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Targeting the Immune Microenvironment in Pancreatic Cancer

by

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer-related death worldwide. Despite the improved worldwide living standards and increased access to healthcare, pancreatic cancer incidence has increased over the past few decades. Regardless of the impressive advances in the field of cancer therapeutics, surgery remains the only potentially curative treatment for pancreatic cancer. However, most of the patients present with an advanced stage of pancreatic cancer at the time of diagnosis, for which there are very few available therapies.

Pancreatic cancer is characterized by a high degree of tumour heterogeneity, with the tumour microenvironment, also known as stroma, accounting for 80% of the total tumour volume. Pancreatic tumour stroma is one of the main hallmarks of pancreatic cancer and is characterized by extensive deposition of extracellular matrix (ECM) components, abundance of proliferating cancer-associated fibroblasts (CAFs), a significant myeloid cell compartment, but a distinct exclusion of T cells. Although deposition of desmoplastic tumour stroma was originally considered as a bystander in carcinogenesis, recent studies have highlighted its role during tumour progression and in facilitating therapeutic resistance.

Improved understanding of the immune system and its role in cancer development and progression has led to impressive advances in the field of cancer immunotherapy over the last decade. However, the success of immunotherapy has not translated to the treatment of pancreatic cancer. Tumour associated macrophages (TAMs) are recognized as critical drivers of immune escape in the tumour microenvironment. Thus, strategies that can abrogate this effect serve as an attractive option for cancer therapeutics. Our group has previously demonstrated that macrophage targeting, via CSF1 receptor inhibition, significantly increased survival in pancreatic tumour-bearing *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice.

I sought to investigate the role of macrophages in the pancreatic cancer microenvironment. I hypothesised that macrophage infiltration to the pancreas would be dependent on the chemotactic signalling through the C-C motif chemokine receptors (CCRs). Thus, I generated KPC mice lacking chemokine receptors CCR1, 2, 3 and 5. By using *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$. *CCR1-5*$^{-/-}$ (KPC CCR1-5$^{-/-}$) mice I aimed to establish the role of infiltrating macrophages in tumour initiation, development, and progression.

Through a series of *in vivo* experiments I characterized KPC CCR1-5$^{-/-}$ mice, demonstrating the absence of CCR2 in primary tumour tissue as well as significantly reduced levels of

Ly6C^{hi} inflammatory monocytes in peripheral blood. Assessment of tumour initiation in KPC and KPC CCR1-5$^{-/-}$ mice at 6 weeks of age revealed no significant changes in the number or grade of early precursor lesions, also known as pancreatic intraepithelial neoplasias (PanINs), between the cohorts. Most importantly, an aging experiment demonstrated that KPC mice lacking CCR1,2,3 and 5 have no survival benefit when compared with KPC mice. Extensive studies of the tumour microenvironment showed no indication of reduced desmoplasia in tumours from KPC CCR1-5$^{-/-}$ mice. Moreover, immunohistochemical analysis of end point tumours revealed high macrophage abundance in KPC CCR1-5$^{-/-}$ mice. This indicated that tissue resident macrophages are sufficient to sustain tumour growth and maintain the fibrotic tumour microenvironment observed in KPC mice. Further studies using pharmacological approaches to inhibit macrophages from an early timepoint in KPC and KPC CCR1-5$^{-/-}$ mice revealed significantly reduced survival in both genotypes compared with the untreated controls of the same genotypes. These data suggest that during the early stages of tumourigenesis, macrophages may play a tumour suppressive role.

Our previous study of CSF1R inhibition in tumour-bearing KPC mice, revealed an upregulation of molecules associated with immune activation. What is more, we observed an increase in infiltration of CD19^{+} B cells in the treated tumours. Together, these data indicated an increase in local adaptive immunity. In contrast, recently published papers focusing on the role of B cells in pancreatic cancer reported a pro-tumourigenic role of B cells. It was shown that transplanted pancreatic ductal epithelial cells exhibit reduced growth in B cell deficient mice when compared with wild-type mice. Due to the conflicting data, I aimed to assess the effect of B cells on pancreatic cancer in a more clinically relevant model. I generated KPC mice deficient in mature B cells, by crossing *Pdx1-Cre LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ mice with *Ighm*$^{-/-}$ mice. This B cell deficient KPC mouse model was characterized using flow cytometry and immunohistochemical approaches to confirm the absence of B cells. Interestingly, survival analysis revealed that the lack of B cells had no effect on tumourigenesis. The immune profiling of the end point tumours revealed no apparent T cell defects.

To rule out the possibility that any tumour-suppressive effects of B cell depletion in established tumours could be masked by tumour-promoting effects of B cell depletion during tumour initiation, I used a pharmacological approach to deplete B cells in mice with established tumours. Mice with confirmed tumours were treated with the anti-CD20 antibody. Analysis of both tumour growth, as well as survival data, demonstrated no evidence of advantageous effects of B cell depletion. Finally, I wanted to further address the

differences observed in the role of B cells seen between the published articles and the data I generated. Therefore, I aimed to investigate the effect of B cells in syngeneic transplant experiments in the B cell-deficient mice using KC and KPC primary cell lines. I observed that mice implanted with KC cells survived consistently longer than mice implanted with KPC cells. This observation was made for WT and *Ighm*$^{-/-}$ mice. However, I did not observe any survival differences between WT and *Ighm*$^{-/-}$ mice transplanted with either KC or KPC tumour cells. Overall, this indicates that B cells do not play a significant role in tumour development in syngeneic allograft models, at least in our hands. This further supports the results observed in the B cell deficient genetically engineered mouse model (GEMM) of pancreatic cancer.

Overall, my data, alongside previously published studies, suggest that depletion of certain immune cell subtypes can elicit opposing effects. Data in this thesis provide further evidence that the selection of study models often lead to discrepancies in the studies of tumour immune microenvironment. The data I present imply that targeting specific signals that promote tumourigenesis rather than specific cell populations might be more beneficial in fighting tumourigenesis. Further studies are needed to allow the development of efficient immune-therapeutic approaches for pancreatic cancer.

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Author’s Declaration

This work is my own throughout. Where I have received assistance from others I have acknowledged these individuals. This work has not been submitted elsewhere.

Definitions/ Abbreviations

ADM, acinar to ductal metaplasia

BM, bone marrow

BTK, Bruton tyrosine kinase

CAFs, cancer associated fibroblasts

CCL, C-C motif chemokine ligand

CCR, C-C motif chemokine receptor

CSF, Colony stimulating factor

CSF1R, Colony-stimulating factor 1 receptor

CTLA-4, cytotoxic T-lymphocyte–associated antigen 4

DCs, dendritic cells

DMEM, Dulbecco's modified eagle medium

ECM, extracellular matrix

FACS, fluorescence-activated cell sorting

FBS, foetal bovine serum

FSC, forward-scattered

H&E, Haematoxylin and eosin

HSCs, Hematopoietic stem cells

IgHM, Immunoglobulin Heavy Constant Mu

IHC, Immunohistochemistry

ISH, *in situ* hybridization

KC, *Pdx1-Cre*, *LSL-Kras*$^{G12D/+}$

KO, whole body knockout

Kras, Kirsten rat sarcoma viral oncogene homolog

MCP-, Monocyte chemoattractant protein-1

MHC, major histocompatibility complex

MRI, Magnetic resonance imaging

NK, natural killer cells

PanIN, pancreatic intraepithelial neoplasia

PD1, programmed cell death protein 1

PDAC, Pancreatic ductal adenocarcinoma

PET, Positron emission tomography

PSCs, pancreatic stellate cells

SSC, side-scattered

TAMs, Tumour-associated macrophages

TILs, Tumour infiltrating lymphocytes

WT, wild type

YS, Yolk sac

α, alpha

αSMA, alpha smooth muscle actin

β, beta

1 INTRODUCTION

1.1 The pancreas

1.1.1 Biology of the normal pancreas

The pancreas is a glandular organ of endodermal origin and is located in the retroperitoneum. It plays a key role in digestion of proteins and carbohydrates (exocrine function) and glucose control (endocrine function). Functioning as an exocrine gland, the pancreas produces zymogens such as trypsin and amylase. Enzymes are produced by the acinar cells of the pancreas that compose 85 percent of the total tissue mass (Campbell, Verbeke et al. 2013). When required, these enzymes are delivered into the gastrointestinal tract by the network of pancreatic ducts.

The endocrine component of the pancreas, which regulates glucose homeostasis, consists of islet cells that cluster together and form the Islets of Langerhans. Endocrine cells of the pancreas produce and secrete two main hormones required to maintain blood glucose levels: insulin and glucagon. Reflecting the complex physiology of the pancreas, there is a spectrum of different pancreatic malignancies that originate from or resemble various normal cellular components of the pancreas. Figure 1.1 shows the histology of the pancreas illustrating the different cell types.

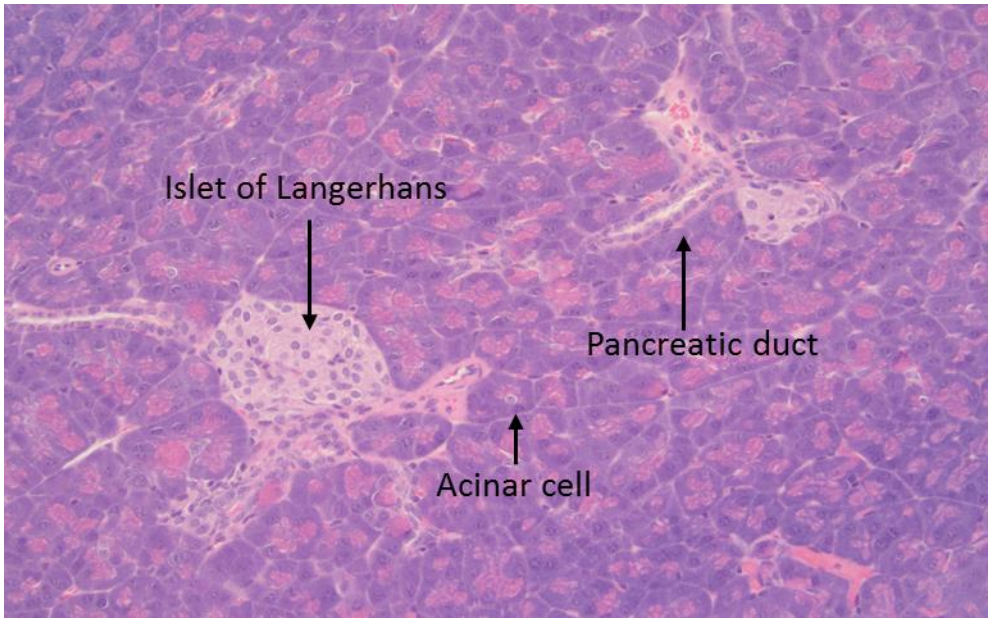

Figure 1.1 Histology of the pancreas.

Haematoxylin and eosin staining of normal mouse pancreas displaying an islet of Langerhans and pancreatic duct surrounded by normal acinar tissue.

1.2 Pancreatic cancer

1.2.1 Epidemiology of pancreatic cancer

Despite the improved worldwide living standards and increased access to healthcare, pancreatic cancer incidence has increased over the past few decades. Pancreatic cancer has the lowest survival of all common cancers. Indeed, less than 7% of people diagnosed with pancreatic cancer survive their disease for five years (CRUK 2018). The incidence of pancreatic cancer varies across regions and populations. However, the highest incidence of 7.7 per 100,000 people was reported in Europe (Rawla, Sunkara et al. 2019). Around 10,300 people are diagnosed with pancreatic cancer in the United Kingdom each year. A slight difference exists in pancreatic cancer incidence rates among genders. The disease is more common in men, with rates being even higher in black males than any other racial group. With a progressively ageing population, unfavourable modern dietary habits, and an increase in risk factors, such as low physical activity and an ageing population, the incidence of pancreatic cancer is predicted to rise by 6% in the UK by 2035.

Mortality rates for pancreatic cancer differ significantly across regions. In 2012, the highest mortality rates from pancreatic cancer in both genders were in Northern America with 6.9 per 100,000 people, followed by Western Europe and other European regions (Ilic and Ilic 2016). Mortality in both genders increases with age, and nearly 90% of all pancreatic cancer deaths occur in people over the age of 55 (Rawla, Sunkara et al. 2019).

Although the causes of pancreatic cancer are not fully understood yet, some risk factors have been recognised. Smoking was identified to increase the risk of pancreatic cancer up to 2.5-fold. The risk correlates with increasing dose and drops off significantly after cessation of smoking (Decker, Batheja et al. 2010). Indeed 25% of deaths associated with pancreatic cancer could be prevented with the cessation of smoking. Heavy drinking also confers increased risk of pancreatic cancer as demonstrated by a pooled analysis of 10 case controlled studies (Lucenteforte, La Vecchia et al. 2012). This study found that people who consume 4 or more alcoholic drinks per day have an increased risk of developing pancreatic cancer, and the odds ratio for people consuming 9 or more drinks per day was 1.6. What is more, obesity has been consistently linked to increased risk of pancreatic cancer. However, the biological mechanism under this association between high BMI and pancreatic cancer is not well defined. Hormonal misbalance and inflammation could be potential mediators (Tsai and Chang 2019).

1.2.2 Current treatment strategies

Early symptoms of pancreatic cancer experienced by patients are usually vague and non-specific. The lack of early clinical signs often results in patients presenting with an advanced stage of pancreatic cancer at the time of diagnosis. Several symptoms that patients present with include back pain, weight loss and poor appetite, jaundice, and changes in bowel movements. Diagnostic assessments of patients with suspected pancreatic cancer include ultrasound imaging, computerized tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) scans. These imaging techniques help to confirm the diagnosis of pancreatic cancer as well as determine the extent of the disease. Usually CT scans of chest, abdomen and pelvis are used to assign the stage of the disease. The stages of pancreatic cancer are indicated on a scale ranging from 0 to IV. The lowest stages indicate that cancer is confined to the pancreas, whereas stage IV indicates that the cancer has spread to other parts of the body. Depending on several factors such as where the main tumour mass is, the stage of cancer, the type of cancer, the general health of the patient and their level of fitness, patients can be assigned to be treated surgically via pancreaticoduodenectomy, with either neoadjuvant or adjuvant chemotherapy, or undergo treatment with chemotherapy alone. However, in the UK, 7 out of 10 patients receive no active treatment whatsoever.

Despite the impressive advances in the field of cancer therapeutics over the last decade, surgery remains the only potentially curative treatment for pancreatic cancer. However, even after the surgery, local and distant relapses are common; up to 75% of patients relapse within 2 years and up to 90% relapse within 5 years. Therefore, surgery is often combined with adjuvant chemotherapy. The European Study of Pancreatic and Ampullary Cancer (ESPAC)-1 trial demonstrated that using 5-fluorouracil (5FU) as an adjuvant chemotherapy significantly improved median survival compared with surgery alone (20.1 versus 15.5 months, respectively; $p = 0.009$) (Neoptolemos, Stocken et al. 2004, Lambert, Schwarz et al. 2019).

Different approaches of adjuvant combination therapy have been tested in order to improve patients' outcomes. The ESPAC-3 clinical trial, comparing 5FU/folinic acid with gemcitabine, demonstrated that these agents are equally effective (Neoptolemos, Stocken et al. 2010). However, patients receiving 5FU and folinic acid exhibited more serious adverse events than patients receiving gemcitabine (14% versus 7.5%), thus establishing gemcitabine as the standard of care adjuvant therapy for patients with resected pancreatic cancer. The ESPAC-4 trial compared gemcitabine combined with capecitabine versus gemcitabine alone. Although the authors reported a significant overall survival benefit in the combination

group (28 months versus 25.5 months in patients compared with those treated with gemcitabine alone), recurrence-free survival were not significantly different (Neoptolemos, Palmer et al. 2017).

Meanwhile, contemporary cytotoxic combination therapies, particularly 5FU, leucovorin, oxaliplatin and irinotecan (FOLFIRINOX), have shown improved outcomes over gemcitabine in metastatic, locally advanced, borderline resectable tumours (Conroy, Desseigne et al. 2011). Therefore, clinical trial PRODIGE24-CCTG PA6 assessed adjuvant FOLFIRINOX (modified) in patients following primary tumour resection. Results from this trial demonstrated the best disease-free survival so far, 21.6 months in the modified-FOLFIRINOX group and 12.8 months in the gemcitabine group (Conroy, Hammel et al. 2018), making FOLFIRINOX the new gold standard adjuvant therapy for those patients fit enough to tolerate the regimen.

The introduction of neoadjuvant treatment for resectable pancreatic cancer is an attractive treatment regime, although data from trials is still lacking. In general, neoadjuvant treatment is well tolerated and recent large-scale studies suggest a survival benefit for patients who receive neoadjuvant treatment for early stage resectable pancreatic cancer (Lutfi, Talamonti et al. 2016). What is more, meta-analysis study has recently confirmed that neoadjuvant therapy results in tumour downsizing and downstaging. Thus, neoadjuvant therapy could potentially allow more patients with borderline resectable pancreatic cancer to undergo resection.

Patients that present with locally advanced pancreatic cancer make up to 30% of all pancreatic cancer patients at diagnosis. Median overall survival ranges from 10 to 30 months. Locally advanced tumours are not metastatic, however, cannot be resected because they often obliterate the portal vein or encase the aorta (Lambert, Schwarz et al. 2019). Currently, standard management of locally advanced pancreatic cancer remains gemcitabine, while FOLFIRINOX can be considered for patients with high performance status.

Treatment strategies for patients with metastatic pancreatic cancer depend on a patient's overall health and preference. Gemcitabine was the standard of care for many years following the original trial that reported improved median survival compared with 5-FU (5.65 months vs. 4.41 months), and alleviation of symptoms in some patients (Burris, Moore et al. 1997). In 2011, a French study reported that the FOLFIRINOX regimen improved median survival in the metastatic setting to 11.1 months compared with 6.8 months on gemcitabine (Conroy, Desseigne et al. 2011). This was closely followed by the phase III study of nab-paclitaxel (Abraxane) in combination with gemcitabine, showing improved

overall survival of 8.5 months compared with 6.7 months in the gemcitabine-treated patients (Von Hoff, Ervin et al. 2013). Routinely, the FOLFIRINOX regimen is recommended for metastatic disease by the National Institute for Health and Care Excellence (NICE) for patients with high performance status, whilst Gemcitabine with or without Abraxane is considered for patients who are not well enough to tolerate FOLFIRINOX ((NICE) 2020).

Improved understanding of the immune system and its role in cancer development and progression has led to impressive advances in the field of cancer immunotherapy over the last decade. The field is rapidly evolving and the list of drugs receiving regulatory approval for the treatment of various cancers is fast growing. However, the success of immunotherapy has not translated to the treatment of pancreatic cancer which has been shown to be unresponsive to anti-programmed death 1 (anti-PD-1) and anti-cytotoxic T-lymphocyte-associated antigen 4 (anti-CTLA-4) (Royal, Levy et al. 2010, Sharma, Dirix et al. 2018). Understanding the role of the tumour microenvironment in facilitating immune escape in pancreatic cancer holds great potential for improving the success of immunotherapy for pancreatic cancer in the future.

