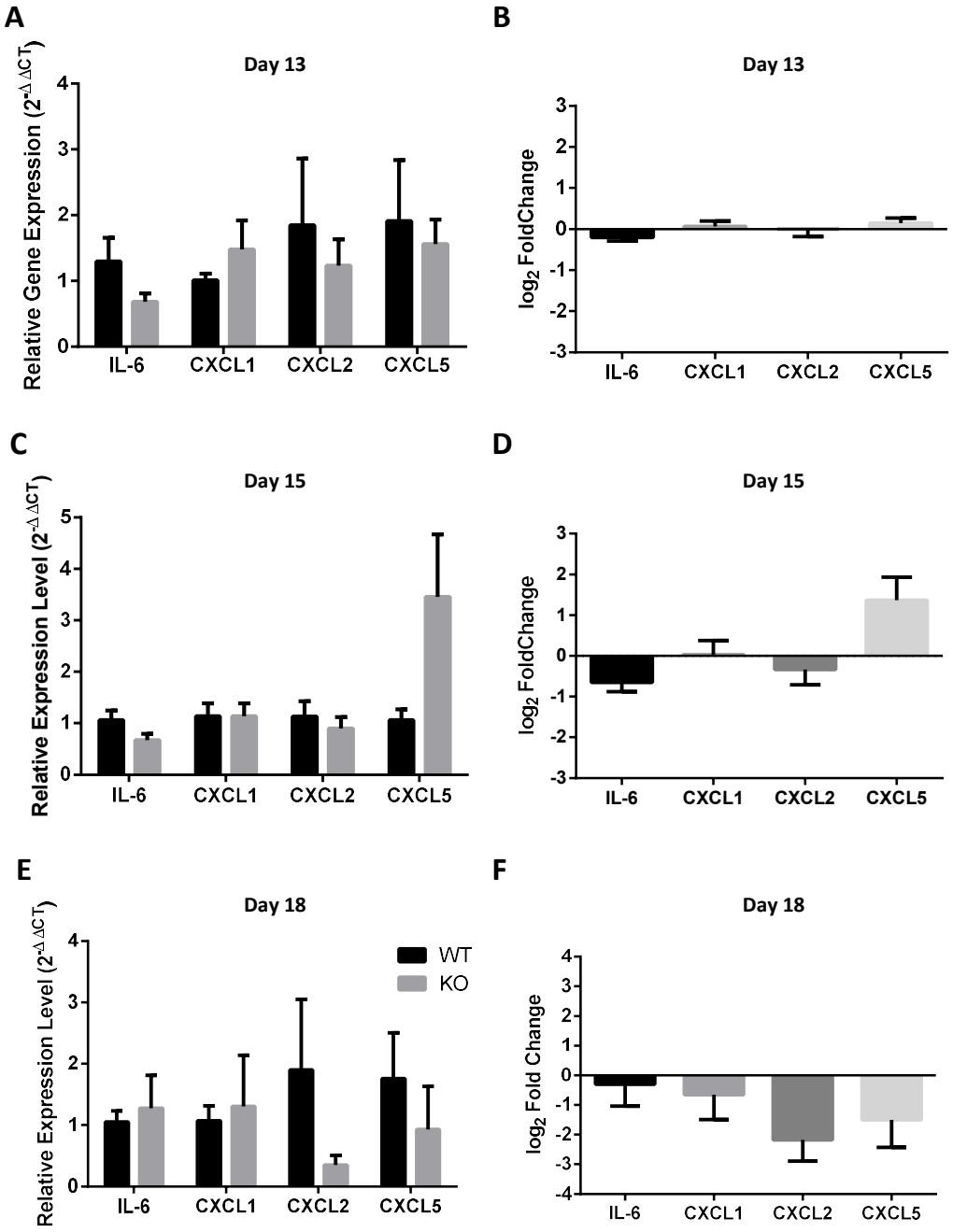


Figure 4.4. Cytokine gene expression in B6BO1 mammary tumours from *Mmp8* KO and wild-type mice across three time points. A C & E) Relative mRNA levels of *IL-6, CXCL1, CXCL2 and CXCL5* at A) Day 13, C) Day 15, E) Day 18. B D & F) Log_2 fold change of ΔΔCT values (KO/WT) at B) Day 13, D) Day 15 and F) Day 18. N≥5. All data are displayed as the mean ±SEM.

4.5. A trend exists for downregulation of *Plin1* mRNA in B6BO1 tumours from *Mmp8* KO mice

In Chapter 3, based on differential lipid metabolism gene expression in spontaneous tumours derived from MMTV-PyMT mice crossed onto an *Mmp8* null background, whether this phenomenon occurred in orthotopically-derived tumours was investigated. Significantly reduced levels of *Plin1* mRNA were detected in DKO tumours. However, now aware that the mice did not possess a functional *Casp11* gene, this analysis was repeated in tumours from *Mmp8* KO mice not harbouring the passenger mutation.

RNA was isolated from the same tumours used for flow cytometric analysis in 4.3. qRT-PCR was undertaken to measure the expression levels of four genes: *lipase E, acyl-CoA synthetase long chain family member 1 (Acsl1), leptin* and *perilipin-1* at Day 13 (Figure 4.5A&B) , Day 15 (Figure 4.5C&D) and Day 18 (Figure 4.5E&F).

At Day 13, expression of 3 of the 4 genes studied could be detected. *Lept* could not be detected in these samples. There was little variation in gene expression in tumours from wild-type and *Mmp8* KO tumours. However, at Day 15, a trend in reduced *Leptin* and *Plin1* mRNA was observed, although unlike in Chapter 3 this was not significant. *LipE* and *Acsl1* mRNA levels remained consistent between genotypes. At Day 18, there was a trend in reduced *Acsl1* and *Plin1* mRNA in tumours from *Mmp8* KO mice though this did not reach significance.

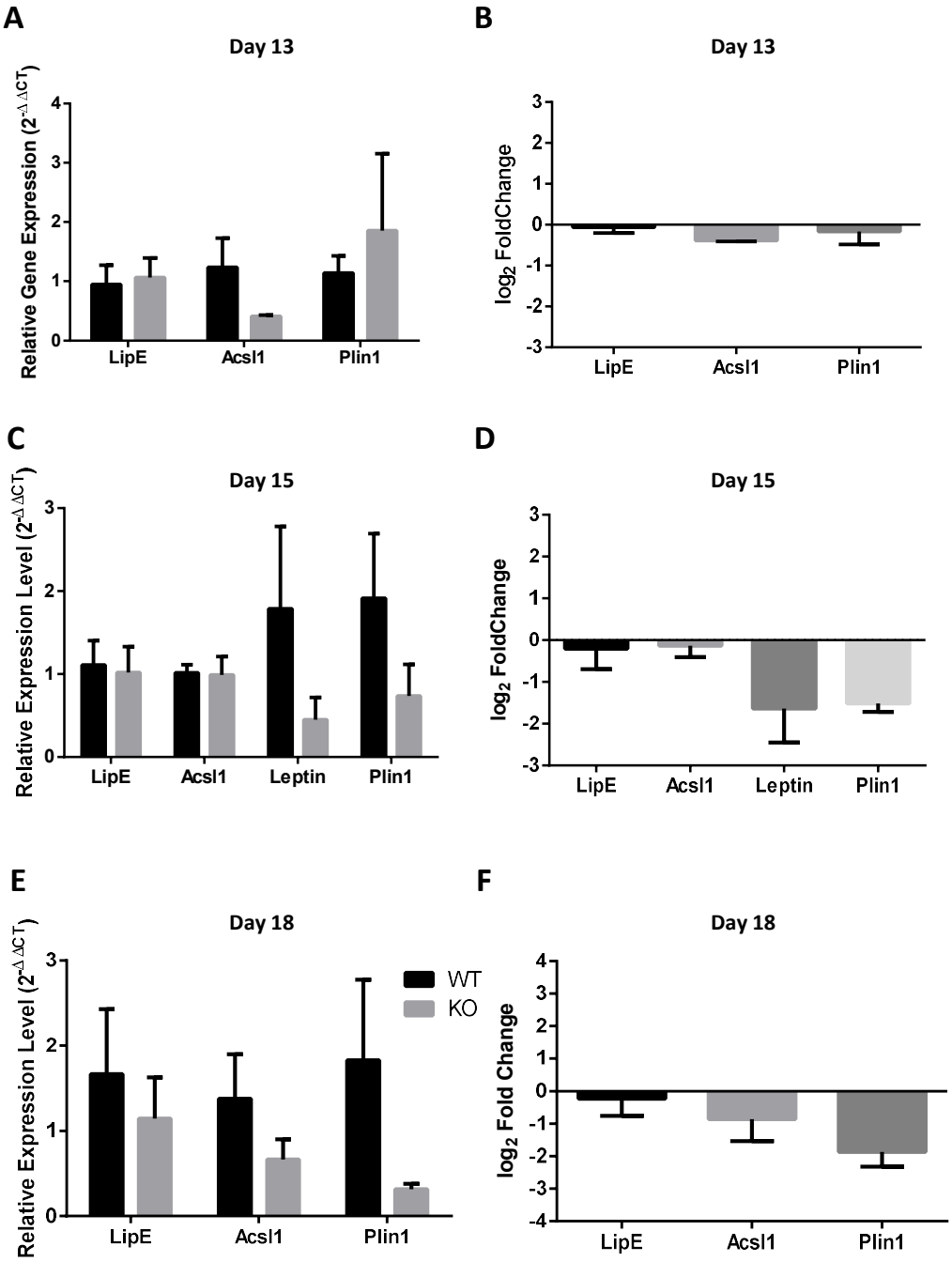


Figure 4.5. Lipid metabolism gene expression in B6BO1 mammary tumours from *Mmp8* KO and wild-type mice across three time points. A C & E) Relative mRNA levels of *LipE, Acsl1, Leptin* and *Plin1* at A) Day 13, C) Day 15, E) Day 18. B D & F) Log_2 fold change of ΔΔCT values (KO/WT) at B) Day 13, D) Day 15 and F) Day 18. N≥5. All data are displayed as the mean ±SEM. N.B. Leptin was not detected at Day 13 or 18.

4.6. The absence of *Mmp8* does not impact tumour progression or volume in a basal-like orthotopic breast cancer model

Results thus far suggested no effect of loss of MMP-8 on tumour growth or intra-tumoural immune populations in ER^+PR^+ B6BO1 tumours of a luminal B subtype. However, to exclude cell-type specific influences such as hormone receptor status or intrinsic subtype tumourigenesis was studied in a different cell line: the E0771 basal-like ER^+PR^- cell line. In order to confirm whether the lack of tumour growth phenotype was not related to *Casp11* status, EO771 BC tumour experiments were carried out in *Mmp8* KO mice.

In a similar fashion to B6BO1 tumour experiments, 100,000 E0771 cells were orthotopically injected into the inguinal mammary fat pad of 8-10 week old female *Mmp8* KO mice and wild-type controls in two separate experiments. E0771 tumours exhibit slower growth than B6BO1 tumours and were harvested at later time points: Day 19 (1 day after the longest time-point for B6B01 tumours) or Day 25. At Day 19 or 25, tumours were excised, weighed and measured using callipers (Figure 4.6A&B). Representative photographs were taken at each time point (Figure 4.6C&D).

Tumours harvested on Day 19 were approximately 300 mm^3 whereas tumours harvested on Day 25 were approaching 1000 mm^3, with very little variation between genotypes. To look at another metric of tumour growth, tumour weight was measured. At Day 19, wild-type tumours weighed on average 0.30 g and *Mmp8* KO tumours weighed 0.34 g. At Day 25, tumours from wild-type mice were on average 0.85 g and tumours from *Mmp8* KO mice were smaller at 0.75 g. Tumour volume or tumour weight was not significantly different between genotypes at Day 19 or Day 25.

Tumour metrics in *Mmp8* KO mice were similar to that of the DKO mice in Chapter 3, although there are only data available at Day 19. Wild-type tumours are on average 200 mm^3 and DKO tumours are approximately 300 mm^3 whilst here, tumours from both genotypes are on average 300 mm^3.

To carry out the same immunological profiling carried out in DKO mice, tumours were subjected to flow cytometric analysis. An identical, optimised myeloid panel was utilised as described in 4.3 to identify numbers of leukocytes, myeloid cells and Mϕs at Day 19 (Figure 4.6E) and Day 25 (Figure 4.6F).

At Day 19, the total percentage of leukocytes within the E0771 tumours was approximately 25% compared to 30% in B6BO1 tumours. By Day 25, this number had reduced further to less than 22% of total cells. Whilst there were no statistically significant differences in amounts of $CD45^+$ leukocytes between genotypes, there was a general trend in reduced numbers of $CD45^+$ cells in tumours from *Mmp8* KO mice.

At Day 25, the myeloid cell population ($CD45^+CD11b^+$ cells) also showed a reduced trend in tumours from *Mmp8* KO mice. Concurrently, there was a trend of reduced Mϕs ($CD45^+CD11b^+Ly6C^-Ly6G^-F4/80^+$cells) in *Mmp8* KO tumours at Day 25.

Overall, at both Day 19 and 25 there were no statistically significant differences between genotypes in any of the myeloid cell populations analysed.

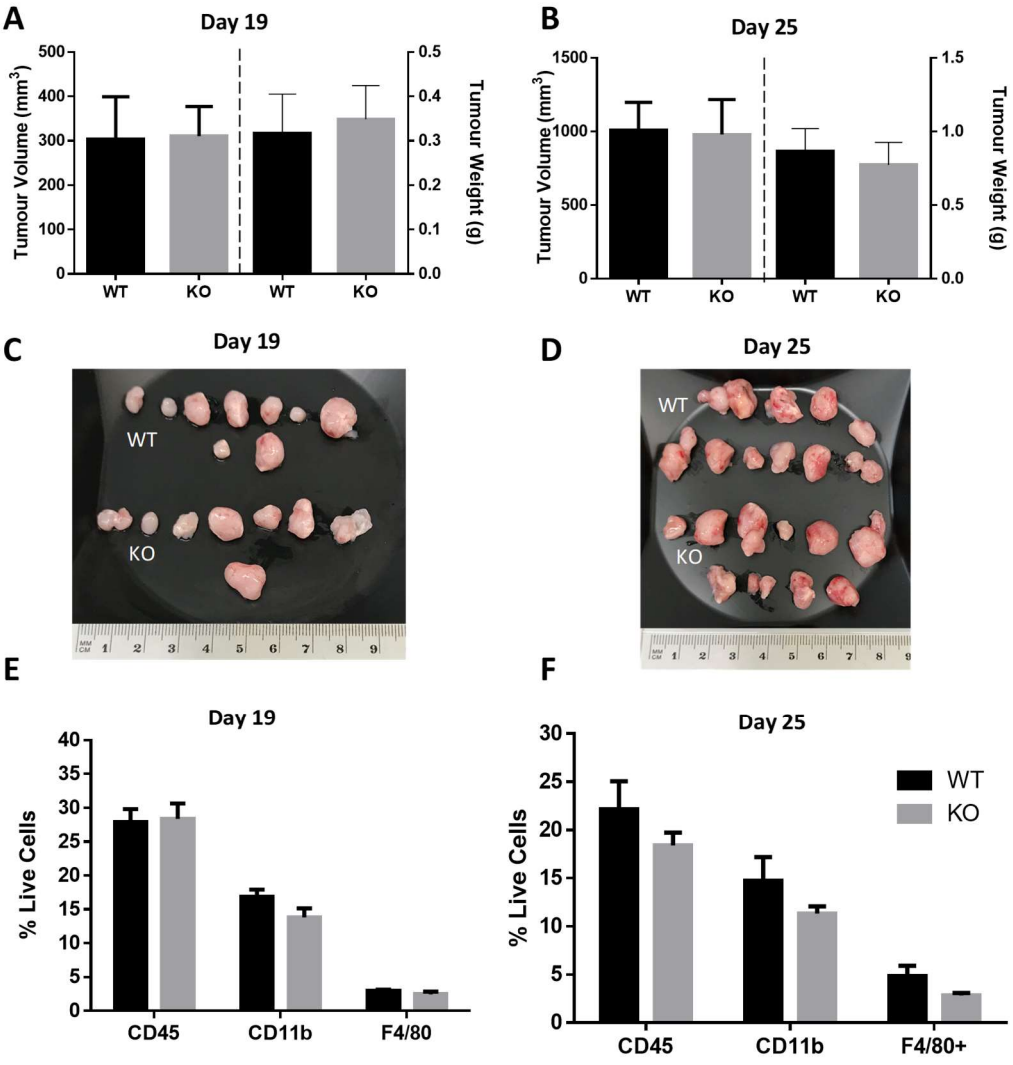


Figure 4.6. Tumour phenotype in wild-type and *Mmp8* KO mice orthotopically injected with E0771 mammary cancer cells. A&B) Tumour volume measurements (mm^3) performed using *ex vivo* calliper measurements and tumour weight (g) on A) Day 19 and B) Day 25. N = 10. C&D) Visualisation of tumours excised C) 19 days and D) 25 days p.i of E0771 mammary carcinoma cells. Ruler shown for scale. Data are presented as mean ±SEM E&F) Flow cytometric analysis of intra-tumoural immune infiltrates at E) Day 19 and F) Day 25. N = 5. Data are presented as a mean percentage of total live cells ± SEM. P.i = post-injection.

4.7. The absence of *Mmp8* does not impact tumour volume or intra-tumoural immune populations in a subcutaneous cancer model

The results obtained in this chapter indicated that MMP-8 does not play a role in inhibiting primary tumour growth in BC, but there is evidence that MMP-8 plays tumour-inhibitory roles in other cancers including skin carcinoma and tongue cancer [292,293]. An alternative model to BC was used to determine whether MMP-8 is involved in tumour progression in other cancer types.

CMT-19T cells, an established lung carcinoma cell line, were subcutaneously injected into both male and female *Mmp8* null mice, DKO mice and wild-type controls (Figure 4.7A). At Day 15, tumours were excised and measured *ex vivo* using callipers to determine tumour volume (Figure 4.7B). Tumours were between 200-250 mm^3 across all genotypes and no significant differences in volume were found.

In order to replicate the intra-tumoural immune population analysis carried out in mammary tumours, cells were stained with the same myeloid antibody panel used in B6BO1 and EO771 mammary tumours and cell populations were analysed using flow cytometry (Figure 4.7C). The number of $CD45^+$ leukocytes was between 20-35% of total cells across all genotypes. There were almost double the number of $CD45^+$ leukocytes in tumours from *Mmp8* KO mice compared to wild-type controls, and this difference was statistically significant. Tumours from DKO mice contained approximately 25% total leukocytes, which was not significantly different from either genotype. However, there were significantly less $CD45^+CD11b^+$ myeloid cells in tumours from DKO animals compared to *Mmp8* KO mice. No other differences in myeloid populations were observed.

Neutrophil numbers were significantly reduced in DKO tumours in comparison to wild-type tumours. There was a trend in reduced neutrophil numbers in *Mmp8* KO tumours compared to wild-type controls but this was not significant.

And lastly, the most differences observed amongst genotypes were in the Mϕ population. In wild-type control animals, tumours contained less than 5% Mϕs in total. This significantly increased to 15% in tumours from *Mmp8* KO animals.

Additionally, tumours from DKO mice had significantly more Mϕs than tumours from wild-type animals. However, tumours from DKO mice contained significantly less Mϕs than those from *Mmp8* KO mice.

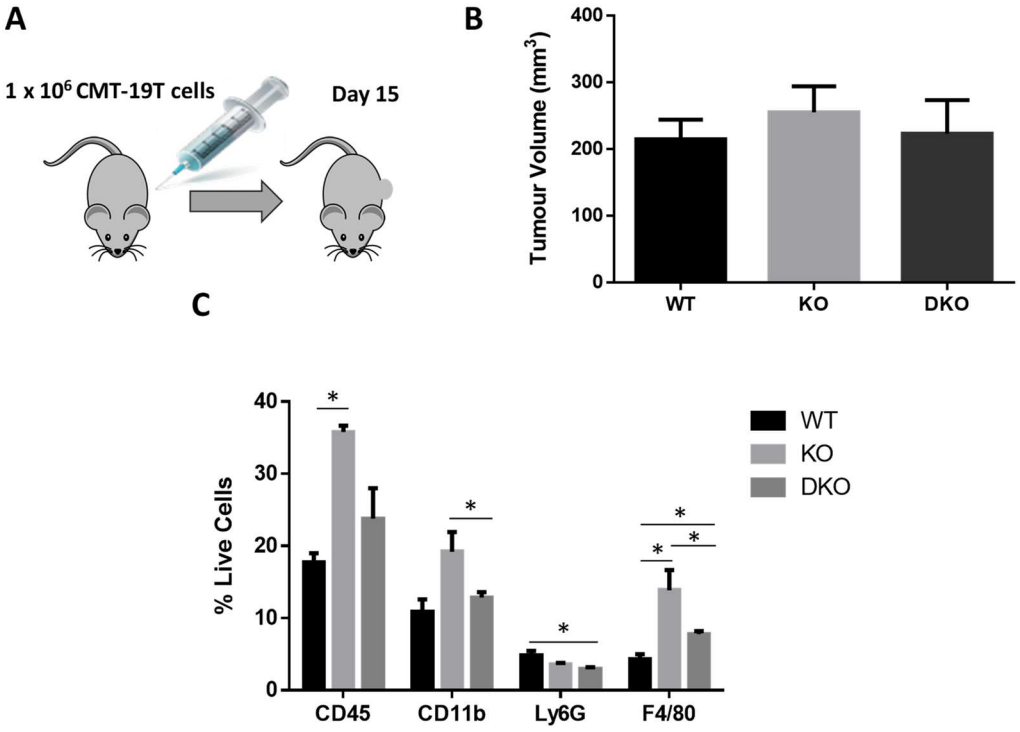


Figure 4.7. Subcutaneous tumour volumes in wild-type, *Mmp8* KO and DKO mice. A) Schematic of experimental design: 1 x 10^6 CMT-19T lung carcinoma cells were subcutaneously injected into male and female mice. **B)** Tumour volume measurements (mm^3) after excision on day 15. N ≥ 5, presented as mean ±SEM. **C)** Myeloid populations from flow cytometric analysis of intra-tumoural immune infiltrates. Data are presented as a mean percentage of total live cells ± SEM. *FDR-adjusted p value<0.05.

4.8. Discussion

In Chapter 3, little evidence was found that supported the hypothesis that MMP-8 inhibits primary BC tumour growth *via* orchestration of the immune system, finding no differences in tumour volume or intra-tumoural immune populations. However, these findings were caveated by the discovery of a loss of function *Casp11* mutation in the DKO mice. To rule out that this impacted discovery of a phenotype, tumour growth assays and phenotypic analyses were repeated in *Mmp8* KO mice containing functional *Casp11*.

To begin to disentangle the contribution that lack of caspase-11 may have made in the DKO model, it was important to acquire mice with a functional *Casp11* gene. Despite extensive backcrossing for at least 10-20 generations, the passenger mutation could not be eradicated, and so carrying out further backcrosses did not seem a sensible option and would have taken a significant amount of time. Through extensive breeding and good fortune, a group in Ghent, The Netherlands, had managed to produce *Mmp8* KO mice devoid of the *Casp11* passenger mutation, and kindly sent mice to enable the establishment of a new colony of *Mmp8* KO mice. In this chapter, these mice without the passenger mutation are referred to as *Mmp8* KO, and mice that contain it are still referred to as DKO as in Chapter 3. .

To confirm that the *Casp11* gene was now functional in the *Mmp8* KO mice colony, PCR and sequencing was carried out to ensure there was no longer a 5bp deletion in the *Casp11* gene. The results reiterated that the *Casp11* gene product was of the correct size for the functional gene and the passenger mutation no longer existed. Furthermore, *Mmp8* homozygote status was confirmed using PCR.

