Transcriptomic Analysis of MMP8 Effects on Murine Mammary Carcinoma Progression

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Abstract

It is well established that Matrix metalloproteinase-8 (MMP8) suppresses growth and metastasis of tumours, and accumulating data suggest that this protective effect is propagated via the immune system. Recent reports links MMP8 to TGFβ signaling, which is known to induce polarization of neutrophils and macrophages towards their pro-tumorigenic phenotypes; N2 and M2. However, the role of MMP8 as a regulator of the TGFβ mediated polarization of myeloid cells is unexplored. The present study analysed the transcriptome of tumours from MMTV-PyMT mice, intercrossed with *Mmp8*-null mice, to explore whether depletion of MMP8 affects myeloid cell polarisation and, to investigate novel pathways affected by MMP8 signaling. Results from qRT-PCR suggests an increased infiltration of both anti- and pro-tumorigenic myeloid cells at later time points, however, these results were not confirmed by RNAseq analysis. In contrast to these results, analysis of the whole transcriptome of MMTV-PyMT tumours showed that depletion of MMP8 induces an up-regulation of gene sets specific for B- and T-lymphocytes and, lipid and glycerol metabolism at 6 and 10 weeks, respectively, as well as inducing a disturbance in the web of proteases. Additionally, several oncogenic proteins showed increased expression in *Mmp8*-null mice. Although further validation is required to confirm the current findings, these results suggest a complex role of MMP8 during tumour progression, in which pleiotropic changes are observed in response to MMP8 ablation. These findings should be further explored in future studies to achieve a better understanding of the mechanistic actions of MMP8, which, in turn, could provide important implications for treatment of breast cancer patients.

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Abbreviations

Acsl-	Acetyl-CoA synthetase long-chain
ADAM	A Disintegrin Metalloproteinase
ADAMTS	A Disintegrin Metalloproteinase with Thrombospondin Motifs
APC	Antigen Presenting Cells
Arg	Arginase
BRCs	B Cell Receptors
Bregs	Regulatory B cells
CCL-	Chemokine (C-C) motif ligand
CCR-	C-C chemokine Receptor
CD	Cluster of Differentiation
CLR	Crohn's-like Lymphoid Reaction
CXCL-	C-X-C motif Chemokine Ligand
DC	Dendritic Cells
ECM	Extracellular Matrix
ER	Estrogen Receptor
FABP-	Fatty Acid Binding Protein
FC	Fold Change
FPKM	Fragments Per Kilobase of exon per Million fragments mapped
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HER-	Human Epidermal growth factor Receptor
IFN	Interferon
IL-	Interleukin
LD	Lipid Droplets
LEP	Leptin
LEPR	Leptin Receptor
LIX	Lipopolysaccharide Induced CXC chemokine
Ly6K	Lymphocyte antigen 6K
M-CSF	Macrophage Colony-Stimulating Factor
MMP	Matrix Metalloproteinase
MMTV-PyMT	Mouse Mammary Tumor Virus-Polyoma Middle T
MPI	Matrix Metalloproteinase Inhibitors
mRNA	messenger RNA
NPY	Neuropeptide Y
PCA	Principal Component Analysis
PDGF	Platelet-Derived Growth Factor
PGP	N-acetyl Pro-Gly-Pro
PLIN-	Perilipin
PR	Progesterone Receptor
QC	Quality Control

qRT-PCR	Quantitative Real-Time PCR
RNA	Ribonucleic Acid
RQS	RNA Quality Score
rRNA	Ribosomal RNA
TAM	Tumour Associated Macrophages
TAN	Tumour Associated Neutrophils
TGF	Transforming Growth Factor
Th-	T helper
TIL	Tumour Infiltrating Leukocytes
TIMP	Tissue Inhibitors of Metalloproteinases
TME	Tumour Microenvironment
TNBC	Triple Negative Breast Cancer
TNF	Tumour Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

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1. INTRODUCTION

1.1. Breast Cancer

Breast cancer is one of the major causes of female deaths and represents the most frequent malignancy in women worldwide (Torre et al., 2015; Siegel et al., 2015). Together with *in-vitro* and *in-vivo* models, comprehensive studies of tumours from patients with different clinical parameters have led to important discoveries in the field of breast cancer, and also significantly improved our understanding of the underlying mechanisms of the malignancy. Despite this, breast cancer still remains a scientific and clinical challenge, which is mainly due to the variety and complexity of malignant cells *and* their interactions with the host environment. Thus, future studies still need to address and resolve the current major gaps in breast cancer research (Eccles et al., 2013).

The normal breast is composed of adipose, connective and gland tissues, in which the latter is divided into lobes that are connected to the nipple through a network of milk ducts (Cancer Research Uk, 2016). Malignant cells most commonly develop from cells in the lobules or milk ducts with subsequent evolution, initially, into ductal carcinoma *in situ* and further into invasive breast cancer, which finally results in metastatic disease (Burnstein et al., 2004).

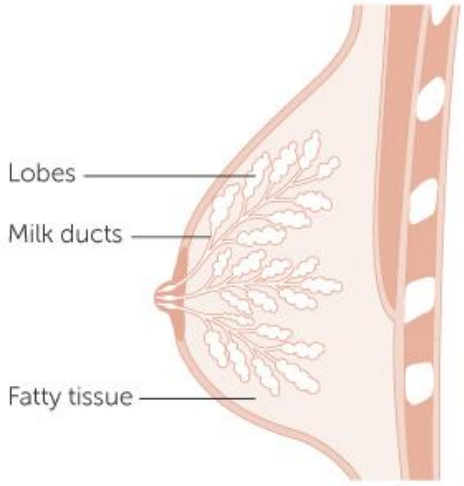


Figure 1.1: Anatomy of the female breast. Carcinogenesis commonly arises from lobules or the milk ducts. Adapted from Cancer Research UK.

Breast cancer is highly heterogeneous and has been divided into distinct subgroups to facilitate the study and clinical management of the disease. Although a universal classification is not in place, most studies divide breast cancer into subgroups based on receptor status, which results in four main subtypes: luminal A, luminal B, HER-2 (human epidermal growth factor receptor 2) type, and triple-negative/basal-like breast cancer. Luminal A and luminal B tumours are estrogen receptor (ER) and/or progesterone receptor (PR) positive, in which luminal A is HER-2 negative and have low grade, whereas luminal B is highly proliferative and/or HER-2 positive. Also, luminal B tends to be node-positive, have larger tumour size and predict poorer outcome. In contrast, HER-2 types are negative for both ER and PR, while also being node-positive and have poorer tumour grade. Further, triple-negative and basal-like tumours are negative for ER, PR and HER-2. Receptor negative tumours are considered to be the most aggressive breast cancer subtypes, which have poorer prognosis compared to receptor positive breast cancers (Fan et al., 2006; Voduc et al., 2010; Foukakis & Bergh, 2015; Carey et al., 2014; Howlader et al., 2014; Perou et al., 2000).

The primary treatment of cancer has previously relied solely on surgery in combination with cytotoxic chemotherapy and radiation therapy (Tinoco et al., 2013). For the last two decades however, a better understanding of the underlying biology of cancer cells has led to more individualised and targeted treatment strategies. In breast cancer, such therapy involves tailored strategies based on the absence or presence of receptors, specifically ER, PR, and HER-2. For instance, patients positive for hormone receptors can be treated with endocrine therapy to inhibit the effect that estrogen and progesterone has on tumour growth. Further, patients with tumours overexpressing HER-2 typically receive therapy that inhibits HER-2 that, in turn, suppress growth and proliferation of tumour cells. Both strategies are given in combination with cytotoxic chemotherapeutic agents and/or other treatment options, such as radiation therapy. Patients with triple negative breast cancer (TNBC) however, are insensitive to these targeted treatment options as they lack expression of all

three receptors. TNBC patients are thus primarily treated with cytotoxic agents (Tinoco et al., 2013; Hernandez-Aya et al., 2011; Ismail-Khan & Bui, 2010).

1.2. The Tumour Microenvironment

Previously, cancer was considered to be masses of malignant cells, in which abnormal cell growth and metastasis were thought to be solely driven by genetic mutations (Hanahan & Coussens, 2012; Hanahan & Weinberg, 2011). Recent advances however have revealed that tumours are complex organs comprised of a variety of cells that can be re-educated by malignant cells to drive tumour progression and metastasis (Balkwill et al., 2012).

Specifically, the tumour microenvironment (TME) consists of non-malignant cells, including tumour-infiltrating leukocytes (TILs), stromal cells, fibroblasts and cells of the vasculature, in addition to secreted factors such as chemokines, cytokines and growth factors (Whiteside, 2008). In the context of the immune system, it was previously thought that tumour-infiltrating immune cells solely recognized and eliminated cancer cells, a process described as immune surveillance (Burnet, 1957). However, later research has revealed that, in addition to killing tumour cells, the immune system can even promote cancer progression as a response to the tumour-immune cross-talk. This process is described as immunoediting and is divided into three phases; elimination, equilibrium and escape (Schreiber et al., 2011, Dunn et al., 2004).

The eliminating phase, also termed immune surveillance, includes efficient elimination of tumour cells by innate and adaptive immune responses. In the equilibrium phase, tumour cells evolve in response to the immune pressure and new phenotypes resistant to the immune system emerges. As a consequence, an equilibrium between immunogenic and non-immunogenic tumour cells occur. This battle between the tumour and the immune system might occur for several years and is described as the longest of the three phases. In the escape phase however, the balance tilts towards tumour progression due to an extensive emergence of tumour cell variants that are capable of evading the immune response. These cells do not only become non-responsive to the

immune system's defence but also secrete factors that suppresses the function of or kill leukocytes (Dunn et al., 2006).

Researchers increasingly emphasise the potential of targeting components of the TME, in which the immune cell repertoire has been of particular interest (e.g. Fang & Declerck, 2013; Shekarian et al., 2015; Quail & Johanna, 2013). This type of therapy includes re-programming of immune cells towards phenotypes capable of specific and enhanced responses against the tumour. Importantly, targeting immune cells not only reduces the probability of resistance, but also generally results in fewer side effects (Mittendorf & Hunt, 2015; Soliman, 2013). However, a better understanding of the characteristics of the immune cell repertoire and its interactions with other components of the TME, as well as the underlying mechanisms of action, is required in order to develop efficient immune therapy strategies that can improve the life of breast cancer patients.

1.2.1. The Inflammatory Cell Repertoire

Tumour-related inflammation can be induced through two different circuits; the extrinsic or intrinsic pathways (Mantovani et al., 2008). The extrinsic pathway is triggered by an inflammatory condition, such as inflammatory bowel disease or prostatitis, which can induce cancer initiation. In contrast, the intrinsic pathway is triggered by oncogenic events that induce expression of inflammatory pathways, such as leukocyte recruitment and survival, angiogenesis and tumour-cell homing to lymph nodes (Mantovani et al., 2008). Immune cells infiltrating the TME include innate immune cells such as macrophages, neutrophils, dendritic cells and myeloid derived suppressor cells, as well as T-lymphocytes and B-lymphocytes, which are part of the adaptive immune system.

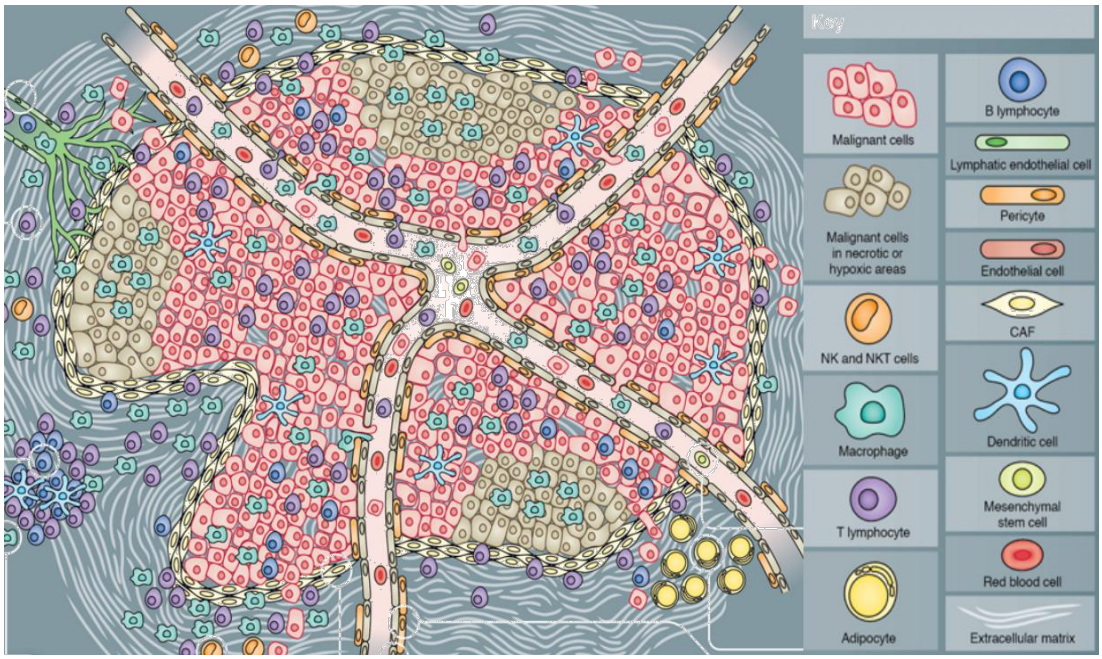


Figure 1.2: Leukocyte subpopulations within the tumour microenvironment. Leukocytes play both anti- and pro-tumorigenic roles during tumour progression. Adapted from Balkwill et al., 2012

The tumour inflammatory repertoire has shown to play a key role in tumour progression due to its characteristics of high plasticity. Interestingly, as the tumour evolves, inflammatory cells can switch from anti-tumorigenic towards a more pro-tumorigenic function in response to cues from the microenvironment (Lin & Karin, 2007; Smyth et al., 2008). In early malignancies, the balance between pro- and anti-tumorigenic inflammation is generally tilted towards an anti-tumorigenic state, which protects the host against tumour progression. However, in established tumours, a more pro-tumorigenic inflammation that supports progression of the tumour is evident. A consequence of the immune cells gradually acting as tumour-promoting cells is a decreased expression of pro-inflammatory proteins, reduced antigen-presenting capabilities and secretion of factors that promote growth and metastasis of tumour cells (Grivennikov et al., 2010). This process of immune cell transformation influences the tumour's ability to escape the immune system which, in turn, allows the tumour to rapidly progress into a more aggressive phenotype.

1.2.1.1. Dendritic Cells

Denditric cells (DCs) play an important role in the communication between innate and adaptive immunity by acting as antigen presenting cells (APCs). In cancer, DCs initiate an adaptive immune response against the tumour by recognising mutated proteins as foreign (Zitvogel et al., 2008). These tumour antigens are captured and processed by DCs before they cross-present the antigen to T helper lymphocytes (CD4+) and cytotoxic T-lymphocytes (CD8+) which, in turn, initiate a highly specific immunological response against the tumour (Steinman et al., 2007; Banchereau et al., 2000; Banchereau et al., 2005). Accordingly, studies have found a positive association between tumour-infiltrating DCs and prolonged survival in a number of different malignancies including breast, head and neck, lung and prostate cancers (Lotze, 1997).

During tumour progression, the number of DCs within the TME generally decreases, which might be explained by either tumour driven secretion of DC apoptotic factors or by a decreased differentiation of monocytes into DCs, or a combination of these two mechanisms (Koido et al., 2005). Rather than guiding differentiation of monocytes into DCs, breast cancer cells favor differentiation of monocytes into macrophages. More specifically, breast cancer cells have shown to secrete IL-6, which acts together with fibroblast secreted M-CSF to promote the macrophage directed differentiation (Chomarat et al., 2000). Additionally, a reduction in functionality has been observed for resident DCs that is partly due to a recruitment of immature DCs.

Mature DCs suppress tumour progression whereas immature DCs promote tumour growth and angiogenesis (Fainaru et al., 2010). Mature DCs can also change functionality in response to cues from the TME towards more pro-tumorigenic phenotypes (Cunha et al., 2014, Markiewski et al., 2008; Korkaya et al., 2011). Additionally, tumour cells can indirectly suppress DC functionality by recruiting and induce generation of immunosuppressive cells, such as Tregs (Liu et al., 2011). For instance, breast cancer cells have shown to induce expression of TGFβ and/or IL-10 in DCs which, in turn, expand the number of immunosuppressive Treg cells (Aspord et al., 2007). In summary, DCs are a

critical mediator between the innate and adaptive immune system that are crucial for a sophisticated and specific immune response against the tumour. Reduced tumour-infiltration, combined with a change in functionality of resident DCs as a result of a pro-tumorigenic microenvironment, is characteristic during tumour progression.

1.2.1.2. B-Lymphocytes

B-lymphocytes are part of the adaptive immune system where they play crucial roles in the humoral immune response. More specifically, they bind specific antigens via B-cell receptors (BCRs) and subsequently differentiate into plasma cells that produce and secret large amounts of antibodies. Additionally, B-lymphocytes act as antigen-presenting cells and secrete various cytokines (Browning, 2006). In cancer, B-lymphocytes are most commonly found in lymphoid structures adjacent to the TME or in draining lymph nodes and are less abundant at the invasive margin of tumours (Balkwill et al., 2012).

The presence of B-lymphocytes within the TME has been associated with better survival in breast and ovarian cancer (Coronella et al., 2001; Milne et al., 2009). However, other studies have shown that B-lymphocytes are capable of promoting tumour development (Qin et al., 1998; Andreu et al., 2010; de Visser et al., 2005). For instance, a subpopulation of B-lymphocytes described as regulatory B-cells (Bregs) suppress immune responses and increase tumour burden in skin cancer (Schioppa et al., 2011). Accordingly, Olkhanud et al. (2011) found an association between the presence of Bregs and increased metastasis to the lungs when employing a breast cancer mouse model. Additionally, subpopulations of B-lymphocytes have been shown to secrete factors, such as IL-2, IL-4 and IL-6, that support tumour-promoting activity of T helper cells (Nelson, 2010). Interestingly, research thus shows that B-lymphocytes can either protect the host from tumour development *or* support tumour progression. The mechanisms of action appear to be suppression and modulation of other immune cells rather than a direct effect on the cancerous cells.

1.2.1.3. T-Lymphocytes

T-lymphocytes are a broad and complex group of lymphocytes that is further divided into subtypes based on cell surface receptors. T-cells found to infiltrate the tumour site includes cytotoxic T-cells (CD8+ CD45RO+), and T helper cells (CD4+); Th1, Th2, T-regulatory cells (Tregs) and Th17 cells, and γδ T-cells (Fridman et al., 2012). The different roles of T cells are highly complex and highly dependent on the tumour context, in which they are in close interaction with the tumour and other cells of the stroma. For instance, CD8+ T-cells are able to recognise specific antigens on the surface of tumour cells and to kill tumour cells by inducing apoptosis (Maher & Davides, 2004). In support of these findings, correlational studies have identified an association between the presence of CD8+ cells and good prognosis in breast cancers, as well as in multiple other cancer types (Fridman et al., 2012; Mahmoud et al., 2011).

Th1 and Th2 cells, as well as Treg and Th17 cells, are all derived from a common T helper progenitor (Th0). Th1 cells have been shown to suppress tumour growth by supporting CD8+ cells and other immune cells, and by secretion of cytotoxic cytokines (such as IL-2, IL-12, TNFα and IFNγ) (Burkholder et al., 2014). Moreover, Hung et al. (1998) reported that Th1 cells secrete cytokines involved in recruitment and activation of macrophages. In contrast to Th1 cells, Th2 cells are generally found to suppress host protective immunity and to promote angiogenesis. Th2 cells are induced by IL-4 and IL-13 and are characterised by high expression of IL-4, IL-5, IL-6, IL-10 and IL-13. Further, Th2 cytokines supports polarisation of macrophages towards an M2 phenotype. Once Th0 cells have derived into Th1 or Th2 cells, their plasticity decreases and they tend to preserve their phenotype (Burkholder et al., 2014).

Treg cells (Tregs) (CD4+, CD25+, FoxP3+) normally play a crucial role in the regulation of autoimmunity by maintaining tolerance to self-antigens (Legoux et al., 2015). In cancer, Tregs have immunosuppressive properties and inhibit Th1 polarisation by secretion of IFNγ and IL-2 (Bettelli et al., 2006). Accordingly, a positive correlation has been found between Tregs infiltration and invasive and metastatic cancers (Sakaguchi, 2004), and the presence of

Tregs in the TME has been reported as a predictor of poor prognosis in many cancers (Oleinika et al., 2012). Although it is yet not fully understood how Tregs are recruited to the tumour, trafficking is presumably driven by cytokines secreted from the tumour. For instance, Tregs express the chemokine receptor CCR4 that binds to CCL22, which is produced by many types of tumours. Differentiation and expansion of Tregs within the TME has been shown to be induced by TGFβ (Lippitz, 2013).

The last type of T helper cells is defined as Th17 cells due to its high production of IL-17. However, Th17 cells are also characterised by the expression of IL-2, IFNγ and GM -CSF/CS2. Th0 cells are differentiated into Th17 cell in response to TGFβ, IL-1α, IL-1β, IL-6 and IL-23 (Zou et al., 2006). Depending on the context, Th17 cells has shown to perform both anti-tumorigenic and pro-tumorigenic functions (DeNardo et al., 2010, Gnerlich et al., 2010). For instance, tumour infiltration of Th17 cells has been associated with poor prognosis in prostate, ovarian, colon and hepatocellular carcinoma (Miyahara et al., 2008; Sfanos et al., 2008; Zhang et al., 2009), whereas a study using a mouse model of human melanoma (B16) reported a host protective effect of Th17 via activation of cytotoxic T cells (Martin-Orozco et al., 2009).

A more distinct population of T-cells, termed γδ T-cells, has shown an extensive interest in recent literature (see Silva-Santos et al., 2015 for review). Most T-cells express T-cell receptors (TCR), which is made up of an α and β chain, and are thus described as αβ T-cells. γδ T-cells however, have distinct TCRs composed of an γ and δ chain, which in turn provides a unique antigen specificity.

In similarity to other subgroups of T-cells, γδ T-cells has found to play dual roles in tumour contexts (Silva-Santos et al., 2015). Studies have reported that γδ T-cells not only rapidly recognize and kill tumour cells, but also secrete high levels of IFNγ (Gao et al., 2003). However, sub-phenotypes of γδ T-cells have also been found to highly express IL-17, which promote tumour growth

(Wakita et al., 2010; Silva-Santos et al., 2015). Further, Coffelt et al. (2015), showed that IL-17 producing γδ T-cells promote metastasis of mammary carcinoma in mice. The functions of γδ T-cells and their interconnections with the immune cell network, as well as the surrounding stroma are not yet fully understood and requires further elucidation to gain a more comprehensive understanding of the role of γδ T-cells during tumour progression.

1.2.1.4. Tumour Associated Macrophages

Macrophages located within or in close proximity to the tumour are defined as tumour associated macrophages (TAMs) and represent the major type of immune cells infiltrating the tumour microenvironment (Biswas et al., 2008; Mantovani et al., 2011a). TAMs may be polarised from resident tissue macrophages, but the majority of TAMs derive from circulating monocytes that are recruited to the site of inflammation in response to signals from the tumour microenvironment (Mantovani et al., 1992; Franklin et al, 2014). More specifically, a wide variety of soluble factors produced by tumour and stromal cells recruit monocytes to the microenvironment and differentiate them into macrophages. CCL2 was the first molecule identified as a tumour secreted chemotactic factor (Mantovani et al. 1986), and later investigations showed that CCL2 also induce polarisation and survival of TAMs (Gazzaniga et al., 2007). CCL5 and CXCL1 has also shown to play an important role in monocyte/macrophage recruitment (Bierie & Moses 2010). Moreover, growth factors (VEGF, PDGF, TGFβ, M-CSF/CSF-1) (Bierie & Moses 2010; Linde et al., 2012) and non-canonical chemotactic peptides such as urokinase plasminogen activator (uPa), basic fibroblast growth factor (bFGF), lectin Reg3b, and the antimicrobial peptide b-defensin-3 (Reed et al., 2012; Brierie & Moses, 2010; Gironella et al., 2013; Mantovani, 2013) have been shown to participate in the recruitment and polarisation of monocytes/macrophages.

The monocyte to macrophage lineage show high diversity and plasticity, in which different environmental signals direct the cells into distinct phenotypes (Biswas & Mantovani, 2010; Sica & Bronte, 2007). TAMs either display an anti-tumorigenic phenotype defined as classical M1 activated macrophages or a pro-

tumorigenic alternatively M2 activated phenotype. It is however important to note that M1 and M2 states only represents the opposite of two extremes in a continuum with a range of different phenotypes. As part of the process of immunoediting, anti-inflammatory and pro-tumorigenic factors, released by tumour and stromal cells, skew TAMs along the continuum towards more pro-tumorigenic phenotypes (Biswas et al., 2008; Mantovani et al., 2013). IFNγ alone, or in combination with cytokines (TNF, GM-CSF) or microbial components (e.g. LPS), induce polarisation towards an M1 phenotype (Sica & Bronte, 2007; Galdiero et al., 2013). Classical M1 macrophages possesses inflammatory functions and are involved in Th1 responses, resistance against microbes and tumours, and perform tissue disruptive actions. They produce and secrete high levels of pro-inflammatory cytokines (e.g. IL-12, IL-23, IL-6, IL-1β, TNF) and show low expression of the anti-inflammatory cytokine IL-10. Additionally, M1 cells show high production of reactive oxygen species and reactive nitrogen intermediates, higher expression of major histocompatibility complex class II and Th1 cell-attracting chemokines such as CXCL9 and CXCL10 (see Figure 1.3; Biswas & Mantovani, 2010).

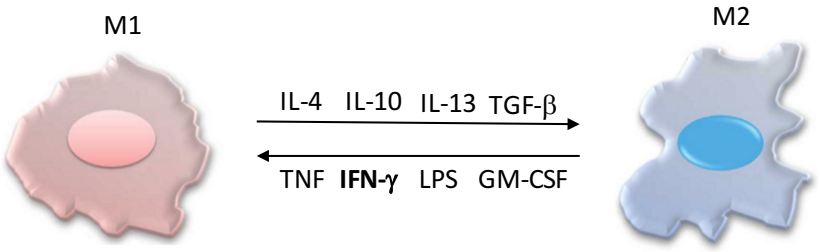


Figure 1.3: A simplified illustration of anti- and pro-tumorigenic macrophages. Information retrieved from Biswas & Mantovani, 2010.

In contrast to M1 macrophages, the alternative M2 activated macrophages are induced by the Th2 cytokines IL-4 and IL-13 (Gordon & Taylor, 2005). TGFβ has also been identified as a key molecule involved in the polarisation towards alternative macrophage activation (Gong et al., 2012). M2 cells release chemokines such as CCL17, CCL22, CCL24, which is involved in recruitment of Treg cells, Th2 cells, eosinophils and basophils (Mantovani et al., 2008;

Martinez et al., 2006). They also show high expression of the anti-inflammatory cytokine IL-10, scavenger mannose and galactose-type receptors, low expression of the pro-inflammatory cytokines IL-12 and IL-23, and have poor antigen presenting capacity (Noel et al., 2004; Mantovani et al., 2013). M2 macrophages participate in the Th2 response, suppress Th1 adaptive immunity and inflammation, they promote wound healing, tissue remodelling and angiogenesis, and have a low tumoricidal activity (Biswas & Mantovani, 2010). Several research groups have demonstrated that the M2-like pro-tumorigenic phenotype is reversible (e.g. Guiducci et al., 2005; Buhtoiarov et al., 2005). For instance, Duluc et al. (2009) purified M2-like TAMs from ovarian cancer and treated them with IFNγ, which caused the TAMs to switch towards an M1-like immunostimulatory phenotype. Additionally, up-regulation of Notch signalling in macrophages has shown to favour M1 polarisation and pro-inflammatory functions (Wang et al., 2010). This, together with the evidenced plasticity of TAMs, provides hope in developing treatment strategies that re-educate TAMs towards tumour-destructive phenotypes.