1.2.3 Molecular mechanism and histological progression of pancreatic ductal adenocarcinoma

Remarkable progress has been made in recent years trying to depict the molecular mechanisms of pancreatic cancer. Although around 10% of all pancreatic cancer cases are associated with an inherited predisposition (Habbe, Langer et al. 2008, Wescott and Rustgi 2008), most are caused by somatic mutations in proto-oncogenes and tumour suppressor genes. These high-frequency mutations include activating mutations in the small GTPase *KRAS* and alterations in genes such as *CDKN2A, TP53* and *SMAD4* that act as tumour suppressors. Activated *KRAS* is observed in more than 90% of all Pancreatic Ductal Adenocarcinomas (PDAC), which is the most common and most lethal pancreatic malignancy (Almoguera, Shibata et al.). Point mutations in codons G12, G13 or Q61 of KRAS locks the protein in a constitutively active state. Mutated KRAS protein is unable to hydrolyse GTP and persistently stimulates downstream signalling pathways that are involved in proliferation, inhibition of cell death, cell migration and metastasis (Rodriguez-Viciana, Tetsu et al. 2005).

Due to its high frequency in PDAC, mutated *KRAS* was proposed as an initiating event in the development of tumours. This has been borne out in mouse models showing that targeting *Kras* mutation specifically to the mouse pancreas is sufficient to induce pancreatic intraepithelial neoplasia (PanIN) that eventually develops into PDAC, thus mimicking the human disease progression (Hingorani, Petricoin et al. 2003) (Figure 1.2). Based on the degree of cellular and nuclear atypia, PanINs are classified into three stages: PanIN-1A/B, PanIN-2, and PanIN-3 (Campbell, Verbeke et al. 2013).

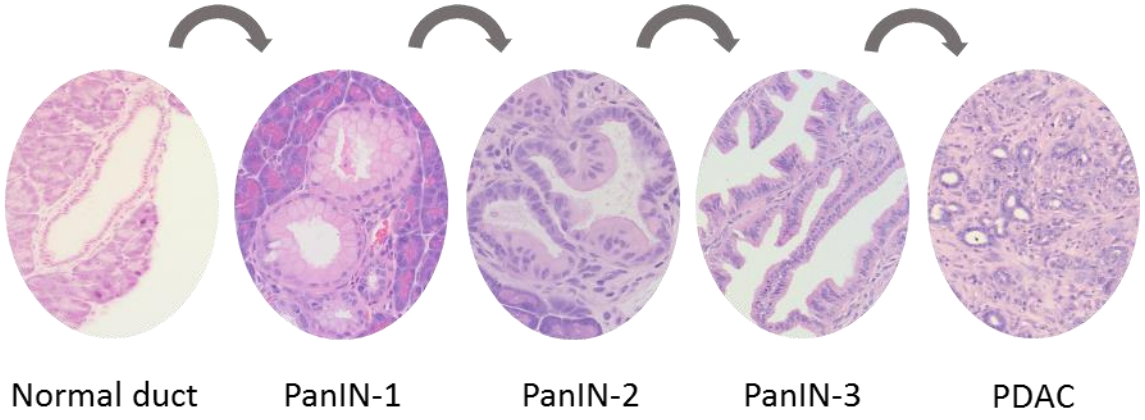

Figure 1.2 Histological progression of PanIN lesions.

Kras mutation is sufficient to induce early pancreatic intraepithelial neoplasia (PanIN) in mouse pancreas that eventually develop into PDAC, thus mimicking the human disease. Early PanIN lesions progress through stages as a result of accumulating mutations in tumour suppressor genes and eventually develop into adenocarcinoma.

Studies have shown that histological progression of PanINs correlates with the accumulation of somatic mutations. Molecular alterations in *KRAS* are classified as an early event in the formation of these preneoplastic lesions, further supporting the initiating role of mutated *KRAS* in the development of PDAC (Feldmann, Beaty et al. 2007). The loss of the *TP53* tumour suppressor gene is observed with increasing frequency in later-stage PanINs that have acquired significant features of atypia (Morris, Wang et al. 2010). In more than 50% of all PDAC cases, mutations occur within the gene region encoding the DNA-binding domain of p53. Missense mutations in *TP53* abolish its anti-proliferative activities, whereas gain-of-function activities of TP53 can promote tumour development and drive metastasis (Weissmueller, Manchado et al. 2014). Studies show that mutated *TP53* in human PDAC correlates with worse survival (Soussi and Beroud 2001) and can confer drug resistance in tumour cells (Masciarelli, Fontemaggi et al. 2014). Recently, it has been demonstrated to drive metastasis in murine models (Weissmueller, Manchado et al. 2014). The progression of PanINs is also associated with acquired mutations in *CDKN2A* and *SMAD4*. These mutations promote malignant transformation of PanINs (Wang, Liu et al. 2013). However,

as with *Trp53*, mice with germline mutations still require constitutively active KRAS for PDAC development, and loss of function of these tumour suppressors has no effect on pancreatic tumourigenesis in the absence of mutant *Kras* (Hezel, Kimmelman et al. 2006). On top of these four high-penetrance genetic alterations there are many other low-penetrance mutations that are observed in PDAC, resulting in significant heterogeneity between tumours. Detailed analysis of exome sequencing data from 99 early sporadic pancreatic ductal adenocarcinomas identified substantial heterogeneity, with 2,016 non-silent mutations and 1,628 copy-number variations. The study identified 16 significantly mutated genes, confirming the importance of known mutations such as *KRAS, TP53, CDKN2A* and *SMAD4*, and revealing novel mutated genes involved in genes involved in axon guidance (*SLIT2*, *ROBO1* and *ROBO2*), chromatin remodelling (*EPC1* and *ARID2*), DNA damage repair (*ATM*) amongst others (Biankin, Waddell et al. 2012).

Studies of specific signalling pathways in PDAC has culminated in comprehensive integrated transcriptomic analyses of RNA expression profiles that led the way to the identification of PDAC subtypes and the various gene programs (GPs) that define them (Collisson, Sadanandam et al. 2011, Moffitt, Marayati et al. 2015, Bailey, Hendley et al. 2016). Most notably, Bailey et al. used integrated genomic analysis of 456 PDAC samples collected after surgical resection and identified frequently mutated genes linked to 10 pathways. Further analysis defined 4 distinct subtypes of PDAC: squamous, progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX) (Bailey, Chang et al. 2016). The discovery of the core gene programmes that characterized individual subtypes opened opportunities for therapeutic development of personalised treatments.

1.3 PDAC microenvironment

1.3.1 Desmoplastic stroma

Alongside the accumulation of mutations in tumour cells, the tumour stroma plays a vital role during tumour progression and also facilitates therapeutic resistance. As PDAC develops, the acinar parenchyma is gradually replaced by fibrotic stroma, which is one of the main hallmarks of pancreatic cancer (Clark, Hingorani et al. 2007). The stromal compartment can make up over 80% of all tumour mass (Hu, Jiao et al. 2015). Pancreatic tumour stroma is characterized by extensive deposition of extracellular matrix (ECM) components, abundance of pancreatic stellate cells (PSCs), and proliferating cancer-associated fibroblasts (CAFs) that produce structural proteins such as fibronectin and

collagen. In addition, the stroma consists of endothelial and inflammatory cells, different growth factors, chemokines and cytokines that play an important role in cancer cell-stroma crosstalk. Although deposition of desmoplastic tumour stroma was originally considered as a bystander in carcinogenesis, recent studies have highlighted the role of tumour stroma in tumour initiation as well as progression (Neesse, Algul et al. 2015). Once established, this tumour microenvironment has also been reported to play a role in resistance to chemotherapy (Waghray, Yalamanchili et al. 2013). However, other studies have suggested a tumour-suppressive role for the pancreatic cancer stroma, and it appears the situation is more complex than previously thought. In the next section I will discuss the role of different stromal components in PDAC development and progression and the potential for targeting tumour stroma as a treatment strategy.

1.3.2 Cancer-associated fibroblasts

One of the main cellular components of PDAC stroma are the fibroblasts, also referred to as cancer associated fibroblasts (CAFs). A major source of fibroblasts in pancreatic cancer are PSCs (Bachem et al. 2005). In a quiescent state they store vitamin A and express fibroblast-activating protein α (Campbell, Verbeke et al. 2013). Once activated, PSCs lose their vitamin A stores, start expressing the myofibroblast protein, α-smooth muscle actin (αSMA), and produce ECM components (Öhlund, Handly-Santana et al. 2017). However, other sources might contribute to the fibroblast population, including mesenchymal stem cells and peri-vascular fibroblasts that might be activated upon tissue injury (Waghray, Yalamanchili et al. 2013). Early observations proposed the stroma to be a physical barrier that impairs the delivery of cytotoxic drugs to the tumour cells, although the stroma may also provide nutrients and numerous survival signals to confer chemo-resistance (Beatty, Werba et al. 2021).

Efforts have been made to modify desmoplastic stroma by directly targeting CAFs. In 2009, work by the Tuveson lab found that targeting Sonic hedgehog (Shh) signalling, a known driver of fibrosis in pancreatic cancer, could deplete the tumour stroma and enhance drug delivery into tumours, resulting in increased survival (Olive, Jacobetz et al. 2009). What is more, a study by Froeling and colleagues hypothesised that restoration of retinol (Vitamin A) stores in PSCs should return them to a quiescent state. Indeed, the group demonstrated that PSCs treated with various isoforms of retinoic acid became quiescent. With the use of organotypic models and mouse models of PDAC, they showed that retinoic acid

administration induced a reduction in proliferation and an increase in apoptosis in surrounding cancer cells (Froeling, Feig et al. 2011). Similarly, it was shown that calcipotriol, a ligand for the Vitamin D receptor (VDR, expressed on PSCs) reduces fibrosis and inflammation in mice with induced chronic pancreatitis. Compared with untreated mice, VDR ligand treated animals had significantly lower PSC activation (Sherman, Yu et al. 2014). On the other hand, *in vivo* depletion of CAFs by selectively killing proliferating αSMA positive fibroblasts in a PDAC mouse model resulted in significantly more invasive and undifferentiated tumours when compared with controls. Depletion of around 80% of tumour resident CAFs was associated with significant reduction in survival, although it should be noted that induction of apoptosis on this scale within the tumour would likely result in immense, potentially tumour-promoting inflammation. Interestingly, depletion of fibroblasts and reduction in tumour tissue stiffness did not result in improved efficacy of Gemcitabine (Özdemir, Pentcheva-Hoang et al. 2014). Thus, CAFs do not seem to serve as physical barrier preventing drug delivery. In a similar study, Rhim et al found that deletion of the known driver of fibrosis, Sonic Hedgehog (Shh), reduced stromal content and led to more aggressive tumours in a mouse model of PDAC (Rhim, Oberstein et al. 2014), however, in this case it should be noted that tumour initiation occurred in the absence of stroma, and likely underwent tumour cell intrinsic changes to adapt. More recently, separate CAF subtypes have been observed, suggesting that targeting CAFs for drug development may not be so straightforward. A study led by Ohlund demonstrated a CAF population (MyCAFs) with elevated expression of αSMA present in PDAC. The location of these fibroblasts was shown to be restricted to the close proximity to cancer cells and be contact-dependent. On the other hand, inflammatory CAFs (iCAFs) become activated by factors secreted from cancer cells, express significantly lower levels of αSMA, but show elevated expression of cytokines and chemokines, particularly IL-6 which can in turn stimulate STAT3 in cancer cells. Importantly, it was demonstrated that these subtypes are transient and can be reverted (Öhlund, Handly-Santana et al. 2017). The Jorgensen lab also recently identified distinct CAF lineages, based on CD105 expression, with these subtypes stable in the long-term and unable to inter-convert. They found that $CD105^{+}$ fibroblasts were permissive for tumour growth, but that the CD105-negative fibroblasts restrained tumour growth, an effect dependent on the adaptive immune system (Hutton, Heider et al. 2021). Taken together, these studies propose PSCs and CAFs as molecular targets, however, highlight the need for caution and subtle targeting of specific CAF populations.

1.3.3 Tumour-associated neutrophils

Although neutrophils were thought to be anti-tumourigenic and coordinate cytotoxic T cells to attack cancer cells, mounting evidence suggests a potential role of neutrophils in cancer development and metastasis. Tumour associated neutrophils have been shown to exhibit anti-tumourigenic (so-called N1) or pro-tumourigenic (N2) characteristics. In vitro studies have shown that anti-tumour effects of neutrophils include expression of immunostimulatory cytokines and chemokines and ability to target and kill cancer cells (Clark and Klebanoff 1975, Yan, Kloecker et al. 2014). Many more studies suggest that neutrophils have pro-tumourigenic activity in pancreatic cancer. It has been demonstrated that TGF-β is responsible for polarization of TANs towards an N2-like phenotype. TGF-β signalling can inhibit neutrophil activity and cytotoxicity. Experiments inhibiting the type I TGF-β receptor demonstrated an increase in the percentage of CD11b+Ly6G+ neutrophils that subsequently show a more profound immunostimulatory mRNA profile than neutrophils from untreated animals (Fridlender, Sun et al. 2009). However, the proposed neutrophil classification into pro- and anti-tumourigenic subtypes could be an oversimplification and further research is needed to determine complete spectrum of neutrophil polarization and signalling pathways involved.

Until recent years, neutrophils were simply seen as short-lived effector cells of the immune system that play an initial role in inflammation by performing phagocytosis and recruiting other effector cells. More attention was paid to the tumour immune microenvironment once it became apparent that chronic inflammation can play an essential part in tumour initiation by damaging tissue cells. Hence it is not surprising that human epidemiological studies link chronic pancreatitis with an increased risk of developing PDAC (Lowenfels and Maisonneuve 2006). Due to their role in inflammation, neutrophils are considered a potential target for treating pancreatitis and consequently preventing inflammation-induced PDAC. Studies with mouse models that use chemical carcinogens to induce inflammation-induced cancer have demonstrated the importance of neutrophils in tumour promotion. It was shown that neutrophils are attracted to the damaged tissue via CXCR2 ligands (Jamieson, Clarke et al. 2012, Seth B. Coffelt 2016). Moreover, inhibition of CXCR2 protects wild-type mice against induced chronic pancreatitis and can even reverse established inflammation in the pancreas (Steele, Karim et al. 2015). In addition to this, CXCR2 inhibition in tumour bearing *Pdx1-Cre, LSL-Kras*$^{G12D/+}$*, LSL-Trp53*$^{R172H/+}$ (KPC) mice (discussed later in this chapter), enhanced survival in combination with chemotherapy, and prevented metastasis by inhibiting neutrophils in the metastatic niche. Interestingly, CXCR2 inhibition or deletion

could also affect the immunosuppressive tumour micro-environment to allow T cell infiltration, providing an opportunity for immunotherapy. Indeed, combination of CXCR2 inhibition with immunotherapy resulted in dramatically extended survival in a number of tumour-bearing KPC mice (Steele, Karim et al. 2016), and these findings were supported by a later study by the Vonderheide lab (Chao, Furth et al. 2016). Taken together these data show that the use of CXCR2 inhibitors has a potential therapeutic benefit for chronic pancreatitis patients and relevant inhibitors are now in clinical trials.

1.3.4 B lymphocytes

Despite the fact that just like other immune cells, B lymphocyte infiltration is observed in human tumours, the role of B cells in the development and progression of PDAC is not widely studied yet (Yang, Lee et al. 2013, Chang, Jiang et al. 2016). While the localization and spatial distribution of T lymphocytes within tumours have been studied and correlated with clinical outcome, B cells which represent a primary cellular constituent of tertiary lymphoid tissue have been largely excluded from these studies.

Recently, $CD20^+$ B lymphocytes were shown to display a distinct spatial organization within the tumour stroma. A study by Castino et al., demonstrated that B cells are not only unevenly interspersed in human pancreatic cancer stroma, but also form dense aggregates resembling tertiary lymphoid tissue. This retrospective study evaluated the distribution of B cells in 104 tissue specimens from PDAC patients. It was shown that B cell confinement within lymphoid tissue correlates with better prognosis, whereas scattered B cells associated with worse prognosis. What is more, the confinement of B cells in a lymphoid site correlated with $CD8^+$ T cell infiltration, suggesting that B cells within these structures could be engaged in T cell responses (Castino, Cortese et al. 2016). Representing human cancer, tumours from pancreatic cancer mouse models were also shown to be infiltrated with B cells.

Interestingly, pancreatic ductal epithelial cells harbouring activating *Kras* mutation exhibited reduced growth when transplanted into B cell deficient mice compared with wild-type mice (Pylayeva-Gupta, Das et al. 2016). Lee et al. studied the role of hypoxia-inducible factor 1α (HIF1α) in pancreatic cancer and showed that its deletion leads to increased B cell infiltration in PanIN lesions in $Kras^{G12D}$-driven GEM models, and drastically accelerated tumourigenesis. When compared with $Kras^{G12D}$-expressing Hif1α wild-type mice, mice with conditional deletion of *Hif1α* had reduced number of conventional B2 cells ($CD19^+CD43^-CD5^-$) but increased numbers of B1 ($CD19^+CD43^+IgM^{hi}$) cells. As a part of innate immunity,

B1 cells produce the majority of natural antibodies against a broad spectrum of infections (Lee, Spata et al. 2016). However, the role of B1 cells in cancer settings is not yet defined. In a third study, B cells were found to promote pro-tumour, immunosuppressive behaviour of macrophages by a Bruton tyrosine kinase (BTK)-dependent mechanism (Gunderson, Kaneda et al. 2016). Overall, these observations suggest that B cells contribute to PDAC progression. However, further studies that would define the complete immune environment and the exact role of B cells in PDAC are needed.

1.3.5 Dendritic cells

Dendritic cells (DCs) are antigen presenting cells (APCs) that play essential roles in the initiation and regulation of innate and adaptive immune responses. DCs are derived from the bone marrow (BM) via consecutive steps that involves common myeloid progenitors (CMPs) and macrophage/DC progenitors (MDPs). Dendritic cells sample the microenvironment and present antigens and co-stimulatory signals to cells of the adaptive immune system. In the steady state, dendritic cells exist as mainly immature and weak APCs with high capacity to engulf antigens. By engulfing and proteolytically degrading proteins from tumour material, DCs generate peptide antigens and present them to $CD4^+$ and $CD8^+$ T cells. Activated DCs are characterized by the increased expression of major histocompatibility complex II (MHC class II), costimulatory molecules, cytokine production (Veglia and Gabrilovich 2017).