After confirming the colony were *Mmp8* KO but not *Casp11* deficient, investigation was carried out to determine whether the passenger mutation contributed to the lack of phenotype. The central finding in the DKO mice was that the loss of MMP-8 did not affect primary BC growth and nor were there any consistent immunological changes within the tumours. However, all findings in Chapter 3. were caveated by the passenger mutation. It was therefore important to rule out through repeat

experiments without the passenger mutation, that it could be the contribution of the passenger mutation that somehow masked the true function of MMP-8 in tumourigenesis.

Whilst there is no literature to suggest that caspase-11 influences cancer cells, if any changes did occur to other cell populations or cellular processes in its absence, they may indirectly impact tumour growth and progression. Therefore, B6BO1 tumour growth experiments were repeated in *Mmp8* KO mice. As before in DKO mice, no significant changes to primary tumour volume were observed. If MMP-8 was tumour-suppressive as initially hypothesised, an increase in tumour volume would have been anticipated in the absence of MMP-8. Since this did not occur in either the presence or absence of the *Casp11* passenger mutation, it can be said with confidence that the passenger mutation was not responsible for a lack of growth phenotype.

Nevertheless, there was an expectancy that *Casp11* status might impact immune populations considering its role in inflammatory cell processes, particularly in immune cell death. In the DKO animals, at Days 13 and 18, no differences were observed in intra-tumoural immune populations. However, at Day 15 reduced numbers of intra-tumoural Mϕs were found. Conversely, in the absence of the passenger mutation, *Mmp8* KO mice have no differences in leukocyte, myeloid or neutrophil numbers at any of the time points. Therefore, since the differences in immune cell populations observed in the DKO animals are not reproduced or expanded upon in the *Mmp8* KO mice, it is likely that MMP-8 does not influence intra-tumoural immune populations. Instead, it appears that the significant reduction in Mϕs observed in Day 15 in DKO mice was a consequence of the *Casp11* passenger mutation, and that the immune compartment was somewhat altered in the presence of the *Casp11* passenger mutation. What is important to note here is that analysis of the M1/M2 populations was not carried out in *Mmp8* KO mice, as was performed in the DKO mice. Instead, a Live/Dead marker and Ly6C was used to delineate monocytes from Mϕs. Therefore, there may have been changes to the proportion of cells in each polarisation state in *Mmp8* KO mice, however, work later in this thesis proposes this is not the case.

One other point of consistency is the continued inability to detect neutrophils in B6BO1 tumours. Two primary concerns to address were whether cell death occurred during preparation, or whether Ly6G, the cell surface marker on neutrophils, had been removed. There is evidence to suggest that cells are vulnerable to cell surface receptor cleavage *via* enzymatic digestion [371]. For tissues such as tumours, or fibrous organs like skin, mechanical disruption is not sufficient to liberate all cells [372]. Therefore, enzymatic digestion is necessary. Most protocols utilise collagenase enzymes, and in these experiments, collagenase type IV was used. However, enzymes by their nature will also recognise cleavage sites on cell surface receptors that may result in their removal or alteration. Some receptors will be more sensitive to this. Collagenase D is known to induce the expression of CD11c, F4/80 and MHC Class I on splenocytes for example [373]. Additionally, trypsin can cleave and remove CD4 on dendritic cells [374]. Collagenase IV is devoid of tryptic activity, and whilst there is no evidence to suggest that it can cleave the epitopes studied within the panel utilised here, it cannot be ruled out. Furthermore, cell viability could be jeopardised if enzyme concentration is too high.

To address these issues, additional data collected both by us and by other groups was inspected. Firstly, an alternative tumour type was analysed in an attempt to identify $Ly6G^{+}$ populations. In CMT-19T subcutaneous tumours digested with collagenase IV as in B6BO1 tumours, an abundance of neutrophils was detected, suggesting that collagenase does not affect neutrophil viability or Ly6G expression. Additionally, Kirkup *et al*. found an absence of Ly6G transcripts in RNA-seq data from B6BO1 tumours excised 15 days post-injection, without collagenase digestion [375] . Therefore, there is a high degree of confidence that B6BO1 tumours do not contain neutrophils.

The lack of neutrophils in this model may be of relevance to the absence of phenotype observed since MMP-8, or neutrophil collagenase, has strong ties to neutrophil function. MMP-8 mediates neutrophil chemotaxis through the IL-6/IL-8 signalling axes as well as facilitating timely neutrophil apoptosis [260,320]. Considering there are no neutrophils within the tumours, there may be no effect of

MMP-8 on the cytokines involved in this pathway since neutrophil efflux and influx does not appear to be occurring. Therefore, this is likely why again there is no change in *IL-6* mRNA or the three mouse homologues of IL-8: *CXCL1, CXCL2* or *CXCL5.*

The most interesting result from the B6BO1 tumours in Chapter 3. was the differential expression of lipid metabolism genes, with trending increases in *Acsl1* mRNA and significant reductions in *Plin1* mRNA in DKO mice. To exclude any influence of the passenger mutation on these findings, once again RNA was harvested from the same B6BO1 tumours harboured by *Mmp8* KO mice and compared to those from wild-type controls. The effects on lipid metabolism gene expression were not as pronounced in *Mmp8* KO tumours when compared to the DKO animals, with none of the lipid metabolism genes significantly differentially expressed at any of the days studied. However, a trend of *Plin1* downregulation was observed at all time points in the *Mmp8* KO tumours and this approached significance at Day 15. Due to colony limitations, the number of mice utilised previously could not be replicated which may have impacted on observing the repeat pattern. It does however appear that it is MMP-8 and not caspase-11 that is responsible for the phenotype of reduced *Plin1* expression. There are several interesting avenues to explore regarding the link between perilipin-1 AND MMP-8, including several studies that suggest that perilipin-1 is itself tumour-inhibitory in BC [376].This will discussed in greater detail in 6.6 but in brief, decreased *Plin1* expression in the absence of *Mmp8* may be indicative of a tumour-suppressive lipid web involving MMP-8. There were many more genes that were highlighted as being differentially regulated in the original RNA-seq data set, and these would be interesting to examine.

In experimental BC studies, it is recognised that cell line properties including hormone receptor status and intrinsic subtype influence cell behaviour and tumour progression when orthotopically injected into mice [377]. The B6BO1 tumours used thus far are ER+PR+ cells and may behave differently to cells with an alternative receptor status. In Chapter 3. , another cell line with a different receptor status and a more aggressive subtype was utilised: basal-like $ER^{+}PR^{-}$ E0771 cells. This helped to exclude cell-type specific effects in the absence of *Mmp8*. No effect on tumour

progression or intra-tumoural immune infiltrates was observed in DKO mice. However, for the final time it was important to rule out any impact of the passenger mutation.

In *Mmp8* KO mice, an almost identical pattern in E0771 tumour progression with wild-type mice was observed (data not shown). There were no differences in final tumour volume in two separate experiments that ran for 19 or 25 days. This suggests that the lack of tumour phenotype seen in DKO and *Mmp8* KO mice is not exclusive to B6BO1s, is not influenced by the passenger mutation and is not time point-dependent. Furthermore, the intratumoural immune compartment was probed for myeloid population changes and no significant differences were found at either Day 19 or Day 25. At Day 25 there was a trend in reduced Mϕs, the phenotype seen at Day 15 in B6BO1 tumours in DKO mice, however this was not significant.

And finally, since primary BC growth was unaffected by loss of MMP-8, a different model of cancer was utilised. The CMT19T mouse lung carcinoma cell line is used by many labs to study angiogenesis, is known to metastasise to the lung and can be injected subcutaneously to measure rudimentary tumour growth [378,379]. Using all three genotypes in our possession, tumour volume and intra-tumoural immune infiltration was examined at Day 15. Tumour volume remained consistent at on average 200 mm^3 between all genotypes, suggesting MMP-8 does not impact CMT19T carcinoma growth. However, upon probing the intra-tumoural immune compartment, several population changes were observed between genotypes. The most changes that occurred were in the Mϕ population. A significant increase in Mϕ number was observed in tumours from both *Mmp8* KO and DKO mice in comparison to those from wild-type controls. What is of more interest however, is the significant reduction in Mϕs in DKO tumours compared to *Mmp8* KO tumours. In each immune population analysed, both *Mmp8* null models do not behave in the same way. For example, in *Mmp8* KO mouse tumours, there were significantly more leukocytes than wild-type controls, but this is not true for DKO mouse tumours. Similarly, DKO mice have significantly reduced intra-tumoural neutrophils, and whilst there is a trend in tumours from *Mmp8* KO mice, this is not significant. Taken together, these

inconsistent immune changes suggest that *Casp11* deficiency may play a role in the orchestration of immune cell populations in the absence of MMP-8, despite not impacting tumour volume. Furthermore, the increases observed in leukocyte and Mϕ populations in tumours from *Mmp8* KO mice suggest MMP-8 alone does influence subcutaneous tumour immune populations.

This alternative cancer type is of interest in the study of MMP-8 since both sexes are used. This is in comparison to B6BO1 and E0771 mammary cancer studies where females were exclusively used. In skin carcinoma, absence of *Mmp8* increased tumour burden exclusively in male mice, whereas oestrogen provided protection to female mice [292]. Accounting for sex in this small cohort was only possible in tumour volume due to number restrictions, but there was no evidence of any trend. It would be interesting to repeat this experiment to rule out sex-mediated effects on intra-tumoural immune populations.

To summarise, through repeat experiments in *Mmp8* KO mice, any robust contribution of the absence of caspase-11 to the findings in DKO mice in Chapter 3 has been ruled out. The passenger mutation does not explain why despite previous literature suggesting a tumour-inhibitory role for MMP-8, is not the case in the orthotopic tumour model. The data presented here suggests MMP-8 does not influence BC primary tumour growth, does not orchestrate the immune system within the tumour or impact on IL-6 or IL-8 levels within the tumour. However, an interesting link has been discovered between *Mmp8* and *perilipin-1*, a tumour-suppressive lipid metabolism gene, which may have implications in future work.

After ruling out the contribution of MMP-8 to primary BC growth, but observing some immune population changes in lung carcinoma tumours, it was pertinent to further examine immune compartments within *Mmp8* KO mice.

To summarise:

- It has been confirmed using molecular biology techniques that the entire mouse colony is *Mmp8* null with a functional *Casp11* gene

- MMP-8 does not inhibit primary BC growth in an orthotopic mouse model using two separate cell lines with differential hormone receptor statuses

- Absence of *Mmp8* does not influence intra-tumoural myeloid immune infiltrates

- Lipid metabolism gene expression is again impacted by the loss of *Mmp8* including a reduction in *perilipin-1* mRNA that was not confounded by the *Casp11* passenger mutation

- Loss of *Mmp8* does not impact subcutaneous lung carcinoma growth, but does influence myeloid immune populations within tumours

5. The influence of MMP-8 on innate immunity

Whilst there is a wealth of information pertaining to the role of MMP-8 in tumourigenesis, there have also been numerous clinical and experimental observations linking MMP-8 with inflammatory disorders.

The alternative name for MMP-8 is neutrophil collagenase, and this title reflects the functionality of the enzyme. Firstly, as a collagenase, MMP-8 can cleave triple helical collagen fibrils, utilised in remodelling of the ECM. And secondly, in reference to the 'neutrophil' nomenclature, originally, neutrophils were thought to be the sole source of MMP-8; storing it as an inactive proenzyme in specific granules. However, it is now recognised that cells belonging to both the adaptive and innate immune system, including plasma cells, T cells, neutrophils and Mϕs, are also producers [380–383].

Perhaps owing to the contribution of these diverse immune cell types, MMP-8 is implicated in a wide range of inflammatory diseases including arthritis, sepsis and periodontal disease [310,384–386]. The latter has been extensively studied, and MMP-8 has even been targeted using the broad-spectrum MMP inhibitor doxycycline [314].

In general, high levels of MMP-8 are associated with progression of inflammation, however, it is not as simple as to say that MMP-8 solely drives pathophysiology, since it also aids in the resolution of inflammation [385]. Although, as a caveat to studies linking MMP-8 to the immune system, most animal studies utilised the knockout mouse model that is highly likely to contain the *Casp11* passenger mutation. One example of this is in in the study of wound healing [298]. During the process of wound healing, the inflammatory response following tissue injury is essential. MMP-8 has been shown to be nonredundant in skin wound closure, in part due to driving neutrophil infiltration, but also at later stages through resolving inflammation to allow the healing process to continue. Since cancer is often described as 'a wound that never heals', there may well be parallels to be drawn between the underlying mechanisms, that could implicate MMP-8.

This chapter will summarise the impact of MMP-8 on immune cell biology both in homeostatic immunity, and in the context of cancer, to further probe the link between MMP-8 and the immune system.

5.1. Bone marrow monocytes can be differentiated into an almost pure population of macrophages

Macrophages are the most abundant immune cell type present in B6B01 and E0771 mammary tumours, as determined using flow cytometry in Chapters 3. and 4. . A useful *ex vivo* model exists to study BMM in culture, where upon plating mouse bone marrow, monocytes strongly adhere to the culture surface whilst other cells such as dendritic cells and neutrophils remain free-floating. Upon the addition of M-CSF, over 7 days, monocytes differentiate into mature macrophages and can be subsequently utilised for phenotypic studies including stimulation with IL-4 or LPS, subjected to flow cytometry or lysed to quantitatively measure protein or RNA (Figure 5.1A) [387].

To determine whether loss of MMP-8 impacts monocyte to macrophage differentiation, bone marrow was obtained from *Mmp8* KO mice and the percentage of macrophages obtained after 7 days was compared to wild-type bone marrow.

On Day 7, BMM were detached and stained for CD11b and F4/80. Double positive cells represent macrophages. Upon flow cytometric analysis, double positive $CD11b^{+}F4/80^{+}$ macrophages were gated on and >98% of cells were found to be BMM in both genotypes, suggesting an almost pure population of macrophages (Figure 5.1B). To account for spread of fluorochromes into the other channel, fluorescence minus one controls (FMOs) were also run consisting of each stain individually. There was no FITC signal in APC-labelled cells and no APC signal in FITC-labelled cells. Similarly, neither APC or FITC signal can detected in unstained cells (US) which suggests positive and negative gates were set correctly, and any signal observed is due to autofluorescence.

The remaining <2% of cells could have been monocytes or dendritic cells, since these are also present within bone marrow cultures.

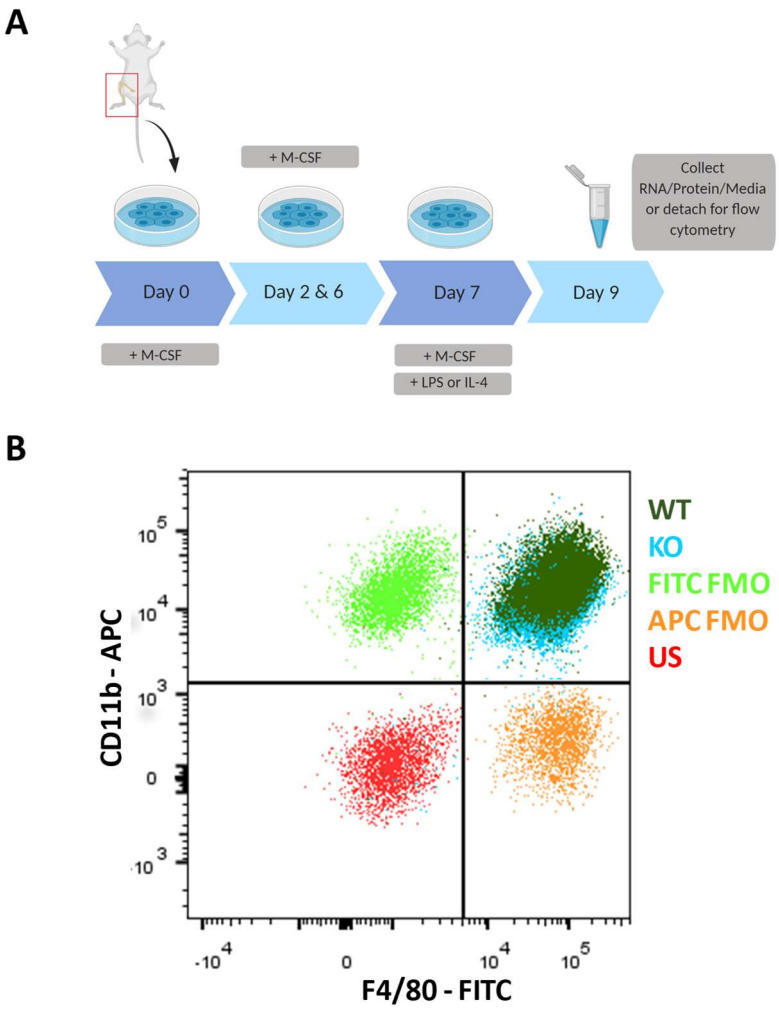


Figure 5.1. Differentiation into bone marrow derived macrophages. A) Experimental design removing mouse bone marrow from the femur and tibia, subsequent stimulation with M-CSF and polarisation with LPS or IL-4 before collection. B) Flow cytometric analysis of CD11b and F4/80 to confirm BMM purity in wild-type and *Mmp8* KO mice.

5.2. The absence of *Mmp8* does not impact M1 or M2 polarisation in bone marrow-derived macrophages

Bone marrow monocytes can be differentiated into bone marrow-derived macrophages (BMM) in culture, and subsequently polarised into 'M1 pro-inflammatory' or 'M2 anti-inflammatory' phenotypes. Other groups have shown that MMP-8 drives the polarisation of Mϕs towards an M2 anti-inflammatory phenotype [326]. To investigate whether absence of MMP-8 influences Mϕ polarisation in either direction, levels of M1 and M2 markers from polarised and unstimulated BMM were investigated from *Mmp8* KO, DKO and wild-type mice.

Monocytes derived from pooled mouse bone marrow were cultured with macrophage colony-stimulating factor (M-CSF) for 7 days where they became an almost pure population of Mϕs (98%). Next, they were subjected to molecules known to induce polarisation towards two ends of a spectrum known as: M1 (lipopolysaccharide (LPS)), M2 (IL-4) or left unstimulated for 48 hours. Cell lysates were Western blotted for M1 and M2 markers: iNOS and arginase-1 respectively to ascertain polarisation state (Figure 5.2A). There was no evidence of iNOS or arginase-1 expression in unstimulated BMM from all genotypes. Arginase-1 could be detected in lysates from all cells stimulated with IL-4, with similar expression across genotypes. iNOS was detected in lysates from all LPS-stimulated cells, again at similar levels across the genotypes.

In order to study short-term depletion of MMP-8 and caspase-11 in wild-type BMDM, siRNA transfection was carried out. BMDM treated with either MMP-8, caspase-11 or non-targeting control siRNA were stimulated and polarised in identical conditions as above. Lysates of cells that were stimulated with LPS were Western blotted for the 'M1' marker iNOS and MMP8 (Figure 5.2B) and blots of those stimulated with IL-4 were probed for the 'M2' marker Arginase-1 (Figure 5.2C). In cells treated with MMP-8 siRNA, reduced levels of iNOS were detected compared to all other conditions except for BMDM derived from *Mmp8* null mice. In cells polarised towards the 'M2' end of the spectrum, treatment with siRNA increased amounts of arginase-1. This effect appeared to be siRNA specific since the non-targeting control siRNA-treated cells also exhibited this phenomenon.

Knockdown efficiency was confirmed at the RNA level using qRT-PCR for both MMP-8 (Figure 5.2D) and caspase-11 (Figure 5.2E), and compared to levels in wild-type and non-targeting control siRNA-treated cells.

These data indicate that MMP-8 does not influence polarisation of BMM in culture, even in the presence of the *Casp11* passenger mutation.

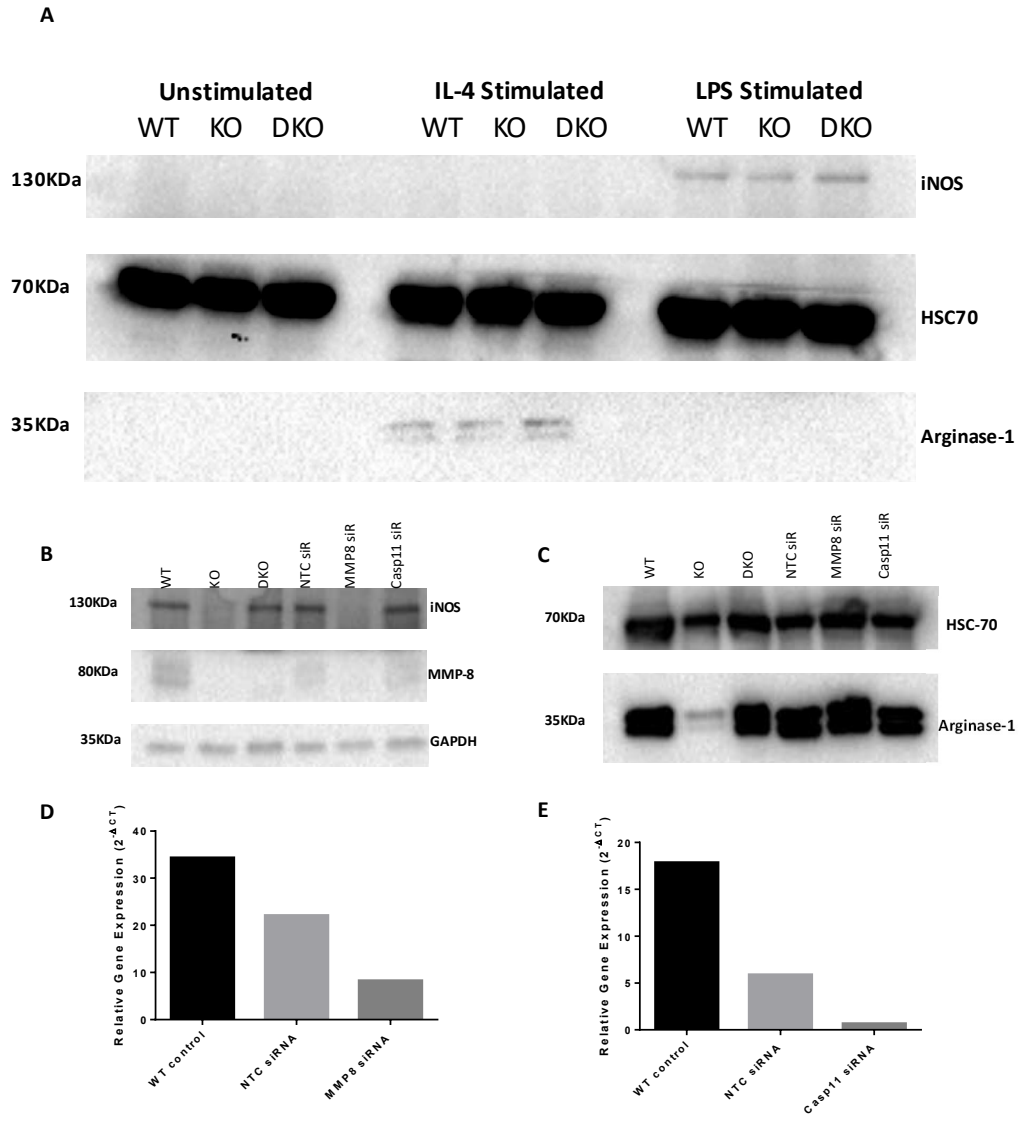


Figure 5.2. Macrophage polarisation marker expression in bone marrow-derived macrophages from *Mmp8* KO, DKO and wild-type control mice. A) Lysates from BMM either unstimulated, stimulated with LPS or IL-4, were run on a Western blot and probed for macrophage polarisation markers: iNOS (M1) and Arginase-1 (M2). Heat shock protein 70 was used as a loading control. Each sample = 2 mice pooled. B) BMM from WT, *Mmp8* KO and DKO mice, and siRNA-treated WT BMM were stimulated with LPS, lysates were run on a Western blot and probed for the M1 polarisation marker: iNOS (M1) and MMP-8. C) BMM from WT, *Mmp8* KO and DKO mice, and siRNA-treated WT BMM were stimulated with IL-4, lysates were run on a Western blot and probed for M2 polarisation marker Arginase-1 (M2). Heat shock protein 70 and GAPDH were used as a loading control. D&E) mRNA levels in siRNA-treated BMM of D) *Mmp8* and E) *Casp11.*

5.3. IL-6 expression is consistent between *Mmp8* KO and wild-type mice in co-cultures of B6BO1s and bone marrow-derived macrophages

Previous literature has indicated that overexpression of *Mmp8* leads to upregulation of *IL-6* in cultured BC cells [296]. Therefore, whether this occurred within the TME in B6BO1 mammary tumours, and if absence of MMP-8 led to opposite effects on IL-6 production was investigated.