It is important to note that the nature of TAMs is complex and highly dependent on location, tumour type and grade (Biswas & Mantovani, 2010). Interestingly, although the presence of TAMs in the majority of human and mouse tumours is correlated with poor prognosis, TAM infiltration in some tumour types is associated with a favorable outcome (Galon et al., 2006; Lewis & Pollard, 2006). For example, analysis of clinical specimen from colorectal cancer patients showed that TAM infiltration was positively associated with anti-metastatic behavior and overall survival (Funada et al., 2003). Additionally, analysis of TAMs from gastric cancer patients identified a host-protective phenotype reflected by high cytotoxicity and antigen presentation capabilities (Ohno et al., 2003). Macrophage phenotype has also shown to be dependent on the localisation or compartmentalisation within the tumour. In a mouse mammary tumour model, M2-like TAMs were found to be localised in hypoxic tumour areas, whereas the anti-tumorigenic M1 phenotypes was enriched in normoxic tumour tissues. They also reported an increase in number of pro-tumorigenic macrophages as the tumour progressed (Movahedi et al., 2010). These findings

demonstrate that results must be interpreted with caution and that a phenotypic profiling of TAMs is necessary in prognostic and therapeutic contexts. In conclusions, several lines of evidence show that tumour cells exploit the plasticity of TAMs by secreting factors that re-educate the immune cells towards phenotypes that supports growth and metastasis of the tumour.

1.2.1.5. Tumour Associated Neutrophils

In the circulatory system, neutrophils account for 50 to 70% of total leukocytes and are the first recruited immune cells at the site of inflammation and infections (Fridlender et al., 2012). However, neutrophils represent only a small proportion of the leukocyte tumour infiltrate, with macrophages as the predominating subpopulation (Biswas et al., 2008; Mantovani et al., 2011a). Generally, neutrophils are involved in elimination of pathogens, immune regulation and cleavage of damaged tissue, which is achieved by secretion of proteases (Kolaczkowska & Kubes, 2013; Mantovani et al., 2011b). Neutrophils have been implicated in cancer research since its early days, as they were observed in close proximity to tumour cells *in-vivo* (Welch et al., 1989). Despite the fact that TAMs dominate the infiltrate, tumour associated neutrophils (TANs) have been shown to play an important role in tumour initiation and progression. Later research has identified TANs as a prognostic marker for recurrence free and overall survival in localised and metastatic clear cell renal cell carcinoma, as well as in head and neck squamous cell carcinoma (Donskov et al., 2006; Jensen et al., 2009; Trellakis et al., 2011). TAN infiltration has been found to correlate with a more aggressive phenotype in pancreatic cancer (Reid et al., 2011), with a higher grade in human gliomas (Fossati et al., 1999), and with a poorer survival in metastatic melanomas (Schmidt et al., 2005). In contrast, and as observed for TAMs, some malignancies show a more favourable outcome in response to neutrophilia (e.g. gastric cancer) (Caruso et al., 2002).

The mechanisms responsible for neutrophil extravasation, translocation and subsequent TAN polarisation are poorly understood. Normally, neutrophils extravasate and translocate to tissues under the influence of specific cytokines

(e.g. IFNγ and TNFα) and chemokines (e.g. MIP2a/CXCL2 and KC/CXCL1), and by interaction of their own surface adhesion molecules with adhesion molecules on the surface of endothelial cells (Kobayashi, 2008). Several mediators released from tumour and stromal cells have been suggested to play a role in the recruitment to tumour sites. More specifically, high levels of GM-CSF have been associated with neutrophil infiltration in lung, pancreas, melanoma and breast cancer (McGary et al., 1995). In addition, G-CSF, IL-1β, IL-6 and VEGF expression have been suggested to induce neutrophilia (Lechner *et al.,* 2010). TANs have also been shown to initiate a positive feedback loop by producing CXCL2, CXCL1, and CCL3 that attracts more neutrophils to the tumour site (Fridlender et al., 2012). Moreover, elevated expression of CXCL6 has been associated with neutrophil recruitment in melanoma tumours (Verbeke et al., 2011). TGFβ has also shown to play a role in recruitment, as an increase in neutrophil counts was observed after blockade of the TGFβ receptor. This might be due to an impaired neutrophil transmigration along the endothelium, as TGFβ is shown to inhibit endothelial adhesiveness for neutrophils (Smith et al., 1996; Allen et al., 2008). Further, several studies have found that T-lymphocytes and macrophages play an important role in neutrophil recruitment by releasing neutrophil chemo-attracting factors such as IL-17, CXCL8, CXCL2 and CXCL1 (Coffelt et al., 2015; Benevides et al., 2015; Himmel et al., 2011; Richards et al., 2010).

TANs have recently been shown to possess similar plasticity as described for TAMs, as they are able to polarise into either anti-tumorigenic (N1) or pro-tumorigenic (N2) phenotypes in response to different external stimulus (Fridlender et al., 2009). This observation can thus explain the contradictory roles reported for TANs (e.g. Caruso et al., 2002; Donskov et al., 2006; Jensen et al., 2009). TANs are polarised towards an N2 phenotype in response to TGFβ, whereas IFNβ or depletion of TGFβ promote polarisation towards an anti-tumorigenic N1 phenotype (Fridlender et al., 2009).

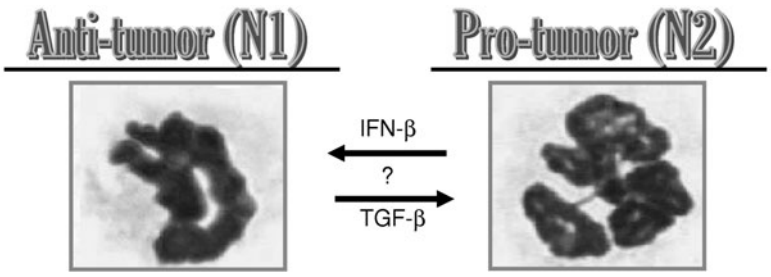


Figure 1.4: A simplified illustration of anti- and pro-tumorigenic neutrophils. IFNβ and TGFβ are key players in polarisation of neutrophil phenotypes. Adapted from Fridlender & Albelda (2012).

Host protective N1 neutrophils are cytotoxic and immuno-stimulatory, whereas pro-tumorigenic N2 states are immunosuppressive and promote angiogenesis. N1 phenotypes are typically characterised by high expression of chemokines (e.g. Ccl3, CXCL9, CXCL10) and cytokines (e.g. IL-12), GM-CSF, VEGF, and low expression of Arginase I. In contrast, N2 phenotypes show a strong down-regulation of cytokine and chemokine expression, but a high expression of Arginase II (Mantovani et al., 2008; Fridlender et al., 2012). In summary, TAN have been shown to play similar dual roles in the tumour microenvironment as observed for TAMs, in which they are implied to have an important impact on tumour progression. However, a precise understanding of the characteristics and mechanisms of action, including its interactions with the tumour and its surrounding stroma, is lacking and requires further elucidation.

1.3. Matrix Metalloproteinases

TAMs and TANs produce high levels of matrix metalloproteinases (MMPs) that have been described as crucial modulators of the tumour microenvironment. Importantly, accumulating evidence implies MMPs as potential players in the cross-talk between tumour and stromal cells. MMPs comprise a large family of extracellular endopeptidases that fulfil a variety of functions in tissue remodeling processes, organ development, inflammatory processes and in carcinomas (Page-McCaw et al., 2007). Twenty-three members have been identified and described for the MMP family in humans, in which the basic structure is made up of a characteristic pro-peptide, a catalytic domain and a

hemopexin-like C-terminal domain. Additionally, a flexible hinge region links to the catalytic domain to the C-terminal domain. Based on variations in domain arrangements as well as substrate specificity, MMPs has been further divided into five subgroups; collagenases, stromelysins, gelatinases, matrilysins and membrane-type MMPs.

Collagenases (MMP1, MMP8, MMP13, and in some species, MMP18) are best known to cleave collagen I, II and III into ¼ and ¾ length fragments (Sun et al., 2000). The collagenases recognize a sequence within the collagen which span about 30 residues (Fields, 1991; Minond et al., 2006; Minond et al., 2007). The N-terminal of this cleavage site consist of a tight wound helix rich in imino amino acids, whereas the C-terminal side contains a loos triple-helix poor in imino acids. The loos triple-helix are unwound by collagenases prior binding and subsequent cleavage of the α chains (Fields, 1991). In addition to collagens, collagenases have shown to digest other molecules and soluble proteins in the extracellular matrix (ECM) (Visse & Nagase, 2003). Stromelysins (MMP3, MMP10, MMP11) are similar to collagenases by domain arrangement but have different substrate specificity. For instance, MMP3 and MMP10 cleave the majority of ECM molecules and activate proMMPs (Murphy et al., 2002). In contrast, MMP11 weakly degrades ECM molecules, but play important roles in the cleavage of serpins (Murphy et al., 1993; Pei et al., 1994).

Gelatinases (MMP2 and MMP9) cleave gelatins and various ECM molecules, including laminin, collagens (type IV, V and XI) and aggregan core protein (Nagase et al., 2006). Further, MMP2 cleaves collagen I, II, and III in a similar fashion as collagenases (Aimes & Quigley, 1995). Furthermore, Matrilysins (MMP7 and MMP26) are classified based on their lack of a hemopexin domain (Ló pez-Otín et al., 2009). Mechanistically, MMP7 processes ECM molecules and cell surface molecules such as pro-a-defensin, E-cadherin and pro-tumour necrosis factor α. MMP26 also processes several ECM components, but in contrast to most MMPs, the proteinase is stored intracellularly (Marchenko et al., 2004). Lastly, membrane-type MMPs (MMP14, MMP15, MMP16, MMP17, MMP24, MMP25) are transmembrane proteins, except for MMP17 and MMP25,

which are glycosylphosphatidylinositol-anchored proteins. However, all membrane-type MMPs, which have a furin recognition sequence, are activated intracellularly before they are expressed on the cell surface. Further, all membrane-type MMPs, with the exception of MMP17, participate in the activation of proMMP2 (Murphy et al., 2002; English et al., 2000).

In general, MMPs are secreted as inactive pro-enzymes that are dependent upon other molecules to become proteolytic activated. Mechanistically, the enzyme is maintained inactive by a pro-domain consisting of a cysteine residue interacting with a Zn^{2+} ion at the active site. Modification of the cysteine thiol group, or removing the pro-domain by proteolysis, will disrupt the blocking mechanism and convert the enzyme into its active form, a process described as the cysteine-switch mechanism (Visse & Nagase, 2003) (see Figure 1).

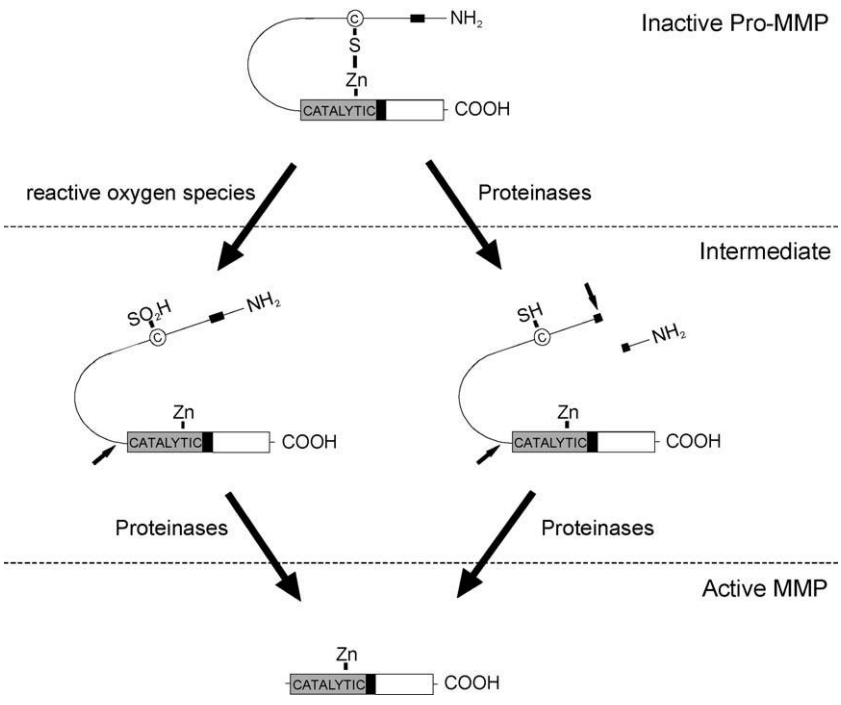


Figure 1.5: MMP activation by the cysteine-switch mechanism.
The MMP is activated upon removal of the cysteine thiol group.
Adapted from VanLint & Libert (2006).

Reactive oxygen species and some proteases (such as MMPs and trypsin) are among molecules that have been shown to activate MMPs (Van Wart & Birkedal-Hansen, 1990; Knauper et al., 1993; Balbin et al., 1998; Knauper et al., 1996; Holopainen et al., 2003; Okamoto et al., 1997). Providing a spatial control of enzymatic activity, MMPs can be cleaved at both the cell surface and in the peri-cellular environment. Additionally, MMPs are controlled by specific inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), RECK, alpha-macrogobulins and some serine protease inhibitors (e.g. Egeblad & Werb, 2002; Oh et al., 2001; Barrett, 1981; Herman et al., 2001). Four different TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) are expressed by vertebrates, in which all types form 1:1 stoichiometric complexes with proteolytic active MMPs. Consequently, enzyme activity is inhibited (Egeblad & Werb, 2002). TIMPs also inhibits other metalloproteinase families, including a disintegrin metalloproteinase (ADAM) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families. These metalloproteinases are in close relation to MMPs and also play important roles in tissue remodeling processes (see Edwards et al., 2008 and Porter et al., 2005 for extensive reviews).

RECK is a GPI-anchored glycoprotein that inhibits MMP2, MMP9 and membrane type-1 MMP (MT1-MMP) and, that has been shown to downregulate the levels of and activate MMP9 and MMP2, respectively (Oh et al., 2001; Takahashi et al., 1998). Further, the serine protease inhibitor tissue factor pathway inhibitor-2 (TFPI-2), has been shown to inhibit several MMPs, including MMP1, MMP2, MMP9 and MMP13 (Herman et al., 2001). The mechanism of action of both RECK and TFPI-2 is poorly understood. Plasma alpha2-macroglobulins however, are known to inhibit endopeptidases by proteolysis of the 'bait' region causing a conformational change that traps the proteinase within the macroglobulin. This inhibitor in able to trap both MMPs and ADAMs (Barrett, 1981). Together with other proteases and protease inhibitors, metalloproteinases form a complex network in the ECM, in which proteolytic activity is dependent upon direct interaction between different proteases and their interaction with protease inhibitors. This interconnected

network is described as the 'protease web' and is responsible for maintaining homeostasis in the ECM. Importantly, dysregulation of the protease web has been linked to several pathologies including inflammatory conditions and cancer (Decock et al., 2011).

1.3.1. MMPs in Cancer

MMPs has been implicated in cancer research for almost half a century. Initially, MMPs were thought to mediate degradation of the ECM, leading to tumour cell invasion and metastasis (Liotta et al., 1980). Inhibition of MMPs was then shown to suppress the invasive and metastatic behaviour of tumours in mice (Reich et al., 1988; Sledge et al., 1995). Due to these compelling results, synthetic metalloproteinase inhibitors (MPIs) were developed and routed into clinical trials (e.g. see Coussens et al., 2002 for an extensive review). Surprisingly, the inhibitors failed the trials, in which an increase of patient survival was not identified as predicted (e.g. Bramhall et al., 2001; Brown, 2000) and some patients even showed poorer survival rate compared to the placebo group (e.g. Moore et al., 2003).

The disappointing results from the clinical trials led MMP research into a new paradigm. MMPs were no longer considered solely as pro-tumorigenic, as MMPs were shown to possess more complex functions within the tumour microenvironment during tumour progression. For instance, in addition to their roles in physiological processes such as tissue remodelling, MMPs were shown to regulate a variety of signalling molecules in the ECM involved in cell proliferation, apoptosis, vascularisation, inflammation, tissue invasion and metastasis (see Kessenbrock et al., 2010 and López-Otín & Matrisian, 2007 for detailed reviews). In addition, by acting as regulators of the tumour microenvironment, MMPs also provided suppressive effects against the tumour, which was identified as a major reason of MPIs failing the clinical trials (López-Otín et al., 2009). Interestingly, studies revealed that some MMPs possessed both pro- and anti-tumorigenic functions, depending on the source of the MMPs, disease stage and tumour type (Lopez-Otin & Matrisian, 2007; Martin & Matrisian, 2007). For instance, MMP3 was first shown to promote

mammary tumorigenesis in mice (Sternlicht et al., 1999), whereas a more recent study using *Mmp3*-null mice reported a reduced initial growth rate of squamous cell carcinoma tumours (McCawley et al., 2004). Such dual roles have also been described for MMP9, MMP11 and MMP19 (Egeblad & Werb, 2002; Coussens et al., 2000; Andarawewa et al., 2003; Pendas et al., 2004; Jost, 2006). Additionally, some MMPs such as MMP8, MMP12, MMP26, were shown to solely provide protective effects during tumour progression (e.g. reviewed in López-Otín et al., 2009). Of these, MMP8 turned out to be the most extensively studied tumour-suppressive MMP, in which accumulating evidence show that MMP8 play an important role in the regulation of immune cell infiltration (e.g. Balbin et al., 2003a; Decock et al., 2015).

1.4. Matrix Metalloproteinase 8

MMP8 is a member of the collagenase subfamily of MMPs and is also known as collagenase-2 or neutrophil collagenase. This enzyme is expressed by neutrophils and stored within intracellular granules prior to secretion, which causes a high degree of glycosylation (Hasty et al., 1990). Later studies have however revealed the protein is not only produced by neutrophils, but also by macrophages, fibroblasts, epithelial cells, keratinocytes, endothelial cells, chondrocytes and plasma cells. Importantly however, secretion from these cells are mainly associated with an inflammatory condition. The cells lack the ability to store in granules and thus promptly secrete the protein following expression, which is reflected by its poor degree of glycosylation and smaller molecular weight compared to neutrophil derived MMP8 (Kiili et al., 2002; Kostamo et al., 2005; Hanemaaijer et al., 1997; Bergmann et al., 1989; Devarajan et al., 1991; Hasty et al., 1990). MMP8 is most frequently secreted as a soluble protein localised in the ECM. In some cases, however, the proteinase appears as a membrane-bound protein that has shown to be more stable and resistant to inhibition by TIMPs (Owen et al., 2004).

MMP8 is secreted as an inactive pro-peptide that requires breakage of a peptide bond to open the catalytic site (Cauwe & Opdenakker, 2010). The peptide bond can be disrupted via proteolysis or autocatalysis by proteolytic

cleavage or oxidative-/nitrosative stress, respectively. For instance, pro-MMP8 has been shown to be activated by MT1-MMP (Holopainen et al., 2003), chemotrypsin and the neutrophil serine protease, Cathepsin G (Knauper et al., 1999). Hydrogen peroxide (H2O2) and reactive nitrogen intermediates such as nitrogen dioxide (NO2) and peroxynitrite (ONOO-) has also been shown to convert pro-MMP8 into its active form (Okamoto et al., 1997; Cauwe & Opdenakker, 2010; Saari et al., 1990).

Once activated, MMP8 efficiently cleaves type I, II and III collagens conferring a critical function in tissue remodeling processes during wound healing and inflammatory conditions (VanLint & Libert, 2006). In addition, MMP8 has shown to cleave other extracellular proteins, such as chemokines, growth factors, cell adhesion proteins, and protease inhibitors (e.g. Van den Steen et al., 2003a; Van den Steen et al., 2003b; Tester et al., 2007). Cleavage results in activation or inhibition of the substrate that, in turn, activates or inhibit a biologic activity. Due to a wide range of substrates, MMP8 is involved in a variety of processes in the extracellular matrix, including breakdown of the ECM as well as release and activation of signaling factors.

1.4.1. MMP8 in Wound Healing

Wound healing is a natural response to tissue injury were damaged and lost cellular structures and tissue are replaced. Crucial in this process is the MMP-driven degradation of ECM components. The most abundant protein in the ECM is collagens, which consists of three polypeptide alpha chains that can only be degraded by collagenases, especially MMP1, MMP8 and MMP13 (Xue & Jackson, 2015).

MMP8 is the main collagenase produced by healing wounds and has thus been suggested to play a crucial role in wound healing (Pirila et al., 2001; Nwomeh et al., 1999). Interestingly, studies using Mmp13-*null* mice has reported an increased secretion of MMP8 at the site of wounding, which is suggestive of a compensation mechanism that results in a restoration of wound healing (Hartenstein et al., 2006). Gutiérrez-Fernández et al. (2007) investigated

MMP8's individual contribution to the healing process by knocking out MMP8 in a mouse model for cutaneous wound healing. They reported a delay in wound closure and re-epithelialization in response to the MMP8 ablation. They also found a delay in neutrophil infiltration at early stages and a sustained inflammation at later time points, suggesting that the MMP8 effect on wound closure is propagated via innate immune pathways. The altered immune response was accompanied by an alteration in the TGFβ1 signaling pathway, an up-regulation of MMP9 and an inhibition of neutrophil apoptosis. Bone marrow transplantation from *Mmp8*-wild-type mice rescued the observed delay in wound closure in *Mmp8*-null mice suggesting that the effect is solely due to absence of MMP8 in inflammatory cells derived from the bone marrow.

Taken together, these results demonstrate that MMP8 play an essential role in wound healing. Although results suggest that MMP8 acts via immune regulatory pathways, the precise role of MMP8 in the course of cutaneous wound repair is still largely unclear and requires further investigations.

1.4.2. MMP8 in Cancer

In addition to its role during normal wound healing, MMP8 has also been implied in a variety of inflammatory conditions, including cancer. The first study to demonstrate its anti-tumorigenic role during tumour progression was performed in the context of a skin cancer mouse model. Balbin et al. (2003) showed that knocking out MMP8 in male mice strongly increased skin tumour incidence. Interestingly, a similar change was not observed in female knock-out mice. However, removal of the ovaries or treatment with the estrogen receptor antagonist, tamoxifen, resulted in an increased tumour susceptibility compared to wild type animals. These results suggest that ovarian estrogen protects against tumour formation in female *Mmp8-null* mice, at least in skin carcinoma. Furthermore, bone marrow transplantation restored the effect, indicating that MMP8 produced from bone marrow hematopoietic stem cells are responsible for the primary effect.

In line with the findings by Balbin et al. (2003), Korpi et al. (2008) reported a positive association between MMP8 expression and improved survival in tongue cancer, in which the tendency was stronger in females. The same research group revealed that estrogen induces MMP8 expression in HSC-3 tongue carcinoma cells, and that MMP8 cleaves both α and β estrogen receptors. In addition to a link between MMP8 and estrogen, Balbin et al. (2003) showed, by histopathological analysis of tumour sections, that depletion of MMP8 induces a sustained inflammation at later stages of tumour development, which suggests a defect in restoring the cancer-related inflammation. MMP8 dependent regulation of the inflammatory response is in line with results generated from wound healing mouse experiments (Gutiérrez-Fernández et al., 2007). Altogether, these results suggest that MMP8 have an estrogen related role in tumour growth, as well as, acting as a regulator if inflammatory pathways.

Tumour-suppressor effects of MMP8 have also been reported for human breast cancer, in which down-regulation and up-regulation of MMP8 *in-vitro* has resulted in increased or decreased metastatic behavior, respectively (Montel et al., 2004). Analysis of clinical breast tumour specimen has supported these findings, in which an inverse correlation between lymph node metastasis and MMP8 expression has been identified (Decock et al., 2007). Further, MMP8 expression has been shown to facilitate cell adhesion to ECM that might directly suppress metastasis (Gutierrez-Fernandez et al., 2008). A more recent study reported a positive association between MMP8 serum levels and disease stage, degree of necrotic tumour tissue and blood neutrophil count in colorectal cancer. MMP8 serum levels were also positively associated with tumour-destroying inflammatory infiltration, low grade Crohn's-like lymphoid reaction (CLR), distant metastasis and low body mass index (BMI) (Väyrynen et al. 2011).

In concurrence with these findings, Decock et al. (2015) reported a significant increase in tumour growth and lung metastasis in MMTV-PyMT; *Mmp8*-null mice compared to wild-type littermates. They also identified an increase in

angiogenesis and neutrophil infiltration at later stages of development, in which the latter confirms findings reported by earlier wound healing and skin tumour mouse experiments (Decock et al.,2015; Balbin et al. 2003; Gutierrez-Fernandez et al., 2007). In summary, these results clearly demonstrate the suppressive role of MMP8 in breast cancer growth and metastasis, additionally, they suggest that MMP8 exerts such effects by inducing pleiotropic changes within the tumour microenvironment. However, a better insight into the mechanistic actions of MMP8 is required to understand the precise role of MMP8 during mammary carcinoma progression.

Although the underlying mechanisms responsible for the effect on immune cell infiltration is largely unknown, recent studies have implied that MMP8 act as a modulator of neutrophilic chemotactic factors. Studies have reported that MMP8 activates both human interleukin-8 (IL-8) and its mouse orthologue lipopolysaccharide induced CXC chemokine (LIX), which is necessary for the recruitment of neutrophils to the site of tissue damage or infection (Tester et al., 2007; Philippe et al., 2003). Later investigations have shown that catalytic active MMP8 induce expression of IL-6 and IL-8 in human breast cancer cell lines via NF- kB, and that IL-8 and IL-6 act through autocrine loops to induce expression of IL-6 and MMP8, respectively (Thirkettle et al., 2013). Additionally, MMP8 has been shown to mediate generation of the fragment N-acetyl Pro-Gly-Pro (PGP), which is a neutrophilic chemotactic peptide acting through CXC chemokine receptors on granulocytes (Weathington et al., 2006; Rocks et al., 2008). Lin et al. (2008) described MMP8 as a facilitator of neutrophil migration through matrix by degradation of collagens and production of PGP. In summary, these results strongly imply MMP8 as a regulator of the neutrophil chemotaxis by biologically activate relevant chemo-attractants that, in turn, orchestrate recruitment of neutrophils to the site of inflammation.

A mutational analysis of human melanoma identified that MMP8 is frequently mutated and that reduced enzyme activity is a consequence of five of the most common mutations (Palavalli et al., 2009). Further, expression of mutant MMP8

inhibited growth of human melanoma cells *in-vitro* and prevented melanoma progression *in-vivo* (Palavalli et al., 2009). Decock et al. (2008) investigated MMP8 gene variations in breast cancer, and identified an association between four single nucleotide polymorphism (SNPs) and lymph node metastasis. Moreover, one of the SNPs (a minor T-allele) located in the promoter region was associated with a reduced relapse and increased survival, in particularly in early stage cancers. Subsequent *in-vitro* experiments revealed a higher promoter activity from the T-allele compared to the C-allele, which is consistent with a more favourable outcome. Accordingly, González-Arriaga et al. (2008) identified an association between polymorphism in the promoter region of MMP8 and a lower risk of developing lung cancer or a decreased progression in lung cancer patients.

In conclusions, several lines of evidence have shown that MMP8 plays an important role in suppressing growth and metastasis of tumour cells. Subsequent studies have implied that MMP8 acts via the immune system by modulating factors involved in myeloid cell recruitment. Despite these findings, the mechanism of action is largely unknown and further investigation is required in order to achieve a precise and holistic understanding of the tumour opposing actions of MMP8.