In tumour microenvironment, DCs exist in mature and immature forms (Palucka and Banchereau 2012). The increased presence of both circulating dendritic cells and dendritic cells within the PDAC microenvironment are associated with prolonged survival. However, DCs infiltrate pancreatic cancer in low numbers and often demonstrate impaired function (Yanagimoto, Takai et al. 2005).

1.3.6 Tumour infiltrating lymphocytes

Tumour infiltrating lymphocytes (TILs) include several subsets with distinct functions, including the $CD8^+$ cytotoxic T cells, the $CD4^+$ T helper cells, the regulatory T cells (Tregs), the memory T cells, the natural killer cells and the gamma delta T cells. TILs form a part of the adaptive immune response and are activated, for the most part, by antigen presentation.

Activated $CD8^+$ T-cells represent the main cytotoxic effector cell type within the tumour microenvironment. Notably, peritumoral infiltration by $CD3^+$, $CD8^+$, and $CD20^+$ TILs is associated with improved overall survival and progression-free survival providing a rationale for the use of immunotherapy for pancreatic cancer (Miksch, Schoenberg et al. 2019).

$CD4^+$ T cells play a vital role in antitumour immunity. Activation of $CD4^+$ naïve T cells leads to their differentiation into $CD4^+$ helper cells. They are able to recognize peptides presented by the MHC II molecules expressed on the surface of antigen presenting cells such as dendritic cells and macrophages. Immunohistochemical staining showed that a higher number of tumour infiltrating $CD4^+$ T cells correlated with better survival in pancreatic cancer (Ino, Yamazaki-Itoh et al. 2013). However, pancreatic cancer cells are able to hinder $CD4^+$ T cell function by inhibiting their proliferation and migration (Fogar, Basso et al. 2011). Five principal subsets or lineages of $CD4^+$ T cells have been identified so far that include T helper 1 (Th1), Th2, Th17, regulatory T cells and follicular helper T cells. Th1 cells produces IFN-γ and induce cell-mediated immune responses, while Th2 cells produce IL4, IL5 and IL13 and assist humoral immune responses. In the tumour microenvironment, Th1 cells are involved in cytotoxic responses, but Th2 cells may promote tumour tolerance. It has been shown that in pancreatic cancer, the Th cell population is shifted towards the Th2 phenotype (Tassi, Gavazzi et al. 2008). Studies suggest that fibroblasts within the pancreatic cancer stroma might be supporting this shift through the secretion of thymic stromal lymphopoietin (TSLP) (De Monte, Reni et al. 2011).

γδ T cells are a rare T cell receptor-expressing cell type with innate-like qualities (Silva-Santos, Mensurado et al. 2019). In human PDAC, γδ T cells infiltrate tumour tissue, but are rarely found in normal pancreatic tissue (Daley, Zambirinis et al. 2016, Seifert, List et al. 2020). Experimental models have shown that γδ T cells can induce pancreatic tumorigenesis (McAllister, Bailey et al. 2014).

Regulatory T cells (Tregs) are highly immunosuppressive cells characterized by the expression of $CD4^+$, $CD25^+$ and the transcription factor forkhead box P3 (FoxP3). These cells accumulate in mouse and human pancreatic cancer and secrete chemokines such as IL10 and TGF-β. What is more, Treg infiltration positively correlates with tumour metastasis and poor prognosis in pancreatic cancer patients (Tang, Xu et al. 2014). Therefore, depletion of Tregs was considered as an attractive approach for the treatment of pancreatic cancer especially in the combination with checkpoint inhibitors. Depletion of Tregs was tested in an orthotopic implantation model of pancreatic cancer in mice, using *KrasG12D* expressing

pancreatic ductal epithelial cells. The authors of this study reported that Treg depletion was sufficient to evoke effective anti-tumour immunity through restoration of immunogenic tumour-associated $CD11c^+$ DCs and an increase in $CD8^+$ cytotoxic T cell activation (Jang, Hajdu et al. 2017). On the other hand, a study using an autochthonous model demonstrated that Treg depletion in the presence of oncogenic *Kras* resulted in harmful inflammation that promoted PanIN formation. Treg depletion was accomplished by the administration of diphtheria toxin in PanIN bearing *Ptf1a-Cre; LSL-KrasG12D; Foxp3^{DTR}* mice and resulted in immune cell infiltration with de-differentiation of pancreata and the formation of acinar-ductal metaplasia (Zhang, Lazarus et al. 2020). What is more, the group identified Tregs as novel regulators of the myofibroblastic CAF (myCAF) population that is driven by TGFβ (Zhang, Lazarus et al. 2020). Interestingly, the same group had previously reported that $CD4^+$ T cell deletion could impair pancreatic tumourigenesis by releasing repression of $CD8^+$ T cells. Although they did not investigate which $CD4^+$ subset of cells was responsible for immunosuppression in this study, Tregs did represent the most abundant $CD4^+$ population in their model (Zhang, Yan et al. 2014).These later data challenge the previous notion where Treg cells are seen as critical executors of immune suppression in pancreatic cancer and shed some light on why therapies targeting Treg cells has not been proven to be effective (Aykut, Chen et al. 2020).

1.3.7 Macrophages

1.3.7.1 *Macrophage function and classification*

Macrophages are a type of white blood cell of the immune system that ingest and clear cellular debris, foreign substances and cancer cells. Monocytes are considered to be the precursor cells of the mononuclear phagocytic system, including macrophages. Macrophages are essential for maintaining the integrity of an organism by directly participating in pathogen elimination or repairing tissue under inflammatory conditions. They can be identified by specific expression of a number of cellular markers such as F4/80, CD11b, CD68, CD14 and Lysozyme M. Overall, macrophages are increased in tumour tissue and high infiltration is related to poor prognosis in many human cancers, including pancreatic cancer (Bingle, Brown et al. 2002, Yu, Guan et al. 2019).

There are different subpopulations of macrophages and each of them have their own characteristics and differ in functionality. Combinations of different stimuli regulate macrophage maturation and activation in tissues. Based on the terminology proposed by

Mills et al. in 2000, macrophages have been classified as being classically activated (M1 or CAM macrophages) or alternatively activated (M2 or AAM macrophages). A lot of our understanding of macrophage polarization has come from *in vitro* studies, where macrophages were stimulated with M1 or M2 polarizing agents. Macrophages become classically activated after exposure to stimuli such as IFN-γ, lipopolysaccharide (LPS) or granulocyte-macrophage colony-stimulating factor (GM-CSF), which stimulate the production of pro-inflammatory cytokines (Chávez-Galán, Olleros et al. 2015). Classically activated macrophages produce inflammatory cytokines, mediate resistance to pathogens and exhibit strong anti-microbial properties. They are characterized by their secretion of cytokines such as IL-1β, TNF, IL-12, and IL-18. They also express high levels of MHC II, CD68 and costimulatory molecules CD80 and CD86. Alternative activation of macrophages is driven by stimuli such as CSF1, IL-4, IL-10, TGF-β and IL-13. Phenotypically, M2 polarized macrophages are characterized by the expression of the macrophage mannose receptor (MMR), also called CD206, and Arg1. However, the M1/M2 nomenclature proved to be confusing and oversimplified due to versatile nature of these cells. More and more evidence is being presented to show that macrophage polarization is a multifaceted process that occurs over a continuum.

1.3.7.2 *Origin of macrophages in the pancreas*

Macrophages are present in all tissues and are critical for immune responses. Large numbers of monocyte/macrophages originate from hematopoietic stem cells (HSCs) and are continuously replenished by monocyte/macrophage precursors. However, studies show that bone marrow (BM) transplantation results in slow and inefficient replacement of tissue macrophages. Moreover, it was observed that defects in BM-derived monocytes resulting from mutations in *GATA2* and *IRF8* had no effect on numbers of tissue macrophages (Bigley, Haniffa et al. 2011, Hambleton, Salem et al. 2011). Therefore, several research groups have focused on studying the origin of pancreatic macrophages. A study led by Calderon, demonstrated the plasticity of myeloid cells in the pancreas under noninflammatory conditions. It was noted that differences exist between the macrophages within the islets of Langerhans and those imbedded in the interacinar stroma (Calderon, Carrero et al. 2015). Lineage tracing studies by Schulz et al. used the deletion of two transcription factors, PU.1 and Myb to track the development of macrophages. The transcription factor PU.1 is required for macrophage development but is unnecessary for the development of HSCs. In contrast, Myb is essential for the development of HSCs, but not for yolk-sac (YS) myelopoiesis. Data

from this study indicated that YS-derived precursors could give rise to populations of F4/80^{hi} macrophages in mouse tissues in the presence of WT HSCs. These macrophages persisted in adult mice independently of HSCs. On the other hand, *Myb*-dependent BM precursors continuously replace classical DCs, F4/80^{low} macrophages as well as a proportion of F4/80^{hi} macrophages, indicating a mixed origin of these populations (Schulz, Perdiguero et al. 2012). Data from these studies indicate that macrophages derived from embryonic haematopoiesis reside exclusively in the pancreatic stroma, whereas macrophages in the islets are derived from adult HSCs (Calderon, Carrero et al. 2015).

What is more, the expression profiles of these two sets of macrophages in the pancreas also differ. Under steady conditions, macrophages in the islets have features of activation: high expression of class II MHC molecules, effective antigen presentation to T lymphocytes and classical activation profiles as suggested by *Il1b* and *Tnfa* transcripts. Stromal macrophages, on the other hand, exhibit expression profiles of alternative activation and are composed of two subsets: one subset having high class II MHC expression and antigen presentation potential, the second having low MHCII expression and reduced antigen presentation (Calderon, Carrero et al. 2015).

1.3.7.3 *Origin of tumour associated macrophages in pancreatic cancer*

Tumour associated macrophages (TAMs) represent a major population of inflammatory cells in the PDAC microenvironment (Noy and Pollard 2014, Cui, Yue et al. 2016). Therefore, TAMs are an attractive target for therapeutic intervention. However, this requires an in-depth understanding of the sources that sustain macrophages in tumours, as well as their phenotype and function. Recent studies in pancreatic cancer have raised questions about the ontogeny of TAMs. The traditional thought was that infiltrating monocytes give rise to TAMs. However, it has now been shown that not all TAMs are derived from hematopoietic stem cells and that they can arise from precursors that are present in the tissues from embryonic stages (Schulz, Perdiguero et al. 2012, Zhu, Herndon et al. 2017). Parabiosis experiments revealed that the chimerism of macrophages in KPC tumours was only 8.8% after 6 weeks of parabiosis, whereas chimerism of Ly6C^{hi} monocytes in the same tissue reached 27%. These data suggested that circulating monocytes are not the sole source of macrophages within pancreatic tumours in KPC mice. The presence of tissue resident macrophages was further supported by bone marrow transplant studies. KPC mice were irradiated at the PanIN stage and CD45.1^{+} cells from wild-type mice were adoptively transferred. It was noted that more than 15% of TAMs in KPC tumours were host derived and that they were able to

expand through *in situ* proliferation as tumours progressed (Zhu, Herndon et al. 2017). Moreover, TAMs of different ontogenies express different levels of cell surface markers and exhibit different functions. TAMs that arise from HSCs are more potent at sampling tumour antigens and exhibit high expression of MHC II, whereas embryonically derived TAMs express pro-fibrotic transcriptional profiles, distinct from those of infiltrating monocytes, and regulate fibrotic responses in PDAC as well as promoting tumorigenesis (Zhu, Herndon et al. 2017). However, during the early stages of tumorigenesis, there is evidence to suggest that tissue-resident macrophages have tumour suppressive properties (Uderhardt, Martins et al. 2019).

1.3.7.4 *The role of macrophages in pancreatic cancer*

Inflammation plays a key role in pancreatic cancer development and progression. Tumour associated macrophages (TAMs) represent a major population of inflammatory cells in PDAC microenvironment (Noy and Pollard 2014, Cui, Yue et al. 2016). In addition, a macrophage transcriptional signature is reported to be particularly high in squamous and immunogenic subtypes of PDAC (Bailey, Chang et al. 2016). TAMs exhibit different phenotypic programs depending on stimuli such as IL-10, TGF-α and other cytokines that are present in local tumour milieu at different stages of carcinogenesis (Movahedi, Laoui et al. 2010). Despite the conventional view of a continuum from classically activated, 'M1'-like macrophages that are pro-inflammatory, to alternatively activated 'M2'-polarized macrophages that exhibit anti-inflammatory properties, recent studies in pancreatic cancer show that TAMs may possess both pro- and anti-inflammatory characteristics and are able to contribute to epithelial-mesenchymal transition (EMT) (Helm, Held-Feindt et al. 2014). TAMs are also critical drivers of immune escape in the tumour microenvironment. Thus, strategies that can abrogate this effect serve as an attractive option for cancer therapeutics. Inhibition of CSF1R, a receptor for macrophage colony-stimulating growth factor enhanced antigen presentation by T- cells and resulted in the downregulation of genes involved in inflammatory response and proteolysis in an orthotopic mouse model of pancreatic cancer (Zhu, Knolhoff et al. 2014). In addition to this, our group have shown that inhibition of CSF1R with AZD7507, a potent selective inhibitor, causes a reduction in size of well-established tumours in the KPC mouse model of PDAC and increased mouse survival (Candido, Morton et al. 2018). The results from this study are striking, as tumours from KPC mice are relatively resistant most therapeutic agents (Alagesan, Contino et al. 2015). Analysis of AZD7507 treated tumours with flow cytometry revealed a significant increase

in cytotoxic and effector T cell populations. Finally, global gene expression signatures of tumours from KPC mice treated with CSF1R inhibitor showed a significant shift from the poorest prognosis, squamous subtype to more "ADEX" and "immunogenic" signatures of PDAC previously described by Bailey et al. (Bailey, Chang et al. 2016). In addition to reports showing that TAMs are directly involved in maintaining the immune-suppressive environment, studies show that macrophages also favour high stromal collagen expression, and this may impair effective anti-tumour immune response. Studies using an orthotopic colorectal cancer model show that TAMs may contribute to tumour development by remodelling of the ECM. It was demonstrated that TAMs promote collagen fibrillogenesis by direct matrix-deposition, crosslinking and linearization of collagen fibres, which was previously thought to be the role of cancer associated fibroblasts (Afik, Zigmond et al. 2016). This observation suggests that immune cells could be targeted not only to improve the anti-tumour immune response but also to manipulate mechanical features of ECM in pancreatic cancer. This may not be limited to the primary tumour since metastasis of PDAC to the liver has been shown to be reliant on recruitment of infiltrating monocytes which activate the hepatic stellate cells to a secretory myofibroblast phenotype to drive a fibrotic tumour-supporting environment (Nielsen, Quaranta et al. 2016). Taken together, recent studies on tumour infiltrating macrophages support their immune-suppressive role in tumour development and progression, and present experimental evidence that TAMs, or the pathways they regulate, may serve as beneficial clinical targets for treating pancreatic cancer.

1.4 The role of C-C Chemokine receptors in monocytic recruitment

Chemokines represent a large family of small, secreted proteins that signal through a large family of G-protein coupled chemokine receptors that are known as seven transmembrane (7TM) proteins. G-protein coupled chemokine receptors are subdivided into four groups that are defined by the subgroup of chemokines they bind. In 2000, systemic nomenclature was introduced that sub-grouped chemokines into: CC, CXC, CX_3C and XC. These subgroups are defined by the arrangement of conserved cysteine residues within the mature protein. Conserved cysteines form disulphide bonds and maintain protein structure. The CC subgroup of chemokines have these cysteine residues juxtaposed. Receptors for CC chemokines are named accordingly, CC Chemokine Receptors (CCRs). Currently, there are 10 CC chemokine receptors identified. However, receptor specificity is very complex and

many CCRs have multiple ligands. Also, many chemokines bind to several CCRs (Hughes and Nibbs 2018). Like other G-protein coupled receptors, CCRs form active homodimers.

Chemokines and their receptors are best known for their ability to facilitate the migration of cells. Consequently, they play a significant role in the homeostasis of the immune system and its inflammatory responses (Hughes and Nibbs 2018). A study led by Dyer, reported a novel mouse model, which they named iCCR, that lacks the chemokine receptors *Ccr1, Ccr2, Ccr3* and *Ccr5*. Using this mouse they demonstrated the role of the above-mentioned CCR receptors in controlling monocytic and eosinophilic recruitment to resting and inflamed sites, but also found evidence of considerable redundancy (Dyer, Medina-Ruiz et al. 2019).

In mice, the chromosomal locus containing the genes for receptors CCR1, CCR2, CCR3 and CCR5 is located on chromosome 9. The organisation of mouse orthologs is very similar to that in humans. (Figure 1.3) (Nomiyama, Osada et al. 2011). As in humans, this locus is considered to be "pristine" as it contains no other genes, except for *Ccr1l1* which is closely related to *Ccr1*. CCR1 was the first C-C chemokine receptor isolated in 1993 (Neote, DiGregorio et al. 1993). *CCR1* is on human chromosome 3p21 in a cluster together with *CCR2, CCR3, CCR4, CCR5, CCR8, CCR9, XCR1, CX3CR1*, and several orphan genes (Murphy, Baggiolini et al. 2000). *CCR1* is broadly expressed on both hematopoietic and nonhematopoietic cells and binds to several inflammatory CC chemokines. All chemokines that bind to CCR1 also bind to other chemokine receptors. Upon the binding of the ligand, CCR1 activates the classic chemokine signalling pathway through G-proteins (Bachelerie, Ben-Baruch et al. 2014). CCR1 signalling is involved in calcium mobilization, inhibition of adenyl cyclase and chemotaxis (Murphy, Baggiolini et al. 2000).

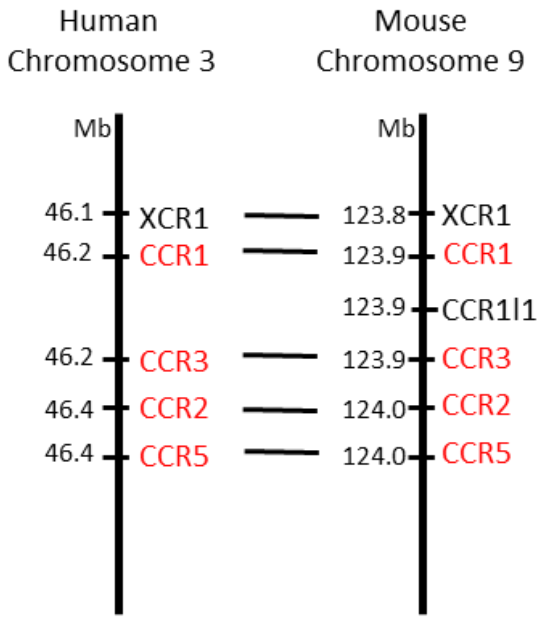

Figure 1.3 Comparative genomic map of C-C chemokine receptors in human and mouse.