To replicate this interaction *in vitro*, B6BO1 mammary cancer cells were co-cultured with unstimulated BMM either directly or separated by a 0.4 µM transwell insert. The amount of IL-6 was quantified in the media after 48 hours and relative *IL-6* mRNA levels were analysed.

Upon direct co-culture of B6BO1s with BMM from both wild-type and *Mmp8* KO mice, no significant difference was observed in media IL-6 levels between genotypes as measured by ELISA. This was also reflected at the RNA level, as the expression of *IL-6* was not significantly different between genotypes. (Figure 5.3A&B).

Whilst these results suggest that the loss of *Mmp8* in BMMs does not impact priming of inflammatory immune responses in response to tumour derived Damage Associated Molecular Patterns (DAMPs), technical limitations prevent a definitive answer to this question. Using co-cultures is a useful method to obtain information on how cells interact with one another, however since the population is homogeneous, it is difficult to determine gene expression for each cell type. To resolve differences in either Mϕs or B6BO1s definitively and to determine the impact of soluble factors in cross-talk, transwells were employed. BMM were seeded into 6-well plates underneath transwells containing B6BO1s. Culture media and RNA was collected from each separate compartment and used for IL-6 protein and gene analysis. Media from BMM-containing wells had an IL-6 concentration of 60-70 pg/mL and B6BO1 containing wells was between 30-40 pg/mL (Figure 5.3C). Relative mRNA levels of *IL-6* were not statistically different from B6BO1s or BMM regardless of BMM origin genotype, though there was a trend for reduced levels of IL-6 in B6BO1s from *Mmp8* KO mice (Figure 5.3D).

One other observation of note was the detection of *Mmp8* in B6BO1 cells in transwells above Mϕs at the RNA level (Figure 5.3E). Whilst the C_T value was high at between 35-37, in comparison to approximately 25 in co-cultures, this finding could be of significance in interpreting the effects upon loss of *Mmp8,* if *Mmp8* is indeed present.

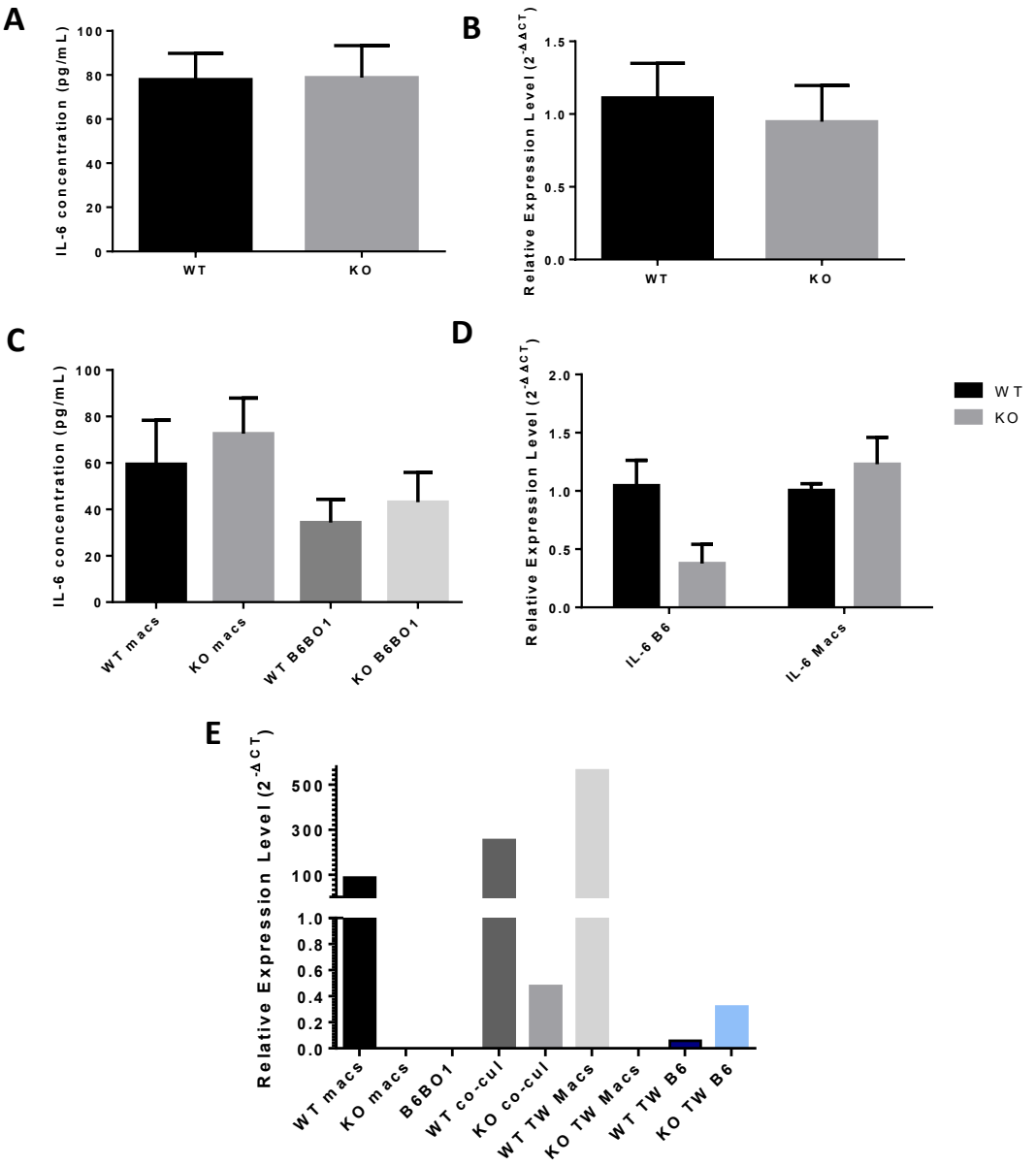


Figure 5.3. Media concentration and mRNA levels of IL-6 and MMP-8 in co-cultures of B6BO1 and bone marrow-derived macrophages after 48 hours. A&B) IL-6 quantities in direct co-cultures A) in the media as measured by ELISA and B) at the mRNA level. C&D) IL-6 quantities in each compartment of transwell co-cultures E) Expression of *Mmp8* as measured by qRT-PCR in direct co-cultures within each compartment of transwell co-cultures.

5.4. Unchallenged *Mmp8* KO mice have a reduced percentage of bone marrow neutrophils

As consistent changes in intra-tumoural immune populations could not be detected in the absence of MMP-8, changes in immune organ populations were instead pursued. There is a fine balance struck between exit of leukocytes into the periphery and production in the bone marrow – the site of haematopoiesis [388,389]. Differing levels of immune populations can reflect the state of the host including presence of disease or neoplasm.

The immune populations were analysed in the bone marrow of *Mmp8* KO and wild-type mice to ascertain whether MMP-8 drives changes in immune landscape.

First, bone marrow was isolated from healthy mice from both genotypes, 6-8 weeks of age and the cells were subjected to flow cytometric analysis. The cells were stained with an identical panel to that used in the mammary and subcutaneous tumours and it was found that the bone marrow was predominantly composed of myeloid cells in wild-type mice, representing 65% of live cells (Figure 5.4A). However, in *Mmp8* KO mice there were significantly less myeloid cells, accounting for 45% of the total cells. This difference within the $CD11b^+$ myeloid population was not reflected in the number of monocytes since this was almost identical between genotypes, representing less than 10% of total cells. Neutrophils made the largest contribution to the myeloid compartment, representing 40% of all cells in wild-type bone marrow. However, in *Mmp8* KO mice, less than 30% of the bone marrow was composed of neutrophils, significantly less than their wild-type counterparts.

Some phenotypes observed in *Mmp8* null mice have been attributed to sex, with female sex hormones thought to play a role [292,293,305]. An investigation into whether some immune cell population differences were influenced by sex was carried out by comparing female vs male mice from both genotypes. Whilst a more pronounced reduction in myeloid cells and neutrophils was found between *Mmp8* KO and wild-type males than with females (Figure 5.4B), no significant differences between sexes within the same genotype were found. No differences between monocyte populations were observed when accounting for gender.

Based on our observations that unchallenged *Mmp8* KO mice have a reduced number of bone marrow myeloid cells and neutrophils, mice bearing tumours were also analysed to see whether the immune compartment was altered during disease (Figure 5.4C).

Within both genotypes, mice bearing tumours had more immune cells in general, and significantly more myeloid cells. This effect was more pronounced in wild-type animals. Wild-type tumour-bearing mice had significantly more myeloid cells and neutrophils than their non-tumour bearing counterparts. *Mmp8* KO mice bearing tumours had significantly more myeloid cells than null mice without tumours, and whilst there was a trend for increased neutrophils, this was not significant.

Looking between genotypes, a trend was observed in reduced myeloid cells and neutrophils in *Mmp8* KO mice without tumours within this experiment. However, upon pooling these data with the previous unchallenged mice, a significant decrease in myeloid cells and neutrophils was observed in *Mmp8* KO mice without tumours (Figure 5.4D).

The gating strategy used to analyse stained cells alongside percentages of each parent gate can be found in Figure 5.4E.

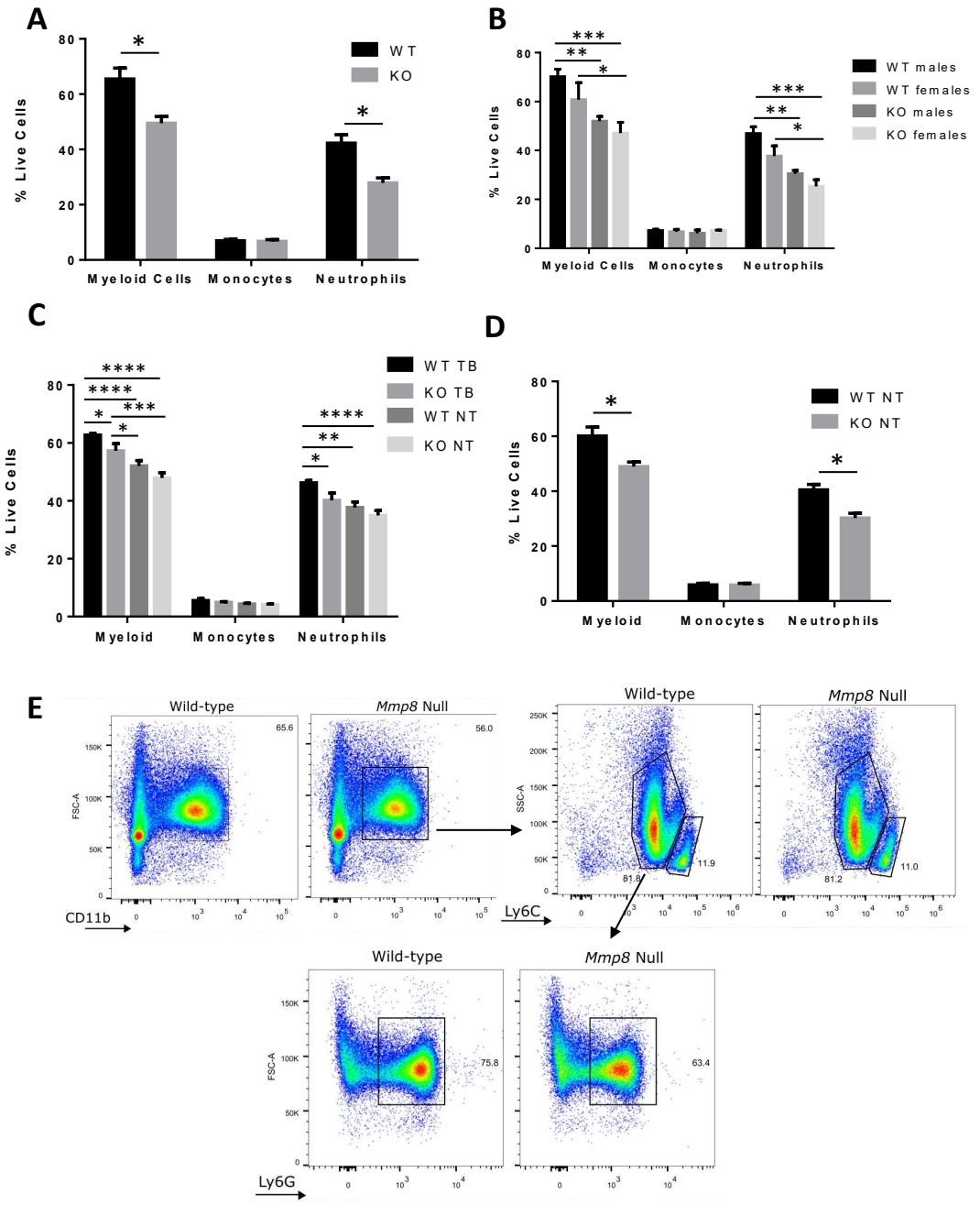


Figure 5.4. Flow cytometric analysis of bone marrow populations. A&B) Bone marrow myeloid cell populations in healthy mice from A) both sexes combined and B) individual sexes. C) Bone marrow populations in tumour-bearing *vs* non-tumour-bearing mice. D) Pooled data from two independent experiments on non-tumour bearing mice. E) Flow cytometric gating strategy for bone marrow populations including $CD11b^+$, $Ly6C^+$ and $Ly6G^+$ cells. Representative plots from both genotypes as found in A. TB = tumour-bearing, NT = non-tumour-bearing. Data is presented as a mean percentage of total live cells ± SEM. *FDR adjusted p value (A) or Holm-Šídák adjusted p-value (B&E). *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001.

5.5. The splenocyte T cell population is not affected by the absence of *Mmp8*

To complement the study of the bone marrow as a primary lymphoid organ, the spleen was also studied as a crucial secondary lymphoid organ whereby circulating T and B cells search for their cognate antigens [390].

Whilst the majority of MMP-8 is myeloid cell-derived, T-cells do also contribute small amounts. Therefore, to investigate whether absence of *Mmp8* impacted lymphoid immune populations, the spleen was harvested from the same animals used for bone marrow analysis. Subsequently the splenocytes were subjected to flow cytometry with an antibody panel designed to look at T cell populations (Figure 5.5A). In the spleen, T cells accounted for approximately 30% of the cells, staining positive for the pan-T cell marker CD3. Within that population, approximately half were $CD4^+$ T helper cells and the other half were $CD8^+$ T cytotoxic cells.

The effect of sex of the animal was explored by categorising data into male and female populations within each genotype (Figure 5.5B). No significant difference in splenocyte populations were detected both dependent or independent of sex. The gating strategy used to analyse stained cells alongside percentages of each parent gate can be found in Figure 5.5C.

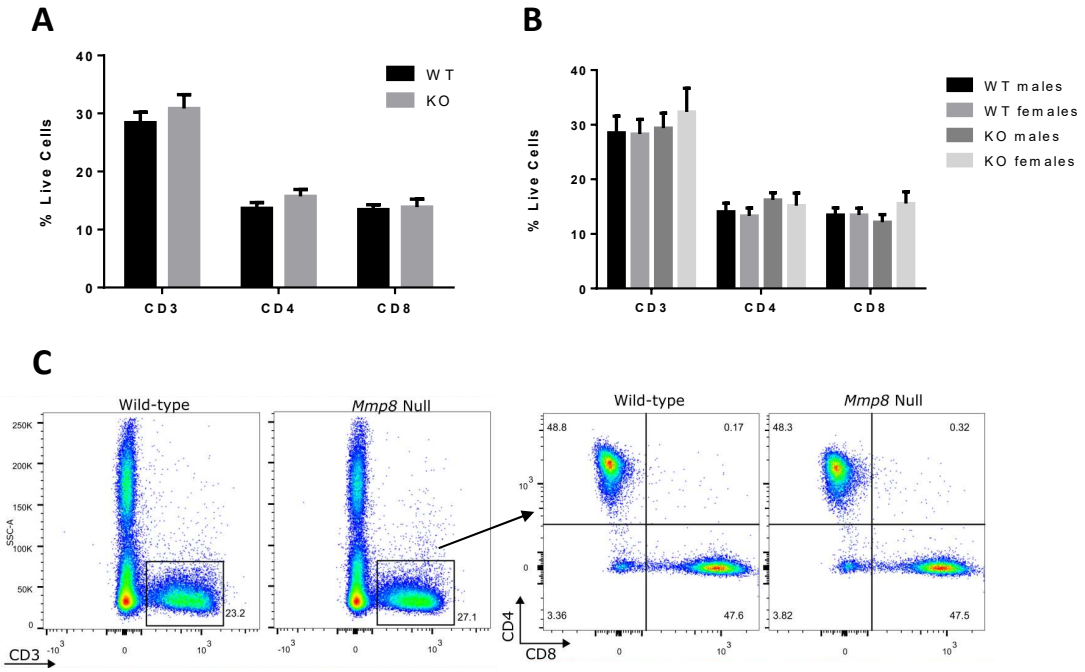


Figure 5.5. Flow cytometric analysis of spleen populations. A&B) Spleen populations in healthy mice from A) both sexes combined and B) individual sexes. C) Flow cytometric gating strategy for splenic populations including $CD3^+$, $CD4^+$ and $CD8^+$ cells. Data is presented as a mean percentage of total live cells ± SEM.

5.6. Discussion

Throughout Chapters 3 and 4, evidence was provided contrary to the hypothesis that MMP-8 inhibits tumour growth through orchestration of the immune system, since no changes were detected in tumour volume in the absence of *Mmp8*, nor consistent immunological changes. The approach taken was to solely investigate the tumour itself, and whilst MMP-8 had little impact in the context of the tumour, there may have been systemic effects of MMP-8. Two different approaches were taken to investigate the role of MMP-8 in both general and cancer immunity. Firstly, to look at how absence of MMP-8 affected the behaviour and phenotype of Mϕs both with inflammatory stimuli and under the influence of cancer cells. And secondly, analysing population level changes in immune organs, additionally searching for potential sex differences.

The focus on Mϕs was for three reasons: 1. They were the most abundant intra-tumoural immune cell. 2. The only immunological change detected in earlier chapters was reduced numbers of intra-tumoural Mϕs and 3. Mϕs can be easily manipulated *in vitro*.

The culture of BMM is a well-established and reproducible technique used to study gene expression, behaviour and many more parameters upon application of various stimuli [387,391]. Since bone marrow-derived monocytes are extremely adherent, they preferentially stick to culture surfaces, whereas the remaining bone marrow cells including dendritic cells and neutrophils float freely. This allows selective culture of monocytes and eventually the differentiation into BMM upon regular addition of the growth factor M-CSF. Adherent Mϕs usually make up over 90% of the population [387]. To confirm the differentiation protocol used in these studies was successful in obtaining an almost pure, homogenous population of Mϕs, the number of $CD11b^{+}F4/80^{+}$ Mϕs was quantified using flow cytometry. This confirmed that the population was almost pure with greater than 98% of cells expressing both markers. It was important at this stage to confirm that MMP-8 did not impact the differentiation of monocytes to Mϕs before carrying out downstream experiments, hence it was confirmed that genotype did not impact the purity obtained.

Mϕs are extremely plastic cells and respond to cues to modulate the immune system, maintain homeostasis and participate in tissue repair [392]. Subsequently, they can adjust and change phenotype including towards each end of a spectrum of polarisation.

There is some debate as to whether the phenotypes and markers associated with in vitro polarisation are definitive and can recapitulated *in vivo* [158]. However, Mϕ polarisation is routinely analysed *in vivo* via flow cytometry for the levels of MHCII and CD206 representing M1 and M2 Mϕs respectively [393]. This analysis was carried out in B6BO1 tumours in Chapter 3 where evidence was found for both populations but no differences between genotypes at any of the three timepoints. However, this was in the context of the tumour itself and to examine the effects of MMP-8 on Mϕ polarisation more generally, the focus was shifted to key immune organs in both tumour-bearing and non-tumour-bearing animals.

There are several lines of evidence that MMPs influence Mϕ polarisation, including MMP-7 and -28. The absence of *Mmp7* resulted in preference to M1 phenotype and absence of MMP-28 impaired M2 polarisation, suggestive that both are important in M2 polarisation. Wen *et al*. demonstrated that MMP-8 influences M2 polarisation. Using BMM derived from *Apolipoprotein-E (Apo-E)*/*Mmp8* null mice, they found that in the absence of *Mmp8*, IL-4 was unable to stimulate differentiation into the M2 phenotype – suggesting MMP-8 drives M2 polarisation [326]. They found this was related to the ability of MMP-8 to cleave fibromodulin to release sequestered TGF-β – an M2 inducer. However, other groups have shown contrasting data linking MMP-8 to TGF-β levels in BC cells. Soria-Valles *et al*. found that MMP-8 is involved in a signalling nexus with decorin and *miR-21* to reduce TGFβ levels, subsequently inhibiting BC growth and metastasis [327].

Unfortunately, the work by Wen *et al*. is likely to be subject to the same caveat as work carried out in Chapter 3. . They used the B6.129X1-MMP8^{tm1Otin}/J strain derived from the SV129 strain known to contain the *Casp11* passenger mutation and crossed it onto an *ApoE*$^{-/-}$ background.

These studies had the privilege to use mice both with (*Mmp8* KO mice) and without the *Casp11* passenger mutation (DKO mice) to investigate whether the absence of

Casp11 contributed to the Mϕ polarisation phenotype. BMM were stimulated with LPS or IL-4 and the levels of iNOS representative of M1 and arginase-1 representative of M2 Mϕs were quantified by Western blot. BMM derived from wild-type, *Mmp8* KO and DKO mice could all be polarised to the M1 or M2 phenotype, with no differences in the amount of iNOS or arginase-1 present. There was an attempt to isolate Mϕs from within tumours using fluorescent-activated cell sorting (FACS) to analyse marker expression at the RNA and protein level, but due to technical reasons this was not possible. Together, the combination of *in vitro* and intra-tumoural polarisation data indicates that MMP-8 is not vital in Mϕ polarisation, independent of *Casp11* status.

There were concerns that absence of *Casp11* could affect Mϕ polarisation by virtue of the fact that it acts as an LPS-binding receptor. It was hypothesised that loss of *Casp11* could prevent LPS-stimulation of Mϕs; however, it appears that this is not the case.

There are several explanations as to why we do not observe differences, when other groups do. Firstly, the mice utilised by Wen *et al.* are both *Mmp8* and *ApoE* null. There is evidence to suggest that apolipoprotein E induces the M2 anti-inflammatory Mϕ phenotype [394]. Therefore, it is difficult to disentangle that contribution from each component in this model. An attempt was made to correct for this using gene silencing techniques in the RAW264.7 Mϕ cell line or wild-type bone marrow. However, the use of viral vectors such as lentiviruses can trigger immunogenicity – particularly in Mϕs and may subsequently affect their response to challenge [395]. An attempt was made to knockdown MMP-8 in wild-type BMM using siRNA gene silencing, but upon stimulation with IL-4, enhanced expression of arginase-1 was observed in BMM both treated with non-targeting control siRNA and MMP-8 siRNA – suggesting an siRNA-induced effect on stimulation.

Secondly, whole-cell protein levels of M1 and M2 markers (iNOS and arginase-1) were quantified *via* Western blot. In contrast, Wen *et al.* confirmed polarisation through qRT-PCR looking at RNA levels of M1 genes (*Arg II, Mcp-1* and *Tnf-α*) or M2 genes (*Arg I, Cd163* and *Cd206*).