1.5. Background and Aims

It is well-documented that MMP8 has a suppressive effect on tumour growth and metastasis (Lopez-Otin & Matrisian, 2007; Decock et al., 2011). In the context of breast cancer, *in-vivo* models and analysis of clinical breast cancer specimen have shown a positive correlation between MMP8 expression and improved survival of mammary carcinoma (Decock et al., 2007; Gutierrez-Fernandez et al., 2008). Further, Decock et al. (2015) showed that MMP8 depletion promoted tumorigenesis and lung metastasis in a MMTV-PyMT mammary carcinoma model. However, the mechanism affected by MMP8 during tumorigenesis is poorly understood. MMP8 has recently been linked to regulation of TGFβ signaling (Thirkettle et al., 2013; Soria-Valles et al., 2013; Wen et al., 2015). TGFβ has been identified as a key player in the process of

polarisation of macrophages and neutrophils towards their tumour promoting phenotypes M2 and N2, respectively. Further, blockade of TGFβ has shown to switch the polarisation towards their M1 and N1 counterparts (Fridlender et al., 2009; Gong et al., 2012), but the mechanism promoting this switch is poorly understood. A recent study suggests a mechanism of a MMP8 mediated TGFβ blockade, in which MMP8 cleaves the ECM protein Decorin that results in release of a core protein that, in turn, acts to sequester TGFβ (Soria-Valles et al., 2013). Based on these findings, MMP8 is hypothesised to oppose tumour malignancy through an effect on the innate immune system. More specifically, MMP8 is thought to sequester TGFβ that in turn switches neutrophil and macrophage polarisation of pro-tumorigenic N2 and M2 phenotypes towards their anti-tumorigenic counterparts, N1 and M1. Studying the role of MMP8 *in-vivo* provides a model in which all immune components are present, which allows for a comprehensive investigation of the immune regulatory changes occurring in the TME at different stages of tumour development.

In summary, the project will test the hypothesis that MMP8 protects the host from mammary carcinoma progression by having an effect on innate immune defenses. More specifically, by acting via TGFβ signaling to switch polarization of neutrophils and macrophages towards anti-tumorigenic phenotypes, N1 and M1. The specific aims and objectives for the present investigation are as follows:

Aim 1: To characterise phenotypes of tumour-associated neutrophils (TANs) and macrophages (TAMs) in primary tumours from MMTV-PyMT; Mmp8-knock-out versus MMTV-PyMT; Mmp8-wild-type mice.

- Objective 1: Quantify RNA expression of M1, M2, N1 and N2 markers at different stages of development.
- Objective 2: Stain tumor sections for specific immune cell markers.

Aim 2: To evaluate the TGFβ signaling axis and novel pathways in MMTV-PyMT;Mmp8-knock-out and MMTV-PyMT; Mmp8-wild-type tumours.

- Objective 1: Identify differentially expressed genes between *Mmp8*-null and wild-type tumours using whole genome RNAseq transcriptomic profiling.

2. METHODS

2.1. Mmp8-*null* mice

All mammary carcinoma mouse tumours used in the present study were generated previously by Decock et al. (2015). They intercrossed *Mmp8*-null mice (described in Gutiérrez-Fernández et al., 2008; Balbin et al., 2003) with MMTV-PyMT mice (Charles River Laboratories, Margate, UK) to generate a robust MMP8 knock-out model for mammary carcinoma. Tumours were generated for multiple experiments and were harvested and portioned by members of the Edwards lab in the period between 2009 – 2011.

The *Mmp8*-null mice were generated previously by Balbin et al. (2003). They used pKO scrambler V916 (Lexicon Genetics) to construct the targeting vector, which contained a 5kb *Hind*III fragment flanked at 5' end with a 3kb region, exon1, intron 1 and part of exon2. The 3'end comprised a 1.3-kb PCR fragment that contained intron 4 and exon 5. A PGK-neo cassette were used to replace most of exon 2, intron 2, exon 3, intron 3 and exon 4. The cassette, with transcriptional orientation opposite to that of MMP8, were cloned into an AscI site of the vector. The construct was then linearized prior electroporation into RW4 embryonic cells (129/ScJ derived). Genotyping and subsequent injection into blastocysts are described in Balbin et al. (2003).

2.2. RNA Isolation

Frozen tumour tissue was pulverised with a chilled mortar and pestle in liquid nitrogen prior tissue homogenization/lysis in RNAbee. RNA was then isolated using a phenol-chloroform extraction method followed by purification using a SV Total RNA Isolation System (#Z3100, Promega, Madison, WI, USA). The samples were then quantified and evaluated for quality by A260/A280nm and

A260/A230nm ratios using a Nanodrop system (Thermo Fisher Scientific, Frederick, MD, USA). Samples were stored at -80°C.

2.3. Agarose Gel Electrophoresis

Total RNA was separated on a 1.2% EtBr stained agarose gel. Briefly, 10µl of sample stained with 2ul of 6x loading dye (#B7024S, New England Biolabs, Ipswich, UK) was loaded into each wells of the agarose gel, and a volume of 5ul of HyperLadder™ 1kb (BIO-33053, New England Biolabs, Ipswich, UK) was used for size orientation. Electrophoresis was run at 60V for 1.5 hour prior visualisation and gel imaging.

2.4. Quantitative RT-PCR

cDNA was synthesised with 1µg of total RNA using MMLV-Superscript which resulted in a final concentration of 50ng/µl (Promega, Madison, WI, USA). Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was conducted using specific Taqman primer/probe sets (Applied Biosystems, California, USA) for Ccl3, VEGFA, Arginase I, Arginase II, CD163, MMP8, MMP2, MMP3, MMP13, Syndecan-4, ADAMTS-1, and ADAMTS-15 (#mm00441259_m1 #mm00475988_m1 #mm00477592_m1 #mm00437306_m1 #00477355_m1 #mm00488527_g1 #mm01176187_m1, Applied Biosystems, Foster City, USA).

As an internal control for the amount of cDNA obtained, gene expression was normalised to 18S rRNA (#mm03928990, Applied Biosystems, Foster City, USA). 18S was selected as standard normalization gene as done in all previous studies by the Edwards lab, including gene expression studies using mouse as model organism (e.g. Decock et al., 2015). In contrast to other housekeeping genes (e.g. GADPH and β-catenin), rRNA gene expression has shown high stability and has frequently been used as an endogenous control in qRT-PCR experiments (e.g. Zhong & Simons, 1999; Schmittgen & Zakrajsek, 2000; Bhatia et al., 1994). Experiments performed by Pennington et al. (2008) also shows that 18S serves as a good quality control.

Detection of selected genes was performed using 5ng cDNA for all genes of interest (1ng for 18S), 8.33µl of Taqman mastermix, 1.25µl of P2P mix and 5.42µl of dH2O (13.42µl for 18S). The cycle conditions for the polymerase chain reaction was as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 90°C and 1 min at 60°C.

2.5. Cryosectioning and Immunohistochemical Staining

The frozen tumours, stored in Optimal Cutting Temperature medium (OCT), were sectioned at 10 µm using a cryostat, and sections were collected on Colorfrost™ Plus Microscope Slides (Thermo Scientific, Cheshire, UK) and stored at -20°C. Dry, defrosted sections were fixed in 100% acetone prior antigen retrieval in citric acid, pH6. Sections were blocked in phosphate buffered saline (PBS)/0.2% Triton with 10% normal goat serum to avoid unspecific staining. Macrophages and blood vessels were detected using primary antibody against F4/80 (MCA497R, Bio-Rad, Kidlington, UK) and Endomucin (Sc-65495, Santa Cruz Inc., Texas, USA), respectively, all at a 1:200 dilution in blocking solution. Donkey-anti-rat Alexa-488-labelled and Alexia-555-labelled secondary antibodies were used for visualisation of F4/80 and Endomucin, respectively, at a 1:500 dilution in PBS/1% normal horse serum. Section were mounted using Vectashield® Mounting Medium with DAPI (Vector Laboratories, Inc., California, USA). Sections were imaged at 10X magnification with Zeiss Axioplan II Fluorescence Microscope (Carl Seizz, Jena, Germany), followed by processing using the Zeiss Axio Vision Imaging Software (Carl Zeiss, Jena, Germany) and Fiji.

2.6. RNA Sequencing and Bioinformatics Analysis

Total RNA was quantified on a Tecan Platereader prior sizing and quality control (QC) using a LabChip GX Instrument (Perkin Elmer®). Quality was assessed based on RNA quality Scores (RQS) (see Table 3.1). Pair-end RNA libraries were prepared according to standard protocols at the Earlham Institute. The libraries were sequenced on an Illumina HiSeq2000, using two lanes per sample. Raw data was quality controlled using FastQC (fastqc-0.11.2, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and a

kontaminant (http://www.tgac.ac.uk/kontaminant/) was used to check for contamination in raw reads. As the data QC was good, no trimming was performed on the raw data.

RNAseq reads were aligned against the mouse genome reference build 38 (ftp://ussd- ftp.illumina.com/Mus musculus/ Ensembl/GRCm38/) using TopHat (tophat-2.1.0, http://ccb. jhu.edu/software /tophat/manual.shtml) (Trapnell et al., 2009) with –min-anchor-length 12 and –max-multihits 20. To control for between- and within-sample variations, the expression level of each gene was normalised by calculating fragments per kilobase per million mapped reads (FPKM). Alignments against the MMP8 allele were visualized using the Integrative Genome Browser (Robinson et al., 2011; Thorvaldsdóttir et al., 2013) to confirm the Mmp8-knockout.

Transcript reconstruction and differential expression was performed in Cufflinks (Cufflinks-2.2.1, http://cole-trapnelllab.github.io/cufflinks/cufflinks/index.html), previously described in Trapnell et al. (2012). A mean log2 ratio (FPKM of gene in sample X/FPKM of gene in sample Y) was calculated and the FDR method was used to identify differentially expressed genes between groups. Genes were considered as significantly differentially expressed when FDR value was less than 0.05.

The R package cummerbund (http://bioconductor.org/packages/ release/ bioc/html/ cummeRbund.html) (Goff et al.,2013) was used to visualise the results of Cufflinks. Further, DAVID Bioinformatics Resources 6.7 (Huang et al., 2009a; Huang et al., 2009b) were used as functional annotation tool to explore functions of genes significantly differentially expressed between conditions. Furthermore, Venny 2.1 (http://bioinfogp .cnb.csic.es/tools/venny/) was used to construct Venn Diagrams and, Gene Cluster 3.0 (described in de Hoon et al., 2004) and Java TreeView 1.1.6r4 (Saldanha, 2004) were used for heatmap construction.

2.7. Statistical Analysis

If not stated otherwise, the data was analysed using the two-way ANOVA test and are represented as mean ± standard error of the mean (SEM).

3. RESULTS

The current investigation builds on previous work that was recently published by Decock et al. (2015). They intercrossed *Mmp8*-null mice with MMTV-PyMT mice to generate a robust *Mmp8*-knock-out model for mammary carcinoma. As a response to MMP8 ablation, this study reported an accelerated tumour progression and lung metastasis, in addition to, an increased infiltration of neutrophils at later time points of tumour development. Moreover, they reported a change in the vasculature, as well as a differential expression of several members of the protease web. Thus, MMP8 appears to suppresses tumour progression by inducing pleiotropic changes within the tumour microenvironment.

Tumours that were generated by Decock et al. (2015), including MMTV-PyMT; Mmp8-knock-out, MMTV-PyMT; Mmp8-heterogenous and MMTV-PyMT; Mmp8-wild type tumours, were stored at -80°C for future investigations. These tumours have been used in the current study to continue the work of Decock et al. (2015) by exploring, on a more mechanistic level, the tumour suppressive functions of MMP8 during mammary carcinoma progression. More specifically, the transcriptome has been analysed to assess if MMP8 acts via innate immune pathways and to explore novel pathways affected by MMP8 signaling.

3.1. Quality controls reveals good RNA integrity

Decock et al. (2015) extracted RNA from MMTV-PyMT tumours to profile for gene expression levels of various members of the protease web. RNA from this experiment was stored together with the tumours at -80°C for future studies. The present investigation aimed at using the same RNA samples for qRT-PCR analysis and whole genome RNA sequencing and, thus, started off by verifying the integrity and quality of these RNA samples. RNA integrity was firstly checked by qRT-PCR by looking at 18S rRNA abundance. Several RNA samples

showed 18S Ct values ranging from 16-18, which is indicative of degraded ribosomal 18S rRNA. Although these results suggested a poor quality of the underlying mRNA population, the RNA was sent to the Earlham Institute for a more in-depth quality assessment.

At the Earlham Institute, A LabChip GX instrument was used to measure RNA integrity by electrophoretic separation on microfluidic sipper chips via laser induced fluorescence. An electropherogram displayed the raw data, which was then converted into a gel-like image for visualization. An RNA quality score (RQS) was calculated based on different parameters of the electropherogram (e.g. peak heights and peak areas) and sample concentration, and was reported as a score between 1-10, where a score >7 is considered as passed (CaplierLifeScience, 2009). The results revealed a generally poor quality across samples, in which several samples did not shown detectable 18S, 28S and 5S (see Supplementary Material). Accordingly, out of 49 samples, only 4 samples showed an RQS score >7 and only 6 passed the overall quality control. This strongly indicates that the integrity and quality of the RNA was not sufficient for downstream gene expression analysis.

The current study concluded that using RNA samples from the Decock et al. (2015) study would provide a high risk of unsuccessful gene expression experiments. Thus, the study went on to isolate fresh RNA from the frozen MMTV-PyMT tumour tissue. Initial quality assessments were again performed in the lab by qRT-PCR, but also by agarose gel electrophoresis to look at 28S/18S rRNA ratios. The Ct values for 18S rRNA ranged from 13 to 15, indicating that ribosomal 18S rRNA was intact which, in turn, should reflect good quality of the underlying mRNA population. Further, visualisation of the agarose gel after electrophoretic separation of total RNA revealed, in the majority of samples, an upper band (28S) approximately twice as intense as a lower band (18S) (Figure 3.1). A 28S:18S ratio of approximately 2:1 indicates good quality RNA and thus verified the results obtained by qRT-PCR. Of note, RNA was separated on two different agarose

gels, however, due to issues during imaging, only one of the gels is shown here (Figure 3.1).

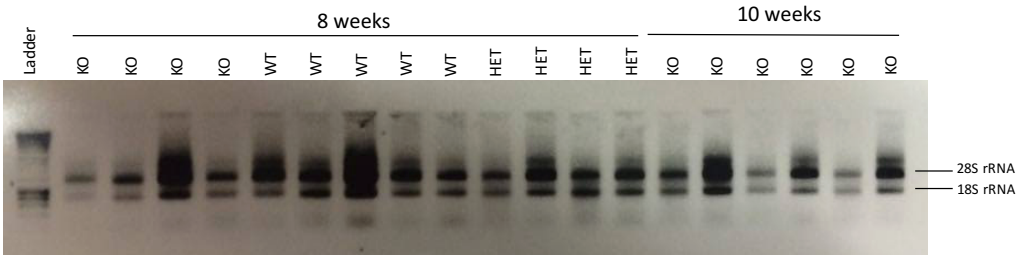


Figure 3.1: Agarose gel electrophoresis of total RNA from individual MMTV-PyMT tumours. Total RNA was separated using 1.2% EtBr-stained agarose gel and integrity was assessed based on 28S and 18S abundance.

The samples were sent to the Earlham Institute to further verify the results obtained by qRT-PCR and agarose gel electrophoresis, and to ensure that the quality of the RNA samples was sufficient for subsequent RNA sequencing. Figure 3.2 shows the gel image from the GX experiment, in which 28S, 18S and 5S is detected in most samples. Electropherogram data of all RNA samples are included in Supplementary Materials for a detailed visualisation of the rRNA peaks. A summary of various parameters, including RQS scores and final QC, measured and calculated by the Earlham Institute, is shown in Table 3.1. Out of 42 RNA samples, 26 passed the QC and all samples passed concentration except sample 12. Subsequent Nanodrop measurements revealed a significant reduction in concentration compared to earlier measurements, indicating that Sample 12 had been subject for degradation. Further, Sample 36 and 42 had very low RQS scores and was thus excluded from downstream RNAseq experiments together with Sample 12 and all heterogeneous groups. A total of 27 samples was thus selected for library construction and subsequent RNA sequencing at the Earlham Institute.

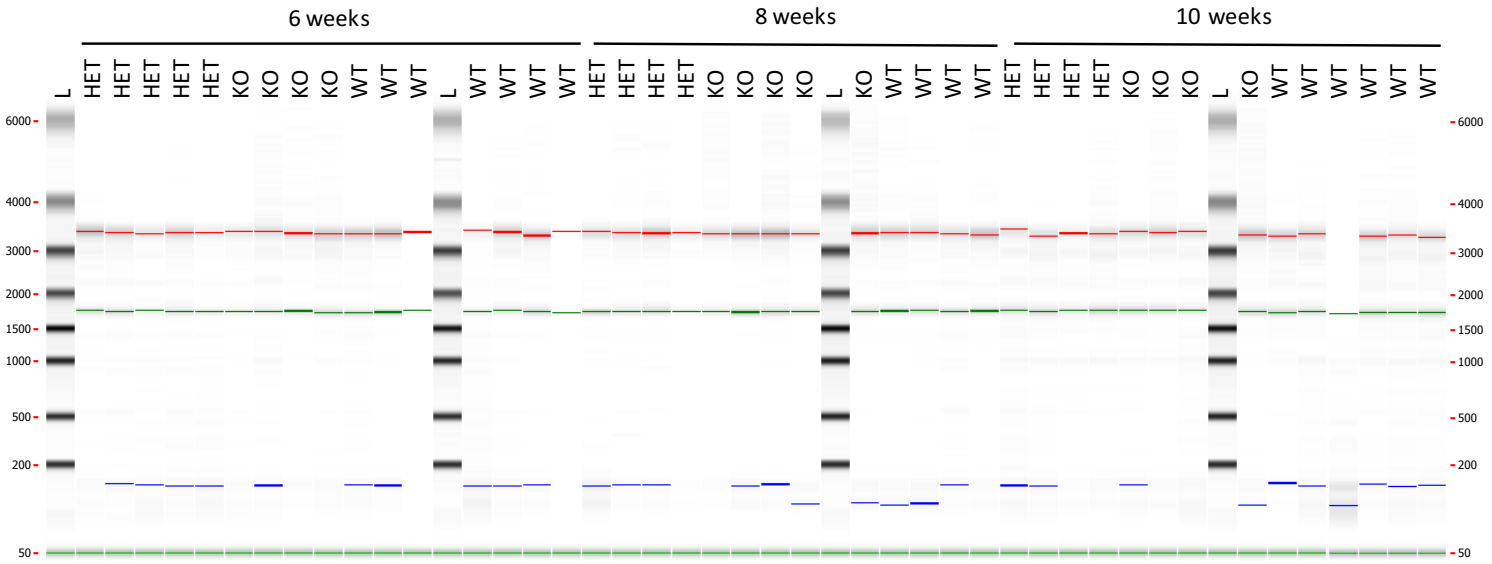


Figure 3.2: Digital gel generated from a LabChip GX assay, reflecting RNA integrity. Total RNA was isolated from MMTV- PyMT; Mmp8-wild-type, MMTV-PyMT; Mmp8-heterozygote and MMTV- PyMT; Mmp8-knock- out tumours prior separation on microfluidic sipper chips in an electrophoretic manner. Red, green and blue bands represent 28S, 18S, and 5S rRNAs, respectively. Source: The Earlham Institute.

Table 3.1: *LabChip GX quality control of RNA extracted from MMTV-PyMT mouse tumours*

			RNA Plate reader		DNA Platereader		GX values				
Timepoint	Genotype	Sample ID	ng/ul	Pass/Fail	ng/ul	ercentage DNA	RQS	Pass/Fail	Vol. of sample (ul)	Total Amount	Overall Pass/Fail
6weeks	KO	6	44.0	Pass	4.1	9.4	7.2	Pass	55	2.4	Pass
6weeks	KO	7	62.8	Pass	<Min	N/A	7.4	Pass	55	3.5	Pass
6weeks	KO	8	39.9	Pass	<Min	N/A	8.5	Pass	55	2.2	Pass
6weeks	KO	9	51.0	Pass	3.7	7.3	8.1	Pass	55	2.8	Pass
6weeks	WT	10	53.3	Pass	3.7	6.9	8.1	Pass	30	1.6	Fail
6weeks	WT	11	48.8	Pass	3.4	7.0	9.0	Pass	30	1.5	Fail
6weeks	WT	14	57.8	Pass	4.4	7.6	8.1	Pass	55	3.2	Pass
6weeks	WT	15	66.5	Pass	6.0	9.0	8.6	Pass	100	6.7	Pass
6weeks	WT	16	51.8	Pass	<Min	N/A	10.0	Pass	10	0.5	Fail
8weeks	KO	21	43.1	Pass	2.9	6.8	8.8	Pass	55	2.4	Pass
8weeks	KO	22	52.2	Pass	3.7	7.0	8.8	Pass	55	2.9	Pass
8weeks	KO	23	63.6	Pass	5.5	8.7	9.0	Pass	55	3.5	Pass
8weeks	KO	24	47.4	Pass	3.3	6.9	8.2	Pass	55	2.6	Pass
8weeks	KO	25	56.5	Pass	4.4	7.8	8.1	Pass	55	3.1	Pass
8weeks	WT	26	51.6	Pass	3.6	7.0	8.1	Pass	55	2.8	Pass
8weeks	WT	27	55.5	Pass	3.7	6.7	8.0	Pass	55	3.1	Pass
8weeks	WT	28	49.6	Pass	2.8	5.6	7.1	Pass	100	5.0	Pass
8weeks	WT	29	61.8	Pass	3.9	6.3	8.9	Pass	55	3.4	Pass
10weeks	KO	34	48.8	Pass	1.9	3.8	7.6	Pass	55	2.7	Pass
10weeks	KO	35	48.0	Pass	3.3	6.9	8.3	Pass	30	1.4	Fail
10weeks	KO	36	53.0	Pass	3.5	6.6	6.8	Fail	55	2.9	Fail
10weeks	KO	37	51.9	Pass	4.4	8.5	8.6	Pass	55	2.9	Pass
10weeks	WT	38	50.7	Pass	3.3	6.6	7.9	Pass	55	2.8	Pass
10weeks	WT	39	54.3	Pass	4.6	8.5	7.4	Pass	55	3.0	Pass
10weeks	WT	41	32.7	Pass	1.9	5.8	7.8	Pass	55	1.8	Fail
10weeks	WT	42	51.1	Pass	3.7	7.2	6.9	Fail	55	2.8	Fail
10weeks	WT	43	50.6	Pass	4.4	8.7	7.2	Pass	55	2.8	Pass

RQS = RNA Quality Score

3.2. MMP8 might suppress tumour progression via innate immune responses

While RNA sequencing was carried out at the Earlham Institute, initial experiments were conducted to assess if MMP8 had an effect on the presence of pro- and anti-tumorigenic myeloid cells (i.e. N1, N2, M1 and M2). The phenotypes of myeloid subpopulations are characterised by the expression of a range of marker genes that is specific for each phenotype and a comprehensive approach to assess the presence of pro- and anti-tumorigenic myeloid cells would be to profile for a complete set of such markers genes. As a trial, the current study initiated by selecting a combination of five markers genes, including Ccl3, VEGFA, Arginase I, Arginase II and CD163, for a time-course gene expression profiling by Taqman qRT-PCR. Ccl3 and VEGFA are highly expressed in N1 phenotypes, Arginase I is lowly expressed in N1 and highly expressed in N2 phenotypes. Further, M1 phenotypes are shown to highly express Arginase II, whereas M2 phenotypes express Arginase II at low levels and CD163 at high levels (e.g. Allavena et al., 2008: Sica & Mantovani, 2012: Martinez et al., 2006; Biswas et al., 2006; Pauleau et al., 2004).

The results from qRT-PCR experiments revealed a significant change in expression of all marker genes at 10 weeks in tumours from *Mmp8*-null mice, compared to those from *Mmp8*-wild-type and heterozygote mice (Figure 3.3). This indicates that depletion of MMP8 induced an increased influx of neutrophils and macrophages at later time points, in which a mix of both pro-tumorigenic (N1 and M1) and anti-tumorigenic (N2 and M2) phenotypes are present in the infiltrate. Notably, this is inconsistent with the prediction made, as a polarisation towards pro-tumorigenic phenotypes was expected to be observed in *Mmp8*-null tumours.

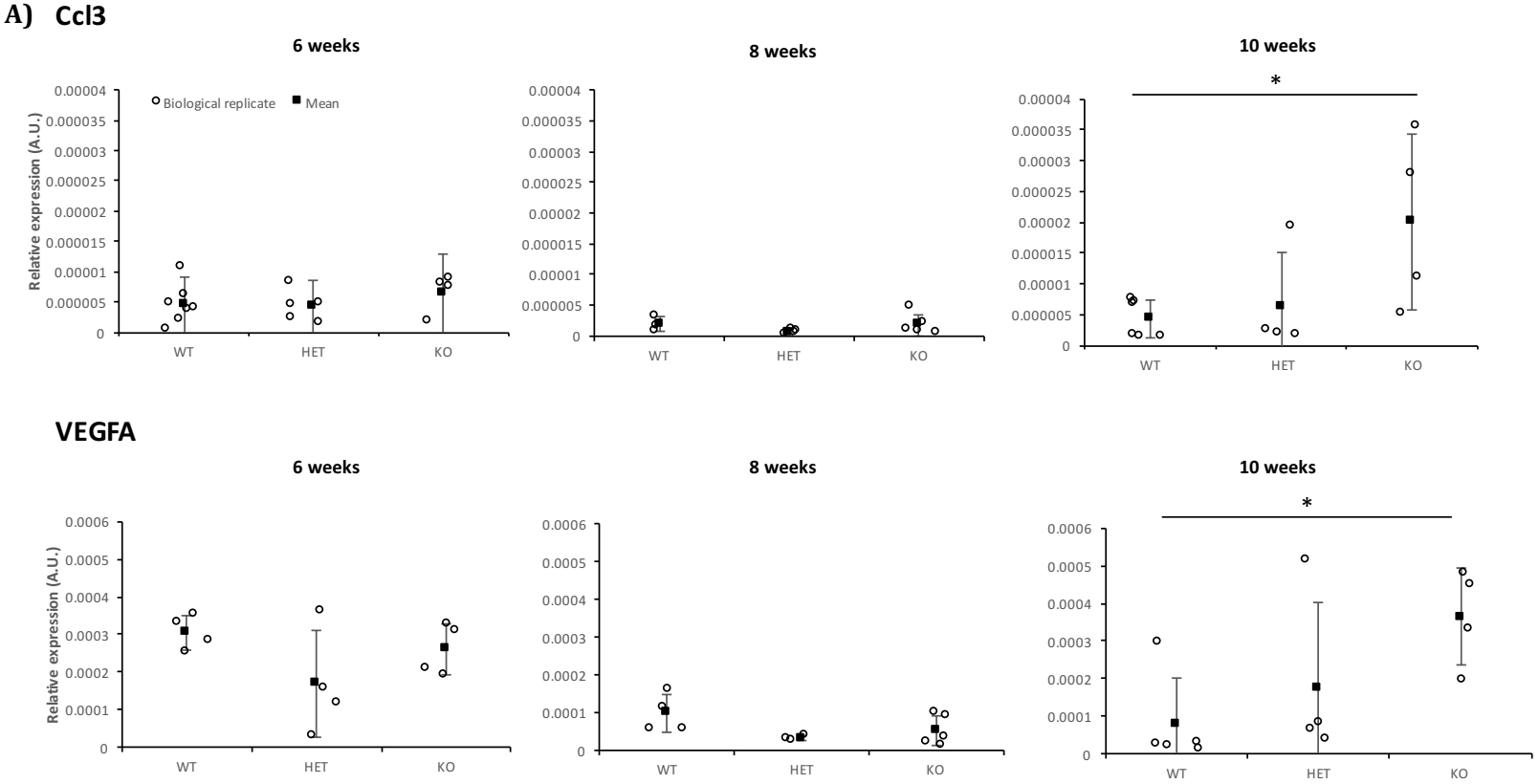
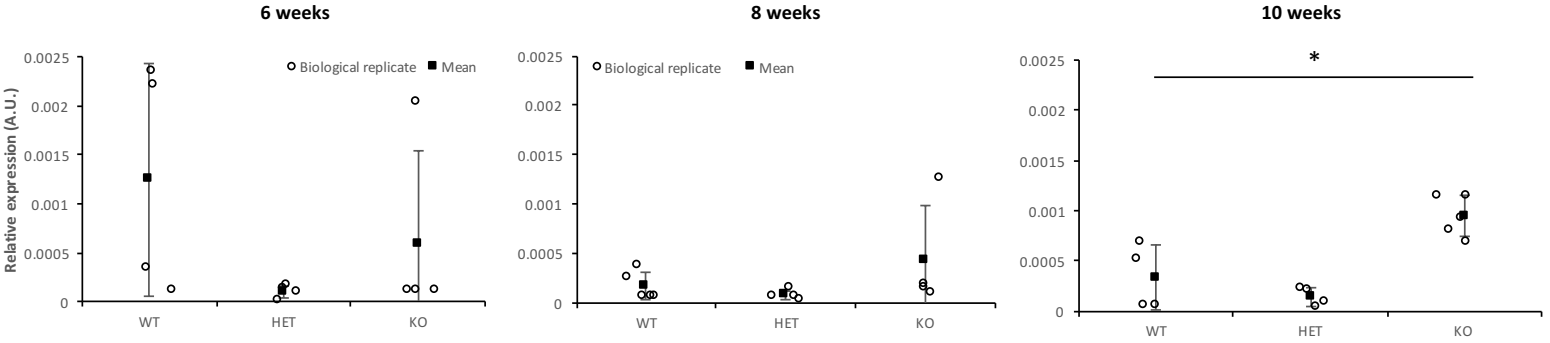
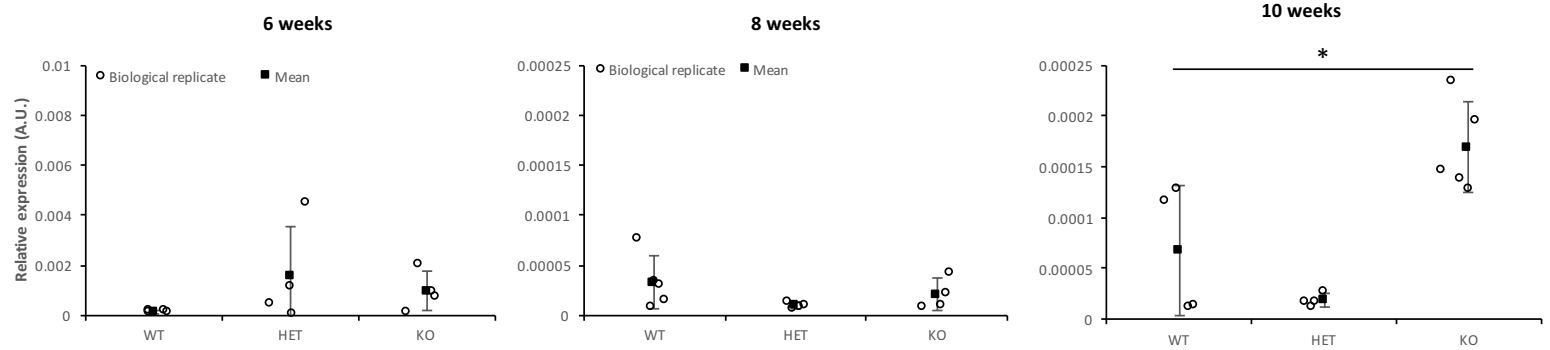


Figure 3.3: Relative expression of selected markers genes for N1, N2, M1 and M2, across all time points. Marker descriptions; Ccl3 and VEGFA is highly expressed in N1 phenotypes, Arginase I (Arg I) is poorly expressed in N1 and highly expressed in N2. Arginase II (Arg II) and CD163 is highly expressed in M1 and M2, respectively. A) Differential expression of Ccl3 and VEGF, and B) of Agr I, Arg II, and CD163. Graphs display mean, standard deviation, and statistical significance (* = < 0.05).