Comparative genomic map of a section of human chromosome 3 and mouse chromosome 9 illustrating gene locations for the CCR1, CCR2, CCR3 and CCR5 receptors. Figure adapted from (Nomiyama, Osada et al. 2011).

CCR2 is evolutionarily the oldest receptor out of CCR1, CCR2, CCR3 and CCR5, with the others arising through gene duplication (Nomiyama, Osada et al. 2011). CCR2 signalling outputs include calcium flux, inhibition of adenyl cyclase and chemotaxis. Multiple ligands are able to bind CCR2, with the only specific ligand being monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a potent chemoattractant for mononuclear leukocytes and is involved in the pathogenesis of atherosclerosis. Studies led by Boring, demonstrated that mice lacking both copies of *Ccr2* are viable and otherwise indistinguishable from wild-type controls. They showed that *Ccr2*$^{-/-}$ mice have an impaired immune response and decreased recruitment of monocytes/macrophages upon stimulation (Boring, Gosling et al. 1997). In homeostasis, the maintenance of islet and pancreatic stromal macrophages is CCR2 independent (Calderon, Carrero et al. 2015).

Human CCR3 is a chemoattractant receptor expressed mainly on eosinophils, dendritic cells, basophils, mast cells and a subset of Th2 T cells. It may play a role in allergic inflammation such as asthma. CCR3 is also a coreceptor for HIV-1. Ligands for human CCR3 include eotaxins, and several inflammatory chemokines such as CCL3L1, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15 and CCL28 (Bachelerie, Ben-Baruch et al. 2014). In addition to

this, murine CCR3 is also bound by MIP-1α/CCL3. The expression of CCR3 in mice is mostly limited to eosinophils (Figure 1.4) (Grimaldi, Yu et al. 1999).

CCR5 is most well-known for controlling susceptibility to macrophage-tropic HIV-1 infection, as it acts as a main coreceptor for the virus. Therefore, it attracted a lot of interest from different groups. In 1996, five different reports were published within one week describing its role in HIV pathogenesis. In humans, it is mainly expressed on dendritic cells, hematopoietic progenitor cells, and a subset of Th1 T cells. Low expression of CCR5 is also reported on monocytes. Ligands for CCR5 include MIP-1α, RANTES, MIP-1β, and MCP-2. However, none of these ligands are selective for CCR5 (Murphy, Baggiolini et al. 2000). Mouse CCR5 has similar ligand selectivity to the human ortholog.

In the Dyer et al study, of the iCCR mouse model, that lacks above-mentioned CCR receptors, it was shown that iCCR mice have profound defects in monocyte recruitment and further highlighted CCR2 as a primary driver of monocytic cell recruitment under acute inflammation settings (Dyer, Medina-Ruiz et al. 2019). Given our previous findings on macrophage inhibition through CSF1R resulting in significantly extended KPC mice survival, we hypothesised that CCR receptors would play an important role in monocyte recruitment to pancreatic tissue, and therefore tumorigenesis and cancer progression.

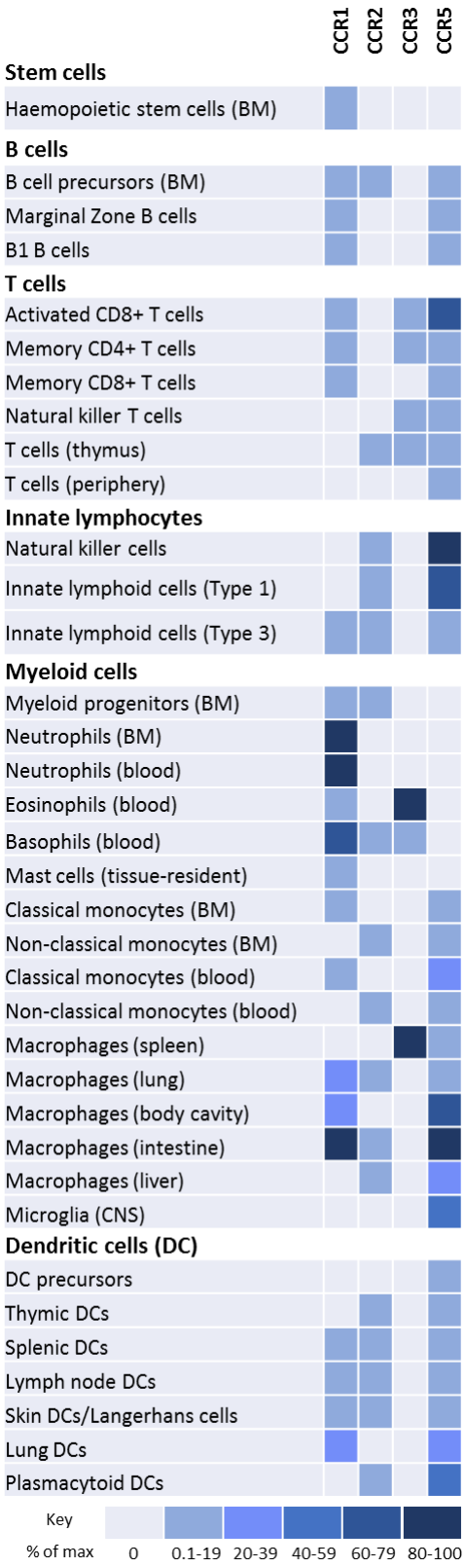

Figure 1.4 Expression of selected chemokine receptors in mouse immune cells.

The figure illustrates the expression of selected C-C motif chemokine receptors in various mouse cells. The figure is based on the transcriptomic data from The Immunological Genome Project database (www.immgen.org). Expression in the specific cell type was assigned a colour according to the percentage of the maximum expression value (Key at the bottom). Figure adapted from (Hughes and Nibbs 2018)

1.5 Immunomodulatory therapies

The progress in immunotherapy has been transformational in the field of cancer care and has brought significant improvements for cancer patients. In general, immunotherapy aims to harness the power of the immune system, enabling recognition and clearance of tumour cells and generation of a long-lasting protection while avoiding inflammatory responses against the host's healthy tissues (Esfahani, Roudaia et al. 2020). Remarkable progress in this field has been made since the late 19th century, when William B. Coley, who is now widely recognized as the father of immunotherapy, first attempted to employ the power of the patient's immune system to fight cancer. He noted that patients with bone sarcomas who had postoperative wound infections would undergo spontaneous regression of the unresected tumours. Thus, Coley started injecting his patients with a mixture of live and inactivated bacteria in order to evoke a strong immune response and hopefully treat the cancer. However, the lack of knowledge on the mechanism of action of such treatment, and the possible detrimental side effects, gave way for alternative therapies. Since then, studies of the immune system have provided a better understanding of the key mediators of the antitumour response and led to the revolutionary wave in cancer immunotherapy. Thus, in following section I will discuss different types of cancer immunotherapy, with a strong focus on their use for pancreatic cancer patients and the limitations encountered.

1.5.1 Immune checkpoint inhibition

Improved understanding of the process of immune surveillance, by which immune cells recognize and eliminate cancer cells, has led to remarkable progress in the field of cancer immunotherapy. The recent discovery of T cell immune checkpoints, such as Cytotoxic T-Lymphocyte–Associated Antigen 4 (CTLA-4) and Programmed Cell Death Protein-1 (PD-1), and development of their inhibitors, has fundamentally changed the treatment strategies for many cancers including melanoma (Wolchok, Kluger et al. 2013) and non-small-cell-lung (Reck, Rodríguez-Abreu et al. 2016). In 2018, the discovery of immune checkpoints was awarded with the Nobel prize to Dr Allison and Dr Honjo.

CTLA-4 is an inhibitory receptor expressed on activated $CD4^+$ and $CD8^+$ T cells. It acts as a major negative regulator of early T cell activation and prevents the hyperactivation of the immune system by competitively inhibiting binding of B7 ligands to the CD28 receptor (Disis 2014). Ipilimumab, a fully humanized CTLA-4 blocking monoclonal antibody was approved in 2011 for clinical use in the USA and Europe. Ipilimumab is used to treat

melanoma and renal cell carcinoma in combination with nivolumab. However, a phase II study in which patients with locally advanced or metastatic pancreatic cancer were treated with ipilimumab as a single agent proved to be unsuccessful (Royal, Levy et al. 2010). Clinical trial data has demonstrated that the combination of ipilimumab with cytotoxic chemotherapy is a safe and tolerable regimen for PDAC. Although one patient in the phase Ib clinical trial for the combination of gemcitabine and ipilimumab had a somewhat durable response of nearly 20 months, adding anti-CTLA-4 antibody to gemcitabine does not appear to be more effective than gemcitabine alone in advanced pancreatic cancer (Kalyan, Kircher et al. 2016).

PD-1 has a more distinct biologic function than CTLA-4. It binds to PD-L1 and PD-L2, which are expressed on the surface of dendritic cells, macrophages or tumour cells, and inhibits the inflammatory activity of the T cells on which it is expressed. Both PD-1 and PDL-1 inhibitors are approved for the treatment of various malignancies such as melanoma, non-small cell lung cancer, Merkel cell carcinoma and urothelial carcinoma. In pancreatic cancer, PD-L1 expression correlates with worse survival (Nomi, Sho et al. 2007). Thus, a clinical trial (NCT02331251) was designed to evaluate the toxicity and efficacy of PD-1 inhibitor, in combination with chemotherapy, in metastatic pancreatic cancer (Weiss, Blaydorn et al. 2018). The efficacy of this combination was slightly improved compared with previously reported results for standard chemotherapy regimens, suggesting that further studies would follow. However, a subsequent phase II randomized clinical trial of PD-L1 antibody with or without CTLA-4 antibody in metastatic PDAC patients who have received one prior chemotherapy regimen, failed to reach efficacy threshold (O'Reilly, Oh et al. 2019).

One of the reasons for the lack of response to immunotherapy in pancreatic cancer is that pancreatic tumours are classed as immunologically ‘cold’. One of the main contributing factors of ‘cold’ tumours is their lack of antigenicity which is usually linked to the mutational burden of the tumours. High mutational burden means that cancer cells make numerous mutated proteins, leading to the release of more neo-antigens that are then recognized by the T cells. Upon detecting tumour antigens, T cells are activated and able to elicit tumour cell destruction. However, in contrast to other cancer types such as melanoma or lung cancer, pancreatic tumours have half as many somatic mutations making them less antigenic, relatively (Torphy, Zhu et al. 2018). High tumour mutational burden has been clinically linked with better response to anti PD-1 in patients with non-small cell lung cancer (Rizvi, Hellmann et al. 2015). A high mutation burden has been hypothesised to evoke an anti-tumour immune response directed against tumour neoantigens which could be improved with the blockage of immune checkpoints. Interestingly, analysis of publicly available

datasets of PDAC genomic profiles demonstrates that PDAC cells express quality neoantigens. Nevertheless, one of the key features of PDAC stroma is a distinct exclusion of T cells. What is more, T lymphocytes infiltrating tumours are actively suppressed in the PDAC tumour microenvironment by the immune-suppressive myeloid compartment. Several groups have focused on investigating the factors mediating immune suppression in the PDAC microenvironment (Liu, Xu et al. 2019). Thus, understanding the role of the tumour microenvironment in facilitating immune escape in pancreatic cancer holds great potential for improving the success of immunotherapy in the future.

1.5.2 Vaccine therapy

The main goal of vaccine-based immunotherapy is to activate and expand tumour specific T cells by increasing the presentation of tumour-associated antigens (TAAs). There are several major categories of cancer vaccines: cell-based vaccines, peptide vaccines and genetic vaccines (Disis 2014). Tumour cell vaccines can also be derived from patient- specific tumour cells or produced from established cell lines. Several vaccines have been trialled for the treatment of pancreatic cancer. Clinical trials have been designed targeting tumour markers such as carcinoembryonic antigen (CEA), mucin 1 (MUC1) (Kaufman, Kim-Schulze et al. 2007), telomerase (Bernhardt, Gjertsen et al. 2006) as well as KRAS, which is mutated in more than 90% of pancreatic cancer patients. Some promising results were demonstrated in clinical trials with synthetic KRAS-derived peptides for patients with resected pancreatic cancer and late stage disease (Abou-Alfa, Chapman et al. 2011). Although vaccines proved to be well tolerated, their use has not translated into clinical use due to low efficacy. More favourable responses were observed using dendritic cell vaccines. Dendritic cells are considered the most potent antigen presenting cells. They prime naïve T cells and generate memory T and B cells that elicit rapid antigen-specific immune responses (Salman, Zhou et al. 2013). Dendritic cell vaccination involves isolation of DCs, stimulation of them *ex vivo* with TAAs, and re-infusion back into patients. Two clinical trials have tested the safety and efficacy of DC-based vaccines in pancreatic cancer patients. Firstly, patients were administered with vaccine upon tumour resection. The follow up at 4 years revealed that 4 out of 12 patients showed no evidence of recurrence. In the second study, a DC-based vaccine, in combination with chemotherapy, was administered to 49 patients with inoperable disease. Complete remission was seen in 2 of these patients, 5 patients had a partial remission, while 10 had stable disease (Lepisto, Moser et al. 2008, Kimura, Tsukada et al. 2012). Results from this trial show that combination treatment is well tolerated and suggests

that further investigation is needed assess the efficacy of this treatment regime in a large-scale study.

1.5.3 Therapies promoting T cell priming

Limited response to immune checkpoint blockade therapies (anti-PD-L1, anti PD-1 and anti-CTLA-4) in pancreatic cancer patients, suggest the presence of additional immunosuppressive mechanisms in the tumour microenvironment. The focus was drawn to costimulatory molecules that amplify initial activating signals to T cells. Recently, new therapeutic attempts demonstrated some promising results. As a result, the costimulatory protein, CD40 is emerging as a promising therapeutic target for pancreatic cancer. It is a member of the tumour necrosis factor superfamily and is mainly expressed by B cells, dendritic cells, monocytes as well as some normal and malignant cells. The main function of CD40 is to activate antigen presenting B cells in response to its ligand, CD40L. CD40L binding to its receptor upregulates surface expression of costimulatory and MHC molecules, induces the release of proinflammatory cytokines, and induces T-cell activation. Agonist mediated CD40 activation was demonstrated to induce macrophage repolarisation. Reprogramming of macrophages was necessary for CD40 induced tumour regression in genetically engineered mouse model of pancreatic cancer (Beatty et al. 2011).

Studies using mouse syngeneic orthotopic models of pancreatic cancer, demonstrated improved overall survival of mice treated with PD-L1 inhibitor and agonist antibody for CD40. It was demonstrated that αCD40 treatment transformed the tumour microenvironment. Upregulation of Th1 chemokines and increased cytotoxic T cell infiltration was observed in treated tumours. Furthermore, αCD40 drove systemic APC maturation, memory T cell expansion, and upregulated tumour and systemic PD-L1 expression (Luheshi, Coates-Ulrichsen et al. 2016). The results from this study along with others (Winograd, Byrne et al. 2015) further support the potential of combining CD40 agonist with immune checkpoint inhibitors.

The phase Ib clinical trial of the agonistic CD40 monoclonal antibody, sotigalimab, was the first in-human trial that evaluated the use of two different doses of CD40 agonists in patients with untreated metastatic pancreatic cancer. Treatment was given in combination with chemotherapy and with or without the PD-1 blocking antibody nivolumab (O'Hara, O'Reilly et al. 2021). Overall, the study demonstrated no synergistic toxicity of combination

treatments and some preliminary efficacy. Promising results indicate that larger and controlled studies will follow.

1.6 GEMM models for studying pancreatic cancer

1.6.1 Modelling the biology of pancreatic cancer in mice

Based on advances in our knowledge of the underlying molecular biology of PDAC, genetically engineered mouse (GEM) models were developed. Importantly, GEM models very closely represent clinical signs of pancreatic cancer such as biliary obstruction, ascites, cachexia, as well as recapitulating the tumour histopathology (Neesse, Algul et al. 2015). One of the most studied murine models of pancreatic cancer is the KPC mouse model which harbours $Kras^{G12D}$ and $Trp53^{R172H}$ mutations targeted specifically to the pancreas using a Cre-loxP based system (Hingorani, Wang et al. 2005). KPC and similar models provided us with further insight into the development and progression of the disease as well as several novel therapeutic approaches.

While the recent improvements in our knowledge of the underlying mechanisms of PDAC has not yet led to improved treatment strategies, this understanding has laid the ground for the development of genetically engineered mouse models that keep providing us with further insight into the disease and allow the robust testing of new therapeutics. Initially, the generation of mice in which pancreas-specific expression of oncogenic *KrasG12D* was engineered led to a model that recapitulated the stepwise development of PDAC seen in humans. The study group, led by David Tuveson, took advantage of a conditional allele containing a transcriptional and translational silencing cassette (STOP) flanked by two loxp sites that was first described in 2001 (Jackson, Willis et al. 2001). The 'LOX-STOP-LOX' cassette prevents non-specific expression of the mutant *Kras* allele, which contains a point mutation and results in a glycine to aspartic acid substitution in codon 12. G12D transition is the most common mutation in *KRAS* is human pancreatic cancer. It results in the constitutive activation of downstream signalling by Ras effector pathways. The expression of oncogenic *Kras* was targeted to pancreatic progenitor cells by crossing *LSL-KrasG12D* mice with mice that express Cre recombinase from pancreas specific promoters such as PDX-1 or P48 (Hingorani, Petricoin et al. 2003). *Pdx-Cre; LSL-KrasG12D* mice (referred to as KC), were viable and exhibited no abnormalities at an early age. However, by 8 weeks of age mice started to develop early PanIN lesions that increased both in number and grade over a period of 2 years. A proportion of these mice developed pancreatic ductal

adenocarcinomas with median survival of 14 months (Hingorani, Petricoin et al. 2003). This model was the first histologically accurate model of PDAC and proved that *Kras* mutations are sufficient to drive the initiation of pancreatic cancer formation in mice. However, the long latency and only occasional progression to invasive tumours made KC mice cost ineffective and limited their use for preclinical studies (Westphalen and Olive 2012). Therefore, further studies have intercrossed KC mice with mice bearing additional tumour suppressor gene mutations observed in human cancer in order to assess whether this could generate a more rapid mouse model for PDAC. Combination of *LSL-KrasG12D* with additional deletion or mutation of tumour suppressor genes such as $Cdkn2a^{fl/+}$ (Aguirre, Bardeesy et al. 2003), LSL-$Trp53^{R172H/+}$ (Hingorani, Wang et al. 2005) and $Trp53^{lox/lox}$ (Bardeesy, Cheng et al. 2006) led to rapid development of highly aggressive and metastatic PDAC in mice. Today, one of the most utilised models is the *Pdx1-Cre, LSL-*$Kras^{G12D/+}$, *LSL-*$Trp53^{R172H/+}$ mouse model (now referred to as KPC). The median survival of KPC mice is ~5.5 months and the tumour phenotype is 100% penetrant. What is more, disease progression and the end point tumours closely resemble what is seen in human pancreatic cancer.