In Chapters 3. and 4. the link was made between MMP-8 and the cytokines IL-6 and IL-8, looking at gene expression levels within B6BO1 tumours. IL-6 is a relevant cytokine to study in BC since it is associated with stem cell maintenance, angiogenesis, cachexia and chemoresistance [396–399]. Since overexpression of *Mmp8* led to upregulation of IL-6 and IL-8 in human BC cells [320], a reduction in expression was anticipated in the absence of *Mmp8*. Whilst a reduced trend in IL-6 mRNA levels was observed at multiple time points, there was minimal effect on the three mouse homologues of *IL-8: CXCL1, CXCL2 and CXCL5*. Therefore, the next action was to attempt to find the cellular source of IL-6 within the TME. Two populations were chosen that were the most likely origins of the IL-6: the BC cells themselves or Mϕs and modelled what may occur using an *in vitro* culture. Through co-culturing, additional information was gleaned on cellular communication that may occur between these cell types – both directly and indirectly, through transwell assays.

Concurrent with the existing literature, IL-6 was detected in both B6BO1 BC cells and BMMs alone [109,400]. Upon co-culture, there were similar amounts of *IL-6* transcripts in both B6BO1 and BMMs but no significant change in the absence of *Mmp8* in BMMs. At the protein level, lower amounts of IL-6 were detected in cultured media from B6BO1 cells *vs*. BMM although this was not significant. In direct co-cultures of BMM and B6BO1 cells, the absence of *Mmp8* in Mϕs did not alter IL-6 levels at the RNA or protein level. Whilst this suggests MMP-8 itself does not influence IL-6 expression in Mϕs or B6BO1 cells, it does not rule out that MMP-8 has an impact on other components of the signalling pathway such as responsiveness to IL-6.

One final observation to discuss regarding the transwell assays is the detection of *Mmp8* transcripts in B6BO1-containg wells. Alone, B6BO1s do not express MMP-8 at the protein or RNA level as discovered in Chapter 3 and confirmed again in transwells. If soluble factors from Mϕs can induce the expression of *MMP-8* from B6BO1 cells, this may occur *in vivo* within tumours and could introduce MMP-8 into the system.

Whilst the *in vitro* and *ex vivo* Mϕ experiments did not reveal a role for MMP-8 in Mϕ activation or IL-6 levels, whether MMP-8 had a broader effect on the immune

system was considered. How absence of *Mmp8* impacted immune populations was explored in two immune organs: the spleen and the bone marrow.

The effects of MMPs are most often considered in the local TME. However, tissue MMPs can enter the bloodstream, and efficient systemic immunity is vital in controlling neoplasms. In inflammatory BC, low plasma levels of MMP-8 are found, suggestive of high levels within the tumour. Within the context of this aggressive cancer, MMP-8 appears to have a pro-tumourigenic role. In parallel to this, MMP-8 plasma levels were high in patients with moderate LN involvement and low in patients strongly disposed to metastasis.

Therefore, it was hypothesised that MMP-8 may have effects on systemic immunity in BC and could ultimately influence disease progression and spread.

The bone marrow is the site of adult haematopoiesis, responsible for generating all cells of the haemopoietic lineage. There is very little literature on absolute percentages of cells in the bone marrow. Most quantitative measures come from traditional bone marrow smears. Flow cytometry was utilised to analyse specific myeloid populations within the bone marrow. It was found that the bone marrow of healthy wild-type mice under steady-state conditions was constituted of 60% myeloid cells of which 40% were neutrophils and less than 5% were monocytes. Other myeloid cells probably included basophils and eosinophils however these populations were not stained for. Whilst there are little data to support bone marrow population findings, differential counts in mouse marrow smears found neutrophils to account for 38-40% of total cells [401]. In bone marrow from *Mmp8* KO mice, significantly reduced numbers of myeloid cells and neutrophils were found under steady-state conditions. Whilst this finding was not followed up on, there are several possible explanations and avenues to explore.

In mice, the bone marrow reserve of neutrophils is thought to be around 120 million cells, and at any one time there are greater than 2.5 million in circulation [402] The balance between production and mobilisation of neutrophils is a finely-tuned homeostatic process and relies heavily on both locally-produced cytokines and distant feedback mechanisms.

The bone marrow microenvironment supports retention of a pool of mature neutrophils via interaction between CXCR4 on the neutrophils themselves and its ligand, stromal-cell derived factor-1 (or CXCL12) [403]. Disruption to this receptor-ligand interaction encourages egress of cells from the bone marrow into the periphery. Several MMPs have demonstrated cleavage activity against CXCL12 including MMP-2, -8, -9 and -14, thus playing a role in regulation of chemokine gradients.

There are several other biological explanations for observing a reduced number of neutrophils: there is less production of neutrophils, more cells are being cleared through apoptosis or there is a shift in populations towards immature precursors.

If the latter explanation is correct, the reduced number of myeloid cells and neutrophils seen could in fact be an artefact of a shift in cell populations that do not express the markers that were stained for in these experiments. This could logically tie to an influence on mobilisation from the bone marrow, since increased mobilisation can cause what is referred to as 'left shift'. This is a process whereby upon depletion of bone marrow stores, myeloid hyperplasia can occur in response, resulting in irregular myelopoiesis and the production and release of immature cells [404]. In the bone marrow, myeloid progenitor and precursor cells do not express CD11b [405]. Therefore, immature cells would not have been included in the analysis. It is possible that MMP-8 leads to a mobilisation of neutrophils from the bone marrow, resulting in erratic production of immature cells in response. However, several groups have carried out WBC counts in the blood of *Mmp8* null mice and have found no significant differences, which suggests the mobilisation theory may not be responsible for the observed phenotype, unless these cells are being transported directly to target organs [308,406].

Another possible explanation for a reduced number of bone marrow neutrophils could be attributed to aberrant apoptosis. In several models of disease, the loss of MMP-8 has been associated with this phenomenon [298,305,308,312]. In the absence of MMP-8, the reduced number of neutrophils observed suggests MMP-8 suppresses neutrophil apoptosis, on the contrary to previous data.

It has been alluded to that incomplete collagen breakdown can lead to sustained signalling, since chemotactic signals are retained in the ECM [407]. Collagen is a major component of the ECM in bone marrow, and cleavage of collagen fibrils could lead to changes in the balance of apoptotic mechanisms, thus impacting upon the bone marrow neutrophil population [408]. For example, there is a collagen fragment called proline-glycine-proline or PGP, that is usually released by a neutrophil-derived enzyme called prolyl endopeptidase [409]. PGP, generated *de novo* from pro-collagen, is a pro-inflammatory molecule that acts as a potent neutrophil chemoattractant. MMP-8 has been shown to regulate neutrophil chemotaxis through generation of PGP from the corneal stroma under the influence of LPS [410]. MMP-8 may help to carve and maintain the bone marrow stromal landscape that results in cell population changes.

Whilst most observed effects of MMP-8 on the immune system are mediated by myeloid cells, there is some evidence to suggest a link between MMP-8 and lymphoid cells. MMP-8 has been detected in T-cells within the central nervous system and in plasma cells [380–382]. Furthermore, there is a relationship between serum levels of MMP-8 and peripheral lymphocytes in Down's syndrome patients with gingivitis, hypothesised to facilitate migration of CD8+ cytotoxic T cells and NK cells [411]. Therefore, the study of lymphoid populations in *Mmp8* KO mice was warranted, and to do this, the spleen was chosen since it is rich in lymphoid cells.

Flow cytometry was carried out using a basic T cell panel in the spleen of *Mmp8* KO and wild-type mice and no differences were found in populations between genotypes. Whilst there is no evidence to suggest MMP-8 plays a role in spleen biology, to our knowledge, we are the first group to look at splenocyte populations in *Mmp8* null mice and have confirmed at least within major T-cell populations, MMP-8 has no impact.

And finally, whether sex of the mouse had any impact on bone marrow and spleen immune populations was explored. In some disease models, the phenotypes observed in female *Mmp8* null mice were different to that in males. For example, in a model of arthritis, male *Mmp8* null mice had more severe joint swelling compared to their wild-type counterparts, but this was not the case in females [305].

Additionally, male *Mmp8* null mice had an increased incidence of carcinogen-induced skin tumours compared to wild-type mice, whereas *Mmp8* null female mice were protected from this increase [292].

Therefore, the bone marrow and spleen of male and female WT and *Mmp8* KO mice was chosen to investigate whether sex had any impact on bone marrow populations. Despite again finding a reduced number of myeloid cells and neutrophils in the bone marrow of *Mmp8* KO mice, no significant differences were found between sexes within the same genotype. This suggests that it is the absence of *Mmp8* that is responsible for this phenotype, with no male- or female- specific effect.

Whilst at least three groups have found sex-specific differences in *Mmp8* null mice, so far there has been no detailed mechanism described. Balbin *et al.* found that in the absence of *Mmp8*, female mice were protected from skin carcinoma through an oestrogen-dependent mechanism since ovariectomy or tamoxifen treatment abrogated this protection [292]. Korpi *et al*. delved further into this oestrogen and MMP-8 association in tongue carcinoma, revealing a role for MMP-8 in ERα and ERβ cleavage to enable dimerization and stabilisation of receptor complexes that promote activation of oestrogen signalling pathways [293]. Furthermore, oestrogen can induce the expression of *Mmp8*, thought to be related to the structure of the *Mmp8* gene promoter, which has a C/EBP element known to associate with ER-α [294].

Our results so far suggest that if there are sex-specific differences in *Mmp8* KO mice in inflammation-related diseases, this is most likely not due to changes in immune populations in the bone marrow or spleen.

In summary, we found that lack of *Mmp8* did not shape Mϕ phenotype upon LPS or IL-4 stimulation, nor impact IL-6 signalling in BC cell co-cultures. However, upon profiling of immune organs, despite finding no changes in splenocytes, significantly reduced neutrophil numbers were found in the bone marrow of both unchallenged and tumour-bearing mice, independent of sex. We believe these observations warrant further study.

To summarise:

- MMP-8 does not influence bone-marrow derived Mϕ differentiation or polarisation towards M1 and M2 phenotypes

- In direct or indirect (transwell) co-cultures of B6BO1 and BMM, the absence of *Mmp8* in BMM does not influence IL-6 protein or RNA levels

- Unchallenged *Mmp8* KO mice have reduced numbers of bone marrow myeloid cells and neutrophils compared to wild-type controls, and this trend exists in mice bearing orthotopic mammary tumours

- The number of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the spleen are not impacted by absence of *Mmp8*

6. Concluding Remarks and Future Work

The MMP family consists of many important players in tumour growth and metastasis. For the most part, MMPs promote tumourigenesis by enzymatically modulating the ECM to facilitate cell migration, invasion and survival. However, one member of this family: MMP-8, appears to play a contrasting role as a tumour-suppressive protease. Previous data has shown that high MMP-8 expression correlates with improved survival in BC patients, and loss of *Mmp8* enhanced metastasis in the MMTV-PyMT spontaneous BC mouse model [286,296]. Concurrently, other groups have demonstrated a link between MMP-8 and the immune system. MMP-8 is involved in both initialising and resolving inflammation through chemotaxis and apoptosis of innate immune cells. Furthermore, MMP-8 modulates TGF-β signalling; TGF-β being a major immunosuppressive molecule in the TME through polarising Mϕs and neutrophils towards pro-tumourigenic phenotypes [326,327,412]. By amalgamating these concepts, this thesis sought to investigate whether MMP-8 inhibits primary BC growth through orchestration of the immune system.

To investigate immunological changes at specific time points in tumour growth, as well as measuring tumour growth itself, the orthotopic injection model was utilised. Using this mouse model of human BC, there was insufficient evidence to suggest that the hypothesis that *Mmp8* orchestrates anti-tumour immunity was correct. At each of the three time points studied, absence of *Mmp8* did not increase tumour volume, and only changes in one immune population could be observed within the tumours: reduced Mϕs at Day 15.

So why does the tumour-inhibitory function of *Mmp8* in the MMTV-PyMT spontaneous model not manifest itself in this alternative orthotopic model of BC? Several possible explanations have been alluded to throughout Chapters 3 and 4, but to briefly summarise:

- tumour initiation vs tumour growth and progression are different processes
- the background strain used in animal models can hugely impact outcome

- littermate controls were not used in both models
- is there inducible expression of *Mmp8* in the orthotopic model that obscures the 'absence' of *Mmp8*?
- is there influence of a potential *Casp11* passenger mutation in the spontaneous model?

Each of these topics will be evaluated in turn throughout this chapter.

6.1. Tumour Initiation *vs* Tumour Growth

Whilst an increase in tumour volume would have been expected in the absence of *Mmp8*, there are some differences in this orthotopic model in comparison to the previous spontaneous model that may in part explain a lack of phenotype. Use of the MMTV-PyMT model is a well-established technique for studying tumour initiation since hyperplastic lesions develop spontaneously and progress without the need for exogenous stimuli. This model mimics the entire process of tumour initiation and growth into metastasis [295]. However, the orthotopic model utilised here does not mimic all stages of tumour growth, since normal cells do not become cancerous spontaneously. Instead, a mass of 100,000 cancer cells are introduced into the mammary gland in the presence of Matrigel which contains a plethora of growth factors and matrix components known to enhance tumour pathophysiology [413]. Therefore, this orthotopic model does not well represent tumour initiation and could explain a lack of tumour phenotype. This differentiation between initiation and progression has been pertinent before in skin carcinoma whereby it was specifically the amount of time to malignancy and the incidence of skin tumours and not tumour progression that was affected by the absence of *Mmp8* [292].

Furthermore, if tumour latency is important in observing a role for MMP-8, other mouse models could be considered. PyMT is just one of many oncogenes that when driven under the MMTV promoter can be used to model BC tumourigenesis. The MMTV-*neu* model for example has a mean latency of 100 days, almost double that of MMTV-PyMT mice, that develop tumours after 53 days on average [414,415].

Therefore, utilisation of this alternative model might allow earlier events in tumourigenesis to be unpicked.

6.2. Model and Background Strain

It is not only differences in tumour dynamics that may explain the lack of observed phenotype. Mouse genetics contribute hugely to experimental responses, particularly strain variations. Transgenic mouse models of BC are a prime example of this. Most MMTV-PyMT experiments are carried out on the FVB/N (FVB) background, and this is partly because similar signalling pathways are activated to that in humans, including those downstream from *erbB2*, an oncogene overexpressed in 30% human BCs [416]. This helps replicate the complex stages that occur during tumour progression. Furthermore, pulmonary metastases are predictable and the tumour latency period is relatively short [295].

However, whilst the MMTV-PyMT spontaneous BC model is extremely useful in identifying gene loci that contribute to tumourigenesis or metastasis, tumour responses to PyMT virus transfection do vary amongst inbred strains [417]. It has been hypothesised that the murine MHC gene locus (H-2) could be responsible for this [418]. Murine H-2 varies between strains, resulting in mice with diverse H-2 haplotypes [419]. FVB mice have a $H\text{-}2^{q}$ haplotype that is more susceptible to PyMT-induced tumourigenesis than C57Bl strains that are of a $H\text{-}2^{b}$ haplotype [420]. One group has shown that PyMT tumourigenesis is delayed by 39 days in C57Bl mice compared to FVB mice [415]. Additionally, they found that tumour kinetics vary between the strains with the exponential growth phase occurring twice as fast in FVB mice compared to C57Bl.

This information may be of relevance in interpreting the data presented in this thesis versus those obtained previously in the spontaneous model. The results prior to this thesis showing MMP-8 to be tumour inhibitory were obtained using a spontaneous BC model on an FVB background [296]. Given, as discussed, the accelerated growth kinetics of BC tumours in this strain, it is reasonable to assume this as a possible explanation for the lack of difference we observe in the inducible model used in this study. In this project, C57Bl/6 mice were employed to study orthotopic tumour

growth, and absence of *Mmp8* did not impact tumour growth. Strain differences including H-2 haplotype could have been responsible for this discrepancy.

6.3. Experimental Variation

Another comparison to be drawn between previous work using the spontaneous model and the orthotopic model utilised here is the use of littermate controls. In this thesis, due to time and colony management restrictions, the gold standard of littermate controls were not used and instead mice were maintained as homozygotes [421]. For experiments using *Mmp8* KO mice without the *Casp11* passenger mutation in Chapter 4, this standard was almost achieved, using the progeny from the litters of heterozygous breeding pairs, or the littermates which in theory should have reduced genetic variation to some degree. However, it is noticeable from the visible heterogeneity in tumour size that there is an unknown experimental variable that causes this. Some tumours upon harvest were very small or very large despite injections occurring on the same day, the same number of cells and age-matching within 2 weeks.

As well as genetic divergence, an alternative source of experimental variability in mouse studies is the microbiota. Divergence of the microbiota has been shown to drive phenotypic changes. For example, some mice show different susceptibilities to *Salmonella enterica* Serovar Typhimurium infection, and there are even known cancer-inhibitory effects of the microbiome [422]. Mice purchased from The Jackson Laboratory controlled melanoma growth more efficiently than mice obtained from Taconic Farms, and responded better to immunotherapy [423]. To rule out microbiota-driven impacts on tumour growth, it has been proposed that littermate controls should be used or mice should be co-housed, unlike the setup here where mice were separated by genotype and maintained in different cages for their entire lifespan [424]. The effect of cage-driven changes to the microbiota could be considered a caveat to all of the tumour experiments carried out in this thesis [425].

6.4. Inducible Expression of *Mmp8*

All models have weaknesses, for example the multiple foci that arise in the spontaneous model make observing immunological changes challenging. The single tumour that forms in the orthotopic model and progresses at a reliable rate is a good alternative to this. However, there are drawbacks to the orthotopic model. Knockout mice are designed so that the function of the gene of interest is disrupted by designing a genetic construct that prevents mRNA transcript production or in some cases results in an mRNA transcript that is rapidly degraded. Therefore, in *Mmp8* null mice that are crossed onto the MMTV-PyMT background, subsequent homozygous mice are completely devoid of *Mmp8.* In the orthotopic model there is no host-derived *Mmp8* in homozygotes, but the use of injectable cell lines provides opportunity for tumour-derived *Mmp8* to contribute. We confirmed that both the E0771 and B6BO1 cell lines did not endogenously express *Mmp8* at both the protein and RNA level. However, under the influence of other cells in 2D and 3D, some *Mmp8* could be detected both in tumour cells in co-cultures and tumours from homozygous mice. In tumours from *Mmp8* null animals, *Mmp8* mRNA could be occasionally detected at a very high C_T value of at least 37. In B6BO1 and BMM co-cultures, *Mmp8* could be consistently detected at a C_T of 34-36. The simple explanation would be that the TaqMan primers span a region that is not targeted by the construct and a dysfunctional mRNA is being detected. However, this appears not to be the case since scrutiny of the primer sequences revealed that the forward primer spans the exon boundary of exons 4 and 5, and the reverse primer is in exon 5. The *Mmp8* null allele construct is devoid of exon 4, which has been replaced with a neomycin resistance cassette [292]. Therefore, unintended amplification of a non-coding transcript is not possible.

However, what could be happening is that *Mmp8* has been introduced into the system by the B6BO1 cells that possess a functional *Mmp8* gene. Whilst most tumoural MMP activity is host-derived, cancer cells are known to produce MMP-8 [291]. Neighbouring cell-induced expression from the B6BO1 cells is possible. One contender for this is Mϕs since co-culture of B6BO1s and BMM derived from *Mmp8*

KO mice resulted in *Mmp8* expression that did not occur in B6BO1s or BMM alone. To confirm B6BO1 cells within the tumour are induced to produce *Mmp8,* a single cell sequencing approach could be taken to look for expression in tumour cells within *Mmp8* KO tumours. This would raise further questions regarding the use of this model to investigate tumour phenotype in the absence of *Mmp8.*

6.5. Passenger Mutations

Of course, the *Casp11* passenger mutation that was present in the mice utilised in Chapter 3 is a point of contention between the spontaneous and orthotopic model. Whilst within this thesis, it was extensively demonstrated that our results were not confounded by the passenger mutation, the same cannot be said for data generated in the spontaneous model.

As discussed in Chapter 1.5, most transgenic mouse models were created using ESCs from the SV129 strain that was more amenable to manipulation than other strains, having a high propensity for germline transmission. However, SV129 mice had low fecundity and C57Bl mice bred well, so transgenic mice were backcrossed onto the C57Bl strain [335], the strain used throughout this thesis. The DKO mice with the passenger mutation on the C57BL/6 background in Chapter 3 originated from the FVB *Mmp8* null mice used to cross onto MMTV-PyMT mice in the spontaneous model [296]. There is of course a small chance that the breeding pairs continued to harbour the passenger mutation whilst the mice used in the tumour experiments lost the mutation, but this is extremely unlikely. Therefore, the discovery that MMP-8 is tumour-inhibitory in BC is subject to the caveat that the model was also lacking in *Casp11* which may have confounded interpretation of the function of MMP-8.

6.6. Further Discussion

So far, the lack of tumour growth phenotype observed in this body of work has been discussed. Much of this thesis also focussed on dissecting immune changes within the tumour compartment. However, in a similar fashion, there was nothing consistent over tumour evolution. Aside from the notion that tumours were analysed at a much later stage than previous studies, it should be acknowledged that the main cellular source of MMP-8, neutrophils, appeared to be absent within both B6BO1 and E0771 tumours. Much of the evidence linking MMP-8 to the immune system both in inflammatory disorders and in the context of cancer is related to neutrophil function and behaviour. For example, in skin carcinoma, the absence of *Mmp8* increased tumour burden that was accompanied by a delay in neutrophil influx and efflux [292]. Furthermore, MMP-8 is involved in a feed-forward mechanism of neutrophil chemotaxis involving increasing levels of potent neutrophil chemoattractants IL-6 and IL-8 [260,320]. The lack of mRNA changes seen in IL-6 and mouse homologs of IL-8 are most likely explained by the lack of neutrophils present in the orthotopic tumour model, since there is no need to produce further neutrophil chemoattractants. The solution to this issue would be to use a cell line that creates tumours dependent on neutrophils or acted upon by neutrophils. For example, tumours derived from the 4T1 mouse mammary carcinoma cell line are known to contain a population of neutrophils that would enable this study [426].

Whilst our original hypothesis that MMP-8 inhibited tumour progression by guiding the immune system proved incorrect, another interesting observation made within tumours from *Mmp8* null mice was dysregulated lipid metabolism. RNA-seq data generated on tumours from MMTV-PyMT mice revealed several genes involved in lipid anabolism and catabolism were downregulated in younger *Mmp8* null mice and upregulated in older *Mmp8* null mice. This led us to look for a similar phenotype within the orthotopic model. Consistent downregulation of *Plin1* mRNA was detected at all stages in our model in the absence of *Mmp8* that could have interesting connotations.

Perilipin-1 is part of a family of five lipid droplet-associated proteins. Lipid droplets are lipid-rich organelles that act as storage vessels for cellular lipids in adipose tissue. Perlipin-1 is located on the surface of lipid droplets, acting as gatekeeper [427]. In order to break down lipids *via* lipolysis, perilipin-1 must be hyper-phosphorylated by protein kinase A, which is activated upon β-adrenergic receptor stimulation [428]. It then relinquishes its role as gatekeeper and allows access to lipases such as hormone-sensitive lipase to mobilise fats. Therefore perilipin-1 holds the key to accessing building blocks that are essential to rapidly dividing cells such as cancer cells.

Perilipin-1 expression has been linked to BC. It is found almost exclusively in adipose tissue but is not readily detected in normal breast epithelial tissue [429,430]. However, it has been seen to be highly expressed in BC cells in tumours that ultimately become unresponsive to treatment [431]. In these tumours, its expression is also correlated with high levels of other lipid metabolism genes including leptin and adiponectin that are characteristic of a 'lipogenic tumour phenotype'. The ability to regulate *de novo* lipid synthesis has been associated with tumour resilience and aggressiveness and even the ability to survive transit through the lymph nodes that are lacking in the fatty acids provided by the breast tissue – enabling metastasis to occur [432].