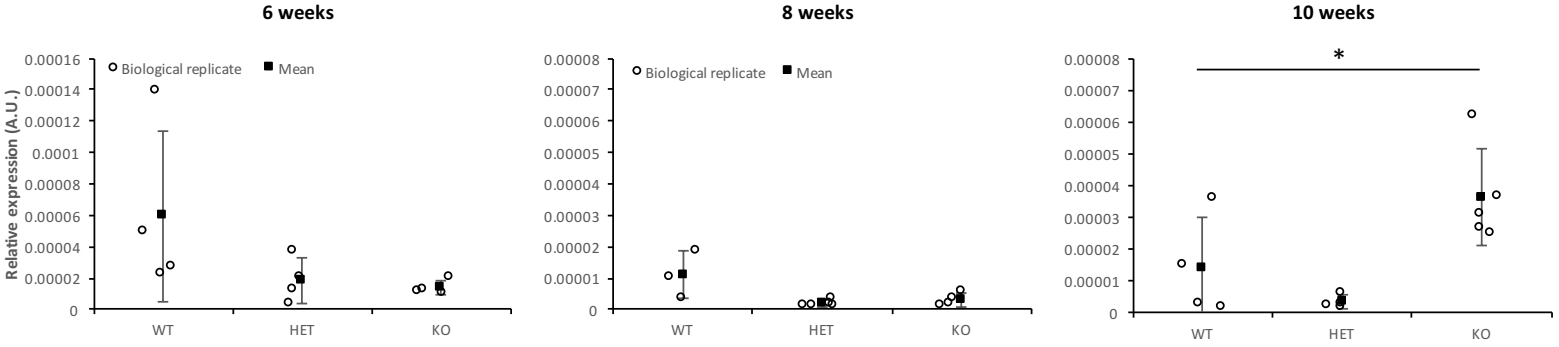
B) Arginase I



Arginase II



CD163



In addition to exploring the expression of myeloid cell markers, qRT-PCR was performed to further investigate if MMP8 ablation induce a disturbance in the protease web. Selected MMP and TIMP members were profiled in a time-course manner by qRT-PCR. Relative expression and fold-change expression was calculated using the ΔΔCt method, in which *Mmp8*-null mice was compared to wild-type when calculating the fold change. *Mmp8*-null mice showed a significant up-regulation of MMP2 and down-regulation of MMP3 and MMP13 at 10 weeks of development (Figure 3.4), compared to wild-type littermates. Further, no significant change in expression was identified for TIMP1, TIMP3 and TIMP4 between *Mmp8*-null, wild-type and heterozygote tumours (Figure 3.4). Altogether, these results strongly demonstrate the interconnections between MMP8 and other MMPs as several members of the MMP family are significantly dysregulated in *Mmp8*-null tumours.

As qRT-PCR revealed that MMP8 might act via innate immune responses to suppress tumour progression, tumour sections were stained for selected immune cell markers for further validation. Initially, tumour sections were stained for M1 and M2 phenotypes using the markers iNOS and CD206, respectively. However, this experiment was unsuccessful as no staining was detected by microscopic visualisation. As no good markers for N1 and N2 were found in previous literature, the study went on to stain for total neutrophils and macrophages using Ly6.B and F4/80 as markers, respectively. No staining for neutrophils was achieved, whereas macrophage staining was successful. Macrophages distribute as hotspots around the tumour edge and quantitative measures was performed by calculating the density of macrophages within these hotspots. Three representative regions of interest were selected and averaged for each tumour section. As illustrated in Figure 3.6, tumour sections from *Mmp8*-null mice showed no significant change in macrophage density at 6, 8 and 10 weeks, compared to wild-type tumours. These results suggest that MMP8 has no direct nor indirect effect on tumour infiltration of macrophages.

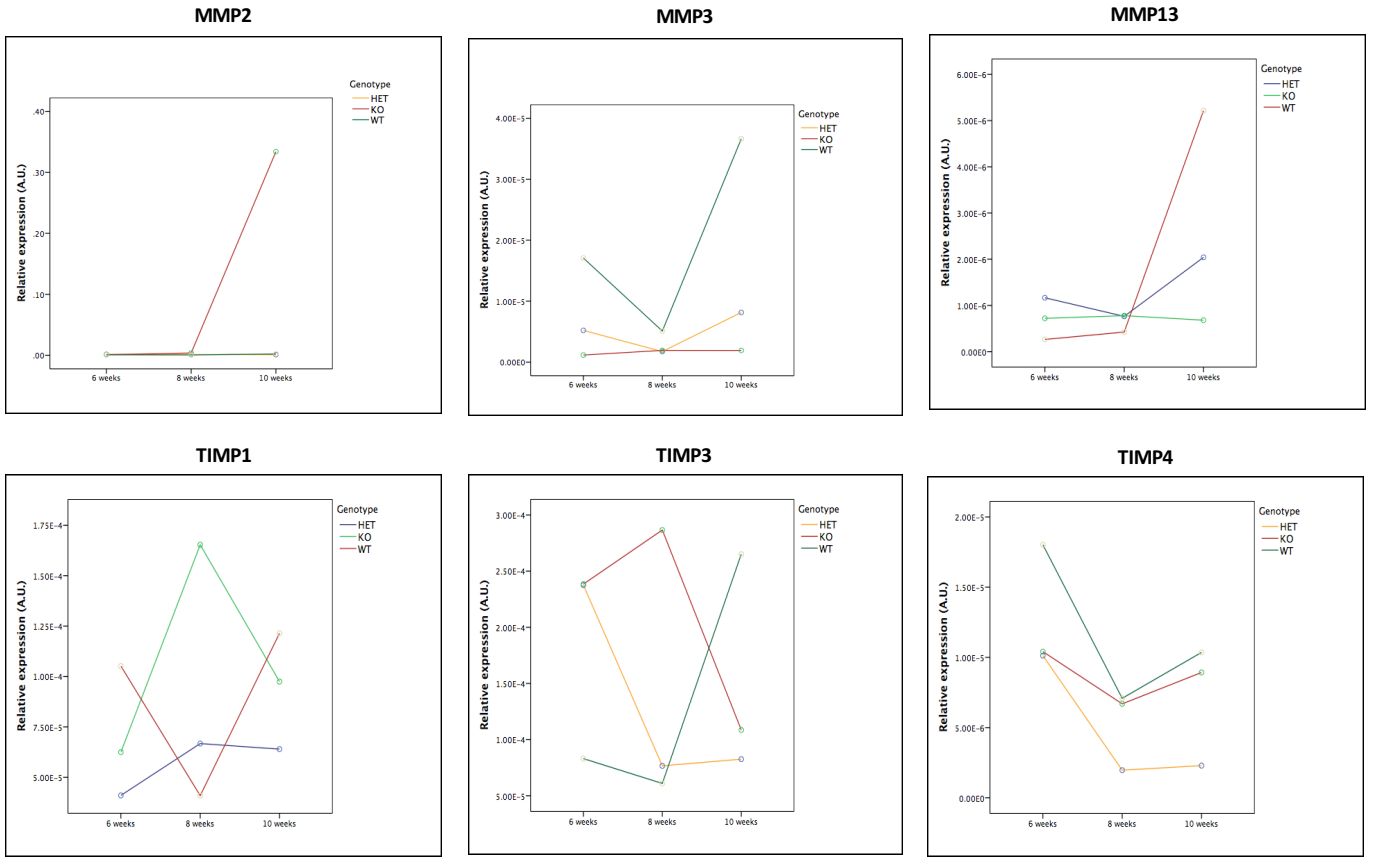
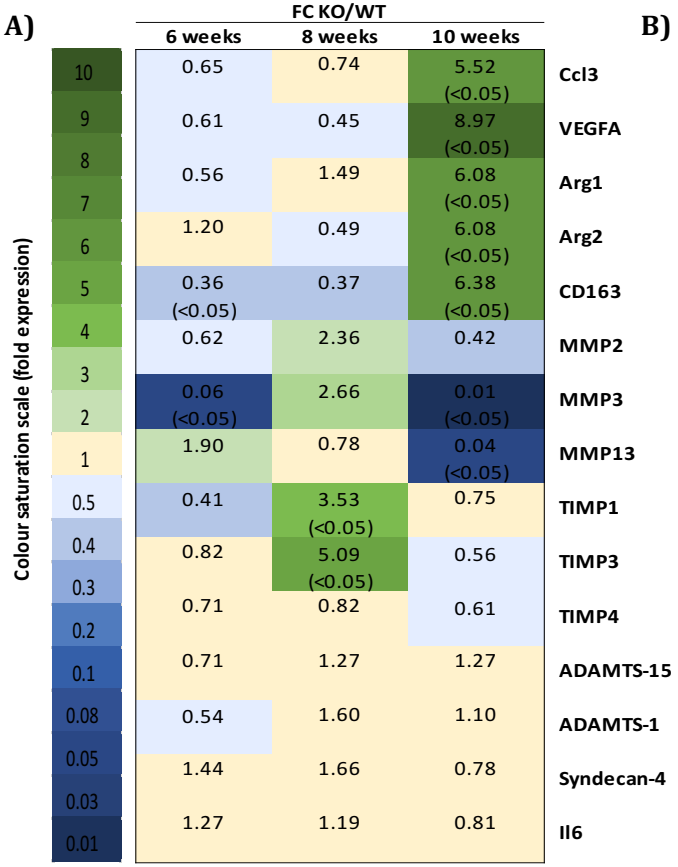


Figure 3.4: Relative expression of selected members of the protease web plotted against time. Mean expression is displayed at 6, 8 and 10 weeks of development for MMTV-PyMT; Mmp8-knock-out (red), MMTV-PyMT; Mmp8-heterozygous (yellow), and MMTV-PyMT; Mmp8-wild-type (green) mice. Expression of MMP2, MMP3, MMP13, TIMP1, TIMP3 and TIMP4 was measured as Ct values using qRT-PCR and relative expression was calculated using the ΔCt method.

A)



B)

	N1	N2	M1	M2
High	Ccl3	Arg I	Arg II	CD163
	VEGFA			
Low	Arg I			Arg II

Figure 3.5: Heat map summarizing fold expression of RNA species in MMTV-PyMT tumours in a time-course manner. A) Fold change between MMTV-PyMT; MMP8-knock-out and MMTV-PyMT; wild-type tumours are indicated with values and by colour. Fold change was calculated using the ΔΔCt method and statistical significance was calculated using the two-way ANOVA (multivariate) test. B) Marker genes for neutrophils and macrophages that display anti- (N1, M1) and pro-tumorigenic (N2, M2) phenotypes. Marker genes were selected based on previous literature (e.g. Allavena et al., 2008: Sica & Mantovani, 2012: Martinez et al., 2006).

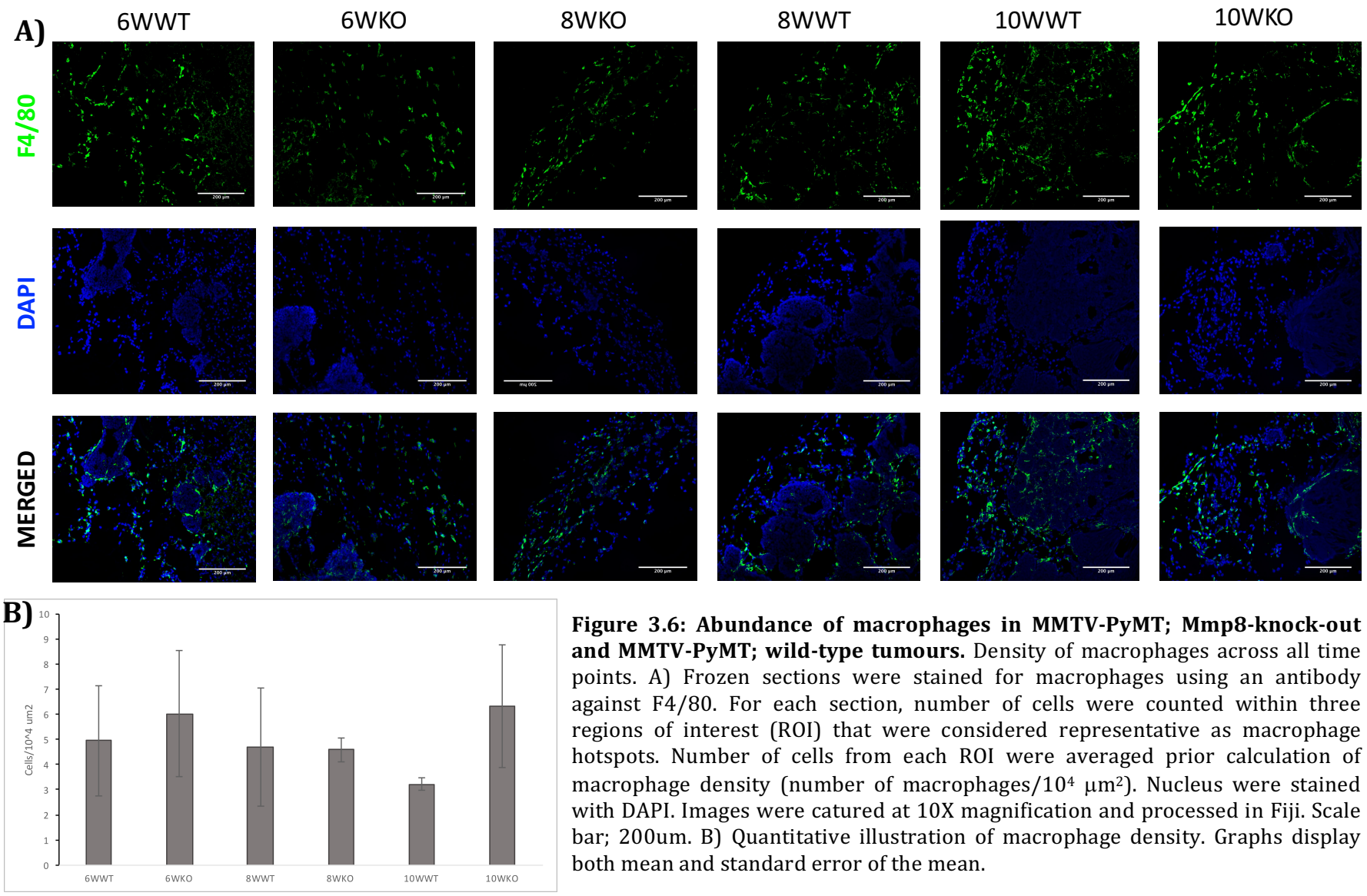


Figure 3.6: Abundance of macrophages in MMTV-PyMT; Mmp8-knock-out and MMTV-PyMT; wild-type tumours. Density of macrophages across all time points. A) Frozen sections were stained for macrophages using an antibody against F4/80. For each section, number of cells were counted within three regions of interest (ROI) that were considered representative as macrophage hotspots. Number of cells from each ROI were averaged prior calculation of macrophage density (number of macrophages/10^4 μm^2). Nucleus were stained with DAPI. Images were catured at 10X magnification and processed in Fiji. Scale bar; 200um. B) Quantitative illustration of macrophage density. Graphs display both mean and standard error of the mean.

In addition to staining for total macrophages, whole tumour sections were stained for blood vessels to determine whether MMP8 ablation has an effect on vascularity. Tumours sections from *Mmp8*-null and wild-type tumours at early and late time points were selected for staining and Endomucin was used as marker for blood vessels. As shown in Figure 3.7, blood vessel density appears to increase, for both *Mmp8* -null and wild-type tumours, from 6 weeks to 10 weeks of development. This indicates that more established late-stage tumours are associated with a more complex vasculature, which concur with the known histology of highly developed tumours. In contrast, no change in blood vessel density was found between genotypes at 6 nor at 10 weeks of disease development. These results suggest that MMP depletion has none or negligible effects on vasculature development during mammary carcinoma progression.

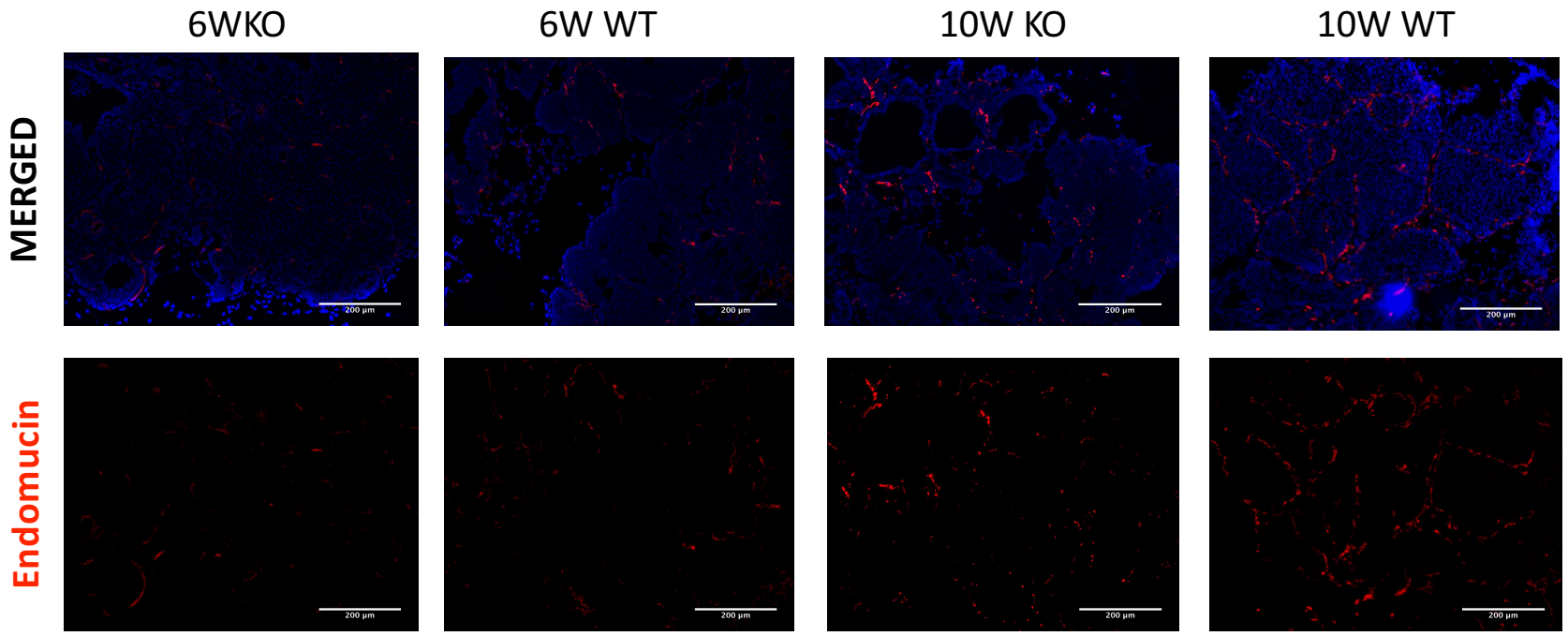


Figure 3.7: Vascular density in MMTV-PyMT tumours at early- and late time points. Representative images of stained sections from MMTV-PyMT; Mmp8-knock-out and wild-type tumours, at 6 and 10 weeks. The vasculature was stained using an antibody against Endomucin and images were captured at 10X magnification, followed by processing in Fiji. Scale bar; 200µm.

3.3. RNA sequencing reveals a more complex role of MMP8

To investigate the action of MMP8 in more depth, the whole transcriptome of tumours from *Mmp8*-null and wild-type mice were sequenced followed by comprehensive bioinformatics analysis. The raw sequence data output from Illumina was checked by assessing the quality of the reads (see Supplementary Materials) and, as the overall read quality was good, no sequence trimming was needed for downstream analysis. Further, TopHat was used as alignment tool to map the reads to the mouse reference genome (GRCm38) prior expression analysis in Cufflinks. Cufflinks reports gene expression results as Fragments Per Kilobase Million (FPKM), which is fragment counts normalised to sequencing depth and gene length (Anders and Huber, 2010).

Global statistics including distribution of FPKM scores, cluster analysis and principal component analysis (PCA) were conducted to screen for potential outliers (see Appendix 1 and Appendix 2). Such comprehensive analysis in addition to visual inspection of the data, identified 3 samples as replicate outliers, one replicate from each of the following conditions; 6 weeks_WT, 6 weeks_KO and 10 weeks_WT. As outliers can have a significant impact on statistical calculations by increasing the likelihood of false positive and negative results, these were excluded from all downstream analysis. Figure 3.8 shows global statistics of normalised data *after* excluding replicate outliers.

To identify significant differentially expressed genes between *Mmp8*-null and wild-type tumours across time points, differential expression analysis was performed using the Cuffdiff algorithm in Cufflinks, followed by statistical computing and graphing in R and other relevant software. Firstly, the level of MMP8 expression were compared between *Mmp8*-null and wild-type tumours. Although the MMP8 gene were edited in *Mmp8*-null mice, no significant differential expression were identified between the two genotypes (see Figure 3.9a). This can be explained by the approach used to generated the *Mmp8*-null mice (see Method section for details), as the knockout were generated via a genetic insertion that replaced most of exon 2, intron 2, exon 3, intron 3, and exon 4. This should result in transcription of a truncated mRNA consisting of

exon 1 and exon 5-10 that encodes for a pro-protein and hemopexin-like domains, respectively (Yates et al., 2015). To explore which regions of the allele that were transcribed in the *Mmp8*-null mice, the present study used the Integrative Genome Browser (IGV) tool to visualize the alignments against the mouse reference genome (GRCm38). As predicted, results showed that fragments were mapped against exon 1 and exon 5-10 (data not shown), which evidences that the gene is transcribed as a truncated protein that are missing the region that encodes for the catalytic domain. Hence, the mRNA is quantified by RNAseq analysis but the protein produced from the truncated mRNA is non-functional.

Venn Diagrams (Figure 3.8) were constructed to explore the relationship between differentially expressed genes across time points. The results revealed that 374, 327 and 415 genes were significantly differentially expressed in *Mmp8*-null tumours at 6, 8 weeks and 10 weeks, respectively. However, only 30 genes changed significantly at all three time points, including 5 and 6 genes that were consistently up-regulated and down-regulated, respectively, across all time points (Figure 3.9 and Table 3.2). Of particular interest, Neuropeptide Y (NPY) and serine (or cysteine) peptidase inhibitor (Serpine2) were significantly *up-regulated*, whereas MMP3 was significantly *down-regulated* across all time points. These results indicate large-scale changes in multiple regulatory systems as a consequence of MMP8 ablation.