1.6.2 Studying the role of macrophages in GEMM of PDAC

Macrophages represent the major inflammatory cell population in pancreatic cancer, both in the human disease and in mouse models of PDAC. However, the exact contribution of macrophages towards tumour initiation and progression is yet to be understood. Several studies have addressed the role of macrophages in pancreatic cancer using various methods.

Pharmacological TAM depletion is among the most widely used study methods to investigate the role of macrophages. Depletion of macrophages is usually performed with clodronate-liposome solution. The clodronate molecules are encapsulated in liposomes, due to their short half-life. The liposomes are promptly recognized and engulfed by macrophages. In macrophages, chlodronate triggers apoptosis (van Rooijen and Hendrikx 2010). However, one caveat to this approach is that it also depletes other phagocytes, including dendritic cells. Therefore, to ensure that the effect observed is specific to macrophage depletion, reconstitution of macrophages by adoptive transfer is needed (Weisser, van Rooijen et al. 2012).

Macrophages can also be targeting using small molecule inhibitors or antibodies for CSF1R. CSF1R inhibitors represent an exciting new class of immune-modulatory drugs and are often used in research to achieve macrophage depletion in various GEMMs.

Systemic macrophage depletion *in vivo* can also be achieved by crossing mice onto a CD11b-diphtheria toxin receptor (DTR) background. These transgenic mice have an inducible system that transiently depletes macrophages in various tissues. To study the role of macrophages in PDAC, CD11b-DTR mice can further be crossed with KPC mice. However, studies investigating brain injury discovered a concerning finding. It was observed that macrophage depletion using the CD11b-DTR model induces brain inflammation in the absence of injury. This is a potentially confounding effect that makes interpretation of results from disease models difficult (Frieler, Nadimpalli et al. 2015). What is more, it is necessary to mention that CD11b is a myeloid cell marker, hence macrophage depletion in CD11b-DTR model is accompanied with depletion of all myeloid cells expressing CD11b. Therefore, the use of this model does not allow the segregation of roles executed by different CD11b expressing myeloid subsets. Finally, as with all GEMMs, the CD11b-DTR model requires the time and expense involved in backcrossing mice. However, the benefits of GEMMs could be considered to outweigh the costs because off target effects can be excluded.

2 MATERIALS AND METHODS

2.1 Animal Work

All animal experiments were performed under UK Home Office licence. Mice were maintained in positively pressurised individually ventilated cages (IVCs) unless enrolled on treatment experiments when they were transferred to conventional caging. These mice were monitored and handled in a laminar flow changing station. Once weaned, mice were genotyped by Transnetyx (Cordoba, TN, USA). All mice were given access to standard diet (CRM (E) expanded diet from Special Diet Services; Cat: 801730) and water ad libitum. Environmental enrichment in the form of nesting materials and fun tunnels was provided. Mice of both sexes were included in all studies.

2.1.1 Generation of spontaneous GEMMs of pancreatic tumourigenesis

Pdx1-Cre, LSL-Kras$^{G12D/+}$*, LSL-Trp53*$^{R172H/+}$ mice were first described by Hingorani et al (Hingorani, Wang et al. 2005) and were bred in house. LoxP site-flanked STOP cassettes preceding the mutant *Kras* and mutant *Trp53* genes are recognised by Cre and excised upon Cre expression, leading to the expression of the transgenes. To drive Cre expression, the KPC model uses the pancreas specific *Pdx1* (pancreatic, duodenal homeobox 1) promoter, which in mouse is expressed from embryonic day 8.5-9.0 and continues until embryonic day 12.0-12.5. Pdx1 expressing cells give rise to the developing pancreatic buds, and eventually, cells of all lineages within the pancreas such as ductal, acinar, and endocrine cells. Thus, upon the action of Cre recombinase, constitutively active *Kras*$^{G12D/+}$ and mutant *Trp53*$^{R172H/+}$ are expressed in all pancreatic cell lineages.

Ccr1-5$^{-/-}$ (CCR1-5KO, also known as iCCR) mice on a C57BL/6 background were kindly gifted by Gerard J. Graham (University of Glasgow) and were previously described by Dyer et al (Dyer, Medina-Ruiz et al. 2019). In house, we crossed *Ccr1-5*$^{-/-}$ mice with *Pdx1-Cre, LSL-Kras*$^{G12D/+}$*, LSL-Trp53*$^{R172H/+}$ (KPC) mice. By breeding appropriate genotypes of these mice, cohorts of *Pdx1-Cre, LSL-Kras*$^{G12D/+}$*, LSL-Trp53*$^{R172H/+}$*, Ccr1-5*$^{+/+}$*, Pdx1-Cre, LSL-Kras*$^{G12D/+}$*, LSL-Trp53*$^{R172H/+}$*, Ccr1-5*$^{+/-}$, and *Pdx1-Cre, LSL-Kras*$^{G12D/+}$*, LSL-Trp53*$^{R172H/+}$*, Ccr1-5*$^{-/-}$ mice were generated.

Ighm$^{-/-}$ (also known as muMt^{-}) mice on C57BL/6 background were obtained from Jackson laboratories (Kitamura, Roes et al. 1991). In house, we crossed *Ighm*$^{-/-}$ mice with KPC mice

described previously. By breeding appropriate genotypes of these mice, cohorts of *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ *Ighm*$^{+/+}$, *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$, *Ighm*$^{+/-}$, and *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$, *IgHm*$^{-/-}$ mice were generated (Figure 2.1).

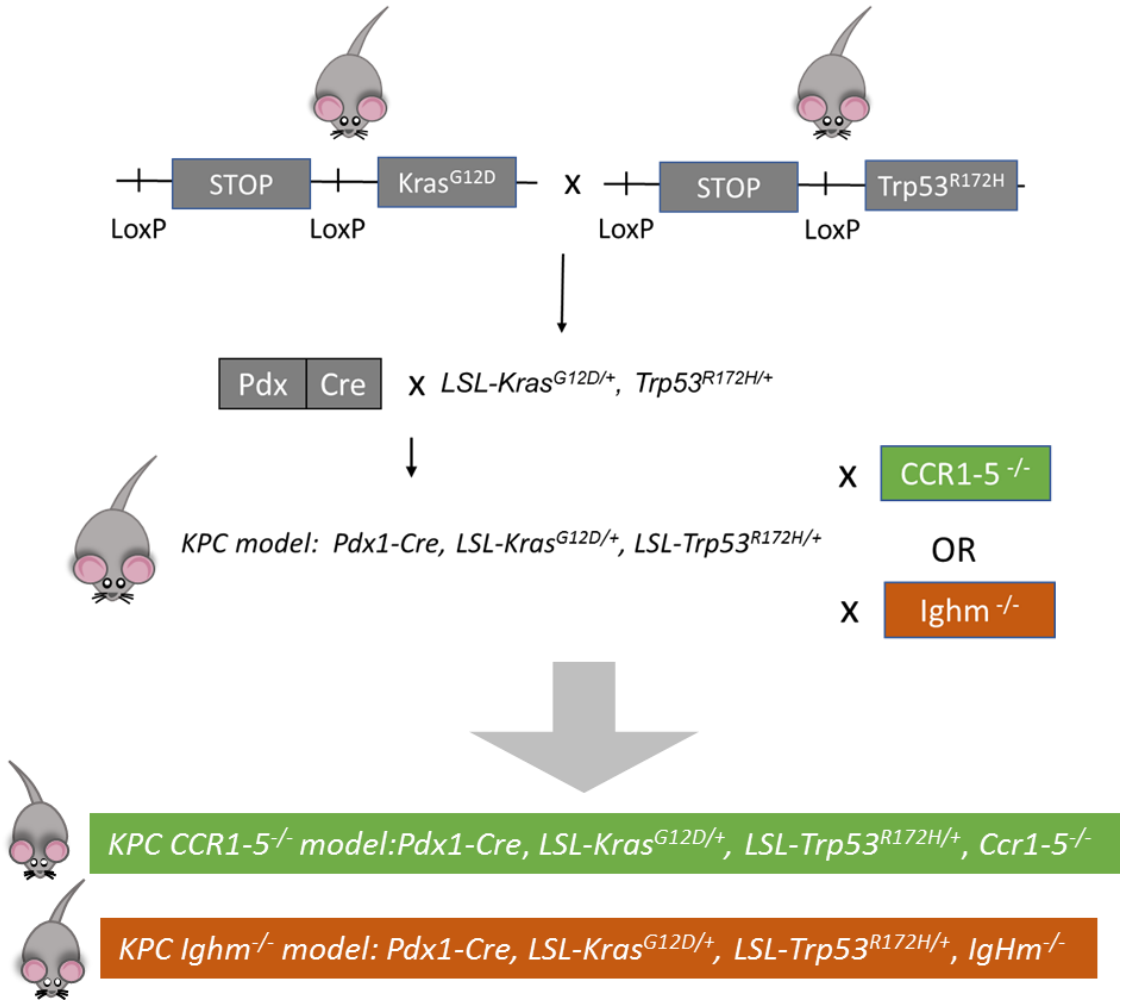

Figure 2.1 Schematic outlining the breeding of the GEMMs used for this study.

2.1.2 Genotyping

Once weaned, mice were ear notched for identification and tissues were sent to Transnetyx (Cordova, TN, USA) for genotyping. Transnetyx uses automated real time PCR to confirm allelic status in ear tissue. Mice of desired genotypes were saved for further matings or enrolled into cohorts, while remaining mice were culled.

2.1.3 Experiments in GEMMs

Four main types of experiment were performed in GEMM cohorts:

1. Analysis of tissue from mice of control and experimental genotypes at time-point.
2. Aging of cohort mice until humane endpoint.
3. Treatment of *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$, *Ccr1-5*$^{+/+}$ (KPC *WT)* or *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$, *Ccr1-5*$^{-/-}$ mice from 6 weeks of age. This time-point was chosen in order to investigate the effect of macrophage depleting treatment on the formation of early PanIN lesions and the development of PDAC in the presence or absence of CCR1-5.
4. Treatment of tumour-bearing *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice. Mice were palpated weekly until the presence of PDAC was observed. PDAC was confirmed using ultrasound imaging and mice were allocated for the treatment cohorts. Treatment of the tumour-bearing mice was performed in order to investigate the impact of the treatment on disease progression and to replicate clinical settings.

2.1.4 Syngeneic Murine Experiments

Primary *C57BL/6* KPC and KC PDAC cell lines were generated in our lab and mycoplasma tested. Cells were grown to ~ 70% confluence in T75 tissue culture flasks. Cells were trypsinised and washed twice with PBS. The cell pellet was resuspended in 10mL PBS and cells were counted using the Countess™ II Automated Cell Counter (ThermoFisher, Cat: AMQAX1000). Cells were then pelleted again and resuspended in ice cold matrigel at a concentration of 1000 cells per 5μL for immediate injection into the tail of the pancreas of *C57Bl/6* WT and *Ighm*$^{-/-}$ mice.

Wild type *C57Bl/6* mice were purchased from Charles River Laboratories. *C57Bl/6 Ighm*$^{-/-}$ mice were generated in house. Prior to the surgery, the mouse abdomens were shaved. Mice received analgesia (Rimadyl®) in their drinking water for 24 hours prior to the surgery. During the surgery, mice were anaesthetised using volatile isoflurane delivered by medical air via nosecone. A left paramedian incision was made and the skin separated from the body wall by blunt dissection. The peritoneum was opened under direct vision and the spleen and tail of pancreas externalised. 1000 KPC or KC PDAC cells in 5μL matrigel were injected directly into the pancreas using a Hamilton® syringe. The body wall was sutured with absorbable, coated VICRYL Plus (Ethicon Inc.). Wound clips (Reflex) were applied to close the wounds, and these were removed 7 days after the procedure. Mice were given a post-operative subcutaneous injection of Buprenorphine and placed in a warmed cage to recover and received analgesia (Rimadyl®) in their drinking water for 48 hours post-surgery.

2.1.5 End Points

Mice were monitored at least 2 times per week and culled when any of these symptoms were observed: abdominal distension, loss of body conditioning, intermittent hunching, reduced mobility or jaundice. Mice displaying any of these symptoms were classed as end point and tumours collected from these animals were termed as end point tumours.

Mice harbouring the *Pdx1-Cre* and *LSL-Kras*^{G12D} alleles can also develop benign papillomas from 8 weeks of age and lymphomas (usually thymic). Mice were culled if they developed lymphoma or if a papilloma exceeded 15mm in size, limited their normal behaviour or became cancerous. Mice culled due to any extra-pancreatic pathologies were censored from analyses.

A more detailed description of clinical signs of disease is outlined in UK Home Office project licence PP8411096 which was always adhered to.

Mice were humanely culled by exposure to carbon dioxide gas in a rising concentration, or by dislocation of the neck at cervical vertebrae 1-3. Death was confirmed by a secondary Schedule 1 method.

2.1.6 Sampling of mice

Post-mortem dissection was performed after euthanasia. Mouse was prepared by the application of 70% ethanol. Blood was collected by cardiac puncture into EDTA-coated tubes. Using scissors, the peritoneum was opened and the organs visually inspected. The presence of pancreatic tumour was confirmed. Liver, diaphragm and lungs were inspected for possible metastases. Organs were removed using scissors and forceps and placed in 10% neutral buffered formalin. Pieces of the tumour were placed in RNAlater (Thermo Scientific, Cat: AM7020) as well as snap frozen on dry ice for further analyses. The rest of the tumour was fixed in 10% neutral buffered formalin. Following fixation in formalin, tumour tissue and organs were paraffin-embedded and 4 µm sections cut and mounted on charged slides. Slides were stained were stained with Haematoxylin and Eosin (H&E) for examination of tumour histology and inspection for the presence micrometastases in organs.

2.1.7 Drug Treatments

Depletion of B cells using anti-CD20 antibody

Pdx1-Cre, LSL-Kras$^{G12D/+}$*, LSL-Trp53*$^{R172H/+}$ mice were monitored for the presence of pancreatic tumours by weekly palpation. The presence of the tumour was confirmed by ultrasound imaging and tumour volume measured. Mice with confirmed tumours were randomly assigned to one of 2 groups: 250µg anti-CD20 antibody (mAb SA271G2; Biolegend, Cat:152104), once every 4 weeks, via intravenous injection; or weekly dose of IgG2α κ isotype control (Biolegend, Cat:400566) given at 200µg by intraperitoneal injection.

CSF1R Inhibition using AstraZeneca CSF1R-inhibiting small molecule

Pdx1-Cre, LSL-Kras$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$, *CCR1-5*$^{-/-}$ and *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ mice were treated with 100mg/kg CSF1R-inhibiting small molecule (AZD7507 (Scott, Dakin et al. 2013)), twice daily, via oral gavage from 6 weeks of age. The structure is available at https://pubchem.ncbi.nlm.nih.gov/compound/25001557. Mice were treated until the clinical end point.

CSF1R Inhibition using Bristol Myers Squibb antibody

Pdx1-Cre, LSL-Kras$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ mice were monitored for pancreatic tumours by weekly palpation. The presence of the tumour was confirmed by ultrasound imaging and tumour volume measured. Mice with confirmed tumours were randomly assigned to one of 2 groups: anti-CSF1R antibody (Bristol Myers Squibb) given at 600µg/100µL by intraperitoneal injection, weekly, or IgG2α κ isotype control (Biolegend, Cat:400566) given at 200µg by intraperitoneal injection, weekly. Mice were treated until the clinical end point.

2.2 Flow Cytometry

Flow cytometry is a cell analysis technique that is used to study individual cell populations in a multi-parametric way. It allows characterization of mixed cell populations from blood and bone marrow as well as solid tissues such as tumours. Cells are distinguished based on their size, granularity, and expression of extracellular and intracellular markers. Immunophenotyping experiments with flow cytometry utilize fluorochrome-conjugated antibodies that are targeted against antigens on the cell surface that are then detected by their emission wavelength light. In this thesis, flow cytometry was used to study immune cell populations from peripheral blood, bone marrow and pancreatic tumours.

2.2.1 Isolation of cells from PDAC

Tumours were isolated from mice using scissors and forceps, placed in ice cold supplemented DMEM (DMEM + 5% FBS + 1% Pen-Strep-Glu) and transported to the lab on ice. In the lab, tumours were finely cut using scalpels and placed in gentleMACS C tubes (Miltenyi Biotec, Cat:130-093-237) together with 2.37mL of DMEM and enzyme mix from Tumour dissociation kit (Miltenyi Biotec, Cat:130-096-730). Tumours were dissociated using gentleMACS™ Dissociator (Miltenyi Biotec, Cat:130-093-235) program '37C_m_TDK_2'. Once dissociated, the substance was passed through a 70µm nylon mesh filter and 10mL of FACS buffer (PBS supplemented with 2% FBS) added to stop the enzymatic reaction. Samples were centrifuged at 1500RPM for 5 minutes. Pellets were re-suspended in PBS followed by the cell debris removal for the macrophage polarization panel or through Percoll density gradient for the T cell panel.

2.2.2 Isolation of cells from the bone marrow

Cells were isolated from the bone marrow by removing the femur and tibia from mouse hind legs. The attached muscles and tendons were then removed before moving to the tissue culture hood. The bones were transferred to the tissue culture hood in PBS. In the hood, the bones were washed with 70% ethanol. The proximal and distal epiphyses were cut using scissors. The bone marrow was flushed out using a 25-gauge needle attached to a 10 mL syringe containing 5 mL complete DMEM (DMEM +5% FBS +1% Pen-Strep-Glu). Cells were passed through a 70µm nylon filter and spun down at 1500RPM for 5 minutes at 4°C. Red blood cell lysis was performed using eBioscience™ 1X RBC Lysis Buffer (ThermoFisher, Cat: 00-4333-57). Cells from a single mouse (4 long bones) were incubated with 1mL of 1xRBC lysis buffer for 5 minutes at room temperature. Cells were then washed with PBS, and pellets used for a downstream staining for flow cytometry.

2.2.3 Isolation of cells from the blood

Blood was collected by cardiac puncture in EDTA coated tubes to prevent coagulation. 50µL of blood was added to 1mL of 1x RBC Lysis Buffer and incubated for 10 minutes at room temperature. Cells in buffer were diluted in 2 mL of FACS buffer to neutralise the lysis buffer and spun down at 1500RPM for 5 minutes at 4°C. Supernatant was aspirated carefully using a 2mL pipette with an attached D200 plastic tip. Pellets were used for downstream staining for flow cytometry.