Nevertheless, most studies have reached the conclusion that *Plin1* is a tumour-suppressor gene. In BC patients, reduced expression of *Plin1* correlates with poorer metastasis-free survival, particularly in ER^+ and luminal A subtypes [376,433]. Furthermore, exogenous expression of perilipin-1 in BC cells inhibited migration, proliferation and invasion, independent of ER status, and using an *in vivo* mouse model, overexpression of *Plin1* in MDA-MB-231 BC cells inhibited tumourigenesis. Taken together, this evidence suggests that perilipin-1 may play a role in BC progression, acting as a tumour-suppressor.

The decrease in *Plin1* mRNA expression seen in the absence of MMP-8 suggests that MMP-8 may be involved in upregulating *Plin1* in BC. This fits with the notion that MMP-8 is tumour inhibitory in BC and may do so by regulating lipid metabolism.

Several associations between MMPs and regulation of lipid metabolism have been made including MMP-2, -8 and -9. MMP-2 and -9 both modulate cholesterol metabolism through phospholipase A2 [434,435]. This enzyme is responsible for liberating arachidonic acid, the precursor to eicosanoids and leukotrienes, key regulators of inflammation [436]. MMP-8 has been shown to degrade apolipoprotein A-1 (apo) A-1– one of the major components of high-density lipoprotein (HDL) particles [437]. Degradation of apoA-1 leads to smaller and unstable HDL particles that are less capable of cholesterol efflux and thus reverse cholesterol transport to prevent accumulation of lipids in Mϕs in vessel walls [438]. MMP-8-mediated degradation of apo A-1 reduces cholesterol efflux efficiency and in the absence of *Mmp8*, mice are less susceptible to atherosclerotic lesion formation [437,439]. Furthermore, *Mmp8* null mice have lower serum triglyceride concentrations and circulating free fatty acids [437,440].

Two hypotheses can be made on the involvement of MMPs in regulation of lipid metabolism. Firstly, cleavage of bioactive molecules such as cytokines by MMPs can lead to up- or down-regulation in their activity – which could be directed towards regulation of lipid availability. Adipocytes themselves produce MMPs which may act in an autocrine or paracrine fashion to modulate lipid metabolism [441]. Secondly, once secreted, some MMPs closely associate with the cell membrane where they can modulate the activity of receptors through sheddase activity. MMP-8 is one example of an MMP found pericellularly on PMNs: 92% of its Type I collagenase activity can be attributed to membrane-bound MMP-8 [301]. This activity is TIMP-resistant and may be responsible for the cleavage of many as-yet unknown molecules from the cell surface. Further examples involving MMP activity, this time in adipogenesis, are MMP-11 and MMP-14. Adipocytes and their precursors exist in a dense type I collagen mesh that is sensitive to enzymatic activity [442]. Bi-directional cross-talk exists between MMP-11 and the TME whereby cancer cells induce the production of MMP-11 in adipose tissue [443]. MMP-11 acts as a negative regulator of adipogenesis, preventing adipocyte maturation and instead promoting de-differentiation into peri-tumoural fibroblast-like cells that support and facilitate tumour progression. Meanwhile, MT1-MMP or MMP-14 is a membrane-bound MMP

that is critical in the development and maintenance of adipose tissue [442]. In mice fed a high-fat diet, MMP-14 modifies the type I collagen architecture surrounding adipocytes, participating in obesity pathogenesis [444].

Therefore, there is ample evidence to suggest that the changes seen in perilipin-1 could be reflective of a wider network involving MMP-8 in regulation of lipid metabolism, specifically in BC. This finding is worth pursuing in future studies with other BC subtypes or different cancers – particularly lipogenic cancers such as prostate and colon [445,446].

Throughout Chapters 3 and 4, despite searching for tumoural immune changes, it was evident that loss of *Mmp8* did not impact anti-tumour immunity. Therefore, we turned our attention to the wider immune system. Alongside being the site of haematopoiesis, the bone marrow is considered an immune regulatory organ [447]. It is home to a plethora of cells including T cells, B cells, dendritic cells, neutrophils, MDSCs and mesenchymal stem cells that use the bone marrow as a nest for carrying out their functions. For some cells such as neutrophils, the bone marrow signifies the beginning of their journey as well as their end, whereby senescent neutrophils receive signals to home back for clearance [403]. We used the bone marrow in our studies throughout Chapter 5 to study the effect of MMP-8 on Mϕ phenotype, communication and population changes.

Alongside the above populations, the bone marrow houses a large monocyte population, the progenitor cell of Mϕs. By modelling Mϕ behaviour in culture, through induced differentiation of bone marrow monocytes, we found there to be no effect of MMP-8 on Mϕ differentiation, polarisation or inflammatory cytokine levels. Since MMP8 has been previously shown to drive Mϕ polarisation towards the M2 phenotype, we speculated that the *Casp11* passenger mutation could have obscured the true MMP-8 phenotype and accounted for this by comparing DKO mice with the mutation to *Mmp8* KO mice without it. However, the results remained the same, that MMP-8 does not appear to be important during Mϕ polarisation independent of the passenger mutation. Furthermore, we investigated cross-talk between Mϕs and tumour cells to mimic cellular interaction within tumours and

measure signalling activity. MMP-8 acts within an immuno-regulatory network involving IL-6 and IL-8. In human BC cells, overexpression of MMP-8 leads to upregulation of both cytokines [320]. To investigate the existence of this network within mouse mammary tumours, IL-6 was measured at both the protein and RNA level in co-culture systems. Again, no role could be found for MMP-8 in modulation of IL-6 levels. It is possible that Mϕs are not the most relevant cell type in the TME to study in regard to IL-6 since neutrophils are the principal producers of IL-6 and indeed MMP-8 [296]. However, no neutrophils were detected in B6BO1 tumours so the study of MMP-8 function within this cancer-cell immune axis would require the use of a cell line that forms tumours containing neutrophils such as 4T1 cells. The involvement of neutrophils could be analysed using neutrophils FACS sorted from tumours or via Ficoll-Paque density gradient centrifugation on mouse bone marrow or indeed more recently the technique has been used in tumours [448].

Perhaps of most interest in this thesis was the finding that *Mmp8* KO mice have lower numbers of bone marrow neutrophils than their wild-type counterparts, and that this phenomenon occurred in both unchallenged mice and to a lesser extent in tumour-bearing mice. The latter result is most intriguing since there is a distinct lack of neutrophils within the tumours harboured by the mice. Many questions remain unanswered. What are the signals involved in population level changes? Where are the origins of the neutrophils and what are their functions? Some speculation has been made in Chapter 5 regarding mobilisation defects, aberrant apoptosis and population shifts, but a definitive explanation remains elusive. If decreased neutrophil apoptosis was responsible, this result directly challenges the central dogma in MMP-8 immunobiology that MMP-8 drives neutrophil apoptosis.

In summary, the lack of phenotypes discovered throughout this thesis casts further doubt on whether MMP-8 is truly suppressive for primary BC growth. We hope that through the extensive work carried out in the orthotopic mouse model of BC, we have ruled out the contribution of MMP-8 in later stages of tumourigenesis. Instead, emphasis should be placed on early stages of tumour initiation and the mechanism behind the inhibition of tumour development. Despite this, we did uncover a

potential role for MMP-8 in lipid metabolism, finding reduced expression of perilipin-1, a tumour-suppressive lipid metabolism gene, in *Mmp8* null tumours. This finding warrants further study into the link between MMP-8 and metabolic regulation within tumours.

Additionally, we have shown that whilst there appears to be no impact of MMP-8 on anti-cancer immunity within mammary tumours themselves, there may be immune changes that occur on a more global scale in the bone marrow. This should be the focus of future research on MMP-8 in cancer immunology.

In terms of impact on the world of MMP-8 biology, this thesis emphasises the importance in fully considering an experimental model. This is especially true for mice, that have complex genetic backgrounds that can influence experimental outcomes. The discovery of the high frequency of germline mutations in the SV129 strain, the origin of countless MMP knockout mouse models in use today, will require many research groups to revisit early mouse models and where necessary recreate them with more modern tools. Whilst within this thesis there was little evidence of the *Casp11* passenger mutation confounding data interpretation, there is a wealth of literature on MMP-8 and MMPs more generally where this may not be the case. The field as it stands should be caveated with this in mind.

Future Directions

There are several areas within this thesis that future work could expand on:

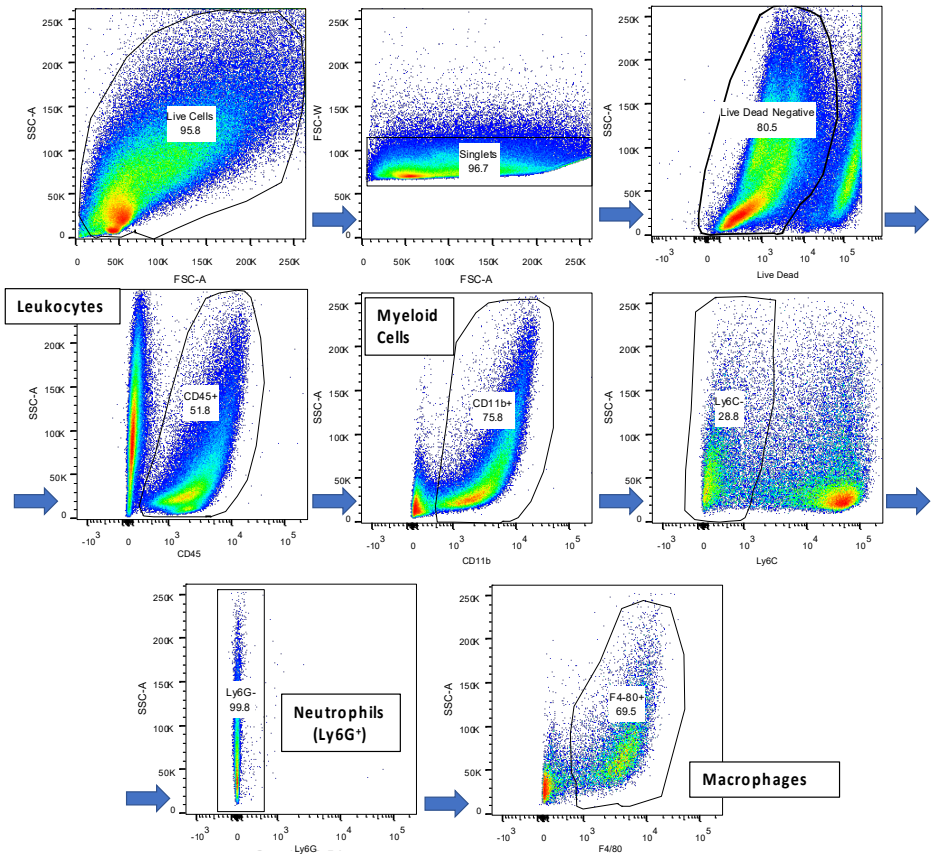
- Carry out repeat experiments utilising *Mmp8* null C57 mice in the spontaneous model and *Mmp8* null FVB mice for orthotopic implantation to determine whether strain differences explain the discrepancies between the two tumour models.

- Increase sample size for tumour experiments to study perilipin-1 phenotype. Histological staining will reveal the location of perilpin-1 to reveal whether it is expressed by breast cancer cells or surrounding healthy adipose tissue.

- Use a mammary cancer cell line that expresses MMP-8 and is known to involve neutrophils to study interaction between MMP-8, neutrophils and breast cancer.

- Explore neutrophils in *Mmp8* null mice by tracking origin and destination of neutrophils, looking at apoptotic markers, and FACS-sorting neutrophil populations to study gene expression and phenotype.

7. Appendix



Supplementary Figure 7.1. Flow cytometric gating strategy for tumour cells. The above gates are used to delineate myeloid populations within tumours and bone marrow. Forward and side scatter parameters are used to remove debris and single cells. Cells that stain positive for the fixable Live/Dead marker are removed as dead cells. Live cells are subsequently gated as CD45+ to separate leukocytes from other cells within the tumour, CD11b+ for myeloid cells, Ly6C- to remove monocytes, Ly6G- to exclude neutrophils and finally as F4/80+ to obtain the Mϕ population

8. Bibliography

1. Fitzmaurice C, Dicker D, Pain A, Hamavid H, Moradi-Lakeh M, MacIntyre MF, et al. The Global Burden of Cancer 2013. *JAMA Oncol*. 2015;1(4):505.

2. Cancer Research UK. Breast cancer mortality statistics | Cancer Research UK [Internet].

3. Howell A, Anderson AS, Clarke RB, Duffy SW, Evans DG, Garcia-Closas M, et al. Risk determination and prevention of breast cancer. *Breast Cancer Res*. 2014;16(5):446.

4. Cancer Research UK. Cancer Statistics for the UK [Internet]. 2016.

5. Inman JL, Robertson C, Mott JD, Bissell MJ. Mammary gland development: cell fate specification, stem cells and the microenvironment. *Development*. 2015;142(6):1028–42.

6. Javed A, Lteif A. Development of the human breast. *Semin Plast Surg*. 2013;27(1):5–12.

7. Ellis H, Mahadevan V. Anatomy and physiology of the breast. *Surg*. 2013;31(1):11–4.

8. Silberstein GB. Postnatal mammary gland morphogenesis. *Microsc Res Tech*. 2001;52(2):155–62.

9. Sharma GN, Dave R, Sanadya J, Sharma P, Sharma KK. Various types and management of breast cancer: an overview. *J Adv Pharm Technol Res*. 2010;1(2):109–26.

10. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin*. 2017;67(1):7–30.

11. White MC, Holman DM, Goodman RA, Richardson LC. Cancer Risk Among Older Adults: Time for Cancer Prevention to Go Silver. *Gerontologist*. 2019;59(Supplement_1):S1–6.

12. Sarri G, Davies M, Lumsden MA, Guideline Development Group. Diagnosis and management of menopause: summary of NICE guidance. *BMJ*. 2015;351:h5746.

13. Dall GV, Britt KL. Estrogen Effects on the Mammary Gland in Early and Late Life and Breast Cancer Risk. *Front Oncol*. 2017;7:110.

14. Collaborative Group on Hormonal Factors in Breast Cancer. Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. *Lancet Oncol*. 2012;13(11):1141–51.

15. Durrani S, Akbar S, Heena H. Breast Cancer During Pregnancy. *Cureus*. 2018;10(7):e2941.

16. Polyak K. Pregnancy and breast cancer: The other side of the coin. *Cancer Cell*. 2006;9(3):151–3.

17. Anothaisintawee T, Wiratkapun C, Lerdsitthichai P, Kasamesup V, Wongwaisayawan S, Srinakarin J, et al. Risk Factors of Breast Cancer. *Asia Pacific J Public Heal*. 2013;25(5):368–87.

18. Victora CG, Bahl R, Barros AJD, França GVA, Horton S, Krasevec J, et al. Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. *Lancet*. 2016;387(10017):475–90.

19. Brewer HR, Jones ME, Schoemaker MJ, Ashworth A, Swerdlow AJ. Family history and risk of breast cancer: an analysis accounting for family structure. *Breast Cancer Res Treat*. 2017;165(1):193–200.

20. Stoppa-Lyonnet D. The biological effects and clinical implications of BRCA mutations: where do we go from here? *Eur J Hum Genet*. 2016;24(S1):S3–9.

21. Evans DG, Graham J, O'Connell S, Arnold S, Fitzsimmons D. Familial breast cancer: summary of updated NICE guidance. *BMJ*. 2013;346:f3829.

22. Jones ME, Schoemaker MJ, Wright L, McFadden E, Griffin J, Thomas D, et al. Menopausal hormone therapy and breast cancer: what is the true size of the increased risk? *Br J Cancer*. 2016;115(5):607–15.

23. E. W. Risk factors and epidemiology of breast cancer. *Women's Heal Med*. 2006;3:8–14.

24. Iversen L, Sivasubramaniam S, Lee AJ, Fielding S, Hannaford PC. Lifetime cancer risk and combined oral contraceptives: the Royal College of General Practitioners' Oral Contraception Study. *Am J Obstet Gynecol*. 2017;216(6):580.e1-580.e9.

25. Russo J, Lareef MH, Tahin Q, Hu YF, Slater C, Ao X, et al. 17Beta-estradiol is carcinogenic in human breast epithelial cells. *J Steroid Biochem Mol Biol*. 2002;80(2):149–62.

26. Russo J, Russo IH. Genotoxicity of steroidal estrogens. *Trends Endocrinol Metab*. 2004;15(5):211–4.

27. Sun Y-S, Zhao Z, Yang Z-N, Xu F, Lu H-J, Zhu Z-Y, et al. Risk Factors and Preventions of Breast Cancer. *Int J Biol Sci*. 2017;13(11):1387–97.

28. Arps DP, Healy P, Zhao L, Kleer CG, Pang JC. Invasive ductal carcinoma with lobular features: a comparison study to invasive ductal and invasive lobular carcinomas of the breast. *Breast Cancer Res Treat*. 2013;138(3):719–26.

29. Cserni G, Chmielik E, Cserni B, Tot T. The new TNM-based staging of breast cancer. *Virchows Arch*. 2018;472(5):697–703.

30. Schwartz AM, Henson DE, Chen D, Rajamarthandan S. Histologic Grade Remains a Prognostic Factor for Breast Cancer Regardless of the Number of Positive Lymph Nodes and Tumor Size: A Study of 161 708 Cases of Breast Cancer From the SEER Program. *Arch Pathol Lab Med*. 2014;138(8):1048–52.

31. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406(6797):747–52.

32. Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, et al. Subtyping of Breast Cancer by Immunohistochemistry to Investigate a Relationship between Subtype and Short and Long Term Survival: A Collaborative Analysis of Data for 10,159 Cases from 12 Studies. Marincola FM, editor. *PLoS Med*. 2010;7(5):e1000279.

33. Haque R, Ahmed SA, Inzhakova G, Shi J, Avila C, Polikoff J, et al. Impact of Breast Cancer Subtypes and Treatment on Survival: An Analysis Spanning Two Decades. *Cancer Epidemiol Biomarkers Prev*. 2012;21(10):1848.

34. Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nat Rev Clin Oncol*. 2016;13(11):674–90.

35. Weigelt B, Mackay A, A'hern R, Natrajan R, Tan DSP, Dowsett M, et al. Breast cancer molecular profiling with single sample predictors: A retrospective analysis. *Lancet Oncol*. 2010;11(4):339–49.

36. Ovcaricek T, Frkovic SG, Matos E, Mozina B, Borstnar S. Triple negative breast cancer – prognostic factors and survival. *Radiol Oncol*. 2011;45(1):46–52.

37. Yersal O, Barutca S. Biological subtypes of breast cancer: Prognostic and therapeutic implications. Vol. 5, World Journal of Clinical Oncology. Baishideng Publishing Group Co., Limited; 2014. p. 412–24.

38. Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, et al. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Mod Pathol*. 2011;24(2):157–67.

39. Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and

transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012;486(7403):346–52.

40. Russnes HG, Lingjærde OC, Børresen-Dale A-L, Caldas C. Breast Cancer Molecular Stratification: From Intrinsic Subtypes to Integrative Clusters. *Am J Pathol*. 2017;187(10):2152–62.

41. National Institute of Clinical Excellence. Overview | Tumour profiling tests to guide adjuvant chemotherapy decisions in early breast cancer | Guidance | NICE.

42. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF, et al. Adjuvant Chemotherapy Guided by a 21-Gene Expression Assay in Breast Cancer. *N Engl J Med*. 2018;379(2):111–21.

43. Golub LM, Lee HM, Greenwald RA, Ryan ME, Sorsa T, Salo T, et al. A matrix metalloproteinase inhibitor reduces bone-type collagen degradation fragments and specific collagenases in gingival crevicular fluid during adult periodontitis. *Inflamm Res*. 1997;46(8):310–9.

44. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AAM, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 2002;415(6871):530–6.

45. Brandão M, Pondé N, Piccart-Gebhart M. Mammaprint™: a comprehensive review. *Futur Oncol*. 2019;15(2):207–24.

46. van Middendorp JJ, Sanchez GM, Burridge AL. The Edwin Smith papyrus: a clinical reappraisal of the oldest known document on spinal injuries. *Eur Spine J*. 2010;19(11):1815–23.

47. Fisher B, Bauer M, Margolese R, Poisson R, Pilch Y, Redmond C, et al. Five-Year Results of a Randomized Clinical Trial Comparing Total Mastectomy and Segmental Mastectomy with or without Radiation in the Treatment of Breast Cancer. *N Engl J Med*. 1985;312(11):665–73.

48. Beatson GT. On the Treatment of Inoperable Cases of Carcinoma of the Mamma: Suggestions for a New Method of Treatment, with Illustrative Cases. *Trans Medico-Chirurgical Soc Edinburgh*. 1896;15:153–79.

49. Gérard C, Brown KA. Obesity and breast cancer – Role of estrogens and the molecular underpinnings of aromatase regulation in breast adipose tissue. Vol. 466, Molecular and Cellular Endocrinology. Elsevier Ireland Ltd; 2018. p. 15–30.

50. Fabian CJ. The what, why and how of aromatase inhibitors: hormonal agents for treatment and prevention of breast cancer. *Int J Clin Pract*. 2007;61(12):2051–63.

51. Perez EA, Romond EH, Suman VJ, Jeong J-H, Sledge G, Geyer CE, et al. Trastuzumab plus adjuvant chemotherapy for human epidermal growth factor receptor 2-positive breast cancer: planned joint analysis of overall survival from NSABP B-31 and NCCTG N9831. *J Clin Oncol*. 2014;32(33):3744–52.

52. Yang M, Büsche G, Ganser A, Li Z. Morphology and quantitative composition of hematopoietic cells in murine bone marrow and spleen of healthy subjects. *Ann Hematol*. 2013;92(5):587–94.

53. Moasser MM, Krop IE. The evolving landscape of HER2 targeting in breast cancer. *JAMA Oncol*. 2015;1(8):1154–61.

54. Torti D, Trusolino L. Oncogene addiction as a foundational rationale for targeted anti-cancer therapy: promises and perils. *EMBO Mol Med*. 2011;3(11):623–36.

55. Dolgin E. Bringing down the cost of cancer treatment. *Nat 2018 5557695*. 2018;

56. Dean L. Blood Groups and Red Cell Antigens. 2005;

57. Jagannathan-Bogdan M, Zon LI. Hematopoiesis. *Development*. 2013;140(12):2463.

58. Galloway JL, Zon LI. Ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo. *Curr Top Dev Biol*. 2003;53:139–58.

59. Cumano A, Godin I. Ontogeny of the Hematopoietic System. *Annu Rev Immunol*. 2007;25(1):745–85.

60. Krantz S. Erythropoietin. *Blood*. 1991;77(3).

61. Kaushansky K. Lineage-specific hematopoietic growth factors. *N Engl J Med*. 2006;354(19):2034–45.

62. McLeod JJA, Baker BN, Ryan JJ. Mast Cell Production and Response to IL-4 and IL-13. *Cytokine*. 2015;75(1):57.

63. Sarma NJ, Takeda A, Yaseen NR. Colony Forming Cell (CFC) Assay for Human Hematopoietic Cells. *J Vis Exp*. 2010;(46).

64. Valerie F.J. Quesniaux, Julian D. Down H-JS. Hematopoiesis, including lymphocyte development and maturation. *Princ Immunopharmacol*. 2005;3–17.

65. Dwivedi P, Greis KD. Granulocyte colony-stimulating factor receptor signaling in severe congenital neutropenia, chronic neutrophilic leukemia, and related malignancies. *Exp Hematol*. 2017;46:9–20.

66. Bannister LH. The interactions of intracellular Protista and their host cells, with special reference to heterotrophic organisms. *Proc R Soc London Ser B Biol Sci*. 1979;204(1155):141–63.

67. van Furth R, Sluiter W. Distribution of blood monocytes between a marginating and a circulating pool. *J Exp Med*. 1986;163(2):474–9.

68. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. *Science (80-)*. 2009;325(5940):612–6.

69. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science*. 2010;327(5966):656–61.