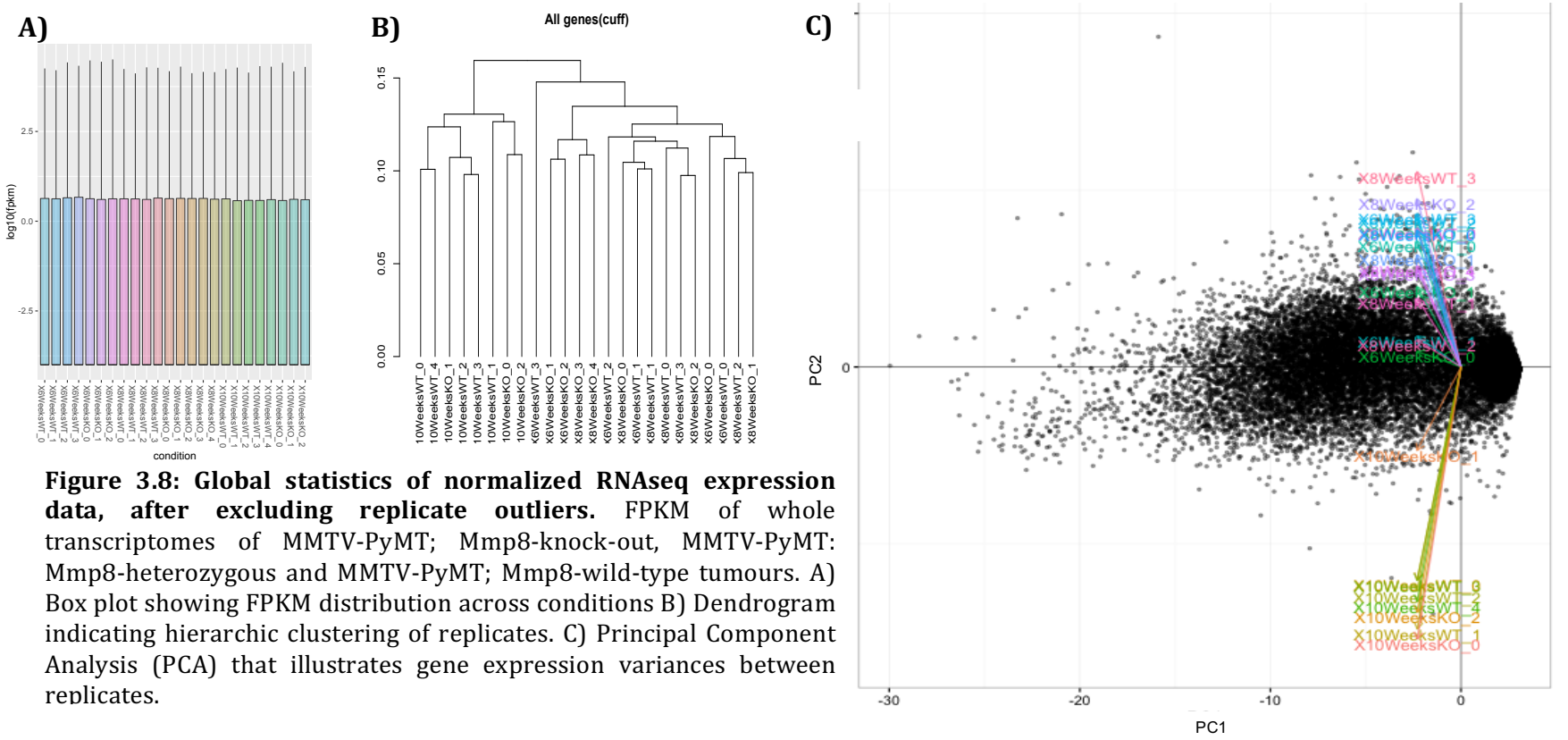
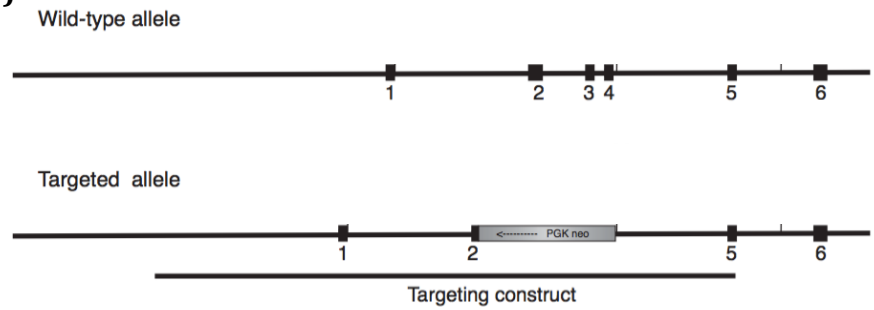


Figure 3.8: Global statistics of normalized RNAseq expression data, after excluding replicate outliers. FPKM of whole transcriptomes of MMTV-PyMT; Mmp8-knock-out, MMTV-PyMT: Mmp8-heterozygous and MMTV-PyMT; Mmp8-wild-type tumours. A) Box plot showing FPKM distribution across conditions B) Dendrogram indicating hierarchic clustering of replicates. C) Principal Component Analysis (PCA) that illustrates gene expression variances between replicates.

A)



B)

	FPKM		log2 FC
Weeks	WT	KO	KO/WT (p-value)
6	0.02	0.14	2.91 (ns)
8	0.05	0.10	0.97 (ns)
10	0.07	0.10	0.54 (ns)

Figure 3.9: Generation of *Mmp8*-null mice A) Schematic representation of *Mmp8*-wild-type and targeted allele. The PGK-neo cassette is inserted into the region that encodes the catalytic domain. Figure adapted from Balbin et al. (2013). B) Table showing FPKM expression value for MMP8 in MMTV-PyMT: Mmp8-knock-out and wild-type tumours at 6, 8 and 10 weeks. FC = fold change.

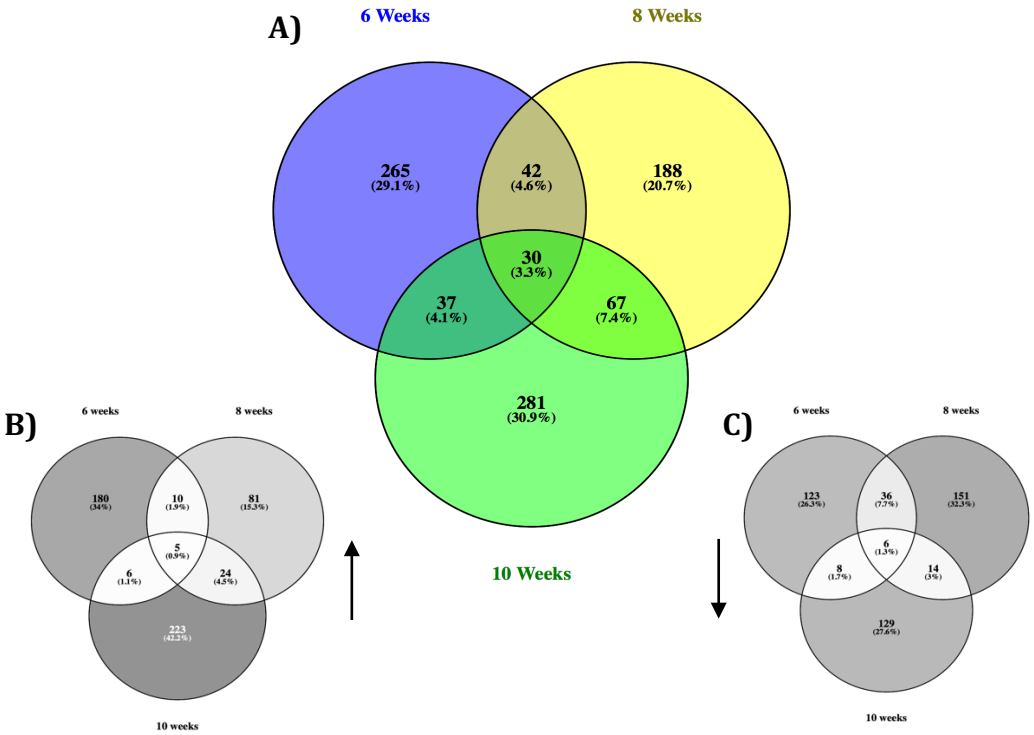


Figure 3.10: Significantly differentially expressed genes in MMTV-PyMT; Mmp8-knock-out compared to MMTV-PyMT; wild-type tumours. A) Venn diagram showing significantly differentially expressed genes at individual and across time points. These were further divided into B) significantly up-regulated and C) significantly down-regulated genes.

Table 3.2*: Significantly up- or down-regulated genes common across all time points.*

		log2 FC KO/WT		
Gene		**6 weeks**	**8weeks**	**10 weeks**
SPARC related modular calcium binding 1	Smoc1	0.61	0.95	0.72
desmoplakin	Dsp	0.63	0.57	0.72
serine (or cysteine) peptidase inhibitor, clade E, member 2	Serpine2	0.83	1.16	0.71
thrombospondin 4	Thbs4	0.98	1.58	1.13
neuropeptide Y	Npy	3.22	2.38	1.30
matrix metallopeptidase 3	Mmp3	-3.73	-1.11	-2.08
troponin I, skeletal, fast 2	Tnni2	-1.40	-3.71	-2.11
ribosomal protein L36	Rpl36	-1.02	-0.81	-1.07
leucine-rich alpha-2-glycoprotein 1	Lrg1	-0.77	-0.59	-0.94
ATPase, Ca++ transporting, type 2C, member 2	Atp2c2	-0.71	-1.12	-0.77
serum amyloid A 2	Saa2	-0.61	-1.01	-1.12

FC, fold change

To investigate the effect of MMP8 ablation in a more time-dependent manner, each set of up- or down-regulated genes at individual time points were annotated using the Gene Ontology categorization tool in DAVID (6.8 Beta). Biological processes were annotated using the GOTERM_BP_FAT category and the 20 most significantly up- and down-regulated biological pathways at 6, 8 and 10 weeks are shown in Figure 3.9-3.11. In *Mmp8*-null tumours at 6 weeks, most up-regulated genes were categorised to be involved in lymphocyte activation and differentiation, which indicates an increased activation of adaptive immune responses at early time points. In contrast, biological processes associated with tumorigenesis were down-regulated at 6 weeks, including cell differentiation and blood vessel development. Mammary gland development was also down-regulated at 6 weeks (Figure 3.9). At 8 weeks, biological processes involved in cell adhesion, locomotion and negative regulation of insulin receptor signaling were up-regulated in *Mmp8*-null tumours. Further, lipid and carbohydrate metabolism and, acute inflammatory responses were down-regulated at this stage (Figure 3.10).

In contrast to these earlier time points, *Mmp8*-null mice at 10 weeks showed an upregulation of lipid and glycerol metabolism, acute inflammatory responses and responses to wounding. Genes involved in regulation of gene transcription and macromolecule metabolic processes were down-regulated at 10 weeks (Figure 3.11). Altogether, these results suggest that MMP8 depletion induce multiple changes in the tumour microenvironment during tumour progression and that MMP8 effects are highly time dependent.

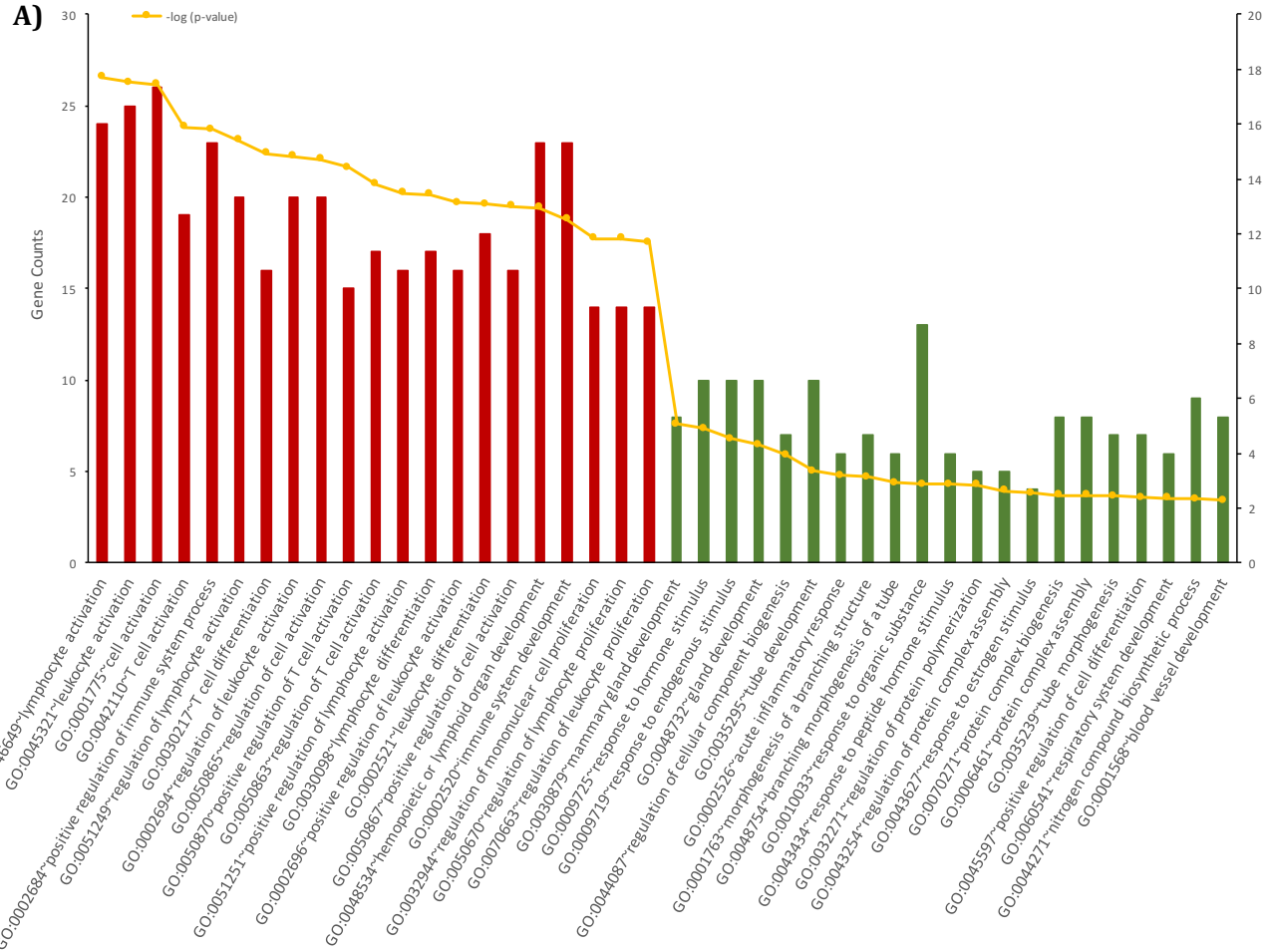
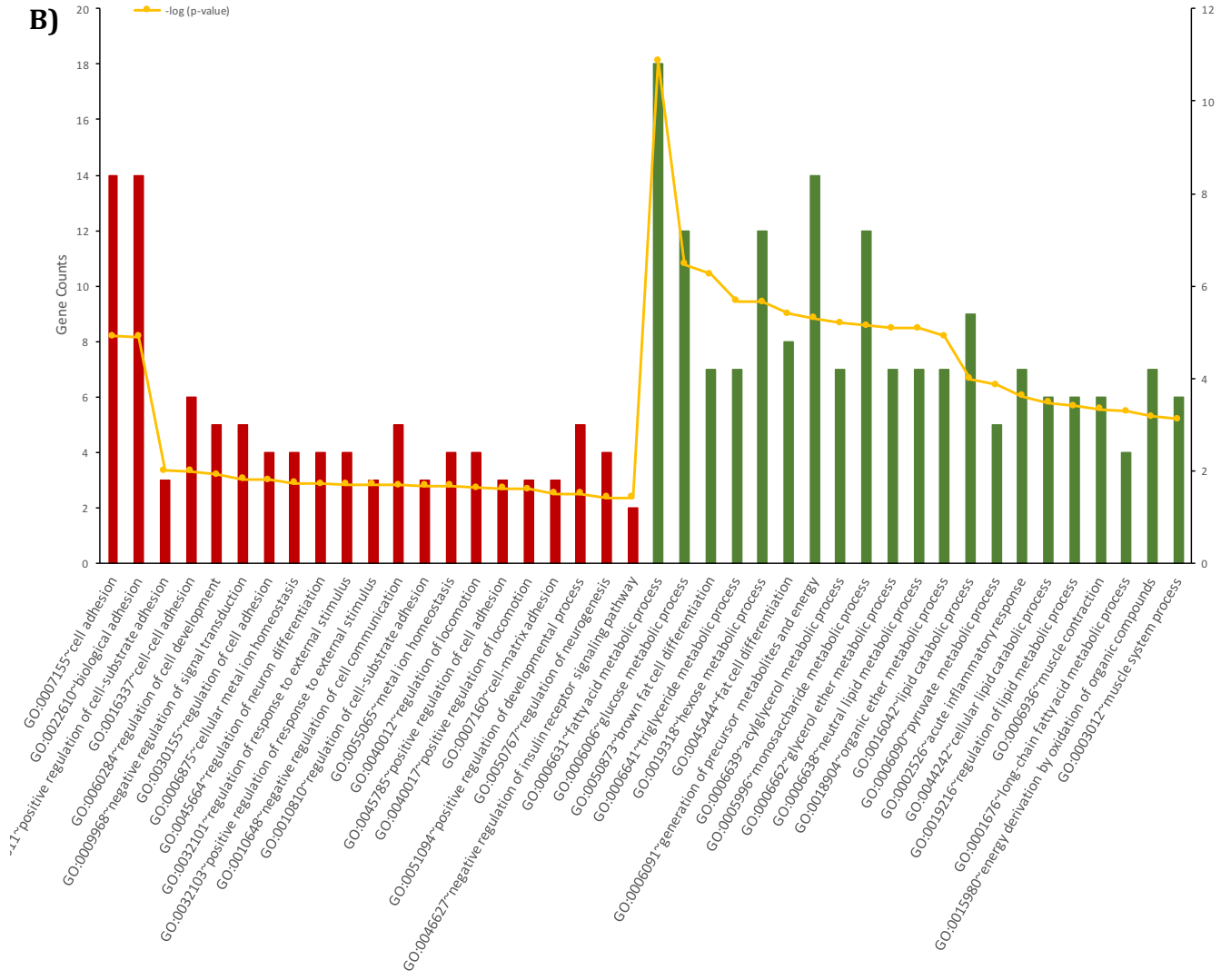
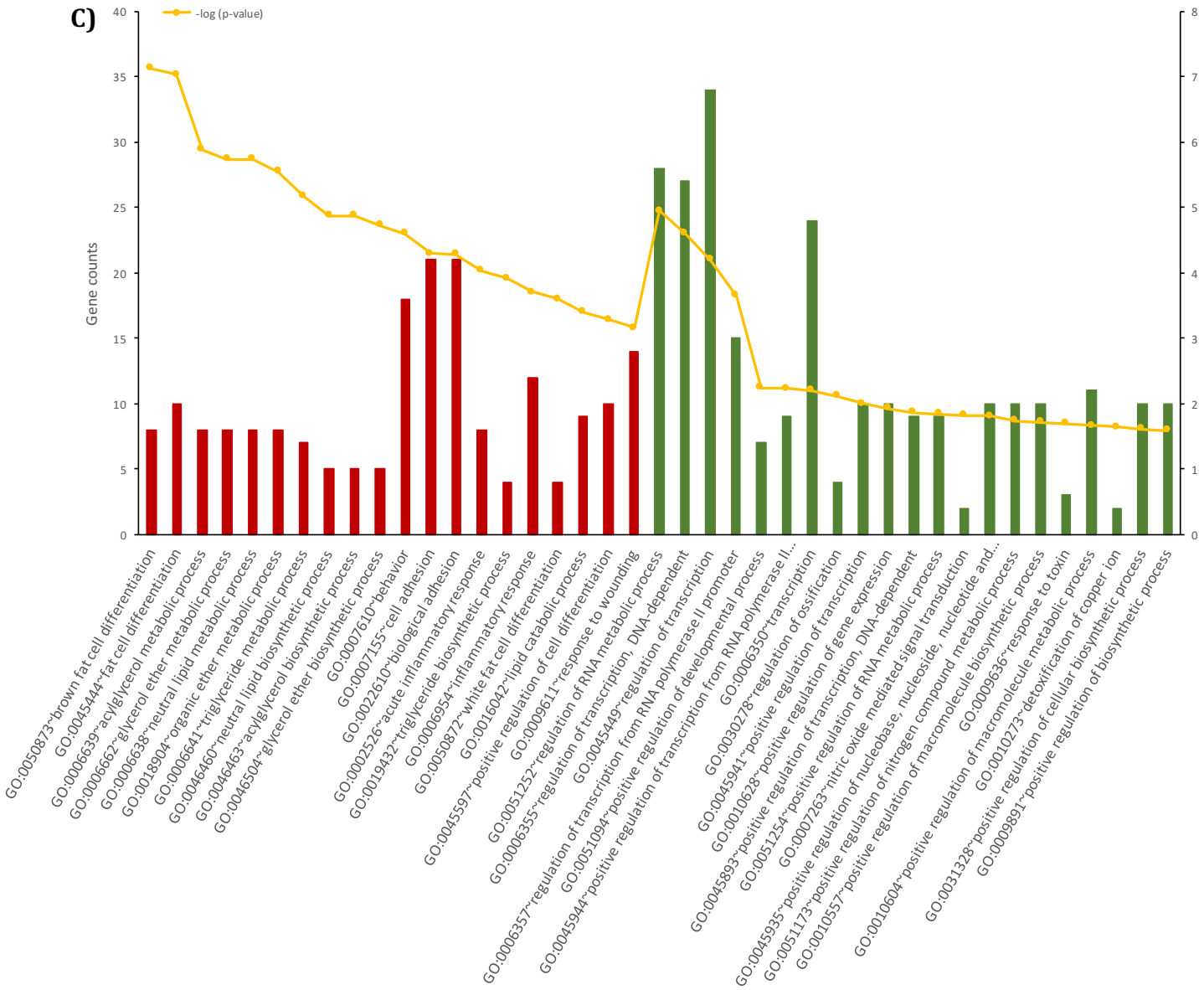


Figure 3.11-3.13: The most significantly changed biological processes in MMTV-PyMT; Mmp8-null tumours at 6, 8 and 10 weeks. Bar chart is showing the 20 most up-regulated biological processes (red) and the 20 most down-regulated biological processes (green) in Mmp8-null tumours at A) 6 weeks, B) 8 weeks and C) 10 weeks of tumour development, compared to Mmp8-wild-typtumours. Bars indicate the number of genes in each biological process. The yellow line indicates the enrichment significance (–log (p-value)) of each biological process, generated using the EASE score. Functional annotation was performed in DAVID using the GOTERM_BP_FAT term for biological process categorisation.

B)


-log (p-value)
Gene Counts
0
2
4
6
8
10
12
14
16
18
20
0
2
4
6
8
10
12
GO:0007155~cell adhesion
GO:0022610~biological adhesion
GO:0007155~cell adhesion
GO:0016337~cell-cell adhesion
GO:0060284~regulation of cell development
GO:0009968~negative regulation of signal transduction
GO:0030155~regulation of cell adhesion
GO:0006875~cellular metal ion homeostasis
GO:0045664~regulation of neuron differentiation
GO:0032101~regulation of response to external stimulus
GO:0032103~positive regulation of response to external stimulus
GO:0010648~negative regulation of cell communication
GO:0010810~regulation of cell-substrate adhesion
GO:0055065~metal ion homeostasis
GO:0040012~regulation of locomotion
GO:0045785~positive regulation of cell adhesion
GO:0040017~positive regulation of locomotion
GO:0007160~cell-matrix adhesion
GO:0051094~positive regulation of developmental process
GO:0050767~regulation of neurogenesis
GO:0046627~negative regulation of insulin receptor signaling pathway
GO:0006631~fatty acid metabolic process
GO:0006006~glucose metabolic process
GO:0050873~brown fat cell differentiation
GO:0006641~triglyceride metabolic process
GO:0019318~hexose metabolic process
GO:0045444~fat cell differentiation
GO:0006091~generation of precursor metabolites and energy
GO:0006639~acylglycerol metabolic process
GO:0005996~monosaccharide metabolic process
GO:0006662~glycerol ether metabolic process
GO:0006638~neutral lipid metabolic process
GO:0018904~organic ether metabolic process
GO:0016042~lipid catabolic process
GO:0006090~pyruvate metabolic process
GO:0002526~acute inflammatory response
GO:0044242~cellular lipid catabolic process
GO:0019216~regulation of lipid metabolic process
GO:0006936~muscle contraction
GO:0001676~long-chain fatty acid metabolic process
GO:0015980~energy derivation by oxidation of organic compounds
GO:0003012~muscle system process


c)
-log (p-value)
Gene counts
0
5
10
15
20
25
30
35
40
0
1
2
3
4
5
6
7
8
GO:0050873~brown fat cell differentiation
GO:0045444~fat cell differentiation
GO:0006639~acylglycerol metabolic process
GO:0006662~glycerol ether metabolic process
GO:0006638~neutral lipid metabolic process
GO:0018904~organic ether metabolic process
GO:0006641~triglyceride metabolic process
GO:0046460~neutral lipid biosynthetic process
GO:0046463~acylglycerol biosynthetic process
GO:0046504~glycerol ether biosynthetic process
GO:0007610~behavior
GO:0007155~cell adhesion
GO:0022610~biological adhesion
GO:0002526~acute inflammatory response
GO:0019432~triglyceride biosynthetic process
GO:0006954~inflammatory response
GO:0050872~white fat cell differentiation
GO:0016042~lipid catabolic process
GO:0045597~positive regulation of cell differentiation
GO:0009611~response to wounding
GO:0051252~regulation of RNA metabolic process
GO:0006355~regulation of transcription, DNA-dependent
GO:0045449~regulation of transcription
GO:0051094~positive regulation of developmental process
GO:0045944~positive regulation of transcription from RNA polymerase II promoter
GO:0006350~transcription
GO:0030278~regulation of ossification
GO:0045941~positive regulation of transcription
GO:0010628~positive regulation of gene expression
GO:0045893~positive regulation of transcription, DNA-dependent
GO:0051254~positive regulation of RNA metabolic process
GO:0007263~nitric oxide mediated signal transduction
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and...
GO:0051173~positive regulation of nitrogen compound metabolic process
GO:0010557~positive regulation of macromolecule biosynthetic process
GO:0010604~positive regulation of macromolecule metabolic process
GO:0031328~positive regulation of cellular biosynthetic process
GO:0009891~positive regulation of biosynthetic process

As the link between MMP8 and immune responses were of particular interest for the present study, genes encoding components of the immune system at early time points were selected for further gene annotation analysis. A list of 61 genes encoding components of the immune system were significantly up-regulated at 6 weeks. To build up a picture of immune networks affected by MMP8 at early time points, pathway analysis of all 61 genes were conducted in DAVID (Figure 3.12). Functional annotation of relevant genes was further used to construct a table of components of the immune system that were significantly differentially expressed in *Mmp8*-null tumours (Table 3.3), compared to wild-type tumours. No significant change was observed for neutrophil and macrophage marker genes (data not shown). However, multiple lymphocyte markers including CD3, CD4, CD8 and CD79, showed a significant fold change in *Mmp8*-null tumours. Further, several B- and T-cell chemotactic factors were significantly up-regulated, including Ccl22, Ccl5, Cxcl13 and Il23a. Genes encoding for cytoskeleton structures, lymphocyte migration and cytokine receptors were also significantly differentially expressed in *Mmp8*-null tumours at 6 weeks. Altogether, these results suggest that MMP8 inhibit adaptive immune responses at early time points, possibly by regulating the chemotaxis responsible for recruitment and activation of B- and T-lymphocytes.

The observed change in adaptive immune responses were not evident at later stages of development. However, several chemo-attractants, including Cxcl9, Ccl9, Ccl8 and Interleukins as well as components of the complement system, were dysregulated at 8 and 10 weeks of development. Although MMP8 depletion appear to have a predominant effect on immune responses at earlier time points, these results suggest a link between MMP8 and immune regulatory networks throughout the time course of tumour development.