2.2.4 Cell debris removal

Single cell suspensions of tumour cells used for the macrophage polarization panel were subjected to cell debris removal. Debris removal solution (Miltenyi Biotec, Cat:130-109-398) was used according to the protocol by Miltenyi. In short, single cell suspensions were washed with ice-cold PBS and centrifuged at 300g for 10 minutes at 4°C. The supernatant was aspirated, and the pellet was resuspended in 3100µL of cold PBS and 900µL debris removal solution. Suspensions were carefully mixed in 15 mL tubes using a pipette and were then overlaid gently with 4mL of cold PBS. Samples were centrifuged at 3000g for 10 minutes at 4°C and three phases were formed. The two top phases were discarded, and the bottom layer washed with cold PBS at 1000g for 10 minutes at 4°C. The pellets were

resuspended in PBS and cell counting performed using the Countess™ II Automated Cell Counter (ThermoFisher, Cat:AMQAX1000).

2.2.5 Percoll density gradient

A Percoll gradient was used on single cell suspensions of tumour cells used for the T cell panel in order to enrich for lymphocytes. First, 15mL tubes were coated with 10% FBS DMEM to ensure smooth movement of liquid down the tube walls when pouring different density layers. Concentrations of 80%, 40% and 20% Percoll (Sigma-Aldrich, Cat:GE17-0891-01) were prepared fresh before use according to the table below (Table 2.1).

	100% Percoll	1x PBS	Total (per sample)
80% Percoll	2.8 mL	0.7 mL	3.5 mL
40% Percoll	1.4 mL	2.1 mL	3.5 mL
20% Percoll	0.3 mL	1.2 mL	1.5 mL

Table 2.1 Dilution of stock solution of Percoll in preparation to perform Percoll density fractionation.

Next 3mL of 80% Percoll was added to the coated 15mL tubes. Single cell suspensions were spun down at 1500RPM for 5 minutes and the pellets were resuspended in 3mL of 40% Percoll. The suspension was gently pipetted onto the 80% Percoll phase. Finally, 1mL of 20% Percoll was pipetted onto the 40% Percoll phase. Samples were centrifuged at 1800RPM for 30minutes at 24°C with the lowest brake setting.

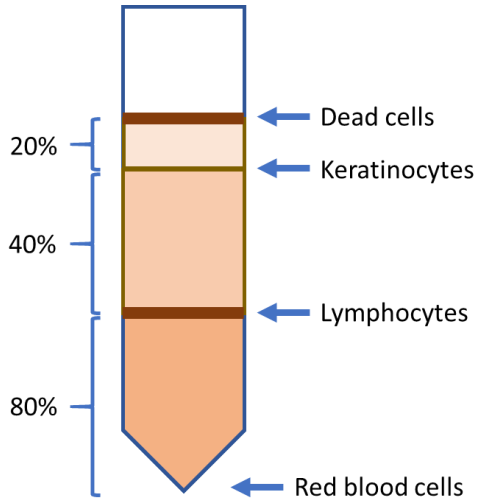

Figure 2.2 Separation of lymphocytes using Percoll gradient.

Separation of lymphocytes using Percoll density gradient and centrifugation. Decreasing concentration of Percoll dilutions were layered as shown in the diagram. After the centrifugation, three distinct layers were visible.

Three layers were visible after centrifugation (Figure 2.2**)**. The top layer of 20% Percoll and a half of the 40% phase were aspirated. 2mL of the 80%/40% interphase containing lymphocytes was carefully collected and transferred to a new 50mL falcon tube. Cells were washed with 8mL FACS buffer and centrifuged at 1800RPM for 5minutes. The pellets were resuspended in PBS for cell counting using the Countess™ II Automated Cell Counter.

2.2.6 Extracellular staining protocol

After cell counting using the Countess™ II Automated Cell Counter (ThermoFisher, Cat:AMQAX1000), up to $1x10^6$ cells were taken for the unstained control and placed in a separate tube and kept on ice until performing flow cytometry. The live-dead stain was added to the rest of the cell suspension in order to exclude dead cells from the analysis. Cells were incubated for 15 minutes in the dark at room temperature. Cells were then washed once with FACS buffer (PBS supplemented with 2% FBS). For the staining of extracellular proteins, up to $1x10^6$ cells were loaded into individual round-bottom polystyrene test tubes (ThermoFisher, Cat:10579511). Cell suspensions were centrifuged at 1500RPM for 5mintes at 4°C and the supernatant discarded. Cells were then incubated for 15 minutes at 4°C with 50µL of an anti-CD16/32 antibody diluted to a final working concentration of 5µg/mL in FACS buffer to prevent non-specific Fc binding. After incubation, 50µL of antibody mixture diluted in FACS buffer was added and samples incubated for a further 25 minutes at 4°C. The cells were then washed with FACS buffer and centrifuged at 1500RPM for 5 minutes and the supernatant was discarded. Cell pellets were resuspended in 400µL of FACS buffer (for the T and B cell panel) or fixed for intracellular staining (macrophage polarization panel).

2.2.7 Intracellular staining protocol

To stain for intracellular cytoplasmic proteins, extracellular staining was first performed as previously described. After the incubation with extracellular antibody mixtures, cells were washed with FACS buffer and resuspended in 200µL of 2x fixation buffer (ThermoFisher, Cat:00-8222-49) diluted in FACS buffer. Samples were kept overnight at 4°C. The following day, 2mL of 1 x permeabilization buffer (ThermoFisher, Cat:00-8333-56) diluted in distilled water was added. Samples were centrifuged at 1500RPM for 5minutes, and the supernatant discarded. Cells were then incubated with 50µL of a mixture of intracellular antibodies

diluted in FACS buffer and incubated for 25 minutes at room temperature, protected from the light. After incubation, samples were washed with 2mL of 1x permeabilization buffer and resuspended in 400µL of FACS buffer. Cells were stored at 4 °C in the dark until performing flow cytometry.

2.2.8 Antibodies

Panel	Antigen	Clone	Conjugated	Company	Cat #	Dilution
Macrophage polarization	F4/80	BM8	BV650	Biolegend	23149	1:50
	CD11b	M1/70	FITC	eBioscienc	11-0112-41	1:250
	MHCII	M5/114.15.	APC-eFluor	eBioscienc	47-5321-82	1:250
	CD80	16-10A1	PerCP-eFluor	eBioscienc	46-0801-82	1:250
	CD86	GL1	PE/Cy7	eBioscienc	25-0862-82	1:250
	CD206	C068C2	APC	Biolegend	141708	1:200
	Arg1	A1exF5	PE	eBioscienc	12-3697-82	1:250
	Live-dead	-	Zombie	Biolegend	423104	1:500
B cell	CD45	30-F11	FITC	eBioscienc	11-0451-82	1:300
	B220	RA3-6B2	APC	eBioscienc	17-0452-82	1:300
	CD3	17A2	PerCP-Cy5.5	Biolegend	100217	1:300
	Ly6C	HK1.4	PeCy7	eBioscienc	25-5932-82	1:300
	Ly6G	1a8	PE	eBioscienc	12-9668-82	1:300
	CD19	6D5	APC	Biolegend	115511	1:300
	DAPI	-	-	ThermoFis	D1306	1:500
T cell	CD3	17A2	FITC	Biolegend	100203	1:150
	CD45	30-F11	Pacific Blue	Biolegend	103125	1:150
	CD4	RM4-5	APC	eBioscienc	17-0042-82	1:200
	CD8	53-6.7	PerCP-Cy5.5	eBioscienc	45-0081-82	1:150
	CD25	PC61.5	PE	eBioscienc	25-0251-82	1:200
	CD69	H1.2F3	PECy7	Biolegend	104511	1:150
Other	CCR2	SA203G11	BV510	Biolegend	150617	1:250
	CD16/32	93	-	Biolegend	101320	1:50

Table 2.2 Antibodies used in flow cytometry.

This table documents the target of the antibody, clone, concentration used, along with manufacturer and catalogue number.

2.2.9 Gating strategy for B cell panel

For B cell identification, debris was initially removed by gating side-scattered (SSC) light intensity high (SSC^{Hi}) and forward-scattered (FSC) light intensity high (FSC^{Hi}) cell populations (Figure 2.3a). Doublets were excluded based on their increased area (Figure 2.3b). Non-viable cells were then excluded based on their uptake of a live-dead dye, DAPI (4′,6-diamidino-2-phenylindole) (Figure 2.3c). Double positive gates were then applied on live cells and used to identify B cells ($CD45^+CD19^+$; $CD45^+B220^+$ Figure 2.3d-e), neutrophils ($CD45^+Ly6G^+$ Figure 2.3f), and monocytes ($CD45^+Ly6C^+$ Figure 2.3g).

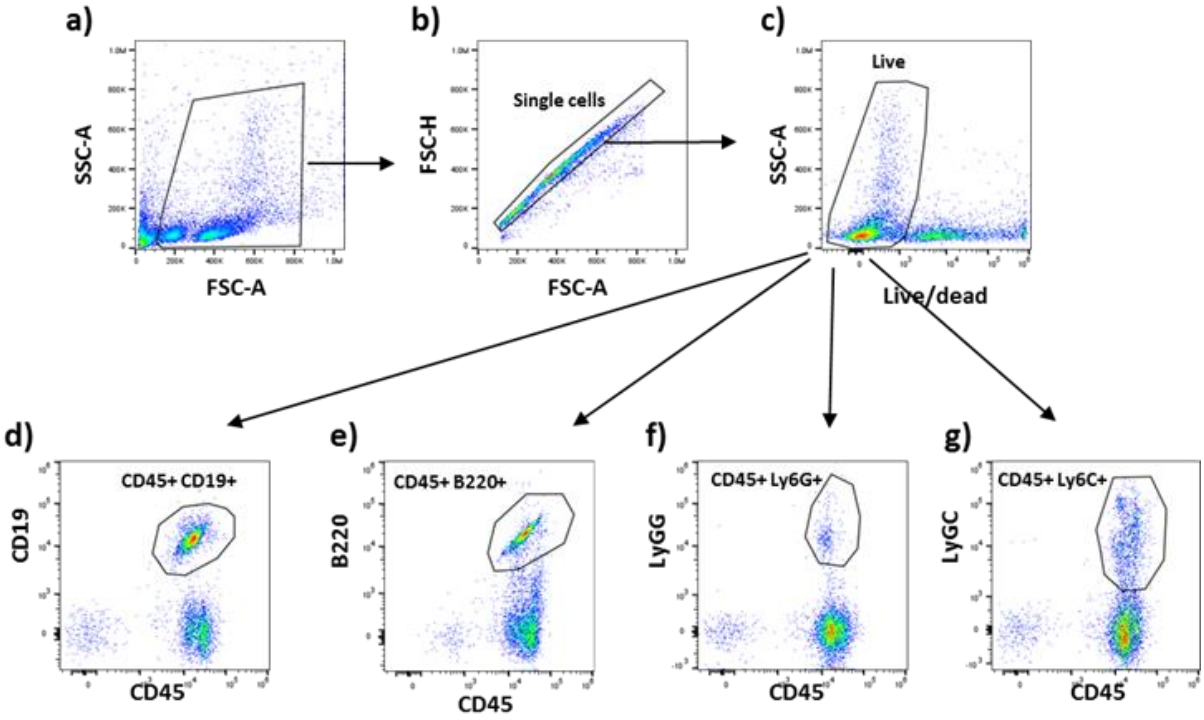

Figure 2.3 Gating strategy for the identification of B cells in the peripheral blood by flow cytometry.

Total B cells, identified as $CD45^+CD19^+$ or $CD45^+B220^+$, were gated as follows: (a) SSC^{Hi} FSC^{Hi}, (b) Single cells, (c) Live cells. (d-e) B cell were identified as double positive cells for $CD45^+CD19^+$ or $CD45^+B220^+$, (f) Neutrophils ($CD45^+Ly6G^+$), (g) Monocytes ($CD45^+Ly6C^+$). All gating strategies were drawn with the help of appropriate controls (FMOs and unstained controls).

2.2.10 Gating strategy for T cell panel

A T cell panel was used to investigate T lymphocyte populations in KPC *Ighm*$^{-/-}$ and KPC control mice. Different T lymphocyte populations were identified first by gating side-scattered (SSC) light intensity high (SSC^{Hi}) and forward-scattered (FSC) light intensity high (FSC^{Hi}) cell populations (Figure 2.4a). Doublets were excluded based on their increased area (Figure 2.4b). Non-viable cells were then excluded based on their uptake of a live-dead dye (Figure 2.4c). T lymphocytes were identified as CD45^{+}CD3^{+} (Figure 2.4d). Expression of CD4, CD8 and CD25 was then used to identify different lymphocyte populations: T helper cells (CD45^{+}CD3^{+}CD4^{+}, Figure 2.4e), T regulatory cells (CD45^{+}CD3^{+}CD4^{+}CD25^{+}, Figure 2.4f) and cytotoxic T cytotoxic (CD45^{+}CD3^{+}CD8^{+},Figure 2.4g).

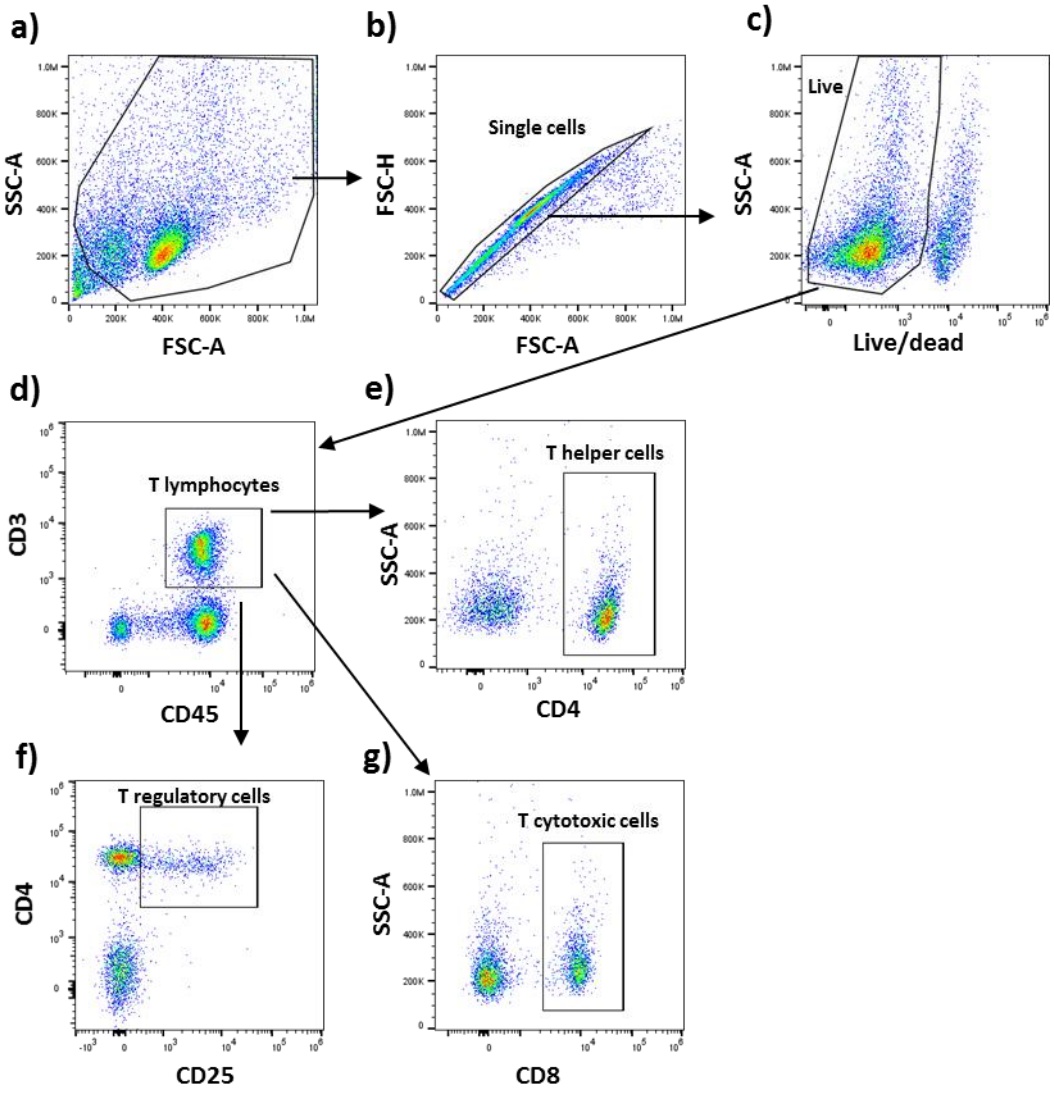

Figure 2.4 Gating strategy for the identification of T lymphocyte populations in tumours by flow cytometry.

T lymphocytes, identified as $CD45^{+}CD3^{+}$, were gated as follows: (a) SSC^{Hi} FSC^{Hi}, (b) Single cells, (c) Live cells. (d) T lymphocytes were identified as double positive for $CD45^{+}CD3^{+}$. This population was further split into different subsets: (e) $CD45^{+}CD3^{+}CD4+$ T helper cells, (f) CD45+CD3+CD4+CD25+ T regulatory cells, (g) cytoxic T cells ($CD45^{+}CD^{3}+CD8^{+}$).

2.2.11 Gating strategy for macrophage polarization panel

A macrophage polarization panel was used to investigate polarization status of macrophages in KPC CCR1-5$^{-/-}$ and KPC control mice. Macrophage populations were identified first by gating side-scattered (SSC) light intensity high (SSC^{Hi}) and forward-scattered (FSC) light intensity high (FSC^{Hi}) cell populations (Figure 2.5a). Doublets were excluded based on their increased area. Non-viable cells were then excluded based on their uptake of a live-dead dye (Figure 2.5c). Macrophages were identified as CD11b^{+}F4/80^{+} (Figure 2.5d). The identified macrophage population was further assessed for the presence of macrophage polarization markers: MHCII, CD80,CD86, CD206, and Arg1 (Figure 2.5e-i).