70. Hettinger J, Richards DM, Hansson J, Barra MM, Joschko A-C, Krijgsveld J, et al. Origin of monocytes and macrophages in a committed progenitor. *Nat Immunol*. 2013;14(8):821–30.

71. Stefater JA, Ren S, Lang RA, Duffield JS. Metchnikoff's policemen: macrophages in development, homeostasis and regeneration. *Trends Mol Med*. 2011;17(12):743–52.

72. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. 2010;

73. Passlick B, Flieger D, Ziegler-Heitbrock H. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*. 1989;74(7).

74. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of Blood Vessels and Tissues by a Population of Monocytes with Patrolling Behavior. *Science (80-)*. 2007;317(5838):666–70.

75. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003;19(1):71–82.

76. Auffray C, Sieweke MH, Geissmann F. Blood Monocytes: Development, Heterogeneity, and Relationship with Dendritic Cells. *Annu Rev Immunol*. 2009;27(1):669–92.

77. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med*.

1994;179(4):1109–18.

78. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. 1992;176(1):287–92.

79. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci*. 2008;13:453–61.

80. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity*. 2014;41(1):14–20.

81. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-Dependent Ly6Clow Monocytes Monitor Endothelial Cells and Orchestrate Their Disposal. *Cell*. 2013;153(2):362–75.

82. Briseño CG, Haldar M, Kretzer NM, Wu X, Theisen DJ, KC W, et al. Distinct Transcriptional Programs Control Cross-Priming in Classical and Monocyte-Derived Dendritic Cells. *Cell Rep*. 2016;15(11):2462–74.

83. Tsou C-L, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, et al. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest*. 2007;117(4):902–9.

84. Jia T, Serbina N V, Brandl K, Zhong MX, Leiner IM, Charo IF, et al. Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during Listeria monocytogenes infection. *J Immunol*. 2008;180(10):6846–53.

85. Gerszten RE, Lim Y-C, Ding HT, Snapp K, Kansas G, Dichek DA, et al. Adhesion of Monocytes to Vascular Cell Adhesion Molecule-1-Transduced Human Endothelial Cells Implications for Atherogenesis [Internet]. 1998.

86. Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA, et al. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J Exp Med*. 2017;214(7):1913–23.

87. Lavin Y, Winter D, Blecher-Gonen R, David E, Keren-Shaul H, Merad M, et al. Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment. *Cell*. 2014;159(6):1312–26.

88. Scott CL, Zheng F, De Baetselier P, Martens L, Saeys Y, De Prijck S, et al. Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun*. 2016;7(1):10321.

89. Cronk JC, Filiano AJ, Louveau A, Marin I, Marsh R, Ji E, et al. Peripherally derived macrophages can engraft the brain independent of irradiation and maintain an identity distinct from microglia. *J Exp Med*. 2018;215(6):1627–47.

90. Meuret G, Bammert J, Hoffmann G. Kinetics of Human Monocytopoiesis. *Blood*. 1974;44(6).

91. Vinh DC, Patel SY, Uzel G, Anderson VL, Freeman AF, Olivier KN, et al. Autosomal dominant and sporadic monocytopenia with susceptibility to mycobacteria, fungi, papillomaviruses, and myelodysplasia. *Blood*. 2010;115(8):1519–29.

92. Poh AR, Ernst M. Targeting Macrophages in Cancer: From Bench to Bedside. *Front Oncol*. 2018;8:49.

93. Krzyszczyk P, Schloss R, Palmer A, Berthiaume F. The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes. *Front Physiol*. 2018;9:419.

94. Takahashi K, Yamamura F, Naito M. Differentiation, Maturation, and Proliferation of Macrophages in the Mouse Yolk Sac: A Light-Microscopic, Enzyme-Cytochemical,

Immunohistochemical, and Ultrastructural Study. *J Leukoc Biol*. 1989;45(2):87–96.

95. McGrath KE, Frame JM, Fegan KH, Bowen JR, Conway SJ, Catherman SC, et al. Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and Provide Functional Blood Cells in the Mammalian Embryo. *Cell Rep*. 2015;11(12):1892–904.

96. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MBB, Leboeuf M, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*. 2013;38(4):792–804.

97. Van Nguyen A, Pollard JW. Colony Stimulating Factor-1 Is Required to Recruit Macrophages into the Mammary Gland to Facilitate Mammary Ductal Outgrowth. *Dev Biol*. 2002;247(1):11–25.

98. Tagliani E, Shi C, Nancy P, Tay C-S, Pamer EG, Erlebacher A. Coordinate regulation of tissue macrophage and dendritic cell population dynamics by CSF-1. *J Exp Med*. 2011;208(9):1901–16.

99. Bain CC, Bravo-Blas A, Scott CL, Perdiguero EG, Geissmann F, Henri S, et al. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol*. 2014;15(10):929–37.

100. Yona S, Kim K-W, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. *Immunity*. 2013;38(1):79–91.

101. Jaiswal S, Jamieson CHM, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 Is Upregulated on Circulating Hematopoietic Stem Cells and Leukemia Cells to Avoid Phagocytosis. *Cell*. 2009;138(2):271–85.

102. Marks SC, Lane PW. Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. *J Hered*. 1976;67(1):11–8.

103. Dai X-M, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, et al. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood*. 2002;99(1):111–20.

104. Niida S, Kaku M, Amano H, Yoshida H, Kataoka H, Nishikawa S, et al. Vascular endothelial growth factor can substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption. *J Exp Med*. 1999;190(2):293–8.

105. Hsu Y-P, Staton CA, Cross N, Buttle DJ. Anti-angiogenic properties of ADAMTS-4 in vitro. *Int J Exp Pathol*. 2012;93(1):70–7.

106. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol*. 2002;196(3):254–65.

107. Gouon-Evans V, Rothenberg ME, Pollard JW. Postnatal mammary gland development requires macrophages and eosinophils. *Development*. 2000;127(11):2269–82.

108. Jäppinen N, Félix I, Lokka E, Tyystjärvi S, Pynttäri A, Lahtela T, et al. Fetal-derived macrophages dominate in adult mammary glands. *Nat Commun*. 2019;10(1):281.

109. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol*. 2014;5:491.

110. Forthal DN. Functions of Antibodies. *Microbiol Spectr*. 2014;2(4):1.

111. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *Int J Mol Sci*. 2017;19(1).

112. Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? *Front*

Physiol. 2018;9:113.

113. von Vietinghoff S, Ley K. Homeostatic regulation of blood neutrophil counts. *J Immunol*. 2008;181(8):5183–8.

114. Baggiolini M. The Enzymes of the Granules of Polymorphonuclear Leukocytes and Their Functions. *Enzyme*. 1972;13(1–3):132–60.

115. Dancey JT, Deubelbeiss KA, Harker LA, Finch CA. Neutrophil kinetics in man. *J Clin Invest*. 1976;58(3):705–15.

116. Hong C-W. Current Understanding in Neutrophil Differentiation and Heterogeneity. *Immune Netw*. 2017;17(5):298–306.

117. Kim HK, De La Luz Sierra M, Williams CK, Gulino AV, Tosato G. G-CSF down-regulation of CXCR4 expression identified as a mechanism for mobilization of myeloid cells. *Blood*. 2006;108(3):812–20.

118. Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. *Trends Immunol*. 2011;32(10):452–60.

119. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. *Trends Immunol*. 2010;31(8):318–24.

120. Bratton DL, Henson PM. Neutrophil clearance: when the party is over, clean-up begins. *Trends Immunol*. 2011;32(8):350–7.

121. Rankin SM. The bone marrow: a site of neutrophil clearance. *J Leukoc Biol*. 2010;88(2):241–51.

122. Uhm TG, Kim BS, Chung IY. Eosinophil Development, Regulation of Eosinophil-Specific Genes, and Role of Eosinophils in the Pathogenesis of Asthma. *Allergy, Asthma Immunol Res*. 2012;4(2).

123. Rothenberg ME, Hogan SP. THE EOSINOPHIL. *Annu Rev Immunol*. 2006;24(1):147–74.

124. Logan MR, Odemuyiwa SO, Moqbel R. Understanding exocytosis in immune and inflammatory cells: the molecular basis of mediator secretion. *J Allergy Clin Immunol*. 2003;111(5):923–32; quiz 933.

125. Mukai K, Galli S. Basophils. In: eLS. Chichester, UK: John Wiley & Sons, Ltd; 2013.

126. Voehringer D. Protective and pathological roles of mast cells and basophils. *Nat Rev Immunol*. 2013;13(5):362–75.

127. Krystel-Whittemore M, Dileepan KN, Wood JG. Mast Cell: A Multi-Functional Master Cell. *Front Immunol*. 2015;6:620.

128. Market E, Papavasiliou FN. V(D)J Recombination and the Evolution of the Adaptive Immune System. *PLoS Biol*. 2003;1(1):e16.

129. Halle S, Halle O, Förster R. Mechanisms and Dynamics of T Cell-Mediated Cytotoxicity In Vivo. *Trends Immunol*. 2017;38(6):432–43.

130. Hoffman W, Lakkis FG, Chalasani G. B Cells, Antibodies, and More. *Clin J Am Soc Nephrol*. 2016;11(1):137–54.

131. Mildner A, Jung S. Development and Function of Dendritic Cell Subsets. *Immunity*. 2014;40(5):642–56.

132. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol*. 2013;31:563–604.

133. Collin M, Bigley V. Human dendritic cell subsets: an update. *Immunology*. 2018;154(1):3–20.

134. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell*. 2010;140(6):883–99.

135. Coley WB. THE TREATMENT OF MALIGNANT TUMORS BY REPEATED INOCULATIONS OF ERYSIPELAS: WITH A REPORT OF TEN ORIGINAL CASES.1: BIBLIOGRAPHY. *Am J Med Sci*. 1893;105(6):487.

136. Fuge O, Vasdev N, Allchorne P, Green JS. Immunotherapy for bladder cancer. *Res reports Urol*. 2015;7:65–79.

137. Karbach J, Neumann A, Brand K, Wahle C, Siegel E, Maeurer M, et al. Phase I Clinical Trial of Mixed Bacterial Vaccine (Coley's Toxins) in Patients with NY-ESO-1 Expressing Cancers: Immunological Effects and Clinical Activity. *Clin Cancer Res*. 2012;18(19):5449–59.

138. Thomas L. On immunosurveillance in human cancer. *Yale J Biol Med*. 1982;55(3–4):329–33.

139. Burnet FM. Immunological Surveillance in Neoplasia. *Immunol Rev*. 1971;7(1):3–25.

140. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256(5517):495–7.

141. Stutman O. Chemical carcinogenesis in nude mice: comparison between nude mice from homozygous matings and heterozygous matings and effect of age and carcinogen dose. *J Natl Cancer Inst*. 1979;62(2):353–8.

142. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNγ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001;410(6832):1107–11.

143. Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. *Curr Opin Immunol*. 2014;27:16–25.

144. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 1996;86(3):353–64.

145. Rosenberg SA. A New Era for Cancer Immunotherapy Review Based on the Genes that Encode Cancer Antigens peptides from tumor cells or from MHC molecules puri-fied from tumor cells and to detect fractions capable of stimulating antitumor T cells after pulsing purified fra [Internet]. Vol. 10, Immunity. 1999.

146. Behrens MD, Wagner WM, Krco CJ, Erskine CL, Kalli KR, Krempski J, et al. The endogenous danger signal, crystalline uric acid, signals for enhanced antibody immunity. *Blood*. 2008;111(3):1472–9.

147. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, et al. Regulation of cutaneous malignancy by gammadelta T cells. *Science*. 2001;294(5542):605–9.

148. Luster AD, Ravetch J V. Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J Exp Med*. 1987;166(4):1084–97.

149. Bromberg JF, Horvath CM, Wen Z, Schreiber RD, Darnell JE. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proc Natl Acad Sci U S A*. 1996;93(15):7673–8.

150. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural Innate and Adaptive Immunity to Cancer. *Annu Rev Immunol*. 2011;29(1):235–71.

151. Lewis CE, Leek R, Harris A, McGee JO. Cytokine regulation of angiogenesis in breast cancer: the role of tumor-associated macrophages. *J Leukoc Biol*. 1995;57(5):747–51.

152. Lin EY, Nguyen A V, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med*. 2001;193(6):727–40.

153. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*. 2002;23(11):549–55.

154. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*. 2014;6:13.

155. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*. 2005;5(12):953–64.

156. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25(12):677–86.

157. Nahrendorf M, Swirski FK. Abandoning M1/M2 for a network model of macrophage function. Vol. 119, Circulation Research. Lippincott Williams and Wilkins; 2016. p. 414–7.

158. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS–) vs. Alternatively Activated Macrophages. *Front Immunol*. 2019;10:1084.

159. Ong S-M, Tan Y-C, Beretta O, Jiang D, Yeap W-H, Tai JJY, et al. Macrophages in human colorectal cancer are pro-inflammatory and prime T cells towards an anti-tumour type-1 inflammatory response. *Eur J Immunol*. 2012;42(1):89–100.

160. Jarosz-Biej M, Kamińska N, Matuszczak S, Cichoń T, Pamuła-Piłat J, Czapla J, et al. M1-like macrophages change tumor blood vessels and microenvironment in murine melanoma. Shiku H, editor. *PLoS One*. 2018;13(1):e0191012.

161. Hanahan D, Weinberg RA. The Hallmarks of Cancer. *Cell*. 2000;100(1):57–70.

162. Gupta RB, Harpaz N, Itzkowitz S, Hossain S, Matula S, Kornbluth A, et al. Histologic Inflammation Is a Risk Factor for Progression to Colorectal Neoplasia in Ulcerative Colitis: A Cohort Study. *Gastroenterology*. 2007;133(4):1099–105.

163. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature*. 2012;491(7423):254–8.

164. Kong L, Zhou Y, Bu H, Lv T, Shi Y, Yang J. Deletion of interleukin-6 in monocytes/macrophages suppresses the initiation of hepatocellular carcinoma in mice. *J Exp Clin Cancer Res*. 2016;35(1):131.

165. Petrova V, Annicchiarico-Petruzzelli M, Melino G, Amelio I. The hypoxic tumour microenvironment. *Oncogenesis*. 2018;7(1):10.

166. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, et al. CD4+ T Cells Regulate Pulmonary Metastasis of Mammary Carcinomas by Enhancing Protumor Properties of Macrophages. *Cancer Cell*. 2009;16(2):91–102.

167. Biswas SK, Gangi L, Paul S, Schioppa T, Saccani A, Sironi M, et al. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). *Blood*. 2006;107(5):2112–22.

168. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res*. 2004;64(16):5839–49.

169. Chen J, Yao Y, Gong C, Yu F, Su S, Chen J, et al. CCL18 from tumor-associated macrophages promotes breast cancer metastasis via PITPNM3. *Cancer Cell*. 2011;19(4):541–55.

170. Carroll MJ, Kapur A, Felder M, Patankar MS, Kreeger PK. M2 macrophages induce ovarian cancer cell proliferation via a heparin binding epidermal growth factor/matrix metalloproteinase 9 intercellular feedback loop. *Oncotarget*. 2016;

171. Tomita T, Sakurai Y, Ishibashi S, Maru Y. Imbalance of Clara cell-mediated homeostatic inflammation is involved in lung metastasis. *Oncogene*. 2011;30(31):3429–39.

172. Yang L, Zhang Y. Tumor-associated macrophages: from basic research to clinical application. *J Hematol Oncol*. 2017;10(1):58.

173. Templeton AJ, McNamara MG, Šeruga B, Vera-Badillo FE, Aneja P, Ocaña A, et al. Prognostic Role of Neutrophil-to-Lymphocyte Ratio in Solid Tumors: A Systematic Review and Meta-Analysis. *JNCI J Natl Cancer Inst*. 2014;106(6):dju124.

174. Shen M, Hu P, Donskov F, Wang G, Liu Q, Du J. Tumor-Associated Neutrophils as a New Prognostic Factor in Cancer: A Systematic Review and Meta-Analysis. Coleman WB, editor. *PLoS One*. 2014;9(6):e98259.

175. Waight JD, Hu Q, Miller A, Liu S, Abrams SI. Tumor-Derived G-CSF Facilitates Neoplastic Growth through a Granulocytic Myeloid-Derived Suppressor Cell-Dependent Mechanism. Blagosklonny M V., editor. *PLoS One*. 2011;6(11):e27690.

176. Shaul ME, Fridlender ZG. Tumour-associated neutrophils in patients with cancer. *Nat Rev Clin Oncol*. 2019;1.

177. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell*. 2009;16(3):183–94.

178. Kim S, Buchlis G, Fridlender ZG, Sun J, Kapoor V, Cheng G, et al. Systemic blockade of transforming growth factor-beta signaling augments the efficacy of immunogene therapy. *Cancer Res*. 2008;68(24):10247–56.

179. Gregory AD, McGarry Houghton A. Tumor-Associated Neutrophils: New Targets for Cancer Therapy. *Cancer Res*. 2011;71(7):2411–6.

180. Deryugina EI, Zajac E, Juncker-Jensen A, Kupriyanova TA, Welter L, Quigley JP. Tissue-Infiltrating Neutrophils Constitute the Major In Vivo Source of Angiogenesis-Inducing MMP-9 in the Tumor Microenvironment. *Neoplasia*. 2014;16(10):771–88.

181. Aldabbous L, Abdul-Salam V, McKinnon T, Duluc L, Pepke-Zaba J, Southwood M, et al. Neutrophil Extracellular Traps Promote Angiogenesis. *Arterioscler Thromb Vasc Biol*. 2016;36(10):2078–87.

182. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. *J Clin Invest*. 2013;123(8):3446–58.

183. Kowanetz M, Wu X, Lee J, Tan M, Hagenbeek T, Qu X, et al. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. *Proc Natl Acad Sci U S A*. 2010;107(50):21248–55.

184. Singh S, Sadanandam A, Nannuru KC, Varney ML, Mayer-Ezell R, Bond R, et al. Small-molecule antagonists for CXCR2 and CXCR1 inhibit human melanoma growth by decreasing tumor cell proliferation, survival, and angiogenesis. *Clin Cancer Res*. 2009;15(7):2380–6.

185. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A*. 2005;102(51):18538–43.

186. Hillen F, Baeten CIM, van de Winkel A, Creytens D, van der Schaft DWJ, Winnepenninckx V, et al. Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary

cutaneous melanoma. *Cancer Immunol Immunother*. 2008;57(1):97–106.

187. Mahmoud SMA, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AHS, et al. Tumor-Infiltrating CD8 + Lymphocytes Predict Clinical Outcome in Breast Cancer. *J Clin Oncol*. 2011;29(15):1949–55.

188. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, et al. Quantification of Regulatory T Cells Enables the Identification of High-Risk Breast Cancer Patients and Those at Risk of Late Relapse. *J Clin Oncol*. 2006;24(34):5373–80.

189. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci U S A*. 2007;104(9):3360.

190. Miracco C, Mourmouras V, Biagioli M, Rubegni P, Mannucci S, Monciatti I, et al. Utility of tumour-infiltrating CD25+FOXP3+ regulatory T cell evaluation in predicting local recurrence in vertical growth phase cutaneous melanoma. *Oncol Rep*. 2007;18(5).

191. Escors D. Tumour immunogenicity, antigen presentation and immunological barriers in cancer immunotherapy. *New J Sci*. 2014;2014.

192. Russell JH, Ley TJ. Lymphocyte-Mediated Cytotoxicity. *Annu Rev Immunol*. 2002;20(1):323–70.

193. Spurrell EL, Lockley M. Adaptive immunity in cancer immunology and therapeutics. *Ecancermedicalscience*. 2014;8:441.

194. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol*. 2008;8(7):523–32.

195. Marsigliante S, Biscozzo L, Marra A, Nicolardi G, Leo G, Lobreglio GB, et al. Computerised counting of tumour infiltrating lymphocytes in 90 breast cancer specimens. *Cancer Lett*. 1999;139(1):33–41.

196. Chin Y, Janseens J, Vandepitte J, Vandenbrande J, Opdebeek L, Raus J. Phenotypic analysis of tumor-infiltrating lymphocytes from human breast cancer. *Anticancer Res*. 1992;12(5):1463–6.

197. Yuen GJ, Demissie E, Pillai S. B lymphocytes and cancer: a love-hate relationship. *Trends in cancer*. 2016;2(12):747–57.

198. Umansky V, Blattner C, Gebhardt C, Utikal J. The Role of Myeloid-Derived Suppressor Cells (MDSC) in Cancer Progression. *Vaccines*. 2016;4(4).

199. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 SYSTEM OF T CELL COSTIMULATION. *Annu Rev Immunol*. 1996;14(1):233–58.

200. Parry R V., Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi S V., et al. CTLA-4 and PD-1 Receptors Inhibit T-Cell Activation by Distinct Mechanisms. *Mol Cell Biol*. 2005;25(21):9543–53.

201. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. *N Engl J Med*. 2010;363(8):711–23.

202. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev*. 2010;236(1):219–42.

203. Eppihimer MJ, Gunn J, Freeman GJ, Greenfield EA, Chernova T, Erickson J, et al. Expression and regulation of the PD-L1 immunoinhibitory molecule on microvascular endothelial cells. *Microcirculation*. 2002;9(2):133–45.

204. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol*. 2002;169(10):5538–45.

205. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci*. 2002;99(19):12293–7.

206. Prasad V, Kaestner V. Nivolumab and pembrolizumab: Monoclonal antibodies against programmed cell death-1 (PD-1) that are interchangeable. *Semin Oncol*. 2017;44(2):132–5.

207. Fellner C. Ipilimumab (yervoy) prolongs survival in advanced melanoma: serious side effects and a hefty price tag may limit its use. *P T*. 2012;37(9):503–30.

208. Sambi M, Bagheri L, Szewczuk MR. Current Challenges in Cancer Immunotherapy: Multimodal Approaches to Improve Efficacy and Patient Response Rates. *J Oncol*. 2019;2019:1–12.

209. Jessurun CAC, Vos JAM, Limpens J, Luiten RM. Biomarkers for Response of Melanoma Patients to Immune Checkpoint Inhibitors: A Systematic Review. *Front Oncol*. 2017;7:233.

210. Maleki Vareki S. High and low mutational burden tumors versus immunologically hot and cold tumors and response to immune checkpoint inhibitors. *J Immunother Cancer*. 2018;6(1):157.

211. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science (80-)*. 2015;348(6230):56–61.

212. Darragh LB, Oweida AJ, Karam SD. Overcoming Resistance to Combination Radiation-Immunotherapy: A Focus on Contributing Pathways Within the Tumor Microenvironment. *Front Immunol*. 2019;9:3154.

213. Hall DA, Jackson DS. International review of connective tissue research. Volume 10. 1983. 448 p.

214. GROSS J, LAPIERE CM. Collagenolytic activity in amphibian tissues: a tissue culture assay*. *Proc Natl Acad Sci*. 1962;48(6).

215. Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. *Curr Opin Cell Biol*. 2004;16(5):558–64.

216. Verma RP, Hansch C. Matrix metalloproteinases (MMPs): Chemical–biological functions and (Q)SARs. *Bioorg Med Chem*. 2007;15(6):2223–68.

217. Van Wart HE, Birkedal-Hansen H. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci U S A*. 1990;87(14):5578–82.

218. Pons T, Gómez R, Chinea G, Valencia A. Beta-propellers: associated functions and their role in human diseases. *Curr Med Chem*. 2003;10(6):505–24.

219. Galea CA, Nguyen HM, George Chandy K, Smith BJ, Norton RS. Domain structure and function of matrix metalloprotease 23 (MMP23): role in potassium channel trafficking. *Cell Mol Life Sci*. 2014;71(7):1191–210.

220. Itoh Y. Membrane-type matrix metalloproteinases: Their functions and regulations. *Matrix Biol*. 2015;44–46:207–23.

221. Fanjul-Fernández M, Folgueras AR, Cabrera S, López-Otín C. Matrix metalloproteinases: Evolution, gene regulation and functional analysis in mouse models. *Biochim Biophys Acta - Mol Cell Res*. 2010;1803(1):3–19.