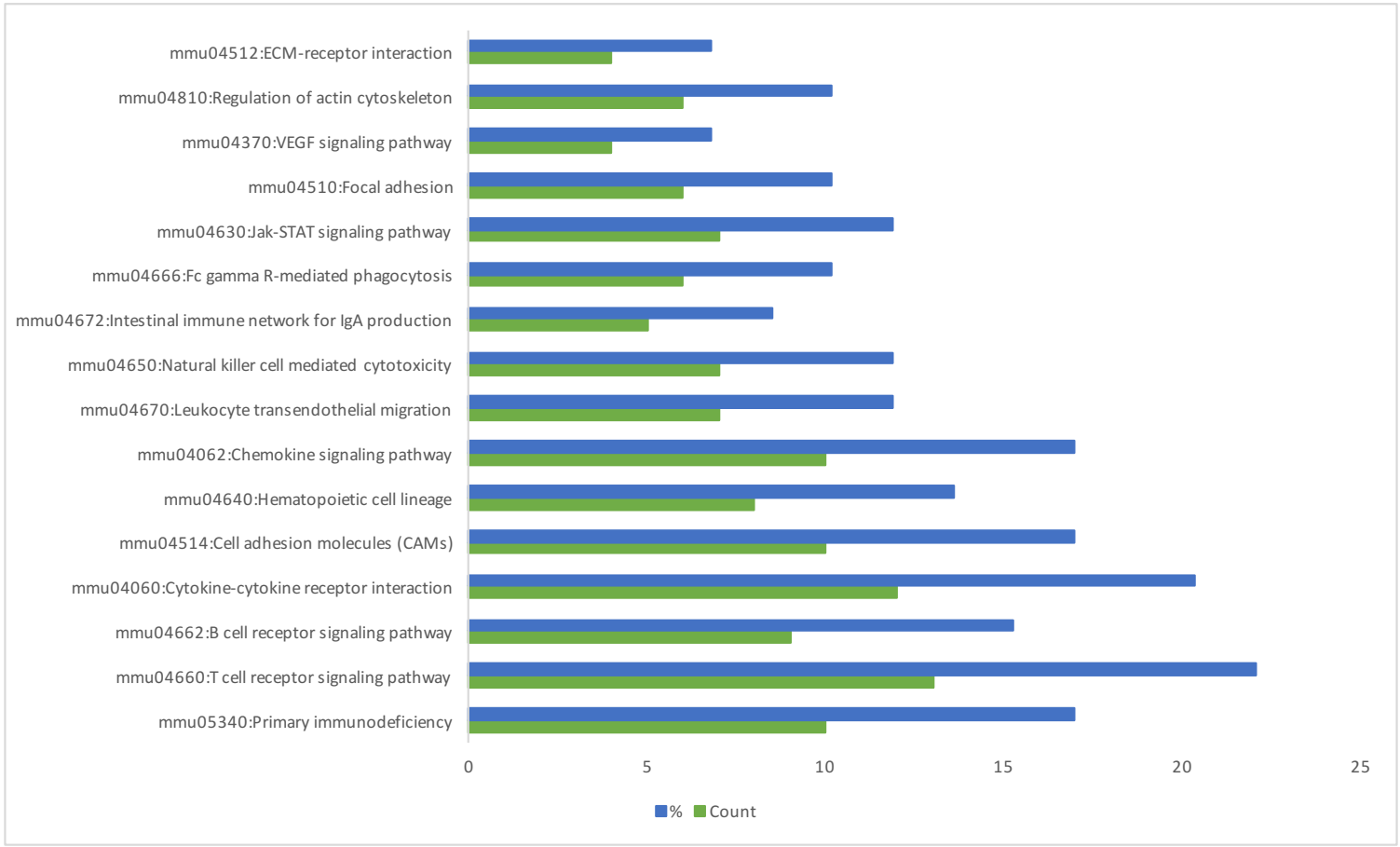


Figure 3.14: Up-regulated immune pathways in Mmp8-null tumours at 6 weeks for development. Significantly differentially expressed genes between MMTV-PyMT; Mmp8-knock-out mice and MMTV-PyMT; Mmp8-wild-type were used for pathway analysis in DAVID. The KEGG_PATHWAY was selected for mapping. Number of genes annotated within each pathway are indicated in blue, whereas the percentage of genes are indicated in blue.

Table 3.3*: Genes annotated as components of the immune system significantly altered in Mmp8-null tumours at 6 weeks.*

		log2 FC KO/WT (FDR-adjusted p-value)		
Gene		**6 Weeks**	**8 Weeks**	**10 Weeks**
B and T lymphocyte associated	Btla	2.16 (0.0017)	0.05 (ns)	0.60 (ns)
CD22 antigen	Cd22	4.21 (0.0017)	1.03 (ns)	0.16 (ns)
CD28 antigen	Cd28	5.15 (0.0116)	-0.11 (ns)	0.22 (ns)
CD3 antigen, delta polypeptide	Cd3d	3.04 (0.0017)	-0.42 (ns)	1.74 (0.0307)
CD3 antigen, epsilon polypeptide	Cd3e	2.96 (0.0044)	-0.07 (ns)	-0.09 (ns)
CD3 antigen, gamma polypeptide	Cd3g	2.38 (0.0017)	-0.35 (ns)	1.25 (ns)
CD4 antigen	Cd4	2.93 (0.0017)	-0.30 (ns)	0.17 (ns)
CD79A antigen	Cd79a	5.15 (0.0215)	0.70 (ns)	-1.28 (ns)
CD79B antigen	Cd79b	2.85 (0.0087)	0.48 (ns)	1.17 (ns)
CD8 antigen, alpha chain	Cd8a	3.70 (0.0017)	0.49 (ns)	0.39 (ns)
CD83 antigen	Cd83	0.98 (0.0441)	0.14 (ns)	0.18 (ns)
CD96 antigen	Cd96	2.08 (0.0087)	0.35 (ns)	0.68 (ns)
chemokine (C-C motif) ligand 22	Ccl22	3.30 (0.0017)	0.47 (ns)	0.64 (ns)
chemokine (C-C motif) ligand 5	Ccl5	2.20 (0.0017)	0.08 (ns)	0.74 (ns)
chemokine (C-C motif) receptor 7	Ccr7	3.98 (0.0017)	0.35 (ns)	0.60 (ns)
chemokine (C-X-C motif) ligand 1	Cxcl1	1.37 (0.0435)	1.00 (ns)	-0.26 (ns)
chemokine (C-X-C motif) ligand 13	Cxcl13	5.23 (0.0323)	-0.53 (ns)	-0.26 (ns)
chemokine (C-X-C motif) receptor 5	Cxcr5	3.12 (0.0031)	0.02 (ns)	0.79 (ns)
ciliary neurotrophic factor receptor	Cntfr	1.97 (0.0159)	0.97 (ns)	-0.36 (ns)
coronin, actin binding protein 1A	Coro1a	1.07 (0.0044)	-0.11 (ns)	0.12 (ns)
cysteine rich protein 61	Cyr61	0.71 (0.0133)	0.39 (ns)	-0.14 (ns)
cytoplasmic FMR1 interacting protein 2	Cyfip2	2.50 (0.0017)	0.14 (ns)	-0.28 (ns)
dedicator of cyto-kinesis 2	Dock2	1.36 (0.0017)	0.03 (ns)	-0.05 (ns)
ectonucleotide pyrophosphatase/phosphodiesterase 2	Enpp2	0.93 (0.0461)	-0.81 (ns)	1.10 (0.0428)
Fc receptor-like 1	Fcrl1	3.90 (0.0031)	0.96 (ns)	1.05 (ns)
Fc receptor, IgG, low affinity IIb	Fcgr2b	0.98 (0.0454)	0.24 (ns)	0.43 (ns)
histocompatibility 2, O region beta locus	H2-Ob	3.23 (0.0017)	0.08 (ns)	0.92 (ns)
IL2 inducible T cell kinase	Itk	3.94 (0.0017)	-0.53 (ns)	1.15 (ns)
integrin alpha 4	Itga4	1.35 (0.0098)	0.43 (ns)	-0.09 (ns)
integrin beta 2	Itgb2	0.96 (0.0289)	0.50 (ns)	0.16 (ns)
integrin beta 7	Itgb7	2.90 (0.0017)	-0.11 (ns)	0.66 (ns)
interleukin 2 receptor, gamma chain	Il2rg	2.04 (0.0017)	0.19 (ns)	0.42 (ns)
interleukin 21 receptor	Il21r	1.52 (0.0098)	0.45 (ns)	0.65 (ns)

Table continues on next page

interleukin 23, alpha subunit p19	Il23a	2.31 (0.0375)	1.82 (0.0242)	0.21 (ns)
interleukin 27 receptor, alpha	Il27ra	2.29 (0.0017)	-0.46 (ns)	0.79 (ns)
interleukin 7 receptor	Il7r	2.51 (0.0017)	0.40 (ns)	0.24 (ns)
lymphocyte antigen 9	Ly9	2.03 (0.0017)	0.22 (ns)	0.47 (ns)
lymphocyte protein tyrosine kinase	Lck	1.67 (0.0031)	0.00 (ns)	0.24 (ns)
lymphocyte transmembrane adaptor 1	Lax1	2.38 (0.0067)	-0.10 (ns)	1.00 (ns)
moesin	Msn	0.62 (0.0107)	0.15 (ns)	-0.06 (ns)
myosin IG	Myo1g	1.57 (0.0125)	-0.41 (ns)	-0.25 (ns)
neuropeptide Y	Npy	3.22 (0.0252)	2.38 (0.0231)	1.30 (0.0423)
nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1	Nfatc1	1.19 (0.0031)	0.00 (ns)	0.01 (ns)
nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2	Nfatc2	1.26 (0.0017)	0.28 (ns)	0.39 (ns)
phosphatidylinositol 3-kinase catalytic delta polypeptide	Pik3cd	1.11 (0.0454)	0.28 (ns)	0.43 (ns)
protein kinase C, beta	Prkcb	1.26 (0.0382)	0.21 (ns)	-0.00 (ns)
protein tyrosine phosphatase, receptor type, C	Ptprc (CD45)	1.90 (0.0017)	0.12 (ns)	0.37 (ns)
RAS guanyl releasing protein 1	Rasgrp1	2.21 (0.0017)	0.20 (ns)	0.71 (ns)
sialic acid binding Ig-like lectin H	Siglech	1.65 (0.0335)	0.60 (ns)	1.33 (ns)
sialophorin	Spn	2.26 (0.0017)	0.19 (ns)	0.19 (ns)
spleen tyrosine kinase	Syk	0.91 (0.0497)	0.07 (ns)	0.13 (ns)
src family associated phosphoprotein 1	Skap1	2.83 (0.0017)	0.00 (ns)	1.51 (0.0407)
suppressor of cytokine signaling 2; predicted gene 8000	Socs2	0.82 (0.0044)	0.62 (0.0443)	0.20(ns)
T cell immunoglobulin and mucin domain containing 4	Timd4	3.49 (0.0017)	-0.46 (ns)	0.29 (ns)
tenascin C	Tnc	1.25 (0.0017)	0.39 (ns)	-0.14 (ns)
tetraspanin 32	Tspan32	2.00 (0.0107)	-0.20 (ns)	0.63 (ns)
thrombospondin 1; similar to thrombospondin 1	Thbs1	0.72 (0.0252)	0.55 (0.0307)	-0.30 (ns)
tumor necrosis factor receptor superfamily, member 13c	Tnfrsf13c	2.01 (0.0375)	-0.42 (ns)	0.10 (ns)
TXK tyrosine kinase	Txk	2.23 (0.0238)	0.25 (ns)	0.03 (ns)
B cell leukemia/lymphoma 6	Bcl6	-0.75 (0.0289)	-0.23 (ns)	-0.43 (ns)
complement factor D (adipsin)	Cfd	-2.09 (0.0017)	-1.00 (0.0014)	0.67 (ns)
indoleamine 2,3-dioxygenase 1	Ido1	-1.93 (0.0017)	-0.70 (ns)	1.50 (0.0182)
interferon regulatory factor 4	Irf4	-1.68 (0.0017)	-1.23 (0.0084)	-0.22 (ns)
pyruvate carboxylase	Pcx	-0.73 (0.0295)	-0.93 (0.0015)	0.68 (ns)
serum amyloid A 1	Saa1	-0.82 (0.0056)	-0.87 (0.0093)	-0.67 (ns)
spondin 2, extracellular matrix protein	Spon2	-1.94 (0.0017)	-0.70 (ns)	0.46 (ns)
transforming growth factor, beta 3	Tgfb3	-0.81 (0.0017)	0.21 (ns)	0.15 (ns)
vanin 1	Vnn1	-1.87 (0.0032)	-0.26 (ns)	1.54 (0.0015)

FC, fold change

Table 3.4*: Genes annotated as components of the immune system significantly altered in Mmp8-null tumours at 8 and 10 weeks.*

		log2 FC KO/WT (FDR-adjusted p-value)		
Gene		**6 weeks**	**8 weeks**	**10 weeks**
arginase, liver	Arg1	0.72 (0.0098)	1.39 (0.0014)	0.39 (ns)
CD3 antigen, delta polypeptide	Cd3d	3.04 (0.0017)	-0.42 (ns)	1.74 (0.0308)
CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	Cd74	0.22 (ns)	0.03 (ns)	0.81 (0.0015)
chemokine (C motif) ligand 1	Xcl1	-0.61 (ns)	-0.39 (ns)	1.50 (0.0428)
chemokine (C-C motif) ligand 8	Ccl8	0.31 (ns)	0.12 (ns)	0.95 (0.0428)
chemokine (C-C motif) ligand 9	Ccl9	-0.09 (ns)	-0.89 (0.0242)	-0.00 (ns)
chemokine (C-X-C motif) ligand 9	Cxcl9	-0.15 (ns)	-0.18 (ns)	1.53 (0.0229)
complement component 1, r subcomponent; predicted gene 8551	C1ra	-0.28 (ns)	-0.26 (ns)	0.91 (0.0358)
complement component 3; similar to complement component C3 prepropeptide, last	C3	-0.63 (ns)	-0.85 (0.0015)	0.67 (ns)
complement component factor h; similar to complement component factor H	Cfh	-0.12 (ns)	0.34 (ns)	0.95 (0.0015)
complement factor D (adipsin)	Cfd	-2.09 (0.0017)	-1.00 (0.0015)	0.67 (ns)
cytokine receptor-like factor 2	Crlf2	-1.89 (ns)	-0.37 (ns)	-2.19 (0.0015)
ectonucleotide pyrophosphatase/phosphodiesterase 2	Enpp2	0.93946	-0.81 (ns)	1.10 (0.0428)
Fc receptor, IgG, alpha chain transporter	Fcgrt	-0.69 (ns)	-0.71 (0.0117)	0.88 (0.0084)
immunity-related GTPase family M member 1	Irgm1	0.22 (ns)	0.64 (0.0207)	0.41 (ns)
immunoglobulin joining chain	Igj	0.18 (ns)	1.27 (0.0413)	0.29 (ns)
indoleamine 2,3-dioxygenase 1	Ido1	-1.93 (0.0017)	-0.70 (ns)	1.50 (0.0183)
interferon regulatory factor 4	Irf4	-1.68 (0.0017)	-1.23 (0.0084)	-0.22 (ns)
interleukin 1 beta	Il1b	0.87 (ns)	1.32 (0.0222)	0.86 (ns)
interleukin 18 binding protein	Il18bp	-0.18 (ns)	0.87 (0.0272)	0.22 (ns)
interleukin 23, alpha subunit p19	Il23a	2.31 (0.0375)	1.82 (0.0242)	0.21 (ns)
lymphocyte antigen 6 complex, locus D	Ly6d	-0.99 (ns)	0.31 (ns)	0.82 (0.0027)
lymphocyte antigen 6 complex, locus K	Ly6k	-0.48 (ns)	-0.19 (ns)	3.58 (0.0015)
macrophage migration inhibitory factor-like	Mif	-0.0 (ns)	0.08 (ns)	-0.87 (0.0015)
orosomucoid 1	Orm1	-0.54 (ns)	-1.47 (0.0015)	1.89 (0.0015)
pentraxin related gene	Ptx3	0.57 (ns)	1.46 (0.0015)	1.09 (0.0015)
pyruvate carboxylase	Pcx	-0.73 (0.0295)	-0.93 (0.0015)	0.68 (ns)
S100 calcium binding protein A8 (calgranulin A)	S100a8	-0.65 (ns)	-0.62 (ns)	-0.67 (0.0403)
serum amyloid A 1	Saa1	-0.82 (0.0056)	-0.87 (0.0093)	-0.67 (ns)
serum amyloid A 3	Saa3	-0.77 (ns)	-1.04 (0.0101)	-1.25 (0.0027)
src family associated phosphoprotein 1	Skap1	2.83 (0.0017)	0.00 (ns)	1.51 (0.0408)
suppressor of cytokine signaling 2; predicted gene 8000	Socs2	0.82 (0.0044)	0.62 (0.0443)	0.20 (ns)
thrombospondin 1; similar to thrombospondin 1	Thbs1	0.72 (0.0253)	0.55 (0.0308)	-0.30 (ns)
vanin 1	Vnn1	-1.87 (0.0032)	-0.26 (ns)	1.54 (0.0015)

FC, fold change.

To control for variances in the number of total leukocytes present in the TME and the surrounding tissue, the FPKM scores of selected immune cell markers genes were normalised to total leukocytes (FPKM levels of CD45). This approach will allow for a more precise measurement of the proportion of specific immune cell populations present in the tumour infiltrate. Marker genes for leukocyte subpopulations were selected based on previous literature (e.g. Gajewski et al., 2013; Quail & Joyce, 2013; Fridman et al., 2012) and are listed in Appendix 3. FPKM scores of the complete set of leukocyte markers, prior and after normalisation, are illustrated in Appendix 3. No significant change in expression was evident for most marker genes between genotypes at 6, 8 nor 10 weeks of development (see Appendix 3 and Figure 3.13). Interestingly, these results include markers for B- and T-lymphocytes that showed a significant increased expression, prior to normalization, in *Mmp8*-null mice at early time points of disease development. Notably, FPKM scores for CD45 shows a significant change at 6 week of development. These results indicate that depletion of MMP8 induces an increased infiltration of total leukocytes and that the proportion of leukocyte subpopulations remains equal between genotypes across all time points.

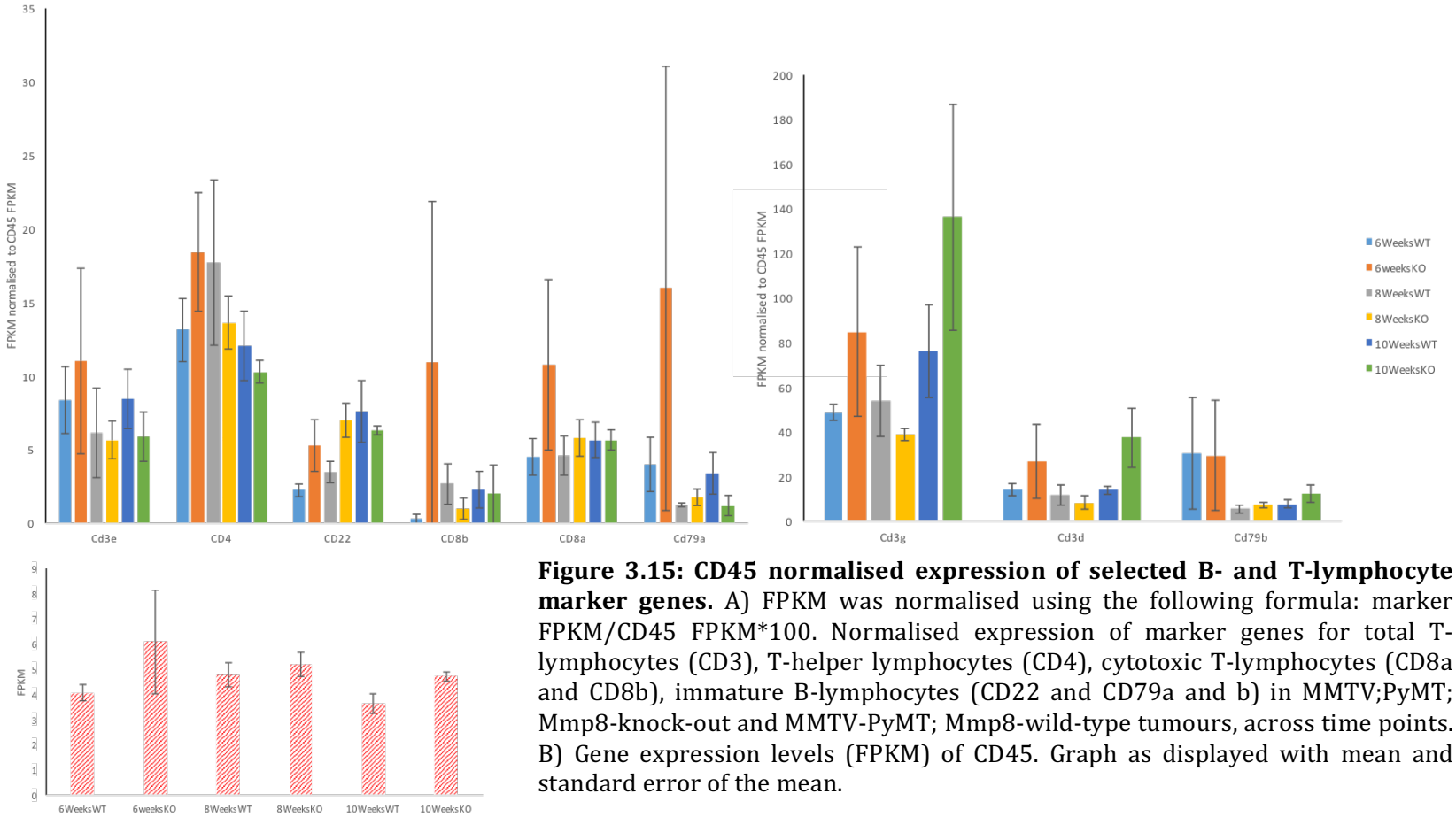


Figure 3.15: CD45 normalised expression of selected B- and T-lymphocyte marker genes. A) FPKM was normalised using the following formula: marker FPKM/CD45 FPKM*100. Normalised expression of marker genes for total T-lymphocytes (CD3), T-helper lymphocytes (CD4), cytotoxic T-lymphocytes (CD8a and CD8b), immature B-lymphocytes (CD22 and CD79a and b) in MMTV;PyMT; Mmp8-knock-out and MMTV-PyMT; Mmp8-wild-type tumours, across time points. B) Gene expression levels (FPKM) of CD45. Graph as displayed with mean and standard error of the mean.

As previous studies have found a disturbance in the protease web in *Mmp8*-null mice and as Integrins are known to interconnect MMP's, the RNAseq data was also analysed for differentially expressed MMPs, TIMPs, ADAM, ADAMTSs and Integrins. Heatmaps were constructed to illustrate fold change expression in *Mmp8*-null tumours in a time-dependent manner (Figure 3.14 and Figure 3.15). The results showed that depletion of MMP8 induces a global disturbance in the protease web as well as a dysregulated expression of various Integrins during tumour progression. Of these, statistical analysis revealed that MMP3 was significantly down-regulated at all time points and that MMP14 was significantly down-regulated at 6 weeks and up-regulated at 10 weeks, whereas MMP2 was only significantly up-regulated at 10 weeks. Further, ADAMTS-5 was significantly up-regulated at 10 weeks, whereas ADAMs and TIMPs showed no significant differential expression at any time points. Fold change in expression and statistical significance for MMPs and TIMPs are indicated in Table 3.5. Altogether, these results further demonstrate the interconnection between MMP8 and other proteinases.

In addition to a dysregulation of proteases and protease inhibitors, several Integrins significantly changed at both early and later time-points (Figure 3.14 and Table 3.5) including Integrin beta-3, Integrin beta-8 and Integrin alpha-7, Integrin beta-4, and Integrin alpha-4, as well as several leukocyte specific integrins. As the RNAseq also identified an increased expression of several markers for leukocyte subpopulations, the dysregulated expression of both regular Integrins and leukocyte specific Integrins might reflect an increased adhesion between leukocytes and endothelial cells due to leukocyte migration.

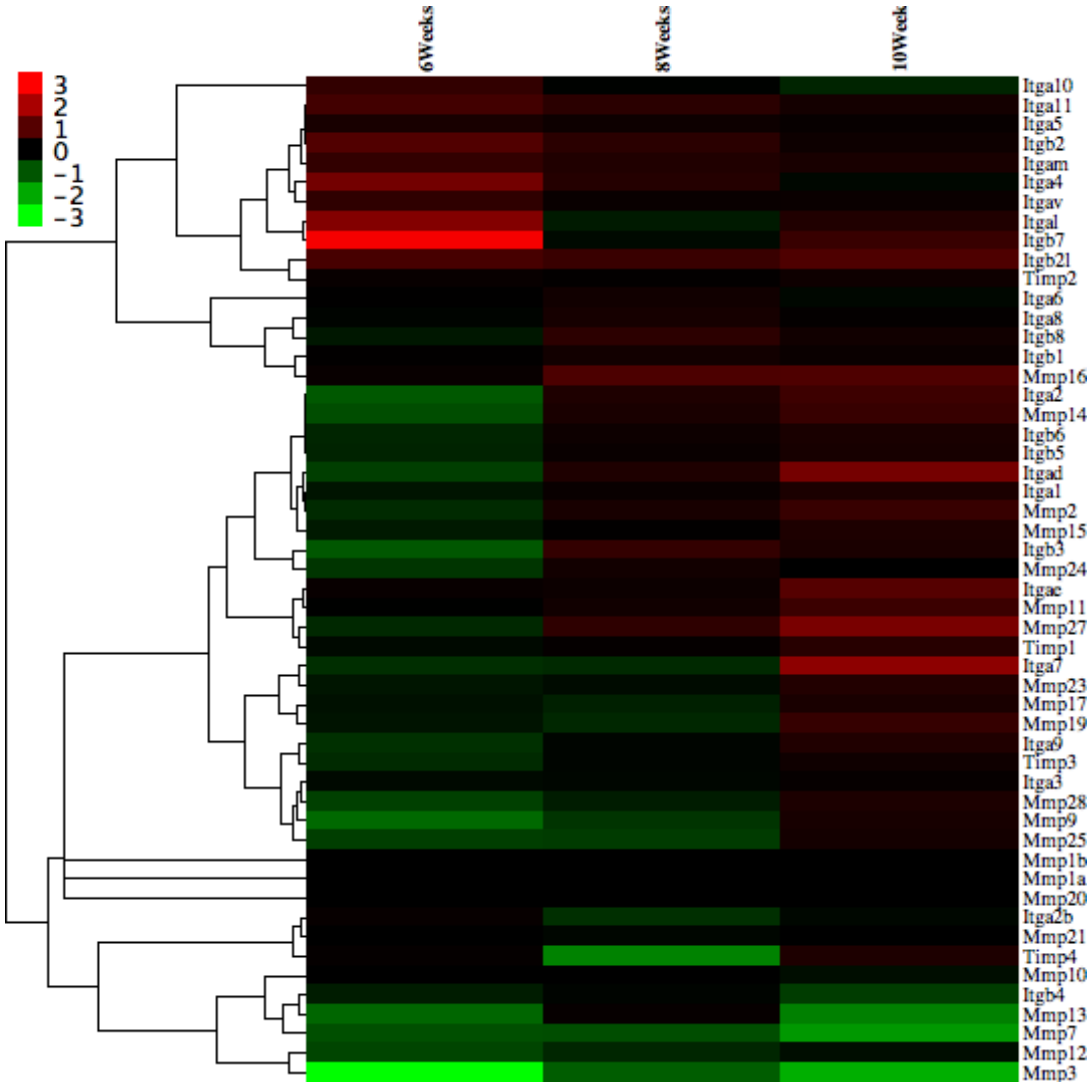


Figure 3.16: Gene expression changes of MMPs, TIMPs and Intergrins in MMTV-PyMT; Mmp8-knock-out tumours. Heatmap illustrates –log2 (fold change) expression of selected genes in MMTV-PyMT; Mmp8-knock-out tumours, compared to MMTV-PyMT; Mmp8-wild-type tumours. Heatmap was constructed using Gene Cluster 3.0, followed by editing in Java TreeView.