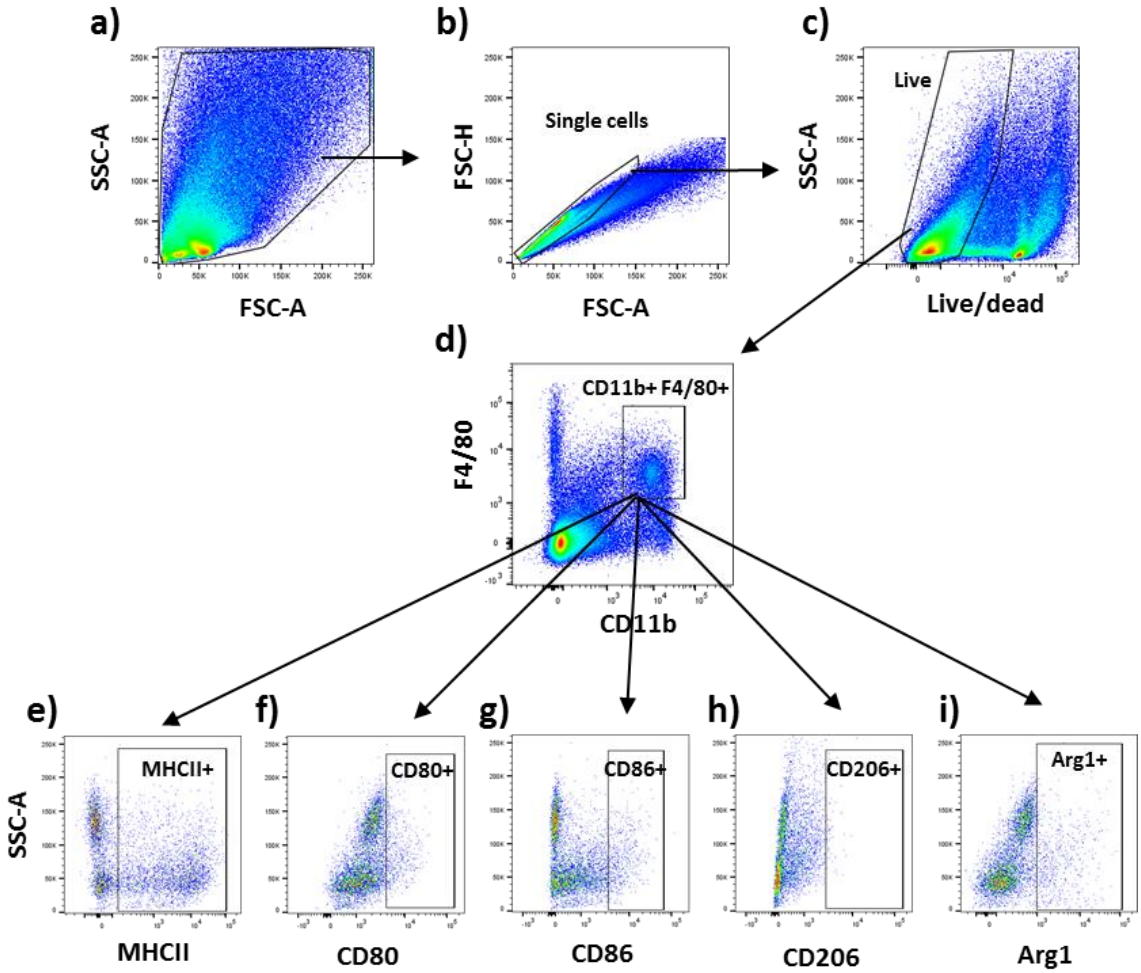

Figure 2.5 Gating strategy for the analysis of macrophage polarization status in tumours by flow cytometry.

Macrophages, identified as $CD11b^{+}F4/80^{+}$, were gated as follow: (a) SSC^{Hi} FSC^{Hi}, (b) Single cells, (c) Live cells. (d) Macrophages were identified as double positive cells for $CD11b^{+}F4/80^{+}$. This population was further analysed for the presence of polarization markers: (e) MHCII (f) CD80 (g) CD86 (h) CD206 (i) Arg1.

2.3 Tissue culture methods

2.3.1 Primary murine pancreatic cancer cell lines

Cell lines were prepared in house from pancreatic tumours harvested from *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ mice, or *Pdx1-Cre, LSL-Kras*$^{G12D/+}$ mice on a pure-bred (at last F10) C57BL/6J background. Cells were grown at 37°C in 5% CO_2 in a humidified incubator.

2.3.2 Pancreatic Culture Medium

All pancreatic cancer cell lines were grown in Dulbecco's modified eagle medium (DMEM) (Gibco, Cat:21969) with 10% foetal bovine serum (FBS) (PAA, Cat:A15-101), 1% Penicillin/Streptomycin (Gibco, Cat:15070), and 2mM L-Glutamine (Gibco, Cat:25030).

2.3.3 Establishing Pancreatic Cancer Cell Lines

Mice displaying signs of clinical end point were euthanized using schedule 1 methods. PDAC tissue was removed using scissors and forceps. A small piece of tumour (approx. 50 μg) was collected and placed in PBS for transportation to the lab. In a laminar flow hood the tumour tissue was minced using disposable scalpels. Cells were then transferred to a small T-25 culture flask with 5mL of culture medium plus an additional 0.5ml FBS (20% total). Cells were maintained at 37°C in a 5% carbon dioxide (CO_2) incubator. Medium was changed twice a week until cells were confluent. Once confluent, all cells were transferred to a T-75 flask using the passaging method described below. Aliquots of cells from passages 1-5 were frozen down as described below. Once established, cells were maintained in T-75 flasks in 15mL pancreatic culture medium and passaged as required.

2.3.4 Passaging Confluent Cells in Culture

Confluency of the cells in culture was confirmed by microscopy. Once ~70-90% confluent, cells were passaged. Passaging of cells was performed by aspirating the medium and washing cells with 5mL of PBS. 1mL of 10% trypsin (Gibco, Cat:15090046) was used for

the detachment of adherent cells. Cells were incubated with trypsin for 2-6 minutes in the incubator until completely detached. Cells were then resuspended in culture medium and split into the required number of flasks at the required dilutions.

2.3.5 Cryopreservation of Cell Lines

Once 70-80% confluent, cells were incubated with 1mL trypsin for 2-6 minutes at 37°C in a 5% carbon dioxide (CO_2) humidified incubator. Once fully detached, cells were resuspended in medium and centrifuged at 1500RPM for 5 minutes. The supernatant was aspirated and the cell pellet resuspended in a solution of 90% FCS;10% DMSO. Cells from a single T-75 flask were resuspended in 3mL of freezing solution. Cells in suspension were transferred to 1mL cryovials, placed in a freezing container and frozen in a -80°C freezer. For long term storage cells were later transferred to liquid nitrogen cold storage.

2.3.6 Re-establishing Cell lines in Culture

Frozen cryovials of cells were defrosted quickly in a 37°C water bath. Cells were diluted in 10mL medium in order to wash out the DMSO, centrifuged at 1500rpm, and the supernatant removed. Cell pellets were resuspended in 15mL pancreatic culture medium and placed in a 37°C; 5% carbon dioxide (CO_2) humidified incubator. The culture medium was changed after 24 hours. Cell lines were then maintained as normal.

2.4 Immunohistochemistry and *In Situ* Hybridization

2.4.1 Immunohistochemistry methods

Immunohistochemistry (IHC) is a microscopy-based method to visualise specific cellular components, usually proteins, within tissue sections, utilising a target-specific antibody.

Following fixation in formalin, tumour tissue and organs were dehydrated through an alcohol gradient of increasing concentration and paraffin-embedded into tissue blocks. Tissue blocks were cut into 4 µm sections and mounted on charged slides. Slides were deparaffinised by immersing slides in xylene 2 times for 10 minutes each. Slides were then rehydrated by washing in decreasing concentrations of alcohol: 2 x 5 minute washes in 100% alcohol; 1 x 5 mins wash in 70% alcohol. Slides were then rinsed and stored in water for transportation to the lab. Heat-induced epitope retrieval was performed using a boiling water bath. Slides were submerged in 10mM pH6 Sodium Citrate in dH_20 and boiled for 20 minutes. After that, slides were cooled down completely at room temperature in the solution and washed in dH_20. To quench endogenous peroxidase activity, slides were incubated in 3% hydrogen peroxide (H_20_2) in water for 15 minutes. This was followed by three 5 minute washes with Tris-buffered saline (TBS) with 0.05% Tween-80 (TBST). Hydrophobic pen was used to draw around the tissue to create a barrier. Tissue blocking was achieved by incubation for 45mins in 5% normal goat or rabbit serum in TBST. Slides were then incubated with primary antibody diluted in 5% serum at 4°C overnight. 3 x 5 minute TBST washes were performed prior to addition of the secondary antibody.

Slides were incubated with the appropriate secondary antibodies from Vectastain ABC peroxidase kits (Vector, Cat: Pk-4004) for 30 min at room temperature. ABC-horse radish peroxidase (ABC-HRP) was made up immediately after adding the secondary antibody and left at room temperature for 30 minutes. 3x 5 minute 0.05% TBST washes were performed after the incubation with the secondary antibody. This was followed by signal amplification by incubating slides with ABC-HRP at room temperature for 30 minutes.

Antibody staining was visualized using 3,3-diaminobenzidine (DAB) (ThermoFisher, Cat: 36000). 1 drop of DAB was added to 2ml of substrate. Each slide was covered with 100-200µl of DAB and incubated for approximately 3-10 minutes. The slides were rinsed with water and counterstained in Haematoxylin for 30-60s and washed again in running tap water.

Slides were dehydrated by incubating in increasing concentrations of alcohol and followed by incubation in xylene prior to covering tissue sections with coverslips.

Antigen	Catalog #	Supplier	Concentration
F4/80	ab6640	AbCam	1:200
Ki 67 SP6	RM-9106	Thermo	1:100
Ly6G (IA8)	BE0075-1	BioXcell	1:60.000
PAX 5	Ab 109443	AbCam	1:1000
Caspase 3 ASP-175	9661	Cell Signalling	1:50
CD3	A0452	Dako	1:50
CD8a	MCA1817T	AbD Serotec	1:30
CD45R	ab64100	AbCam	1:200
CD138	50641-RP02	Sino Biological Inc	1:1000
Smooth Muscle Actin	A2547	Sigma-Aldrich	1:25000

Table 2.3 Antibodies used in immunohistochemistry.

This table documents the target of the antibody, concentration used, along with manufacturer and catalogue number.

2.4.2 *In Situ* Hybridization

In situ detection of PDL-1 transcripts was performed using an RNAscope assay, an *in situ* hybridization assay for detection of target RNA within intact cells. Tissue blocks were cut into 4 µm sections and mounted on slides. Slides were deparaffinised as described above. PDL-1 specific, Mm-CD274 RNAscope 2.5 LS probe, (Advanced Cell Diagnostics, Cat:420508) was obtained from Advanced Cell Diagnostics (Newark, CA, USA). ISH detection for CD274, Mm-PPIB and dapβ (Advanced Cell Diagnostics, Cat:420508; 313918; 312038) mRNA was performed using RNAScope 2.5 LSx (Brown) detection kit (Advanced Cell Diagnostics, Cat:322700) on a Leica Bond Rx autostainer strictly according to the manufactu’er's instructions.

2.4.3 Automated quantification of staining

Whole digital slide images were captured using the Aperio AT2 slide scanner. Staining of each probe was quantified using the image analysis platform HALO (v3.1.1076.363, Indica Labs, Albuquerque, NM, USA) (Figure 2.6). In short, representative tumour areas were selected by excluding tumour margins, tertiary lymphoid structures, and necrotic areas. Tissue sections for the quantification were selected using annotation tools. Table 2.4 shows the summary of markers that were quantified using HALO, measurement parameters and the HALO modules applied.

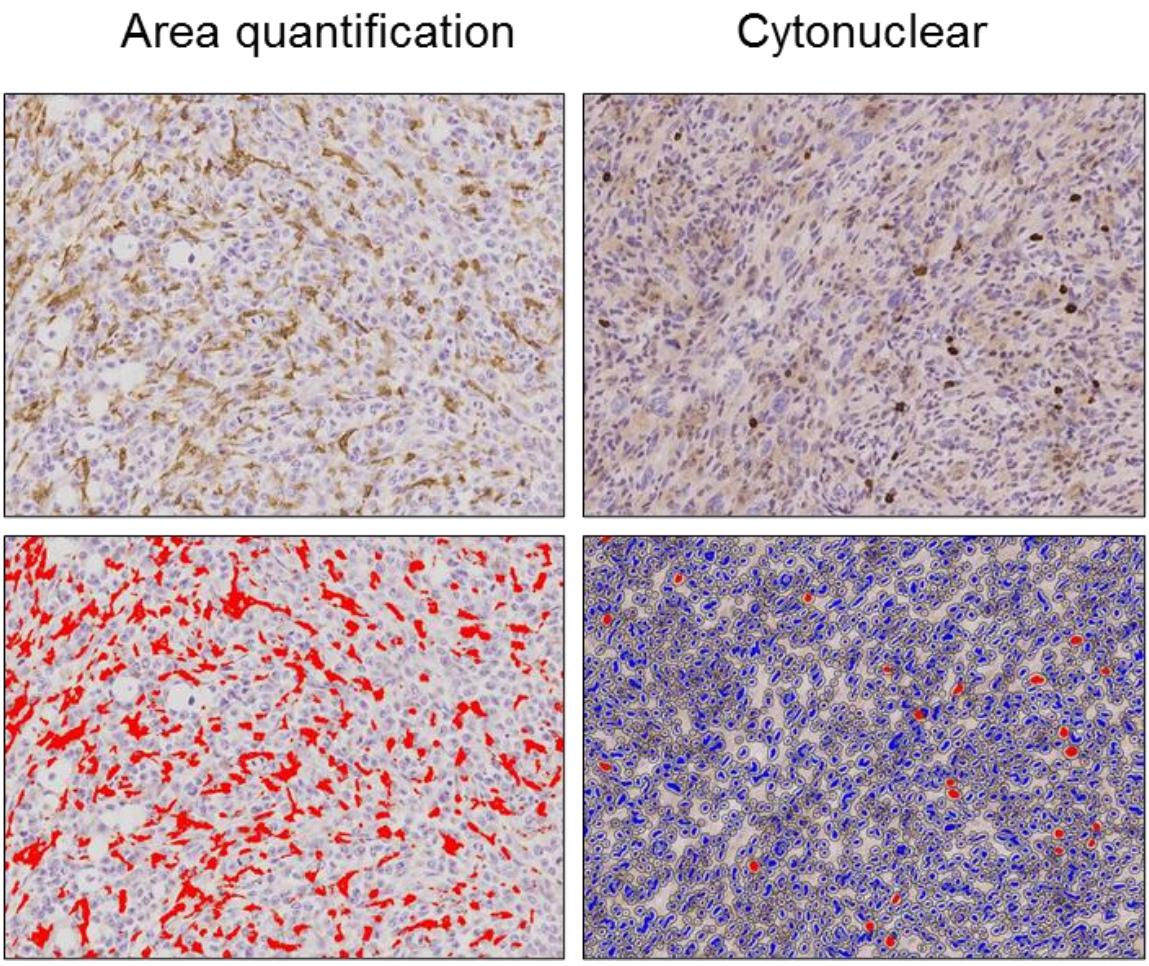

Figure 2.6 Representative images of IHC quantification using image analysis platform HALO.

Representative images show immunohistochemistry staining quantified using Area quantification module (Left) and CytoNuclear module (Right) on image analysis platform HALO.

Marker	Measurement parameter	Module
CD3	Percentage of positive cells	CytoNuclear
CD45R	Percentage of positive cells	CytoNuclear
CD8a	Percentage of positive cells	CytoNuclear
F4/80	Area of positive tissue	Area quantification
Ly6G	Percentage of positive cells	CytoNuclear
Sirius Red	Area of positive tissue	Area quantification
SMA	Area of positive tissue	Area quantification
PDL-1	ISH expression on a cell-by-cell basis	ISH

Table 2.4 Automated quantification of immunohistochemistry staining.

This table documents the markers that were quantified using image analysis platform HALO (v3.1.1076.363, Indica Labs, Albuquerque, NM, USA).

2.4.4 Manual scoring of immunohistochemistry staining

Quantification of positive staining cells by immunohistochemistry in PanINs was performed manually. Pictures of sections of interest were taken using an Olympus BX53 microscope. Pictures were imported to ImageJ and positive cells were counted manually. Ki67 positive cells were scored in each individual PanIN and percentage of positive cells was calculated.

2.5 Statistical analysis

Animal experiments were performed adhering to the principles of the 3Rs: Replacement, Reduction and Refinement. Kaplan-Meier survival analysis was performed to compare survival and significance estimated using Log Rank tests (GraphPad Prism 9.1.2).

Assessment of differences in various parameters between different mice cohorts was performed using nonparametric Mann-Whitney testing and unpaired t tests (GraphPad Prism 9.1.2).

RESULTS

3 The role of macrophages in PDAC initiation, development, and metastasis

3.1 Introduction

Recently, work in our lab revealed an important role for macrophages in pancreatic cancer. Targeting macrophages, via CSF1R inhibition, could extend survival in KPC mice with late stage pancreatic cancer, and could also alter the immunosuppressive microenvironment, reduce fibrosis and tumour 'stiffness', dampen PD-L1 expression and allow activated T cells to accumulate without the need for immune checkpoint inhibition. However, the role of macrophages throughout PanIN development and progression to PDAC is not well characterized.

The progression of pancreatic cancer in *Pdx1-Cre*, *LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ (KPC) mice closely resembles human disease. The pancreas in KPC mice pups is normal and has no neoplastic lesions. However, by 6 to 10 weeks of age, KPC mice start to develop precursor lesions also known as pancreatic intraepithelial neoplasia (PanIN), within the pancreas (Lee, Komar et al. 2016). Based on the degree of cellular and nuclear atypia, PanINs are classified into three stages: PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3 (Campbell, Verbeke, and SpringerLink (Online service) 2013). This early stage of PDAC development is followed by a strong inflammatory response. As early as 6 weeks, infiltration of F4/80 positive macrophages can be seen which persists throughout the disease progression. Macrophage recruitment and accumulation adjacent to pancreatic precursor lesions are regarded as one of the earliest immune cell responses (Clark, Hingorani et al. 2007). Therefore, I sought to investigate the role of infiltrating macrophages on PanIN development and progression using the KPC model.

Pancreatic cancer is a very aggressive disease that is histologically characterized by a dense desmoplastic stroma that is surrounding malignant cells. As PDAC develops, acinar parenchyma is gradually replaced by fibrotic stroma, which is one of the main hallmarks of pancreatic cancer (Clark, Hingorani et al. 2007). The stromal compartment can take up to 80% of all tumour mass (Hu, Jiao et al. 2015) and is mainly composed of TAMs, extracellular matrix (ECM) components, pancreatic stellate cells (PSCs) and proliferating cancer-associated fibroblasts (CAFs) that produce structural proteins such as fibronectin and collagen. Although the desmoplastic tumour stroma was originally considered as a bystander

in carcinogenesis, recent studies have highlighted the role of tumour stroma, in particular macrophages and fibroblasts, in tumour initiation as well as development.

Tumour associated macrophages (TAMs) represent a major population of inflammatory cells in PDAC microenvironment. TAMs accumulate in the tumour and exhibit a variety of functions such as control of immune suppression, tumour cell invasion and resistance to chemotherapy. As shown by recent studies, several subsets of macrophages exist in PDAC. Resident and infiltrating macrophages have been shown to co-exist in PDAC and play distinct functions. The KPC model faithfully recapitulates pathological features observed in human PDAC, including development of fibrotic reaction and extensive infiltration of macrophages (Hingorani, Wang et al. 2005, Zhu, Herndon et al. 2017). Therefore, I sought to investigate the role of macrophages in pancreatic cancer progression using this model.