222. Ujfaludi Z, Tuzesi A, Majoros H, Rothler B, Pankotai T, Boros IM. Coordinated activation of a cluster of MMP genes in response to UVB radiation. *Sci Rep*. 2018;8(1):2660.

223. Raymond L, Eck S, Mollmark J, Hays E, Tomek I, Kantor S, et al. Interleukin-1 beta induction of matrix metalloproteinase-1 transcription in chondrocytes requires ERK-dependent activation of CCAAT enhancer-binding protein-beta. *J Cell Physiol*. 2006;207(3):683–8.

224. Li D-Q, Lokeshwar BL, Solomon A, Monroy D, Ji Z, Pflugfelder SC. Regulation of MMP-9 Production by Human Corneal Epithelial Cells. *Exp Eye Res*. 2001;73(4):449–59.

225. Abba M, Patil N, Allgayer H. MicroRNAs in the Regulation of MMPs and Metastasis. *Cancers (Basel)*. 2014;6(2):625.

226. Loffek S, Schilling O, Franzke C-W. Biological role of matrix metalloproteinases: a critical balance. *Eur Respir J*. 2011;38(1):191–208.

227. Remacle AG, Rozanov D V, Fugere M, Day R, Strongin AY. Furin regulates the intracellular activation and the uptake rate of cell surface-associated MT1-MMP. *Oncogene*. 2006;25(41):5648–55.

228. Nagase H. Activation mechanisms of matrix metalloproteinases. *Biol Chem*. 1997;378(3–4):151–60.

229. Mori K, Uchida T, Yoshie T, Mizote Y, Ishikawa F, Katsuyama M, et al. A mitochondrial ROS pathway controls matrix metalloproteinase 9 levels and invasive properties in RAS -activated cancer cells. *FEBS J*. 2019;286(3):459–78.

230. Nagase H, Suzuki K, Morodomi T, Enghild JJ, Salvesen G. Activation mechanisms of the precursors of matrix metalloproteinases 1, 2 and 3. *Matrix Suppl*. 1992;1:237–44.

231. Ra H-J, Parks WC. Control of matrix metalloproteinase catalytic activity. *Matrix Biol*. 2007;26(8):587–96.

232. Stricker TP, Dumin JA, Dickeson SK, Chung L, Nagase H, Parks WC, et al. Structural analysis of the alpha(2) integrin I domain/procollagenase-1 (matrix metalloproteinase-1) interaction. *J Biol Chem*. 2001;276(31):29375–81.

233. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. *Biochim Biophys Acta*. 2010;1803(1):55.

234. Nothnick WB, Soloway P, Curry TE. Assessment of the Role of Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) during the Periovulatory Period in Female Mice Lacking a Functional TIMP-1 Gene1. *Biol Reprod*. 1997;56(5):1181–8.

235. Murphy G, Nagase H. Progress in matrix metalloproteinase research. *Mol Aspects Med*. 2008;29(5):290–308.

236. Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ, et al. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett*. 1998;435(1):39–44.

237. Kashiwagi M, Tortorella M, Nagase H, Brew K. TIMP-3 Is a Potent Inhibitor of Aggrecanase 1 (ADAM-TS4) and Aggrecanase 2 (ADAM-TS5). *J Biol Chem*. 2001;276(16):12501–4.

238. Pruessmeyer J, Ludwig A. The good, the bad and the ugly substrates for ADAM10 and ADAM17 in brain pathology, inflammation and cancer. *Semin Cell Dev Biol*. 2009;20(2):164–74.

239. Hojilla C V., Jackson HW, Khokha R. TIMP3 Regulates Mammary Epithelial Apoptosis with Immune Cell Recruitment Through Differential TNF Dependence. Lydon JP, editor. *PLoS One*. 2011;6(10):e26718.

240. Leco KJ, Waterhouse P, Sanchez OH, Gowing KL, Poole AR, Wakeham A, et al. Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). *J Clin Invest*. 2001;108(6):817–29.

241. Melendez-Zajgla J, Pozo L, Ceballos G, Maldonado V. Tissue Inhibitor of Metalloproteinases-4. The road less traveled. *Mol Cancer*. 2008;7(1):85.

242. Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci*. 2002;115(Pt 19):3719–27.

243. Sekton B. Matrix metalloproteinases – an overview. *Res Rep Biol*. 2010;2010:1.

244. Rawlings ND, Barrett AJ, Bateman A. MEROPS: the peptidase database. *Nucleic Acids Res*. 2010;38(suppl_1):D227–33.

245. Deryugina EI, Ratnikov BI, Postnova TI, Rozanov D V., Strongin AY. Processing of Integrin α_v Subunit by Membrane Type 1 Matrix Metalloproteinase Stimulates Migration of Breast Carcinoma Cells on Vitronectin and Enhances Tyrosine Phosphorylation of Focal Adhesion Kinase. *J Biol Chem*. 2002;277(12):9749–56.

246. Horiguchi M, Ota M, Rifkin DB. Matrix control of transforming growth factor-β function. *J Biochem*. 2012;152(4):321–9.

247. Zeng Z-S, Cohen AM, Guillem JG. Loss of basement membrane type IV collagen is associated with increased expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) during human colorectal tumorigenesis. *Carcinogenesis*. 1999;20(5):749–55.

248. Hawinkels LJAC, Zuidwijk K, Verspaget HW, de Jonge-Muller ESM, Duijn W van, Ferreira V, et al. VEGF release by MMP-9 mediated heparan sulphate cleavage induces colorectal cancer angiogenesis. *Eur J Cancer*. 2008;44(13):1904–13.

249. Lee S, Jilani SM, Nikolova G V., Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol*. 2005;169(4):681–91.

250. van Hinsbergh VWM, Koolwijk P. Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. *Cardiovasc Res*. 2008;78(2):203–12.

251. Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, et al. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell*. 1999;99(1):81–92.

252. Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, et al. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development*. 2004;131(23):5883–95.

253. Mosig RA, Dowling O, DiFeo A, Ramirez MCM, Parker IC, Abe E, et al. Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth. *Hum Mol Genet*. 2007;16(9):1113–23.

254. Nyman JS, Lynch CC, Perrien DS, Thiolloy S, O'Quinn EC, Patil CA, et al. Differential effects between the loss of MMP-2 and MMP-9 on structural and tissue-level properties of bone. *J Bone Miner Res*. 2011;26(6):1252–60.

255. Muller WA. Getting leukocytes to the site of inflammation. *Vet Pathol*. 2013;50(1):7–22.

256. Coito AJ. Leukocyte Transmigration across endothelial and extracellular matrix protein barriers in liver ischemia/repurfusion injury. *Curr Opin Organ Transpl*. 2011;16(1):34–40.

257. Li Q, Park PW, Wilson CL, Parks WC. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell*. 2002;111(5):635–46.

258. Chen P, Abacherli LE, Nadler ST, Wang Y, Li Q, Parks WC. MMP7 Shedding of Syndecan-1 Facilitates Re-Epithelialization by Affecting α2β1 Integrin Activation. Vij N, editor. *PLoS One*. 2009;4(8):e6565.

259. LAMBERT V, WIELOCKX B, MUNAUT C, GALOPIN C, JOST M, ITOH T, et al. MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. *FASEB J*. 2003;17(15):2290–2.

260. Tester AM, Cox JH, Connor AR, Starr AE, Dean RA, Puente XS, et al. LPS Responsiveness and Neutrophil Chemotaxis In Vivo Require PMN MMP-8 Activity. Unutmaz D, editor. *PLoS One*.

2007;2(3):e312.

261. Gearing AJH, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, et al. Processing of tumour necrosis factor-α precursor by metalloproteinases. *Nature*. 1994;370(6490):555–7.

262. Schönbeck U, Mach F, Libby P. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *J Immunol*. 1998;161(7):3340–6.

263. Agrawal S, Anderson P, Durbeej M, van Rooijen N, Ivars F, Opdenakker G, et al. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. *J Exp Med*. 2006;203(4):1007–19.

264. Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL. T cell gelatinases mediate basement membrane transmigration in vitro. *J Immunol*. 1995;154(9):4379–89.

265. Faveeuw C, Preece G, Ager A. Transendothelial migration of lymphocytes across high endothelial venules into lymph nodes is affected by metalloproteinases. *Blood*. 2001;98(3):688–95.

266. Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, López-Boado YS, Stratman JL, et al. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science*. 1999;286(5437):113–7.

267. Liuzzi GM, Mastroianni CM, Santacroce MP, Fanelli M, D'Agostino C, Vullo V, et al. Increased activity of matrix metalloproteinases in the cerebrospinal fluid of patients with HIV-associated neurological diseases. *J Neurovirol*. 2000;6(2):156–63.

268. Farhad SZ, Aminzadeh A, Mafi M, Barekatain M, Naghney M, Ghafari MR. The effect of adjunctive low-dose doxycycline and licorice therapy on gingival crevicular fluid matrix metalloproteinase-8 levels in chronic periodontitis. *Dent Res J (Isfahan)*. 2013;10(5):624–9.

269. Papazafiropoulou A, Tentolouris N. Matrix metalloproteinases and cardiovascular diseases. *Hippokratia*. 2009;13(2):76–82.

270. Lettner T, Lang R, Klausegger A, Hainzl S, Bauer JW, Wally V. MMP-9 and CXCL8/IL-8 Are Potential Therapeutic Targets in Epidermolysis Bullosa Simplex. Wiche G, editor. *PLoS One*. 2013;8(7):e70123.

271. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646–74.

272. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell*. 2010;141(1):52–67.

273. Vihinen P, Kähäri V-M, Kapp M, Dietl J, Anacker J, Duffy M, et al. Matrix metalloproteinases in cancer: Prognostic markers and therapeutic targets. *Int J Cancer*. 2002;99(2):157–66.

274. Cathcart J, Pulkoski-Gross A, Cao J. Targeting matrix metalloproteinases in cancer: Bringing new life to old ideas. *Genes Dis*. 2015;2(1):26–34.

275. Sparano JA, Bernardo P, Stephenson P, Gradishar WJ, Ingle JN, Zucker S, et al. Randomized Phase III Trial of Marimastat Versus Placebo in Patients With Metastatic Breast Cancer Who Have Responding or Stable Disease After First-Line Chemotherapy: Eastern Cooperative Oncology Group Trial E2196. *J Clin Oncol*. 2004;22(23):4683–90.

276. Fingleton B. MMPs as therapeutic targets—Still a viable option? *Semin Cell Dev Biol*. 2008;19(1):61–8.

277. Decock J, Thirkettle S, Wagstaff L, Edwards DR. Matrix metalloproteinases: protective roles in cancer. *J Cell Mol Med*. 2011;15(6):1254–65.

278. López-Otín C, Palavalli LH, Samuels Y. Protective roles of matrix metalloproteinases: from mouse models to human cancer. *Cell Cycle*. 2009;8(22):3657–62.

279. López-Otín C, Matrisian LM. Emerging roles of proteases in tumour suppression. *Nat Rev Cancer*. 2007;7(10):800–8.

280. Vizoso FJ, González LO, Corte MD, Rodríguez JC, Vázquez J, Lamelas ML, et al. Study of matrix metalloproteinases and their inhibitors in breast cancer. *Br J Cancer*. 2007;96(6):903–11.

281. Köhrmann A, Kammerer U, Kapp M, Dietl J, Anacker J. Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. *BMC Cancer*. 2009;9:188.

282. Benson CS, Babu SD, Radhakrishna S, Selvamurugan N, Ravi Sankar B. Expression of matrix metalloproteinases in human breast cancer tissues. *Dis Markers*. 2013;34(6):395–405.

283. Gonzalez LO, Corte MD, Vazquez J, Junquera S, Sanchez R, Alvarez AC, et al. Androgen receptor expresion in breast cancer: relationship with clinicopathological characteristics of the tumors, prognosis, and expression of metalloproteases and their inhibitors. *BMC Cancer*. 2008;8:149.

284. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF, et al. Prospective Validation of a 21-Gene Expression Assay in Breast Cancer. *N Engl J Med*. 2015;373(21):2005–14.

285. Tian S, Roepman P, Veer LJ van't, Bernards R, Snoo F de, Glas AM. Biological Functions of the Genes in the Mammaprint Breast Cancer Profile Reflect the Hallmarks of Cancer. *Biomark Insights*. 2010;5:129.

286. Decock J, Hendrickx W, Vanleeuw U, Van Belle V, Van Huffel S, Christiaens M, et al. Plasma MMP1 and MMP8 expression in breast cancer: Protective role of MMP8 against lymph node metastasis. *BMC Cancer*. 2008;8(1):77.

287. Gutierrez-Fernandez A, Fueyo A, Folgueras AR, Garabaya C, Pennington CJ, Pilgrim S, et al. Matrix Metalloproteinase-8 Functions as a Metastasis Suppressor through Modulation of Tumor Cell Adhesion and Invasion. *Cancer Res*. 2008;68(8):2755–63.

288. Knäuper V, López-Otin C, Smith B, Knight G, Murphy G. Biochemical characterization of human collagenase-3. *J Biol Chem*. 1996;271(3):1544–50.

289. Van Lint P, Libert C. Matrix metalloproteinase-8: Cleavage can be decisive. *Cytokine Growth Factor Rev*. 2006;17(4):217–23.

290. Agarwal D, Goodison S, Nicholson B, Tarin D, Urquidi V. Expression of matrix metalloproteinase 8 (MMP-8) and tyrosinase-related protein-1 (TYRP-1) correlates with the absence of metastasis in an isogenic human breast cancer model. *Differentiation*. 2003;71(2):114–25.

291. Montel V, Kleeman J, Agarwal D, Spinella D, Kawai K, Tarin D. Altered Metastatic Behavior of Human Breast Cancer Cells after Experimental Manipulation of Matrix Metalloproteinase 8 Gene Expression. *Cancer Res*. 2004;64(5).

292. Balbín M, Fueyo A, Tester AM, Pendás AM, Pitiot AS, Astudillo A, et al. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet*. 2003;35(3):252–7.

293. Korpi JT, Kervinen V, Mäklin H, Väänänen A, Lahtinen M, Läärä E, et al. Collagenase-2 (matrix metalloproteinase-8) plays a protective role in tongue cancer. *Br J Cancer*. 2008;98(4).

294. Khanna-Gupta A, Zibello T, Idone V, Sun H, Lekstrom-Himes J, Berliner N. Human neutrophil collagenase expression is C/EBP-dependent during myeloid development. *Exp Hematol*. 2005;33(1):42–52.

295. Fantozzi A, Christofori G. Mouse models of breast cancer metastasis. *Breast Cancer Res*. 2006;8(4):212.

296. Decock J, Hendrickx W, Thirkettle S, Gutiérrez-Fernández A, Robinson SD, Edwards DR. Pleiotropic functions of the tumor- and metastasis-suppressing matrix metalloproteinase-8 in mammary cancer in MMTV-PyMT transgenic mice. *Breast Cancer Res*. 2015;17(1):1–13.

297. Stadlmann S, Pollheimer J, Moser PLL, Raggi A, Amberger A, Margreiter R, et al. Cytokine-regulated expression of collagenase-2 (MMP-8) is involved in the progression of ovarian cancer. *Eur J Cancer*. 2003;39(17):2499–505.

298. Gutierrez-Fernandez A, Inada M, Balbin M, Fueyo A, Pitiot AS, Astudillo A, et al. Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *FASEB J*. 2007;21(10):2580–91.

299. Lempinen M, Lyytinen I, Nordin A, Tervahartiala T, Mäkisalo H, Sorsa T, et al. Prognostic value of serum MMP-8, -9 and TIMP-1 in patients with hepatocellular carcinoma. *Ann Med*. 2013;45(7):482–7.

300. Sirniö P, Tuomisto A, Tervahartiala T, Sorsa T, Klintrup K, Karhu T, et al. High-serum MMP-8 levels are associated with decreased survival and systemic inflammation in colorectal cancer. *Br J Cancer*. 2018;119(2):213–9.

301. Owen C a, Hu Z, Lopez-Otin C, Shapiro SD. Membrane-bound matrix metalloproteinase-8 on activated polymorphonuclear cells is a potent, tissue inhibitor of metalloproteinase-resistant collagenase and serpinase. *J Immunol*. 2004;172(12):7791–803.

302. Qing C. The molecular biology in wound healing & non-healaling wound. *Chinese J Traumatol = Zhonghua chuang shang za zhi*. 2017;20(4):189–93.

303. de Oliveira S, Rosowski EE, Huttenlocher A. Neutrophil migration in infection and wound repair: going forward in reverse. *Nat Rev Immunol*. 2016;16(6):378–91.

304. García S, Forteza J, López-Otin C, Gómez-Reino JJ, González A, Conde C. Matrix metalloproteinase-8 deficiency increases joint inflammation and bone erosion in the K/BxN serum-transfer arthritis model. *Arthritis Res Ther*. 2010;12(6):R224.

305. Cox JH, Starr AE, Kappelhoff R, Yan R, Roberts CR, Overall CM. Matrix metalloproteinase 8 deficiency in mice exacerbates inflammatory arthritis through delayed neutrophil apoptosis and reduced caspase 11 expression. *Arthritis Rheum*. 2010;62(12):3645–55.

306. Kuula H, Salo T, Pirilä E, Tuomainen AM, Jauhiainen M, Uitto V-J, et al. Local and Systemic Responses in Matrix Metalloproteinase 8-Deficient Mice during Porphyromonas gingivalis-Induced Periodontitis. *Infect Immun*. 2009;77(2):850–9.

307. Emingil G, Atilla G, Sorsa T, Luoto H, Kirilmaz L, Baylas H. The Effect of Adjunctive Low-Dose Doxycycline Therapy on Clinical Parameters and Gingival Crevicular Fluid Matrix Metalloproteinase-8 Levels in Chronic Periodontitis. *J Periodontol*. 2004;75(1):106–15.

308. Gueders MM, Balbin M, Rocks N, Foidart J-M, Gosset P, Louis R, et al. Matrix metalloproteinase-8 deficiency promotes granulocytic allergen-induced airway inflammation. *J Immunol*. 2005;175(4):2589–97.

309. Van Lint P, Wielockx B, Puimège L, Noël A, López-Otin C, Libert C, et al. Resistance of collagenase-2 (matrix metalloproteinase-8)-deficient mice to TNF-induced lethal hepatitis. *J Immunol*. 2005;175(11):7642–9.

310. Vandenbroucke RE, Dejonckheere E, Van Lint P, Demeestere D, Van Wonterghem E, Vanlaere I, et al. Matrix Metalloprotease 8-Dependent Extracellular Matrix Cleavage at the Blood-CSF Barrier Contributes to Lethality during Systemic Inflammatory Diseases. *Neurobiol Dis*. 2012;32(29):9805–9816.

311. Nwomeh BC, Liang H-XX, Cohen IKK, Yager DR. MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers. *J Surg Res*. 1999;81(2):189–95.

312. García-Prieto E, González-López A, Cabrera S, Astudillo A, Gutiérrez-Fernández A, Fanjul-Fernandez M, et al. Resistance to bleomycin-induced lung fibrosis in MMP-8 deficient mice is mediated by interleukin-10. *PLoS One*. 2010;5(10):e13242.

313. Sorsa T, Tjäderhane L, Salo T. Matrix metalloproteinases (MMPs) in oral diseases. *Oral Dis*. 2004;10(6):311–8.

314. Sorsa T, Tjäderhane L, Konttinen YT, Lauhio A, Salo T, Lee H, et al. Matrix metalloproteinases: Contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med*. 2006;38(5):306–21.

315. Koltsova EK, Ley K. The Mysterious Ways of the Chemokine CXCL5. *Immunity*. 2010;33:7–9.

316. Mei J, Liu Y, Dai N, Favara M, Greene T, Jeyaseelan S, et al. CXCL5 regulates chemokine scavenging and pulmonary host defense to bacterial infection. *Immunity*. 2010;33(1):106–17.

317. Rot A. Neutrophil attractant/activation protein-1 (interleukin-8) inducesin vitro neutrophil migration by haptotactic mechanism. *Eur J Immunol*. 1993;23(1):303–6.

318. Schröder JM, Sticherling M, Henneicke HH, Preissner WC, Christophers E, Loof A De, et al. IL-1 alpha or tumor necrosis factor-alpha stimulate release of three NAP-1/IL-8-related neutrophil chemotactic proteins in human dermal fibroblasts. *J Immunol*. 1990;144(6):2223–32.

319. Fortelny N, Cox JH, Kappelhoff R, Starr AE, Lange PF, Pavlidis P, et al. Network analyses reveal pervasive functional regulation between proteases in the human protease web. *PLoS Biol*. 2014;12(5):e1001869.

320. Thirkettle S, Decock J, Arnold H, Pennington CJ, Jaworski DM, Edwards DR. Matrix metalloproteinase 8 (collagenase 2) induces the expression of interleukins 6 and 8 in breast cancer cells. *J Biol Chem*. 2013;288(23):16282–94.

321. Taylor MA, Lee Y-H, Schiemann WP. Role of TGF-β and the tumor microenvironment during mammary tumorigenesis. *Gene Expr*. 2011;15(3):117–32.

322. Santibanez JF, Obradović H, Kukolj T, Krstić J. Transforming growth factor-β, matrix metalloproteinases, and urokinase-type plasminogen activator interaction in the cancer epithelial to mesenchymal transition. *Dev Dyn*. 2018;247(3):382–95.

323. Xu J, Lamouille S, Derynck R. TGF-β-induced epithelial to mesenchymal transition. *Cell Res*. 2009;19(2):156–72.

324. Pickup M, Novitskiy S, Moses HL. The roles of TGFβ in the tumour microenvironment. *Nat Rev Cancer*. 2013;13(11):788–99.

325. Gong D, Shi W, Yi S, Chen H, Groffen J, Heisterkamp N. TGFβ signaling plays a critical role in promoting alternative macrophage activation. *BMC Immunol*. 2012;13(1):31.

326. Wen G, Zhang C, Chen Q, Luong LA, Mustafa A, Ye S, et al. A Novel Role of Matrix Metalloproteinase-8 in Macrophage Differentiation and Polarization. *J Biol Chem*. 2015;290(31):19158–72.

327. Soria-Valles C, Gutiérrez-Fernández A, Guiu M, Mari B, Fueyo A, Gomis RR, et al. The anti-metastatic activity of collagenase-2 in breast cancer cells is mediated by a signaling pathway involving decorin and miR-21. *Oncogene*. 2014;33(23):3054–63.

328. Limaye A, Hall B, Kulkarni AB. Manipulation of mouse embryonic stem cells for knockout mouse production. *Curr Protoc cell Biol*. 2009;Chapter 19:Unit 19.13 19.13.1-24.

329. Gurumurthy CB, Lloyd KCK. Generating mouse models for biomedical research: technological advances. *Dis Model Mech*. 2019;12(1):dmm029462.

330. Rivera J, Tessarollo L. Genetic Background and the Dilemma of Translating Mouse Studies to Humans. *Immunity*. 2008;28(1):1–4.

331. Flaherty L. Congenic Strains. Vol. 1. N.Y Academic Press; 1981.

332. Silver LM. Mouse Genetics: Concepts and Applications. Oxford University Press, Oxford; 1995.

333. Simpson EM, Linder CC, Sargent EE, Davisson MT, Mobraaten LE, Sharp JJ. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nat Genet*. 1997;16(1):19–27.

334. Lusis AJ, Yu J, Wang SS. The problem of passenger genes in transgenic mice. *Arterioscler Thromb Vasc Biol*. 2007;27(10):2100–3.

335. Vanden Berghe T, Hulpiau P, Martens L, Vandenbroucke RE, Van Wonterghem E, Perry SW, et al. Passenger Mutations Confound Interpretation of All Genetically Modified Congenic Mice. *Immunity*. 2015;43(1):200–9.

336. Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, et al. Mice Deficient in IL-lp-Converting Enzyme Are Defective in Production of Mature IL-lp and Resistant to Endotoxic Shock [Internet]. Vol. 80, Cell. 1995.

337. Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, et al. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science*. 1995;267(5206):2000–3.

338. Kayagaki N, Warming S, Lamkanfi M, Walle L Vande, Louie S, Dong J, et al. Non-canonical inflammasome activation targets caspase-11. *Nature*. 2011;479(7371):117–21.

339. Man SM, Karki R, Briard B, Burton A, Gingras S, Pelletier S, et al. Differential roles of caspase-1 and caspase-11 in infection and inflammation. *Sci Rep*. 2017;7(1):45126.

340. Ying W, Cheruku PS, Bazer FW, Safe SH, Zhou B. Investigation of macrophage polarization using bone marrow derived macrophages. *J Vis Exp*. 2013;(76).

341. Neri A, Marrelli D, Megha T, Bettarini F, Tacchini D, De Franco L, et al. "Clinical significance of multifocal and multicentric breast cancers and choice of surgical treatment: a retrospective study on a series of 1158 cases." *BMC Surg*. 2015;15(1):1.

342. Behbod F, Gomes AM, Machado HL. Modeling Human Ductal Carcinoma In Situ in the Mouse. *J Mammary Gland Biol Neoplasia*. 2018;23(4):269–78.

343. Berg H. Transcriptomic analysis of MMP8 effects on murine mammary carcinoma progression [Internet]. University of East Anglia; 2016.

344. Craig VJ, Polverino F, Laucho-Contreras ME, Shi Y, Liu Y, Osorio JC, et al. Mononuclear Phagocytes and Airway Epithelial Cells: Novel Sources of Matrix Metalloproteinase-8 (MMP-8) in Patients with Idiopathic Pulmonary Fibrosis. Mora A, editor. *PLoS One*. 2014;9(5):e97485.

345. Hanemaaijer R, Sorsa T, Konttinen YT, Ding Y, Sutinen M, Visser H, et al. Matrix Metalloproteinase-8 Is Expressed in Rheumatoid Synovial Fibroblasts and Endothelial Cells. *J Biol Chem*. 1997;272(50):31504–9.

346. Sarper M, Allen MD, Gomm J, Haywood L, Decock J, Thirkettle S, et al. Loss of MMP-8 in ductal carcinoma in situ (DCIS)-associated myoepithelial cells contributes to tumour promotion through altered adhesive and proteolytic function. *Breast Cancer Res*. 2017;19(1):33.

347. Leung E, Kim JE, Askarian-Amiri M, Finlay GJ, Baguley BC. Evidence for the existence of triple-negative variants in the MCF-7 breast cancer cell population. *Biomed Res Int*. 2014;2014:836769.

348. Lopez-Tarruella S, Schiff R. The Dynamics of Estrogen Receptor Status in Breast Cancer: Re-shaping the Paradigm. *Clin Cancer Res*. 2007;13(23):6921–5.

349. Su X, Esser AK, Amend SR, Xiang J, Xu Y, Ross MH, et al. Antagonizing integrin β3 increases immunosuppression in cancer. *Cancer Res*. 2016;76(12):3484–95.

350. Johnstone CN, Smith YE, Cao Y, Burrows AD, Cross RSN, Ling X, et al. Functional and molecular characterisation of EO771.LMB tumours, a new C57BL/6-mouse-derived model of spontaneously metastatic mammary cancer. *Dis Model Mech*. 2015;8(3):237–51.

351. Duffy M, Blaser J, Duggan C, McDermott E, O'Higgins N, Fennelly J, et al. Assay of matrix metalloproteases types 8 and 9 by ELISA in human breast cancer. *Br J Cancer*. 1995;71(5):1025–8.

352. Van den Steen PE, Wuyts A, Husson SJ, Proost P, Van Damme J, Opdenakker G. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem*. 2003;270(18):3739–49.

353. Kirkup BM, Mckee A, Makin KA, Paveley J, Caim S, Alcon-Giner C, et al. Perturbation of the gut microbiota by antibiotics results in accelerated breast tumour growth and metabolic dysregulation Running Title: Antibiotics drive breast tumour growth and alter metabolic processes. *Biorxiv Prepr*. 2019;

354. Schreiber RD, Old LJ, Smyth MJ. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. *Science (80-)*. 2011;331(6024):1565–70.

355. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL, et al. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res*. 1996;56(20):4625–9.

356. Pellinen T, Rantala JK, Arjonen A, Mpindi J-P, Kallioniemi O, Ivaska J. A functional genetic screen reveals new regulators of 1-integrin activity. *J Cell Sci*. 2012;125(3):649–61.

357. Neurath MF, Finotto S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev*. 2011;22(2):83–9.

358. Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, et al. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res*. 2000;6(8):3282–9.

359. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. *J Gen Physiol*. 1927;8(6):519–30.

360. Schwartz L, Supuran C, Alfarouk K. The Warburg Effect and the Hallmarks of Cancer. *Anticancer Agents Med Chem*. 2017;17(2):164–70.

361. Badowska-Kozakiewicz AM, Budzik MP. Immunohistochemical characteristics of basal-like breast cancer. *Contemp Oncol (Poznan, Poland)*. 2016;20(6):436–43.

362. Wang J, Shao Y, Wang W, Li S, Xin N, Xie F, et al. Caspase-11 deficiency impairs neutrophil recruitment and bacterial clearance in the early stage of pulmonary Klebsiella pneumoniae infection. *Int J Med Microbiol*. 2017;307(8):490–6.

363. Bell RA V, Megeney LA. Evolution of caspase-mediated cell death and differentiation: twins separated at birth. *Cell Death Differ*. 2017;24(8):1359–68.

364. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol*. 2013;5(4):a008656.

365. Yi Y-S. Caspase-11 non-canonical inflammasome: a critical sensor of intracellular lipopolysaccharide in macrophage-mediated inflammatory responses. *Immunology*. 2017;152(2):207–17.

366. Viganò E, Diamond CE, Spreafico R, Balachander A, Sobota RM, Mortellaro A. Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. *Nat Commun*. 2015;6(1):8761.

367. Zanoni I, Tan Y, Di Gioia M, Broggi A, Ruan J, Shi J, et al. An endogenous caspase-11 ligand

elicits interleukin-1 release from living dendritic cells. *Science*. 2016;352(6290):1232–6.

368. Vandenbroucke RE, Vanlaere I, Van Hauwermeiren F, Van Wonterghem E, Wilson C, Libert C. Pro-inflammatory effects of matrix metalloproteinase 7 in acute inflammation. *Mucosal Immunol*. 2014;7(3):579–88.

369. Vandenbroucke RE, Dejonckheere E, Van Hauwermeiren F, Lodens S, De Rycke R, Van Wonterghem E, et al. Matrix metalloproteinase 13 modulates intestinal epithelial barrier integrity in inflammatory diseases by activating TNF. *EMBO Mol Med*. 2013;5(7):1000–16.

370. Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science*. 2013;341(6151):1250–3.

371. Middlebrook AJ, Austin C, Santos D, Snowden E, Porter W, Hahn F, et al. The effects of enzymatic digestion on epitope detection by flow cytometry. In: Tumor Biology. American Association for Cancer Research; 2018. p. 2113–2113.

372. Botting RA, Bertram KM, Baharlou H, Sandgren KJ, Fletcher J, Rhodes JW, et al. Phenotypic and functional consequences of different isolation protocols on skin mononuclear phagocytes. *J Leukoc Biol*. 2017;101(6):1393.

373. Autengruber A, Gereke M, Hansen G, Hennig C, Bruder D. Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. *Eur J Microbiol Immunol*. 2012;2(2):112–20.

374. Lynch GW, Slaytor EK, Elliott FD, Saurajen A, Turville SG, Sloane AJ, et al. CD4 is expressed by epidermal Langerhans' cells predominantly as covalent dimers. *Exp Dermatol*. 2003;12(5):700–11.

375. Kirkup B, McKee A, Makin K, Paveley J, Caim S, Alcon-Giner C, et al. Perturbation of the gut microbiota by antibiotics results in accelerated breast tumour growth and metabolic dysregulation. *bioRxiv*. 2019;553602.

376. Zhou C, Wang M, Zhou L, Zhang Y, Liu W, Qin W, et al. Prognostic significance of PLIN1 expression in human breast cancer. *Oncotarget*. 2016;7(34):54488–502.

377. Zhang Y, Zhang G-L, Sun X, Cao K-X, Ma C, Nan N, et al. Establishment of a murine breast tumor model by subcutaneous or orthotopic implantation. *Oncol Lett*. 2018;15(5):6233–40.

378. Atkinson SJ, Gontarczyk AM, Alghamdi AA, Ellison TS, Johnson RT, Fowler WJ, et al. The β3-integrin endothelial adhesome regulates microtubule-dependent cell migration. *EMBO Rep*. 2018;19(7).

379. Ellison TS, Atkinson SJ, Steri V, Kirkup BM, Preedy MEJ, Johnson RT, et al. Suppression of β3-integrin in mice triggers a neuropilin-1-dependent change in focal adhesion remodelling that can be targeted to block pathological angiogenesis. *Dis Model Mech*. 2015;8(9):1105–19.

380. Owens T, Toft-Hansen H, Nuttall RK, Edwards DR. Autoimmune Encephalomyelitis Specific Cell Types in Experimental Key Metalloproteinases Are Expressed by. *J Immunol Ref*. 2004;173:5209–18.

381. Clark RT, Nance JP, Noor S, Wilson EH. T-cell production of matrix metalloproteinases and inhibition of parasite clearance by TIMP-1 during chronic Toxoplasma infection in the brain. *ASN Neuro*. 2011;3(1):e00049.

382. Wahlgren J, Maisi P, Sorsa T, Sutinen M, Tervahartiala T, Piril E, et al. Expression and induction of collagenases (MMP-8 and -13) in plasma cells associated with bone-destructive lesions. *J Pathol*. 2001;194(2):217–24.

383. Hasty, K. A., Pourmotabbed, T. F., Goldberg, G. I., Thompson, J. P., Spinella, D. G., Stevens, R.M., and Mainardi CL. Human neutrophil collagenase: A distinct gene product with homology to other matrix metalloproteinases. *J Biol Chem*. 1990;265:11421–4.

384. Vanlaere I, Libert C. Matrix Metalloproteinases as Drug Targets in Infections Caused by Gram-Negative Bacteria and in Septic Shock. *Clin Microbiol Rev*. 2009;22(2):224–39.

385. Dejonckheere E, Vandenbroucke RE, Libert C, E. D, R.E. V, C. L. Matrix metalloproteinase8 has a central role in inflammatory disorders and cancer progression. *Cytokine Growth Factor Rev*. 2011;22(2):73–81.

386. Zhang L, Li X, Yan H, Huang L. Salivary matrix metalloproteinase (MMP)-8 as a biomarker for periodontitis: A PRISMA-compliant systematic review and meta-analysis. *Medicine (Baltimore)*. 2018;97(3):e9642.

387. Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. *Curr Protoc Immunol*. 2008;Chapter 14:Unit 14.1.

388. Opdenakker G, Fibbe WE, Van Damme J. The molecular basis of leukocytosis. *Immunol Today*. 1998;19(4):182–9.

389. Scheiermann C, Frenette PS, Hidalgo A. Regulation of leucocyte homeostasis in the circulation. *Cardiovasc Res*. 2015;107(3):340.

390. Hughes CE, Benson RA, Bedaj M, Maffia P. Antigen-Presenting Cells and Antigen Presentation in Tertiary Lymphoid Organs. *Front Immunol*. 2016;7:481.

391. Pineda-Torra I, Gage M, de Juan A, Pello OM. Isolation, Culture, and Polarization of Murine Bone Marrow-Derived and Peritoneal Macrophages. In Humana Press, New York, NY; 2015. p. 101–9.

392. Huang X, Li Y, Fu M, Xin H-B. Polarizing Macrophages In Vitro. In: Methods in molecular biology (Clifton, NJ). 2018. p. 119–26.

393. Mills CD, Ley K. M1 and M2 Macrophages: The Chicken and the Egg of Immunity. *J Innate Immun*. 2014;6(6):716–26.

394. Baitsch D, Bock HH, Engel T, Telgmann R, Müller-Tidow C, Varga G, et al. Apolipoprotein E Induces Antiinflammatory Phenotype in Macrophages. *Arterioscler Thromb Vasc Biol*. 2011;31(5):1160–8.

395. Nayak S, Herzog RW. Progress and prospects: immune responses to viral vectors. *Gene Ther*. 2010;17(3):295–304.

396. Kim S-Y, Kang JW, Song X, Kim BK, Yoo YD, Kwon YT, et al. Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. *Cell Signal*. 2013;25(4):961–9.

397. Middleton K, Jones J, Lwin Z, Coward JIG. Interleukin-6: An angiogenic target in solid tumours. *Crit Rev Oncol Hematol*. 2014;89(1):129–39.

398. Iwase S, Murakami T, Saito Y, Nakagawa K. Steep elevation of blood interleukin-6 (IL-6) associated only with late stages of cachexia in cancer patients. *Eur Cytokine Netw*. 2004;15(4):312–6.

399. Eichten A, Su J, Adler AP, Zhang L, Ioffe E, Parveen AA, et al. Resistance to Anti-VEGF Therapy Mediated by Autocrine IL6/STAT3 Signaling and Overcome by IL6 Blockade. *Cancer Res*. 2016;76(8):2327–39.

400. Dethlefsen C, Højfeldt G, Hojman P. The role of intratumoral and systemic IL-6 in breast cancer. *Breast Cancer Res Treat*. 2013;138(3):657–64.

401. Chervenick P, Boggs D, Marsh J, Cartwright G, Wintrobe M. Quantitative studies of blood and bone marrow neutrophils in normal mice. *Am J Physiol Content*. 1968;215(2):353–60.

402. Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. *Immunology*. 2008;125(3):281–8.

403. Strydom N, Rankin SM. Regulation of Circulating Neutrophil Numbers under Homeostasis and in Disease. *J Innate Immun*. 2013;5(4):304–14.

404. Seebach JD, Morant R, Rüegg R, Seifert B, Fehr J. The Diagnostic Value of the Neutrophil Left Shift in Predicting Inflammatory and Infectious Disease. *Am J Clin Pathol*. 1997;107(5):582–91.

405. Challen GA, Boles N, Lin KK-Y, Goodell MA. Mouse hematopoietic stem cell identification and analysis. *Cytometry A*. 2009;75(1):14–24.

406. Quintero PA, Knolle MD, Cala LF, Zhuang Y, Owen CA. Matrix metalloproteinase-8 inactivates macrophage inflammatory protein-1 alpha to reduce acute lung inflammation and injury in mice. *J Immunol*. 2010;184(3):1575–88.

407. Pelman GR, Morrison CJ, Overall CM. Pivotal molecular determinants of peptidic and collagen triple helicase activities reside in the S3' subsite of matrix metalloproteinase 8 (MMP-8): the role of hydrogen bonding potential of ASN188 and TYR189 and the connecting cis bond. *J Biol Chem*. 2005;280(3):2370–7.

408. Gordon MY. Extracellular matrix of the marrow microenvironment. *Br J Haematol*. 1988;70(1):1–4.

409. O'Reilly PJ, Hardison MT, Jackson PL, Xu X, Snelgrove RJ, Gaggar A, et al. Neutrophils contain prolyl endopeptidase and generate the chemotactic peptide, PGP, from collagen. *J Neuroimmunol*. 2009;217(1–2):51–4.

410. Lin M, Jackson P, Tester AM, Diaconu E, Overall CM, Blalock JE, et al. Matrix Metalloproteinase-8 Facilitates Neutrophil Migration through the Corneal Stromal Matrix by Collagen Degradation and Production of the Chemotactic Peptide Pro-Gly-Pro. *Am J Pathol*. 2008;173(1):144.

411. Tsilingaridis G, Yucel-Lindberg T, Concha Quezada H, Modéer T. The relationship between matrix metalloproteinases (MMP-3, -8, -9) in serum and peripheral lymphocytes ($CD8^+$, $CD56^+$) in Down syndrome children with gingivitis. *J Periodontal Res*. 2014;49(6):742–50.

412. Yang L, Pang Y, Moses HL. TGF-β and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends Immunol*. 2010;31(6):220–7.

413. Shuhendler AJ, Prasad P, Cai P, Hui KKW, Henderson JT, Rauth AM, et al. Matrigel alters the pathophysiology of orthotopic human breast adenocarcinoma xenografts with implications for nanomedicine evaluation. *Nanomedicine Nanotechnology, Biol Med*. 2013;9(6):795–805.

414. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell*. 1988;54(1):105–15.

415. Davie SA, Maglione JE, Manner CK, Young D, Cardiff RD, MacLeod CL, et al. Effects of FVB/NJ and C57Bl/6J strain backgrounds on mammary tumor phenotype in inducible nitric oxide synthase deficient mice. *Transgenic Res*. 2007;16(2):193–201.

416. Lupu R, Lippman ME. The role of erbB2 signal transduction pathways in human breast cancer. *Breast Cancer Res Treat*. 1993;27(1–2):83–93.

417. Freund R, Dubensky T, Bronson R, Sotnikov A, Carroll J, Benjamin T. Polyoma tumorigenesis in mice: Evidence for dominant resistance and dominant susceptibility genes of the host. *Virology*. 1992;191(2):724–31.

418. Law LW, Ting RC, Leckband E. Prevention of virus-induced neoplasms in mice through passive transfer of immunity by sensitized syngeneic lymphoid cells. *Proc Natl Acad Sci U S A*. 1967;57(4):1068–75.

419. Kamath AB, Alt J, Debbabi H, Taylor C, Behar SM. The Major Histocompatibility Complex Haplotype Affects T-Cell Recognition of Mycobacterial Antigens but Not Resistance to

Mycobacterium tuberculosis in C3H Mice. *Infect Immun*. 2004;72(12):6790–8.

420. Oomen LC, Van der Valk MA, Den Engelse L. Tumour susceptibility in mice in relation to H-2 haplotype. *IARC Sci Publ*. 1983;(51):205–21.

421. Justice MJ, Dhillon P. Using the mouse to model human disease: increasing validity and reproducibility. 2016;

422. Bratburd JR, Keller C, Vivas E, Gemperline E, Li L, Rey FE, et al. Gut Microbial and Metabolic Responses to Salmonella enterica Serovar Typhimurium and Candida albicans. *MBio*. 2018;9(6):e02032-18.

423. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science*. 2015;350(6264):1084–9.

424. Robertson SJ, Lemire P, Maughan H, Goethel A, Turpin W, Bedrani L, et al. Comparison of Co-housing and Littermate Methods for Microbiota Standardization in Mouse Models. *Cell Rep*. 2019;27(6):1910-1919.e2.

425. Miyoshi J, Leone V, Nobutani K, Musch MW, Martinez-Guryn K, Wang Y, et al. Minimizing confounders and increasing data quality in murine models for studies of the gut microbiome. *PeerJ*. 2018;6:e5166.

426. DuPré SA, Redelman D, Hunter KW, Jr. The mouse mammary carcinoma 4T1: characterization of the cellular landscape of primary tumours and metastatic tumour foci. *Int J Exp Pathol*. 2007;88(5):351–60.

427. Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ, Londos C. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J Biol Chem*. 1991;266(17):11341–6.

428. Miyoshi H, Souza SC, Zhang H-H, Strissel KJ, Christoffolete MA, Kovsan J, et al. Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *J Biol Chem*. 2006;281(23):15837–44.

429. Londos C, Brasaemle DL, Gruia-Gray J, Servetnick DA, Schultz CJ, Levin DM, et al. Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells. *Biochem Soc Trans*. 1995;23(3):611–5.

430. Russell TD, Palmer CA, Orlicky DJ, Fischer A, Rudolph MC, Neville MC, et al. Cytoplasmic lipid droplet accumulation in developing mammary epithelial cells: roles of adipophilin and lipid metabolism. *J Lipid Res*. 2007;48(7):1463–75.

431. Harvell DME, Spoelstra NS, Singh M, McManaman JL, Finlayson C, Phang T, et al. Molecular signatures of neoadjuvant endocrine therapy for breast cancer: characteristics of response or intrinsic resistance. *Breast Cancer Res Treat*. 2008;112(3):475–88.

432. Kinlaw WB, Quinn JL, Wells WA, Roser-Jones C, Moncur JT. Spot 14: A Marker of Aggressive Breast Cancer and a Potential Therapeutic Target. *Endocrinology*. 2006;147(9):4048–55.

433. Malvia S, Bagadi SAR, Pradhan D, Chintamani C, Bhatnagar A, Arora D, et al. Study of Gene Expression Profiles of Breast Cancers in Indian Women. *Sci Rep*. 2019;9(1):10018.

434. Hardy E, Hardy-Sosa A, Fernandez-Patron C. MMP-2: is too low as bad as too high in the cardiovascular system? *Am J Physiol Circ Physiol*. 2018;315(5):H1332–40.

435. Hernandez-Anzaldo S, Brglez V, Hemmeryckx B, Leung D, Filep JG, Vance JE, et al. Novel Role for Matrix Metalloproteinase 9 in Modulation of Cholesterol Metabolism. *J Am Heart Assoc*. 2016;5(10).

436. Murakami M, Kudo I. Phospholipase A2. *J Biochem*. 2002;131(3):285–92.

437. Salminen A, Åström P, Metso J, Soliymani R, Salo T, Jauhiainen M, et al. Matrix metalloproteinase 8 degrades apolipoprotein A-I and reduces its cholesterol efflux capacity. *FASEB J*. 2015;29(4):1435–45.

438. Favari E, Chroni A, Tietge UJF, Zanotti I, Escolà-Gil JC, Bernini F. Cholesterol Efflux and Reverse Cholesterol Transport. In 2015. p. 181–206.

439. Laxton RC, Hu Y, Duchene J, Zhang F, Zhang Z, Leung K-Y, et al. A Role of Matrix Metalloproteinase-8 in Atherosclerosis. *Circ Res*. 2009;105(9):921–9.

440. Bult CJ, Blake JA, Smith CL, Kadin JA, Richardson JE, Anagnostopoulos A, et al. Mouse Genome Database (MGD) 2019. *Nucleic Acids Res*. 2019;47(D1):D801–6.

441. Chavey C, Mari B, Monthouel M-N, Bonnafous S, Anglard P, Van Obberghen E, et al. Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. *J Biol Chem*. 2003;278(14):11888–96.

442. Chun T-H, Hotary KB, Sabeh F, Saltiel AR, Allen ED, Weiss SJ. A Pericellular Collagenase Directs the 3-Dimensional Development of White Adipose Tissue. *Cell*. 2006;125(3):577–91.

443. Motrescu ER, Rio M-C. Cancer cells, adipocytes and matrix metalloproteinase 11: a vicious tumor progression cycle. *Biol Chem*. 2008;389(8):1037–41.

444. Chun T-H, Inoue M, Morisaki H, Yamanaka I, Miyamoto Y, Okamura T, et al. Genetic link between obesity and MMP14-dependent adipogenic collagen turnover. *Diabetes*. 2010;59(10):2484–94.

445. Swinnen J V, Esquenet M, Goossens K, Heyns W, Verhoeven G. Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. *Cancer Res*. 1997;57(6):1086–90.

446. Notarnicola M, Messa C, Caruso MG. A significant role of lipogenic enzymes in colorectal cancer. *Anticancer Res*. 2012;32(7):2585–90.

447. Zhao E, Xu H, Wang L, Kryczek I, Wu K, Hu Y, et al. Bone marrow and the control of immunity. *Cell Mol Immunol*. 2012;9(1):11–9.

448. Tan YS, Lei YL. Isolation of Tumor-Infiltrating Lymphocytes by Ficoll-Paque Density Gradient Centrifugation. In: Allen IC, editor. Mouse Models of Innate Immunity: Methods and Protocols. New York, NY: Springer New York; 2019. p. 93–9.

449. American Breast Cancer Foundation. Breast Anatomy - ABCF [Internet]. 2017.

450. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958–69.

451. National Centre for Biotechnology Information. NCBI's genome browser for mouse (Mus musculus) - Genome Data Viewer [Internet].