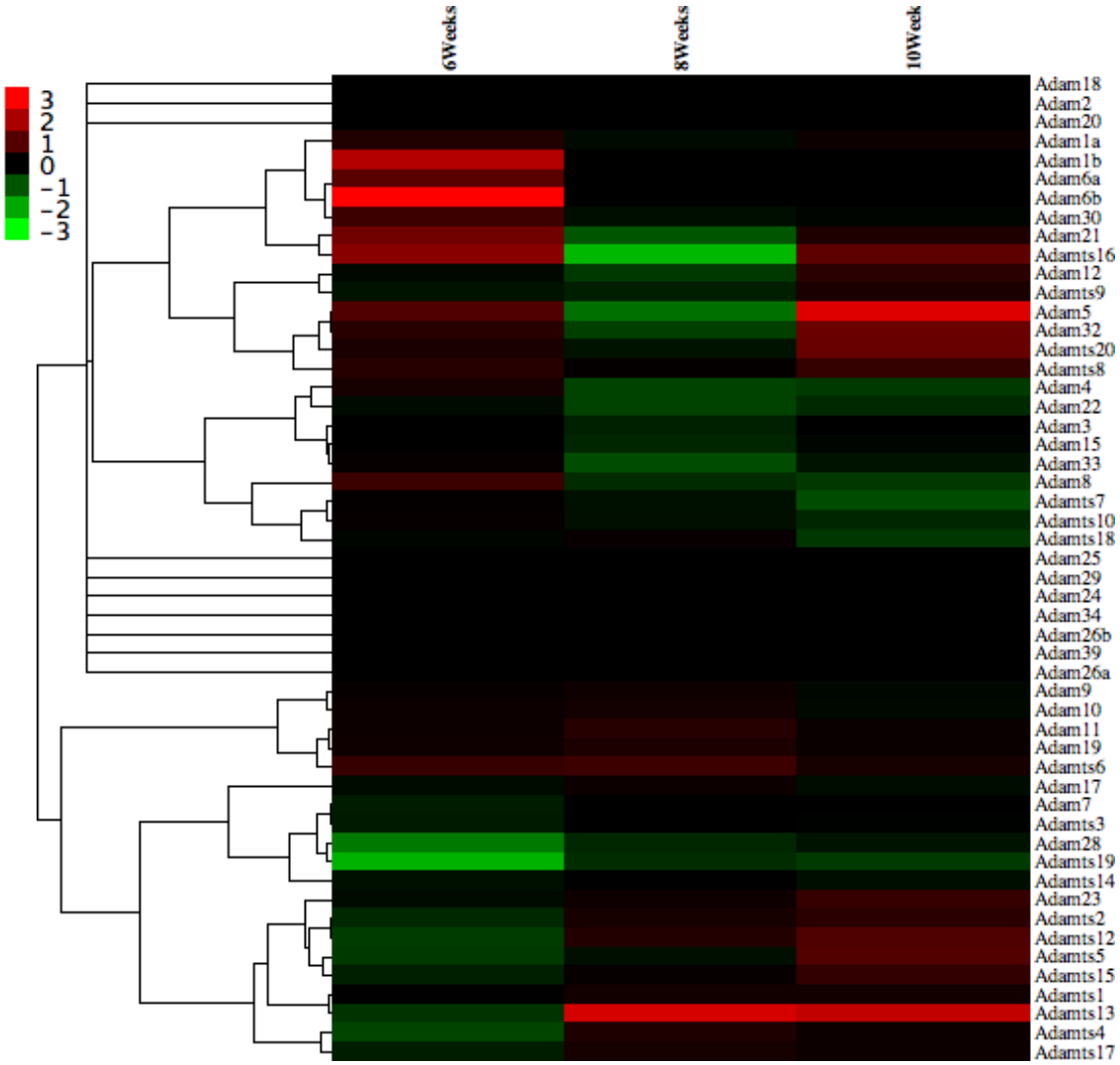


Figure 3.17: Gene expression changes for ADAM and ADAMTS's in MMTV-PyMT; Mmp8-knock-out compared to MMTV-PyMT; wild-type tumours. Heatmap constructed as in Figure 3.14.

Table 3.5: *MMPs, ADAMTSs and Integrins showing significant expression in MMTV-PyMT; Mmp8-knock-out mice.*

	log2 FC KO/WT (FDR-adjusted p-value)		
Gene	**6 weeks**	**8 weeks**	**10 weeks**
Mmp2	-0.50 (ns)	0.28 (ns)	0.65 (0.0189)
Mmp3	-3.73 (0.0017)	-1.10 (0.0291)	-2.07 (0.0014)
Mmp14	-0.91 (0.0017)	0.30 (ns)	0.65 (0.0428)
Adamts5	-0.68 (ns)	-0.18 (ns)	0.97 (0.0229)
Itgal	1.55 (0.0044)	-0.31 (ns)	0.37 (ns)
Itgb2	0.96 (0.0289)	0.50 (ns)	0.16 (ns)
Itgb3	-1.02 (0.0017)	0.60 (0.0093)	0.30 (ns)
Itga4	1.35 (0.0098)	0.43 (ns)	-0.09 (ns)
Itgb4	-0.33 (ns)	-0.05 (ns)	-0.71 (0.0048)
Itga7	-0.55 (ns)	-0.49 (ns)	1.67 (0.0014)
Itgb7	2.90 (0.0017)	-0.11 (ns)	0.66 (ns)
Itgb8	-0.28 (ns)	0.52 (0.0313)	0.20 (ns)

FC, fold change.

In *Mmp8*-null mice at 10 weeks of development, gene annotation in DAVID revealed an up-regulation of multiple genes involved in lipid and glycerol metabolism, including hormone sensitive lipase (LIPE), perilipin (PLIN1), fatty acid binding protein 4 (FABP4), acetyl-CoA synthetase long-chain family member 1 (Acsl1) and leptin (LEP). These results indicate that depletion of MMP8 induces a metabolic switch in MMTV-PyMT tumours at later time points of disease development. The complete list significantly increased genes involved in lipid and glycerol metabolic processes are listed in Table 3.6.

As a final analysis, the output gene list from Cuffdiff of significantly differentially expressed genes was explored to look for other relevant pro-tumorigenic proteins that were affected by the MMP8 ablation. Such visual inspection of the RNAseq data identified two protein of particular interest; the lymphocyte antigen 6K (Ly6K) and neuropeptide Y (NPY). In *Mmp8*-null tumours, Ly6K was 12-fold significantly up-regulated ($p = 0.0015$) at 10 weeks of tumour development, whereas NPY was up-regulated at 6, 8 and 10 weeks with a significant 9- ($p = 0.0252$), 5- ($p = 0.0235$), and 2.5-fold ($p = 0.0423$) increase, respectively. The strong up-regulation of Ly6K and the sustained increased in NPY across all time points suggests that these proteins play important pro-tumorigenic roles during mammary carcinoma progression.

Table 3.6: *Genes involved in lipid and glycerol metabolism that were significantly up-regulated at 10 weeks of disease development in MMTV-PyMT; Mmp8-knock-out mice.*

Gene		log2 FC KO/WT (FDR-adjusted p-value)	Term
acyl-CoA synthetase long-chain family member 1	Acsl1	0.62 (0.0169)	Lipid metabolism
apolipoprotein D	Apod	1.27 (0.0014)	lipid binding
coiled-coil domain containing 80	Ccdc80	1.30 (0.0014)	adipose tissue
diacylglycerol O-acyltransferase 2	Dgat2	1.52 (0.0014)	Lipid metabolism, glycerol metabolism
ectonucleotide pyrophosphatase/phosphodiesterase 2	Enpp2	1.10 (0.0428)	obesity, lipid degradation
fatty acid binding protein 4, adipocyte	Fabp4	1.69 (0.0014)	lipid binding
free fatty acid receptor 2	Ffar2	2.01 (0.0014)	lipid binding
GPI-anchored HDL-binding protein 1	Gpihbp1	1.02 (0.0154)	lipid binding
hydroxysteroid 11-beta dehydrogenase 1	Hsd11b1	1.01 (0.0014)	Lipid metabolism
insulin induced gene 1	Insig1	0.71 (0.0076)	Lipid metabolism
leptin	Lep	2.63 (0.0014)	obesity, adipose tissue, diabetes mellitus
lipase, hormone sensitive	Lipe	0.76 (0.0260)	Lipid metabolism, lipid degradation
lipoprotein lipase; similar to Lipoprotein lipase precursor (LPL)	Lpl	1.19 (0.0014)	lipid degradation
patatin-like phospholipase domain containing 3	Pnpla3	1.38 (0.0014)	Lipid metabolism, lipid degradation
perilipin	Plin1	1.29 (0.0076)	Lipid metabolism
phospholipase A2, group IVA (cytosolic, calcium-dependent)	Pla2g4a	0.63 (0.0025)	lipid degradation
phospholipase B1	Plb1	0.93 (0.0161)	lipid degradation
resistin	Retn	1.94 (0.0014)	obesity, diabetes mellitus
serum deprivation response	Sdpr	0.69 (0.0015)	lipid binding
similar to monoacylglycerol O-acyltransferase 1; monoacylglycerol O-acyltransferase 1	Mogat1	1.52 (0.0109)	Lipid metabolism, glycerol metabolism

4. DISCUSSION

The overall aim of the present study was to explore the underlying mechanisms that drive the suppressive effects of MMP8 during tumour progression in MMTV-PyMT mice. Based on previous results in the current literature, it was hypothesized that MMP8 acts via the innate immune system by polarizing pro-tumorigenic neutrophils and macrophages towards their more anti-tumorigenic counterparts (Fridlender et al., 2009; Gong et al., 2012; Soria-Valles et al., 2014). In line with this, the present study found that depletion of MMP8 induces an up-regulation of markers for anti- and pro-tumorigenic myeloid cells at later time points of tumour development, when quantifying expression by qRT-PCR. However, these changes were not evident by RNAseq, which, in contrast, predominantly identified a strong up-regulation of adaptive immune responses at early time points, in response to the MMP8 ablation (see Table 4.1 for an overview of differentially expressed genes). Whether an up-regulation of such adaptive immune responses is causative of tumour aggressiveness remains unclear as B- and T-lymphocytes can act both as host-protective and tumour-promoting, depending on cues from the tumour microenvironment (e.g. Burkholder et al., 2014; Sakaguchi, 2004; Andreu et al., 2010; de Visser et al., 2005; Milne et al., 2009). A characterization of B- and T-lymphocyte phenotypes present in the infiltrate are thus required for further elucidation.

In addition to dysregulated immune pathways, the current study also found that MMP8 depletion induces a global disturbance in the protease web, as well as, an upregulation of several oncogenic proteins at later time points. For example, several MMPs and ADAMTS-5 were significantly dysregulated in *Mmp8*-null tumours and, neuropeptide Y and Ly6K were up-regulated across all time points and at 10 weeks, respectively. Another interesting finding was a metabolic alteration observed in *Mmp8*-null mice at 10 weeks of development. More specifically, these tumours showed an enhanced lipid and glycerol metabolism, which reflects a more proliferative and aggressive tumour microenvironment (Currie et al., 2013). Taken together, these results suggest that depletion of MMP8 induces pleiotropic changes within the tumour microenvironment that are causative of a more aggressive phenotype.

Table 4.1: *Summary table showing genes significantly differentially expressed in MMTV-PyMT; Mmp8-knock-out compared to MMTV-PyMT; Mmp8-wild-type tumours.*

			log2 FC KO/WT (FDR-adjusted p-value)		
	Gene		**6 Weeks**	**8 Weeks**	**10 Weeks**
Immune pathways	B and T lymphocyte associated	Btla	2.16 (0.0017)	0.05 (ns)	0.60 (ns)
	CD22 antigen	Cd22	4.21 (0.0017)	1.03 (ns)	0.16 (ns)
	CD28 antigen	Cd28	5.15 (0.0116)	-0.11 (ns)	0.22 (ns)
	CD3 antigen, delta polypeptide	Cd3d	3.04 (0.0017)	-0.42 (ns)	1.74 (0.0307)
	CD3 antigen, epsilon polypeptide	Cd3e	2.96 (0.0044)	-0.07 (ns)	-0.09 (ns)
	CD3 antigen, gamma polypeptide	Cd3g	2.38 (0.0017)	-0.35 (ns)	1.25 (ns)
	CD4 antigen	Cd4	2.93 (0.0017)	-0.30 (ns)	0.17 (ns)
	CD79A antigen	Cd79a	5.15 (0.0215)	0.70 (ns)	-1.28 (ns)
	CD79B antigen	Cd79b	2.85 (0.0087)	0.48 (ns)	1.17 (ns)
	CD8 antigen, alpha chain	Cd8a	3.70 (0.0017)	0.49 (ns)	0.39 (ns)
	Chemokine (C-C motif) ligand 22	Ccl22	3.30 (0.0017)	0.47 (ns)	0.64 (ns)
	Chemokine (C-C motif) ligand 5	Ccl5	2.20 (0.0017)	0.08 (ns)	0.74 (ns)
	Chemokine (C-X-C motif) ligand 1	Cxcl1	1.37 (0.0435)	1.00 (ns)	-0.26 (ns)
	Chemokine (C-X-C motif) ligand 13	Cxcl13	5.23 (0.0323)	-0.53 (ns)	-0.26 (ns)
	Interleukin 23, alpha subunit p19	Il23a	2.31 (0.0375)	1.82 (0.0242)	0.21 (ns)
	C-C motif chemokine 3	Ccl3	0.65 (ns)[a]	0.74 (ns)[a]	5.52 (0.006)[a]
	Vascular endothelial growth factor A	VEGFA	0.61 (ns)[a]	0.45 (ns)[a]	8.97 (0.002)[a]
	Arginase-1	Arg1	0.56 (ns)[a]	1.49 (ns)[a]	6.08 (0.031)[a]
	Arginase-2	Arg2	1.27 (ns)[a]	0.49 (ns)[a]	6.08 (0.021)[a]
	Scavenger receptor cysteine-rich type 1 protein M130	CD163	0.36 (ns)[a]	0.37 (ns)[a]	6.38 (0.005)[a]
	Interleukin 1 beta	Il1b	0.87 (ns)	1.32 (0.0222)	0.86 (ns)

Table continues on next page

Integrins	Integrin beta-2	Itgb2	0.96 (0.0289)	0.50 (ns)	0.16 (ns)
	Integrin beta-3	Itgb3	-1.02 (0.0017)	0.60 (0.0093)	0.30 (ns)
	Integrin alpha-4	Itga4	1.35 (0.0098)	0.43 (ns)	-0.09 (ns)
	Integrin beta-4	Itgb4	-0.33 (ns)	-0.05 (ns)	-0.71 (0.0048)
	Integrin alpha-7	Itga7	-0.55 (ns)	-0.49 (ns)	1.67 (0.0014)
	Integrin beta-7	Itgb7	2.90 (0.0017)	-0.11 (ns)	0.66 (ns)
	Integrin beta-8	Itgb8	-0.28 (ns)	0.52 (0.0313)	0.20 (ns)
	Integrin alpha-L	Itgal	1.55 (0.0044)	-0.31 (ns)	0.37 (ns)
Protease web	Matrix metalloproteinase-2	Mmp2	-0.50 (ns)	0.28 (ns)	0.65 (0.0189)
	Matrix metalloproteinase-3	Mmp3	-3.73 (0.0017)	-1.10 (0.0291)	-2.07 (0.0014)
			0.06 (0.0013)[a]	2.66 (ns)[a]	0.01 (0.000)[a]
	Matrix metalloproteinase-13	Mmp13	1.90 (ns)[a]	0.78 (ns)[a]	0.04 (0.000)[a]
	Matrix metalloproteinase-14	Mmp14	-0.91 (0.0017)	0.30 (ns)	0.65 (0.0428)
	A disintegrin and metalloproteinase with thrombospondin motifs 5	Adamts5	-0.68 (ns)	-0.18 (ns)	0.97 (0.0229)
	Tissue inhibitor of metalloproteinases 1	Timp1	0.41 (ns)	3.53 (0.032)	0.20 (ns)
	Tissue inhibitor of metalloproteinases 3	Timp3	0.82 (ns)	0.37 (0.044)	0.20 (ns)
	Serine (or cysteine) peptidase inhibitor, clade E, member 2	Serpine2	0.82 (0.0078)	1.15 (0.0014)	0.71 (0.0101)
Oncogenes	Neuropeptide Y	Npy	3.22 (0.0253)	2.38 (0.0235)	1.30 (0.0424)
	Lymphocyte antigen 6 complex, locus K	Ly6k	-0.48 (ns)	-0.19 (ns)	3.58 (0.0015)
Lipid metabolism	Acyl-CoA synthetase long-chain family member 1	Acsl1	-0.00 (ns)	-0.56 (0.0296)	0.62 (0.0169)
	Fatty acid binding protein 4, adipocyte	Fabp4	-0.33 (ns)	-1.09 (0.0014)	1.69 (0.0014)
	Lipase, hormone sensitive	Lipe	-0.79 (0.0176)	-0.81 (0.0015)	0.76 (0.0260)
	Perilipin	Plin1	-1.04 (0.0088)	-1.05 (0.0015)	1.29 (0.0076)

[a] *= qRT-PCR data showing fold change and p-value*

4.1. MMP8 as a player in immune regulatory networks

MMP8 has previously been described as an important regulator of innate immune responses. For instance, Balbin et al. (2003) and Gutierrez-Fernandez et al. (2007) showed that depletion of MMP8 in mice induces a delayed influx of neutrophils at early time points in skin tumours and wounds, respectively. Both studies also reported an increased infiltration of neutrophils in *Mmp8*-null mice at later inflammatory stages. This is in line with the study by Decock et al. (2015) that found a sustained infiltration of neutrophils in MMTV-PyMT; Mmp8-knock-out tumours at week 10 of development, compared to a decreased infiltration from 8 to 10 weeks in the wild-type littermates. However, a delayed neutrophil infiltration was not evident at earlier time point of disease development, which is inconsistent with the previous findings (Balbin et al. 2003; Gutierrez-Fernandez et al., 2007).

These studies have been further supported on a more mechanistic level. Studies has shown that recombinant MMP8 are able to activate both human interleukin-8 (IL-8) and its mouse orthologue lipopolysaccharide induced CXC chemokine (LIX) that, in turn, is known to recruit neutrophils to the site of inflammation (Tester et al., 2007). Consistent with the current literature, the present study found an increased expression of N1 and N2 markers in *Mmp8*-null tumours at 10 weeks, which suggest an increased influx of both pro- and anti-tumorigenic neutrophils at later stages of tumour development. However, these results are inconclusive as a significant differential expression of N1 and N2 markers where not evident in the RNAseq data. At early time-points however, the RNAseq data revealed an up-regulation of a neutrophil chemo-attractant (Cxcl1), but this was not accompanied by differential expression of markers specific for neutrophils. However, it is important to note that Ly6G, which is an established neutrophil marker, was identified as not expressed in the RNAseq data. Also, the Ly6.B marker that was used for immunostaining of MMTV-PyMT sections were not detected in the RNAseq data, which suggests that this gene is epigenetic silenced or absent in the MMTV-PyMT mice. This might also explain why the immunostaining was unsuccessful. Thus, further characterisation of the presence of neutrophils in the tumour infiltrate are required in order to conclude these findings.

In addition to neutrophil marker genes, the present study found an increase in M1 and M2 markers at 10 weeks in *Mmp8*-null mice. However, and as for N1 and N2 markers, the differential expression was not confirmed by RNA sequencing. In concurrence with this, RNAseq and immunostaining of F4/80 revealed no change in total macrophages between *Mmp8*-null and wild-type tumours at 10 weeks nor at other time points of tumour development. Accordingly, Decock et al. (2015) reported no change in macrophage infiltration in MMTV-PyMT; *Mmp8*-null and heterozygote mice, compared to wild-type, throughout the time course of mammary carcinoma development. It is, however, important to note that the results obtained by Decock et al. (2015) should be interpreted with caution, as the comparison of macrophage density was performed on a limited MMTV-PyMT mice cohort.

The inconsistency between qRT-PCR and RNAseq might be explained by the technical and statistical differences between the two technologies. qRT-PCR detects transcripts based on sequence specific primer/probe sets, whereas RNAseq technologies detects mRNA molecules at a single nucleotide resolution. Alternatively spliced transcripts that lack the primer/probe specific region will not be detected by qRT-PCR, but quantified by RNA sequencing, which will produce conflicting results. Further analysis to assess the presence of multiple transcripts are thus required to investigate whether this is causative of the observed inconsistencies between the two methods. In summary, the increased expression of anti- and pro-tumorigenic myeloid cells at 10 weeks of development are inconclusive due to the inconsistency between the two technologies. Future experiments are thus required to more comprehensively assess the whether MMP8 play a role in the polarisation of myeloid cells towards anti-tumorigenic phenotypes.

In contrast to effects on innate immune pathways, genes sets involved in adaptive immune responses were prevalent among up-regulated genes at 6 weeks in *Mmp8*-null tumours. Up-regulation of B- and T-lymphocyte marker genes and respective chemo-attractants, as well as leukocyte specific integrins, might suggest that MMP8 regulates tumour infiltration of such adaptive

immune responses at early stages of tumour development. An increased influx of lymphocyte subpopulations might be linked to the accelerated tumour growth and metastasis previously observed in MMTV-PyMT; Mmp8-knock-out mice (Decock et al., 2015). However, a phenotypic profiling of lymphocyte subpopulations is required in order to investigate this statement further. The up-regulation of CD8 at 6 weeks is indicative of the presence of cytotoxic T-lymphocytes, which is highly protective against cancer progression (Maher & Davides, 2004) and are, thus, inconsistent with this assumption.

Interestingly, several markers for pro-tumorigenic lymphocyte action were also significantly up-regulated in *Mmp8*-null tumours, including Ccl22, Il-23a, and Il-1b, which might participate in the accelerated tumour progression observed in MMTV-PyMT; Mmp8-knock-out mice (Decock et al., 2015). Tumour-secreted Ccl22 has been shown to recruit Tregs through binding of the chemokine receptor CCR4 that is expressed on Tregs (Gobert et al., 2009). Tumour infiltration of Tregs has been associated with more invasive and metastatic cancer (Sakaguchi, 2004), in which immunosuppression via inhibition of Th1 polarisation has been described as an underlying mechanism (Bettelli et al., 2006). Treg cells are characterised by the expression of CD4, CD25 and FoxP3 (Legoux et al., 2015). CD4 was significantly up-regulated at 6 weeks, but no significant change was identified for CD25 and FoxP3. Although Ccl22 is significantly up-regulated in *Mmp8*-null tumours, these results suggest that the presence of Tregs are similar between genotypes. However, a more precise profiling of Tregs in *Mmp8*-null and wild-type tumours are required to determine whether the up-regulated expression of Ccl22 is associated with an increased tumour infiltration of Tregs in *Mmp8*-null tumours.

In contrast to Ccl22, Il-23a (alpha subunit of Il-23) and Il-1b are known to stimulate differentiation of Th0 cells into Th17 cells. Th17 cells have been described to possess context dependent anti- or pro-tumorigenic functions. For instance, Martin-Orozco et al. (2009) showed, in the context of a mouse B16 melanoma model, that Th17 cells perform host-protective functions by activating cytotoxic T-lymphocytes. In contrast, a positive correlation has been

found between Th17 infiltration and poor prognosis in several cancers, including breast, ovarian and colon cancer (Miyahara et al., 2008; Sfanos et al., 2008; Zhang et al., 2009). Although an up-regulation of Il23a and Il-1b were evident in *Mmp8*-null mice, other markers, including Il-17, IL-2, IFNg and GM-CSF/CS2 (Zou et al., 2006), characteristic for Th17 cells were not significantly differentially expressed. These results might rule out the initial suggestion, however, as these marker genes also can be expressed by other cell types, a more precise phenotyping of immune cells populations are required in order to determine whether MMP8 ablation induces an increased infiltration and polarisation of Th17 cells.

The present study also identified a significant up-regulation of a gene set specific for B-lymphocyte recruitment, activation, and differentiation at 6 weeks of tumour development, in *Mmp8*-null tumours. Analogous to T-lymphocytes, B-lymphocytes has shown to play dual roles during tumour progression, where its actions are highly context dependent (e.g. Coronella et al., 2001; Milne et al., 2009; Qin et al., 1998; Andreu et al., 2010). For instance, subpopulations of B-lymphocytes have been reported to support activity of pro-tumorigenic T-helper lymphocytes by secreting Il-2, Il -4 and Il-6 (Nelson, 2010). In support of this, Olkhanud et al. (2011) found that the presence of a specific B-lymphocyte subpopulation (termed Bregs) positively correlated with an increased metastasis to the lungs in a breast cancer mouse model. However, infiltration of B-lymphocytes has also been associated with a better prognosis in ovarian and mammary carcinoma (Coronella et al., 2010; Milne et al., 2009). Thus, a better insight into the action of B-lymphocytes in MMTV-PyMT; Mmp8-knock-out mice is required in order to explore whether they are causative of the more aggressive phenotype observed in Mmp8-null tumours.

Although an increased expression of marker genes for B- and T-lymphocytes was evident at 6 weeks in *Mmp8*-null mice, subsequent calculations of the proportional relationship of leukocyte subpopulations relative to total leukocytes (CD45+) showed different results. More specifically, when normalising the expression of leukocyte marker genes to CD45, no change in

expression was identified between *Mmp8*-null and wild-type tumours. These results reflect that the proportion of leukocyte subpopulations, including B- and T-lymphocytes, present in the infiltrate are similar in both genotypes. Thus, the increased expression of lymphocyte markers observed at 6 weeks might be explained by an increased tumour infiltration of leukocytes in total. This argument concurs with the significant change in expression of CD45 at 6 weeks, but raises the questions of why other leukocytes subpopulations were not identified as significantly changed. Possibly, looking at subpopulations in isolation, a slightly increased infiltration will not necessarily result in a significant change in expression of marker genes specific for that phenotype, but when looking at CD45 expression in total a significance would be evident. In conclusion, this might suggest that MMP8 directly, or through secondary pathways, accelerates the onset of inflammation in *Mmp8*-null mice. However, a more accurate profiling of populations of immune cells present in the infiltrate are required to assess this in more depth. For instance, by fluorescence-activated cell sorting (FACS) of leukocytes followed by gene expression profiling of marker genes.

4.2. MMP8 ablation induces global changes in the protease web

In addition to an effect on immune regulatory networks, MMP8 ablation has shown to induce changes in the protease web. More specifically, Decock et al. (2015) showed that depletion of MMP8 induces a dysregulated expression of several MMPs as well as Timp2. MMP8 has also been shown to cleave and inactivate Alpha1-Proteinase Inhibitor (α1-PI), which is a potent inhibitor of neutrophil elastase (Knäuper et al., 1990). Such interconnections in the protease web has also been identified for other members of the MMP family (Fortelny et al., 2014) and thus reflects the importance of investigating the effect on the protease web in response to ablation of specific MMP species. Hence, MMP8 ablation might influence the activity of other proteases or protease inhibitors.

In the present study, a global disturbance in the protease web in response to the MMP8 ablation was evident both from qRT-PCR and RNAseq analysis.

However, inconsistencies in differential expression between the two gene expression methods occurred, as for the immune cell marker genes. For instance, qRT-PCR data revealed that MMP3 were significantly down-regulated at 10 weeks, whereas the RNAseq data showed a significant reduced expression of MMP3 at all time points. The latter is consistent with previous findings, of which Decock et al. (2015) reported a significant down-regulation of MMP3 at 6, 8 and 10weeks in MMTV-PyMT; Mmp8-knock-out mice. A down-regulation of MMP3 might contribute to the accelerated tumour progression observed for *Mmp8*-null mice. A study using a squamous cell carcinoma model described MMP3 as a tumour suppressive metalloproteinase; *Mmp3*-null mice showed accelerated initial tumour growth rates compared to wild-type littermates. No difference was observed in tumour onset or incidence, but the enhanced growth rate was accompanied by an elevated proliferative index. *Mmp3*-null mice also showed more undifferentiated tumours and an enhanced metastasis to the lung surface (McCawley et al., 2004). A more recent study supports these findings by showing that tumour produced MMP3 reduces the number of papillomas and carcinomas, whereas no difference was found in tumour onset. Analysis of epidermal biopsies revealed that keratinocyte expression of MMP3 decreased and increased proliferation and differentiation, respectively (McCawley et al., 2008). The effect on proliferation is interesting as the present study found an increase in lipid metabolism, a hallmark of a proliferative microenvironment, at later time points in *Mmp8*-null mice. Thus, the decreased MMP3 expression might contribute to the accelerated tumour progression by regulating proliferative activity. Another study by Witty et al. (1995) found significant reduction in development of breast tumours following 7,12-Dimethylbenz[a]anthracene (DMBA) induced carcinogenesis in MMTV-MMP3 transgenic mice. They suggested that MMP3 act as a tumour suppressor by influencing the rate of cell turnover. More specifically, they found a 4-fold increase in apoptotic cells in MMTV-MMP3 transgenic mice when comparing to non-transgenic littermates. However, no significant effect on apoptosis was found by McCawley et al. (2004) when comparing *Mmp3*-null and wild-type tumours. Future studies are thus required in order to conclude whether MMP3 inhibits tumour growth by regulating apoptosis of carcinogenic cells.

In addition to the down-regulated expression of MMP3, MMP2 was up-regulated at mid-late time-points in both datasets, which concur with previous findings. Decock et al. (2015) reported an increased expression of MMP2 at 10 and 14 weeks of disease development in MMTV-PyMT; Mmp8-knock-out mice. Additionally, *Mmp2*-null mice have shown a reduced angiogenesis and tumour progression compared to wild-type animals (Itoh et al., 1998). This is supported by several studies showing that MMP2 promotes angiogenesis (Fang et al., 2000; Kolb et al., 1997). In the present study, an up -regulation of several proangiogenic factors were detected at 10 weeks, including neurophilin-1, kinase domain receptor and chondroitin sulfate proteoglycan 4 (data not shown). These findings are in line with the proangiogenic properties described for MMP2.

In contrast the indications of a proangiogenic environment, the present study did not identify a difference in vascular density between *Mmp8*-null and wild-type tumours when staining tumour sections for Endomucin. However, a more comprehensive immunostaining study might be required to assess this more accurately. More specifically, selection of tissue sections that matches similar areas of the tumours (e.g. invading margins vs hypoxic regions) will provide a more precise comparison of blood vessel density between the genotypes. Quantification of blood vessel density is also required to more comprehensively assess whether depletion of MMP8 promote angiogenesis via MMP2. MMP2 might also play other roles in the context of the MMTV-PyMT tumour model. During invasion, MMP2 has been shown to re-localize to invadopodia where the proteinase promotes cell invasion together with MMP14 (Nakahara et al., 1997; Brooks et al., 1996). MMP2 and MMP14 also triggers cell motility by cleavage of laminin-5 (Giannelli et al., 1997; Koshikawa et al., 2000). This in consistent with the present findings showing a significant up-regulation of MMP14 at 10 weeks in *Mmp8*-null tumours. MMP2 and MMP14 might thus coordinately trigger invasion at later time points of disease development in the absence of MMP8 in MMTV-PyMT mice. However, verification of the increased MMP14 expression

by qRT-PCR, and further validation at protein level for both MMP2 and MMP14 is required in order to conclude these findings.

TIMPs are major inhibitors of metalloproteinases, which play key roles in the regulation of MMP activity (Nagase et al., 2006). Thus, members of the TIMP family was quantified to investigate if MMP8 ablation had a significant effect on expression of these proteinase inhibitors. TIMP members was profiled based on the availability of primer/probes for qRT-PCR analysis. No significant change in expression was identified for TIMP1, TIMP3 and TIMP4 between *Mmp8*-null and wild-type tumours. This was further confirmed by RNAseq, where non-significant change in expression also was evident for TIMP2.

RNAseq data allowed for a more in-depth investigation into inhibitors of protease web members, and a significant dysregulation of several protease inhibitors were evident. Notably, the results revealed that several serine protease inhibitors (Serpins) changed expression pattern in *Mmp8*-null tumours, at both early and late time points. Serpins are known to target trypsin, thrombin, plasmin, plasminogen activator (Baker et al., 1980, Scott et al., 1985), T cell proteinase-1 (Gurwitz et al., 1989), matriptase (Myerburg et al., 2008), and prostasin (Chen et al., 2004).

In the current study, and of particular interest, Serpine2 was found to be up-regulated throughout the time course of the disease, in *Mmp8*-null mice. This serine peptidase inhibitor has previously been reported to play an important role in tumour growth and metastasis. More specifically, Serpine2 has been implied to promote invasion of breast (Candia et al., 2006), pancreatic (Buchholz et al., 2003), and lung cancer cells (Yang et al., 2009). Accordingly, Serpine2 has been shown to be over-expressed in, colorectal, pancreatic and breast tumours (Candia et al., 2006, Buchholz et al., 2003, Selzer-Plon et al., 2009), and in liposarcoma (Thelin-Jarnum et al., 1999) and oral squamous carcinoma (Gao et al, 2008). In conclusion, these findings suggest that Serpine2 play a role in the observed accelerated growth and metastasis in *Mmp8* -null mice. However, a better insight into its mechanisms of action is required in

order to explore the link between MMP8 and Serpin2 expression, and to further elucidate its role in mammary carcinoma progression.

4.3. Novel pathways affected by MMP8 signaling

4.3.1. The link between MMP8, Ly6K and Neuropeptide Y

In addition to changes in the immune cell repertoire and in the web of proteases, whole-genome RNA sequencing revealed that MMP8 depletion induces a dysregulation of several proteins that has previously been reported to play important roles during tumour progression. Of these, lymphocyte antigen 6K (Ly6K) was significantly up-regulated at 10 weeks of tumour development in *Mmp8*-null mice. Ly6K is a cancer-testis antigen that has been suggested as a diagnostic biomarker and therapeutic target in several cancers, including breast, bladder, esophangeal squamous cell carcinoma and non-small cell lung carcinoma (Lee et al., 2006; Ishikawa et al., 2007; Matsuda et al., 2011). Ly6K has been shown to positively regulate cell growth, migration and invasion of several bladder cancer cell lines (Matsuda et al., 2011). Likewise, Kong et al. (2012) reported an increased cell invasion and metastasis in response to over-expression of Ly6K in breast cancer cells. This effect was shown to be mediated trough the Ras/ERK pathway, and was accompanied by an increased expression of MMP2 and MMP9. Thus, the increased expression of MMP2 observed at 10 weeks in the present study might be induced by the increased expression of Ly6K. These results are highly suggestive of Ly6K and MMP2 as important players in the accelerated metastasis observed in MMTV-PyMT; Mmp8-knock-out tumours. However, the mechanisms of Ly6K action needs to be explored to investigate whether this protein are interconnected with MMP2 and to further evaluate its metastatic effects.

Neuropeptide Y (NPY), which is a sympathetic neurotransmitter, was strongly up-regulated across all time points in *Mmp8*-null tumours. More than two decades ago, neuropeptides were recognized as important mitogens during cancer, in which they function in an autocrine/paracrine manner to stimulate proliferation and migration (Rozengurt, 2002). Accordingly, NPY has been recognised as an important mediator of both growth and metastasis of several

cancers (Antoni et al., 2006; Tilan et al., 2010; Elenkov et al., 2000; Marsland et al., 2002; McEwen et al., 1993). NPY receptors (i.e. Y1R, Y2R and Y5R) are highly expressed in several malignancies, including breast (Reubi et al., 2001), ovarian (Korner et al., 2003) and prostate cancer (Magni & Motta, 2001). Reubi et al. (2001) found that Y1R was more highly expressed compared to Y2R in breast cancers, and that normal breast tissue only express Y2R. The expression of Y5R has not been extensively studied but Sheriff et al. (2010) has reported expression of Y5R in several human breast cancer cell lines. NPY and NPY receptor signaling has been shown to stimulate both proliferation and migration of cancer cells (e.g. Sheriff et al., 2010; Korner & Reubi, 2008) and, thus, might participate in the accelerated tumour growth and metastasis recently observed in MMTV-PyMT; *Mmp8*-null tumours (Decock et al., 2015). Neuroendocrine tumour studies have demonstrated that Y2R activation increases cell proliferation (Korner & Reubi, 2008; Kitlinska et al., 2005). For instance, Sheriff et al. (2010) reported an Y5R mediated cell growth in BT-549 breast cancer cells via MAPK activation, which was accompanied by an increased phosphorylation of ERK1/2 (Sheriff et al., 2010). This was further supported by Medeiros et al. (2011), which showed that treatment of NPY increased proliferation of 4T1 cells in a concentration-dependent manner, and that this effect was mediated via an increased phosphorylation of ERK1/1.

NPY signaling has also been demonstrated to promote migration of multiple cell types, including endothelial and malignant cells (Sheriff et al., 2010, Drell et al., 2003). For instance, Sheriff et al. (2010) showed that treatment with NPY increased the migratory potential to MDA MB-231 cells by acting via Y2R and Y5R. A subsequent study described NPY as a chemo-attractant that acts via Y2R and Y5R to stimulate cell migration (Medeiros et al., 2011). Further, NPY stimulated activation of Y2R and Y5R has been shown to promote migration of endothelial cells (Movafagh et al., 2006). These results imply that NPY and NPY receptors play a crucial role in metastasis of cancer cells, which together with its evident effect on cell proliferation, strongly suggest NPY as an important mediator of growth and metastasis in MMTV-PyMT; *Mmp8*-null tumours. However, subsequent studies exploring the NPY signaling pathway in *Mmp8*-

null tumours more comprehensively are required to firmly establish this statement.

4.3.2. MMP8 ablation affects lipid and glycerol metabolism at later time points of tumour development

Bioinformatics analysis of RNAseq data also revealed a metabolic change in response to the MMP8 ablation, especially at 10 weeks of disease development. The best known metabolic alteration in tumour cells are the Warburg effect, which describes the glycolysis-switch from low-rate oxidative phosphorylation towards high-rate glycolysis and lactate production in the cytosol (Warburg, 1956). More recently, tumour cells have been shown to alter their lipid metabolism in response to high proliferation, a metabolic switch that has been associated with tumour growth and metastasis (Currie et al., 2013). Cancer cells satisfy their increased fatty acid consumption by an increased uptake of exogenous lipids and lipoproteins or by an up-regulation of endogenous synthesis of lipids and cholesterols (Beloribi-Djefaflia et al., 2016). Lipids and cholesterol that are not used immediately are stored in lipid droplets (LDs) which has recently been considered as a hallmark of aggressive cancers (Yue et al., 2014, Bozza et al., 2010, de Gonzalo-Calvo et al., 2015, Abramczyk et al., 2015).

In the present study, *Mmp8*-null tumours appear to have an up-regulated lipid metabolism at later time points, compared to wild-type littermates. These findings are consistent with the recently observed accelerated tumour growth and metastasis reported for MMTV-PyMT; *Mmp8*-null tumours (Decock et al., 2015). Interestingly, several of the genes that were significantly up-regulated are identified as key players of metabolic lipid pathways. For instance, hormone sensitive lipase (LIPE) is an enzyme involved in lipid catabolism and degradation (Liew et al., 2013). Further, Perilipin (PLIN1) and fatty acid binding protein 4 (FABP4) are both important in uptake and transport of fatty acids (Greenberg et al., 1991). Acetyl-CoA synthetase long-chain family member 1 (Acsl1) are involved in synthesis and degradation of cellular lipids (Soupene & Kuypers, 2008).

Furthermore, leptin (LEP), which was strongly up-regulated at 10 weeks in *Mmp8*-null tumours, is a major regulator of energy homeostasis and weight control (Surmacz, 2007) and, induce a variety of signaling pathways upon binding to the leptin receptor (LEPR) in peripheral tissues. In cancer, leptin has been shown to induce proliferation and survival through LEPR activation, which activates Jak/STAT3, ERK1/2 and phospohoinostitide 3-kinase signaling pathways. Additionally, LEPR activation induces expression of cyclin D and causes a hyperphosphorylation of the retinoblastoma protein (Garofalo & Surmacz, 2006, Frankenberry et al., 2006).

Altogether, these results might suggest that MMP8 act as a suppressor of lipid metabolism pathways to protect the host from tumour aggressiveness. However, a link between MMP8 and lipid metabolism has not previously been reported, and a more comprehensive investigation is required in order to assess this assumption. Alternatively, MMP8 might act more directly to drive proliferation (e.g. via Leptin), which, in turn, might reprogram the tumour cells to change their metabolic phenotype as an adaption to a harsh and highly proliferative tumour microenvironment.

4.4. Limitations

It is important to note the methodological limitations of the present study. As previously discussed, the majority of RNA species that showed significant differential expression in CuffDiff were not subject for further validation. Follow-up studies are thus required in order to conclude the findings. Additionally, several mRNA species showed inconsistent expression between RNAseq and qRT-PCR experiments. It is important to characterise the underlying cause if these conflicting results in subsequent studies. This includes an investigation into the presence of multiple transcripts for candidate genes to evaluate whether RNAseq technologies quantifies transcripts that are not detected by qRT-PCR due to alternative splicing events. If this is the case, customised primer/probe sets can be developed for a more accurate quantification of target genes.

Differences in statistical methodologies, such as normalization methods, might also contribute to data variability (Huggett et al., 2005). Normalization against endogenous reference genes is essential in qRT-PCR to control for between-sample variations and errors in sample quantification. Ideally, such reference genes should be stably expressed in all tissues or cells at all time points, even under experimental treatment (Dheda et al., 2005; Bonefeld et al., 2008). However, investigations into the validity of such genes has questioned the reliability of endogenous controls in qRT-PCR experiments and no definite gold standard has been suggested for this purpose (Dheda et al., 2005, de Kok et al., 2004). I lieu, several reports have suggested the use of multiple control genes as references, where the geometric mean between the control genes are used for normalization (e.g. Pérez et al., 2007; Vandesompele et al., 2002). This will enhance the reliability of the quantification process, which might have a significant impact on experimental conclusions. This should be taken into consideration when validating selected candidate genes in future experiments.

The approach used to profile for different leukocyte subpopulations is possibly the primary limitation of this study. The tumour microenvironment is highly heterogeneous consisting of a variety of different cell types, including a range of different immune cells populations (Mantovani et al., 2008). Populations of immune cells are mainly characterised by receptor status and expression patterns of cytokines and growth factors. However, such marker genes can be expressed by multiple cell types within the microenvironment (Gajewski et al., 2013). This might interfere with gene expression results and, thus, enhances the chances of false negative and false positive results. The presence of areas with normal breast and necrotic tissue within the tumour might also contribute to a mis-interpretation of gene expression data and should preferably be removed by microdissection of tumours prior RNA isolation. A more optimal approach however, especially when profiling for immune cell populations, are to separate and quantify cells by FACS based on a combination of cell surface/intracellular marker genes. Complementing this with profiling of selected markers at both RNA and protein levels should provide a more

comprehensive and robust approach for immunophenotyping. Alternatively, a transgenic mouse model whose selected leukocyte subsets express colour-coded proteins can be employed. This allow for *in-vivo* visualization and imaging of leukocytes and, simplifies *ex-vivo* quantification of immune cell populations (Pitsillides et al., 2011; Fan et al., 2010). Using more optimal approaches for immune phenotyping will allow future investigators to build up, in a systemic manner, a picture of the altered immune defense pathways in *Mmp8*-null mice.

It is also important to note the number of biological replicates that were selected for differential expression analysis in Cuffdiff. A number of four-to-five biological replicates were initially selected for sequencing, but due to poor RNA quality of some samples and due to extreme outlier replicates, this number was reduced to three replicates in two of the conditions. Although no universal recommendation of the number of biological replicates for RNAseq experiments are established, several reports have suggested that at least six replicates are required to produce data with high validity, especially when working with heterogeneous systems, such as cancer (Conesa et al., 2016; Schurch et al., 2016). Thus, a higher number of biological replicates should be considered in future studies to ensure high validity of biological interpretation.

In the present study and in the study by Decock et al. (2015), tumour development was measured as number of days from birth, which might challenge the interpretation of significantly changed immune responses between genotypes. The presence of palpable tumours varied both between and within genogroups, of which MMTV-PyMT; Mmp8-knock-out mice developed palpable tumours between 3.6 - 5.7 weeks, whereas wild-type littermates showed palpable tumours between 3.6 and 7.8 weeks (Decock et al., 2015). Also, about 90% of *Mmp8*-null tumours were palpable at 3.6 weeks, whereas only about 67% of wild-type tumours were palpable at this stage. In a tumour environment, host defense responses are initiated by recruitment of NK-cells, dendritic cells and macrophages. Adaptive immune cells are then recruited to the tumour in response to the action of innate immune cells (Finn,

2012; Dranoff, 2004). Thus, the significant up-regulation of markers for B- and T-lymphocytes in *Mmp8*-null tumours at 6 weeks might reflect that these tumours have reached a later stage of development rather than an accelerated influx of lymphocytes at early stages. Pro-tumorigenic leukocytes are generally not present before later stages of tumour development, which explains why these lymphocytes appear to possess anti-tumorigenic phenotypes.

It would be of importance to characterise time of tumour initiation in MMTV-PyMT in future studies and, consider interpreting tumour changes as weeks from tumour initiation rather than weeks from birth. Tumour palpability as measure for tumour initiation has its limitations as tumours have already initiated once they become palpable. To ensure a more precise identification of tumour initiation, a model containing luciferase expressing tumour cells can be employed. This allows for bioluminescence imaging of luciferase activity *in-vivo* that can be utilized to track tumour growth from the stage of initiation to metastasis (Welsh & Kay, 2005).

Finally, it is necessary to evaluate obstacles associated with the time course profiling of expression patterns in the MMTV-PyMT model. Looking at global statistics (i.e. replicate PCA analysis and dendrogram) of data from whole genome RNA sequencing, 6 and 8 weeks genotypes are clustered together whereas groups from 10 weeks are clustered separately. This might suggest that the rate of tumour development varies between replicates at earlier time points and, that such variances equalizes at later stages of development. Such biases might have a significant impact on differential expression analysis and should thus be characterized for future studies in order to achieve a more precise interpretation of gene expression data.

4.5. Concluding remarks

This was the first study to explore the effect of MMP8 ablation by analysing the transcriptome of whole MMTV-PyMT tumours. The overall results suggest that MMP8 plays pleiotropic roles during mammary tumour progression. More specifically, the results found that depletion of MMP8 induces changes in the

inflammatory cell repertoire and the protease web, as well as inducing an up-regulation of important genes involved in metabolism and, growth and metastasis of tumour cells. However, further validation of the present results by more optimal approaches for immunophenotyping and by qRT-PCR for validation of candidate genes are required in order to state final conclusions. The role of MMP8 is complex as multiple genes expression networks appear to be affected by MMP8 depletion. Future studies are thus suggested to investigate such system in isolation (e.g. lipid metabolism) to explore whether MMP8 directly affect these pathways or if this is a secondary consequence of a more inflammatory and tumour aggressive environment. Also, an in-depth profiling of leukocyte subpopulations in *Mmp8*-null mice will build up a better picture of the immune regulatory role of MMP8.

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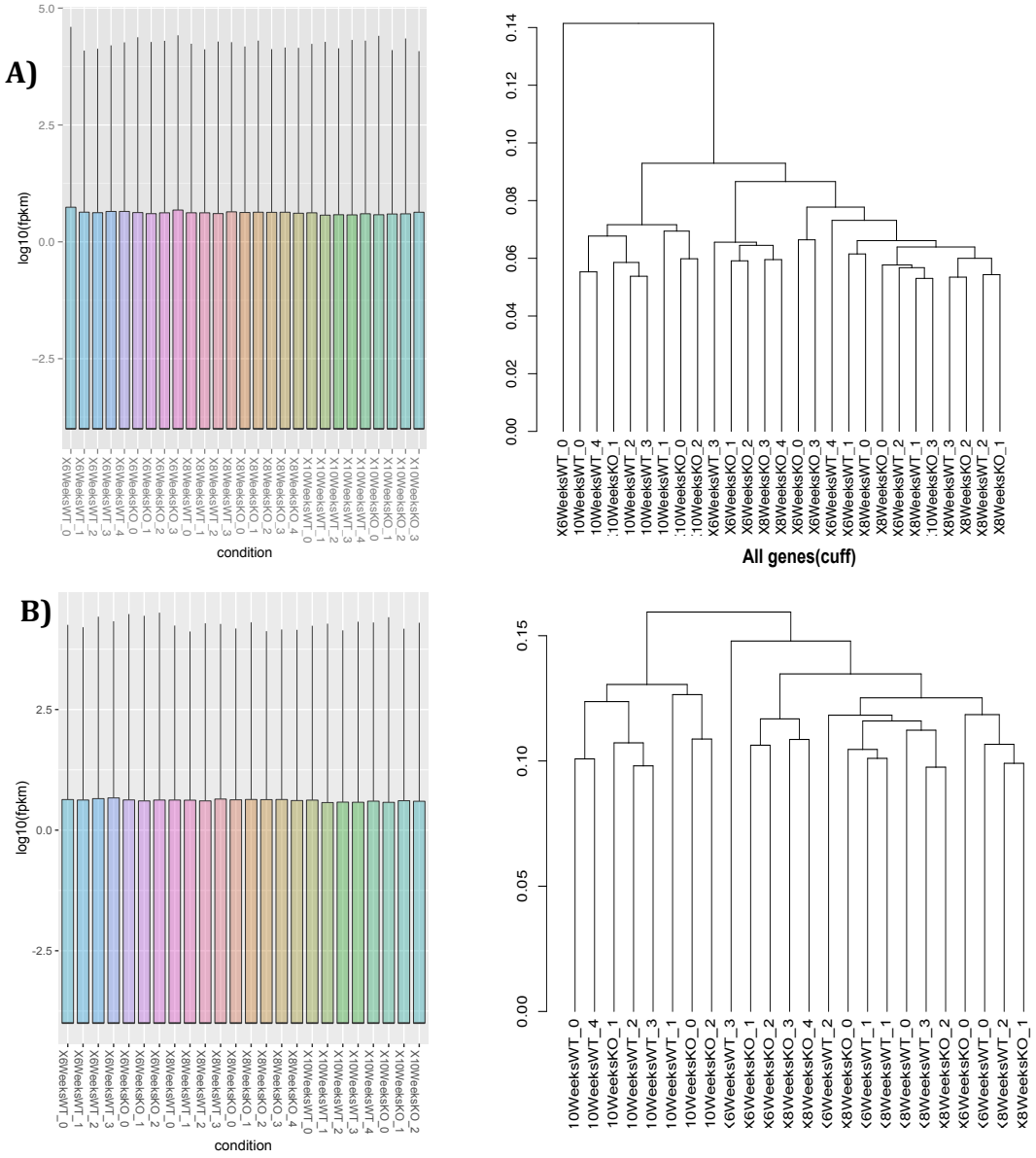
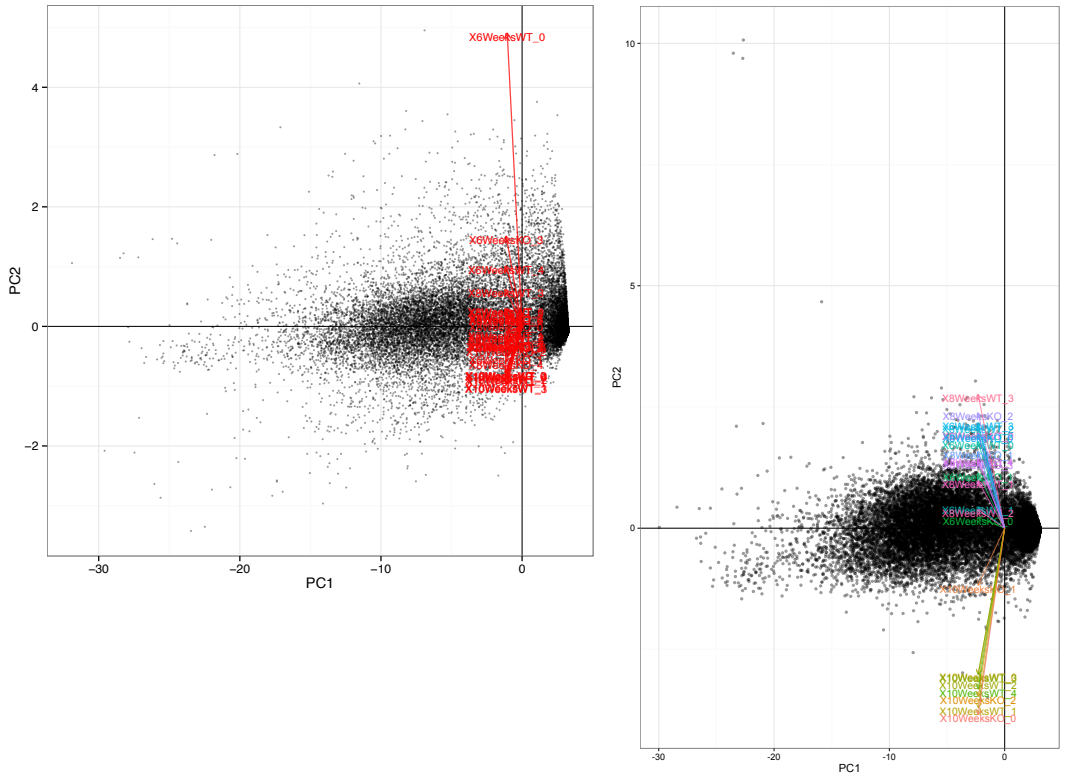
Appendices

APPENDIX 1: FPKM distribution and dendrogram of replicate samples before (A) and after (B) excluding outlier replicates.



APPENDIX 2: Principal Component Analysis (PCA) of replicate samples before (A) and after (B) removing outlier replicates.



APPENDIX 3: Marker genes for leukocyte subpopulations

Ensembl gene ID	Gene	Expressed
ENSMUSG00000000982	CCL3	N1 high N2 low
ENSMUSG00000029417	CXCL9	N1 high
ENSMUSG00000034855	CXCL10	N1 high
ENSMUSG00000024401	TNFα	N1 high M1 high M2 low
ENSMUSG00000018916	GM-CSF	N1 high
ENSMUSG00000027776	Il12a	N1 M1 high M2 low
ENSMUSG00000004296	Il12b	N1 M1 high M2 low
ENSMUSG00000023951	VEGF	N1 high
ENSMUSG00000025929	Il-17a	N1 high
ENSMUSG00000019987	Arginase-I	N1 low N2 high M1 low M2 high
ENSMUSG00000021125	Arginase-II	M1 high M2 low
ENSMUSG00000025383	IL-23a	M1 high M2 low
ENSMUSG00000027398	IL1b	M1 high M2 low
ENSMUSG00000031779	MCP-1 (Ccl2)	M1 high
ENSMUSG00000029417	CXCL9	M1 high
ENSMUSG00000034855	CXCL10	M1 high
ENSMUSG00000025746	IL6	M1 high M2 low
ENSMUSG00000026712	MRC1/CD206 mannose receptor	M2 high
ENSMUSG00000008845	CD163	M2 high
ENSMUSG00000016529	IL-10	M2 high M1 low
ENSMUSG00000031494	cd209a	M2 high
ENSMUSG00000031495	cd209d	M2 high
ENSMUSG00000040165	cd209c	M2 high
ENSMUSG00000051906	cd209f	M2 high
ENSMUSG00000079168	cd209g	M2 high
ENSMUSG00000065987	cd209b	M2 high
ENSMUSG00000040197	cd209e	M2 high
ENSMUSG00000040950	Mgl2	M2 high
ENSMUSG00000061100	Fizz1	M2 high
ENSMUSG00000025044	Macrophage scavenger receptor 1	M2 high
ENSMUSG00000000318	MGL-1	M2 high
ENSMUSG00000079293	Dectin-1	M2 high
ENSMUSG00000026712	Mannose receptor 1	M2 high
ENSMUSG00000026981	IL-1RA	M2 high
ENSMUSG00000023274	CD4	Tregs, Th1, Th2, Th17
ENSMUSG00000039521	FoxP3	Tregs
ENSMUSG00000026770	CD25	Tregs
ENSMUSG00000053977	cd8a	Cytotoxic T cells
ENSMUSG00000053044	cd8b1	Cytotoxic T cells
ENSMUSG00000004730	F4/80	Macrophages
ENSMUSG00000026395	CD45	Leukocytes
ENSMUSG00000032094	CD3d	Total T cells
ENSMUSG00000032093	CD3e	Total T cells
ENSMUSG00000002033	CD3g	Total T cells
ENSMUSG00000003379	CD79a	B-cell
ENSMUSG00000040592	CD79b	B-cell

APPENDIX 4: Relative gene expression of selected immune marker genes after normalising against CD45. Graphs show mean and standard error of mean.