Pancreatic cancer has very poor prognosis largely because it is detected in the late stage of disease progression. Most patients present with metastatic disease at the point of diagnosis. Unfortunately, the treatment options for metastatic patients are very limited. In fact, metastases are estimated to be the cause of approximately 90% of cancer deaths (Hanahan and Weinberg 2000). Metastasis is one of the cancer hallmarks and is a multi-step process where cancer cells spread from the primary tumour and colonise distant sites. In humans, pancreatic cancer most commonly metastasizes to the liver, lungs and/or peritoneum. As discussed previously, the KPC mouse model very closely resembles human disease with metastases observed in up to 80% of cases. The metastasis observed in KPC mice are usually in liver, lungs and/or diaphragm. For cancer cells to spread to the secondary sites, several steps must be achieved, including arrest at the secondary site and extravasation from the blood vessel into the tissue. In order to start colonisation in the secondary site, cancer cells develop resistance to the host tissue immune defences (Hanahan and Weinberg 2000). Literature suggests that systemic changes occur at the distant sites to prepare for the cell colonisation. Most studies have focused on recruitment of myeloid cells to the premetastatic sites. Previously, it was shown that systemic macrophage depletion by liposomal clodronate results in markedly reduced metastasis (Griesmann, Drexel et al. 2017). In a breast cancer model, metastases are almost completely abrogated by genetic knockout of colony stimulating factor 1 (CSF1) (Lin, Nguyen et al. 2001). These data provide an insight into the importance of tumour-associated macrophages for the 'preconditioning' of the distant sites and cancer cell colonisation. Therefore, I also sought to investigate the role of macrophages in metastatic progression.

3.2 Experimental Aims

I hypothesised that macrophage infiltration to the pancreas would be dependent on the chemotactic signalling through the CCRs. Using a murine model of PDAC I aimed to investigate the importance of infiltrating macrophages to the development of pancreatic cancer. By using *Pdx1-Cre, LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$. *CCR1-5*$^{-/-}$ (KPC CCR1-5$^{-/-}$) mice I aimed to establish the role of infiltrating macrophages in tumour initiation, development and progression. Given the significant role macrophages play in the setting of premetastatic sites, I hypothesised that ablation of infiltrating macrophages, could also have an effect on the priming of premetastatic site and tumour cell colonisation. To investigate this question, I also exploited the KPC CCR1-5$^{-/-}$ model.

3.3 Results

3.3.1 Characterization of CCR1-5$^{-/-}$ mice

CCR1-5$^{-/-}$ mice, previously described in (Dyer, Medina-Ruiz et al. 2019) were obtained from Gerard J. Graham's lab. Firstly, I characterized the CCR1-5$^{-/-}$ model in otherwise wild-type mice. CCR1-5$^{-/-}$ and wild-type control mice were aged until 6 weeks of age and culled. The pancreata together with spleen, lobe of liver and lungs were sampled, formalin fixed and paraffin embedded. Slides were obtained and stained with haematoxylin and eosin (H&E), and representative images taken (Figure 3.1). I did not observe any histological differences in any of the organs between the WT and CCR1-5$^{-/-}$ mice, suggesting that these mice develop normally and exhibit no obvious abnormalities within the pancreata or other organs.

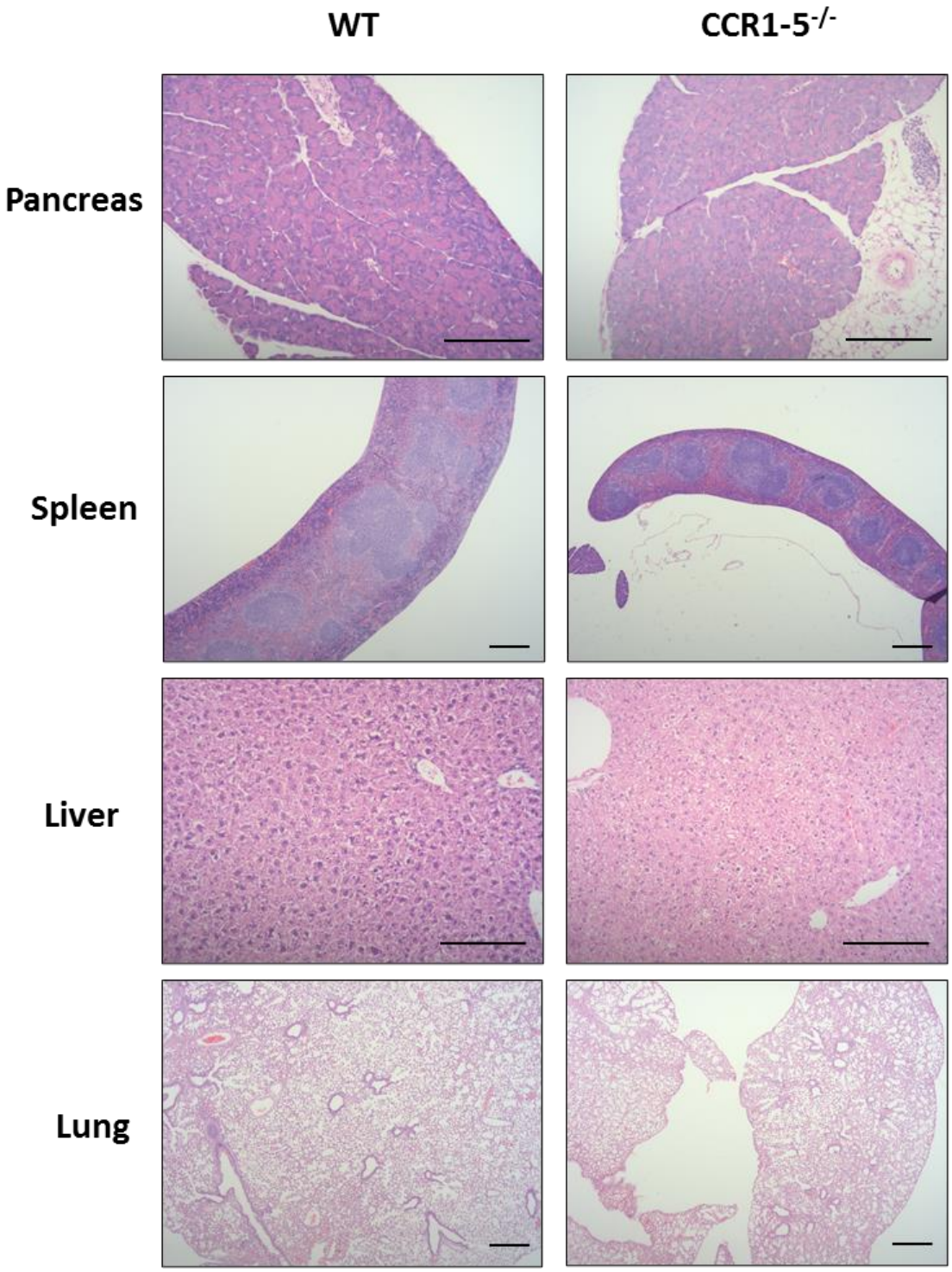

Figure 3.1 Histological comparison of WT and CCR1-5$^{-/-}$ mice.

Representative images of haematoxylin and eosin (H&E) staining of WT (left) and CCR1-5$^{-/-}$ (right) pancreas, spleen, liver and lung.

I also assessed peripheral blood leukocyte populations in these mice using the ProCyte Dx Haematology Analyser (Figure 3.2). Although I observed a trend towards decreased monocyte levels in CCR1-5$^{-/-}$ mice, no statistically significant changes in circulating leukocyte populations were observed.

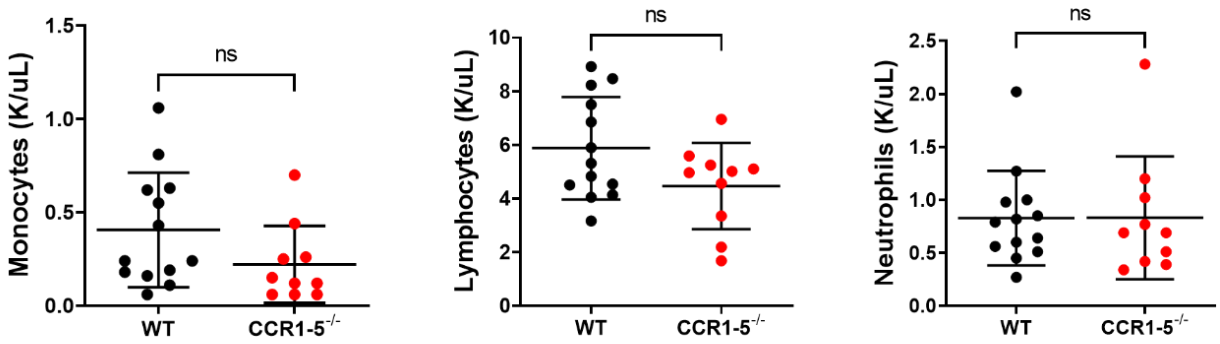

Figure 3.2 Analysis of peripheral blood from WT and CCR1-5$^{-/-}$ mice.

Graphs illustrate peripheral blood leukocyte counts. Blood was taken and stored in EDTA tubes until processed using the ProCyte Dx Haematology Analyser. WT (N=13) vs CCR1-5$^{-/-}$ (N=10), Mann Whitney.

In order to measure monocyte levels in peripheral blood with greater precision, I decided to use flow cytometry. Flow cytometry data clearly shows that CCR receptor-lacking mice have significantly reduced numbers of circulating monocytes (Figure 3.3). I found at least a 50% reduction of CD11b^{+} Ly6C^{hi} inflammatory monocytes, which is consistent with observations made by Dyer et al (Dyer, Medina-Ruiz et al. 2019).

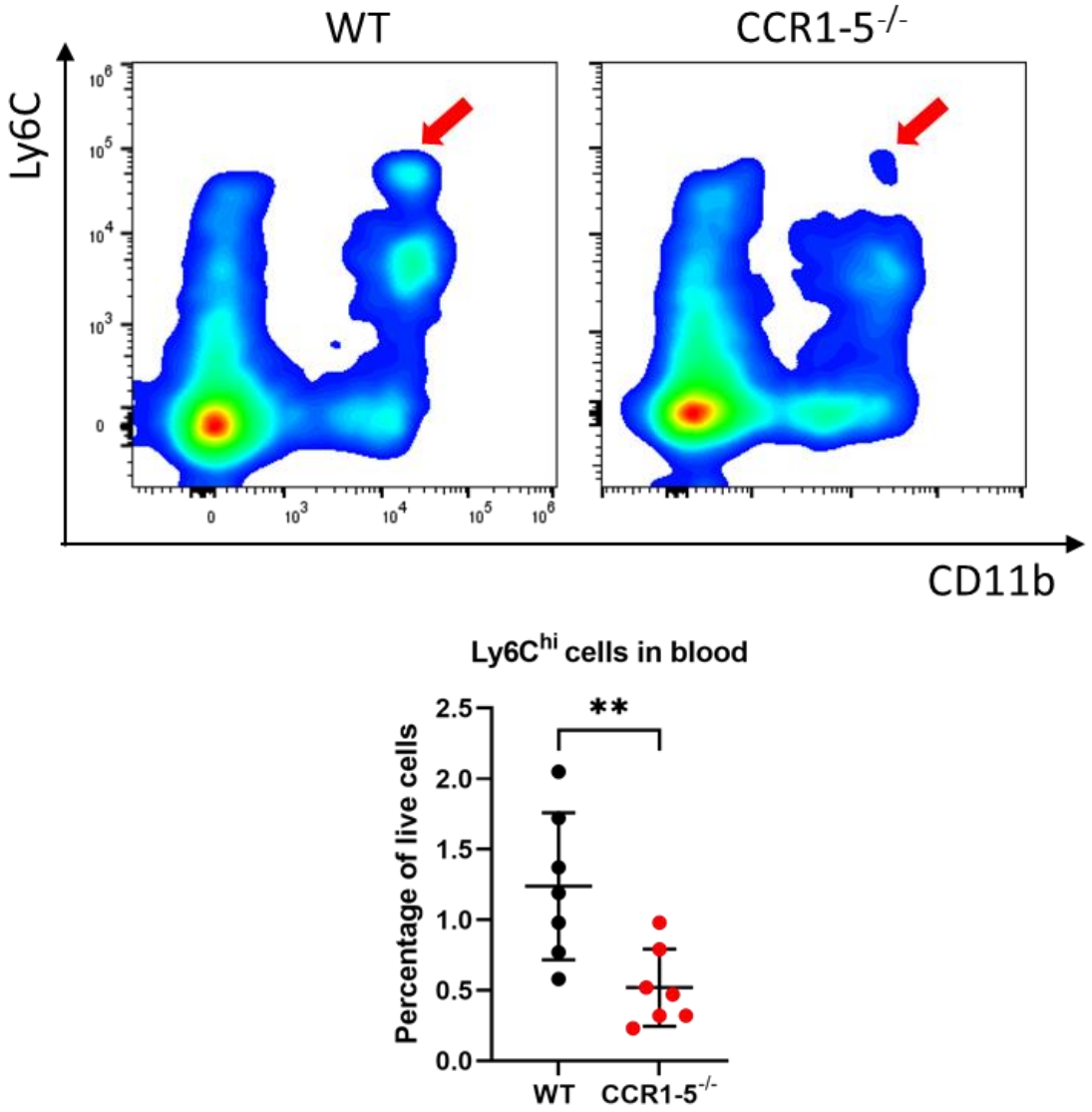

Figure 3.3 CCR1-5$^{-/-}$ mice display reduced numbers of circulating Ly6C^{hi} inflammatory monocytes.

Top: Representative flow cytometry analysis of peripheral blood leukocyte populations from 6 week old WT (left) and CCR1-5$^{-/-}$ mice (right). Blood samples were stained with PECy7-conjugated monoclonal antibody against Ly6C and FITC-conjugated CD11b monoclonal antibody. Quantification graph (bottom), WT N=7 vs CCR1-5$^{-/-}$ N=7, p= 0.0082, Mann Whitney.

I also analysed leukocyte populations in the bone marrow of these mice (Figure 3.4). No changes in leukocyte populations between the cohorts were detected.

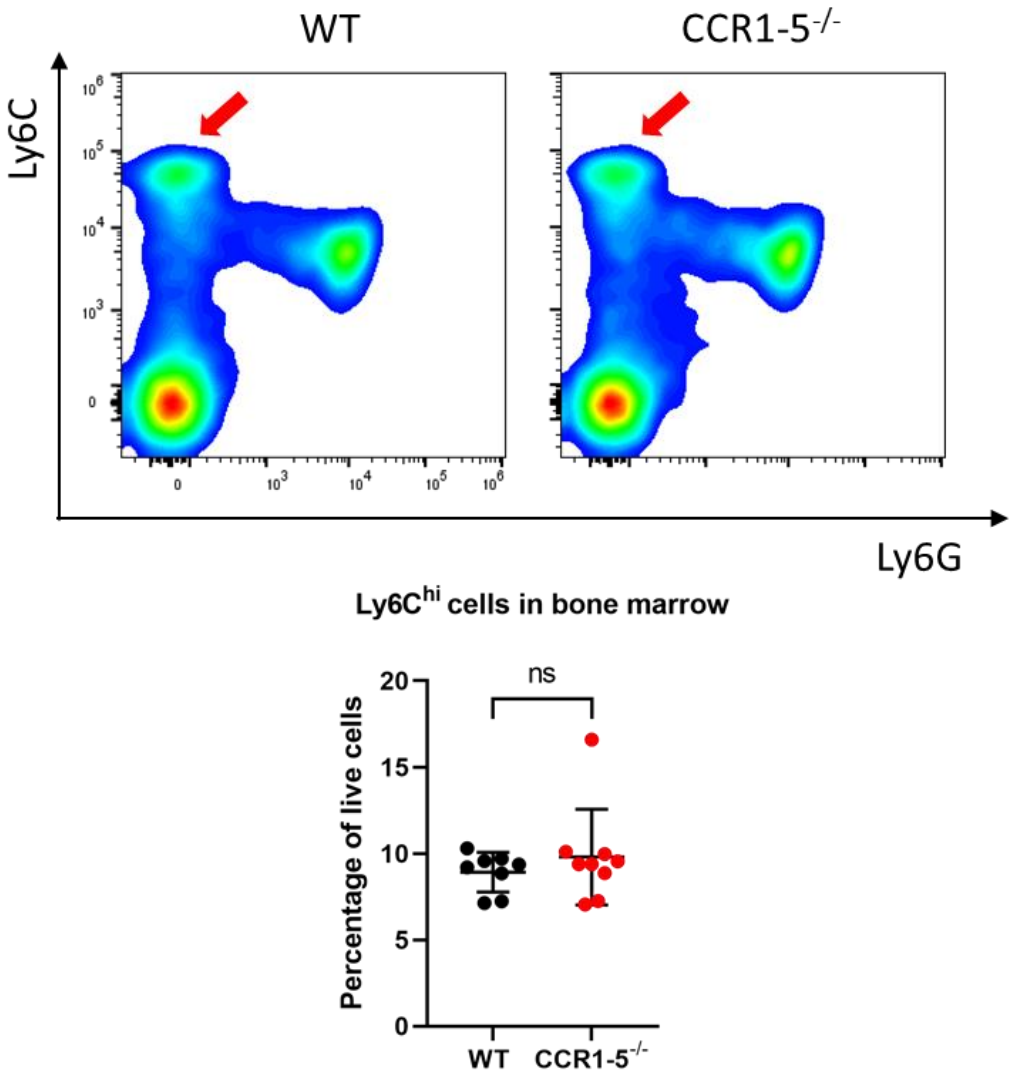

Figure 3.4 Monocytes do not accumulate in the bone marrow in CCR1-5$^{-/-}$ mice.

Top: Representative flow cytometry analysis of bone marrow leukocyte populations from 6 week old mice. Tissues were stained with PECy7-conjugated monoclonal antibody against Ly6C and APC-conjugated monoclonal antibody against Ly6G. Quantification graph (bottom), WT N=7 vs CCR1-5$^{-/-}$ N=7, Mann Whitney.

Therefore, I concluded that the CCR1-5 receptors are playing a role in monocyte egress from the bone marrow into the circulation. However, there is no accumulation of monocytes in the bone marrow of CCR1-5$^{-/-}$ mice under normal conditions. This data is also consistent with the original findings in the CCR1-5$^{-/-}$ model (Dyer, Medina-Ruiz et al. 2019).

Next, I looked at macrophage presence within tissues of 6 week old CCR1-5$^{-/-}$ mice. To assess this, immunohistochemistry staining for F4/80 was performed on slides of pancreas, spleen, liver and lung tissue. It was clear that F4/80 positive cells were present in the spleen and liver of both genotypes of mice. In the pancreata and lung, we could see very few macrophages present in either genotype (Figure 3.5).

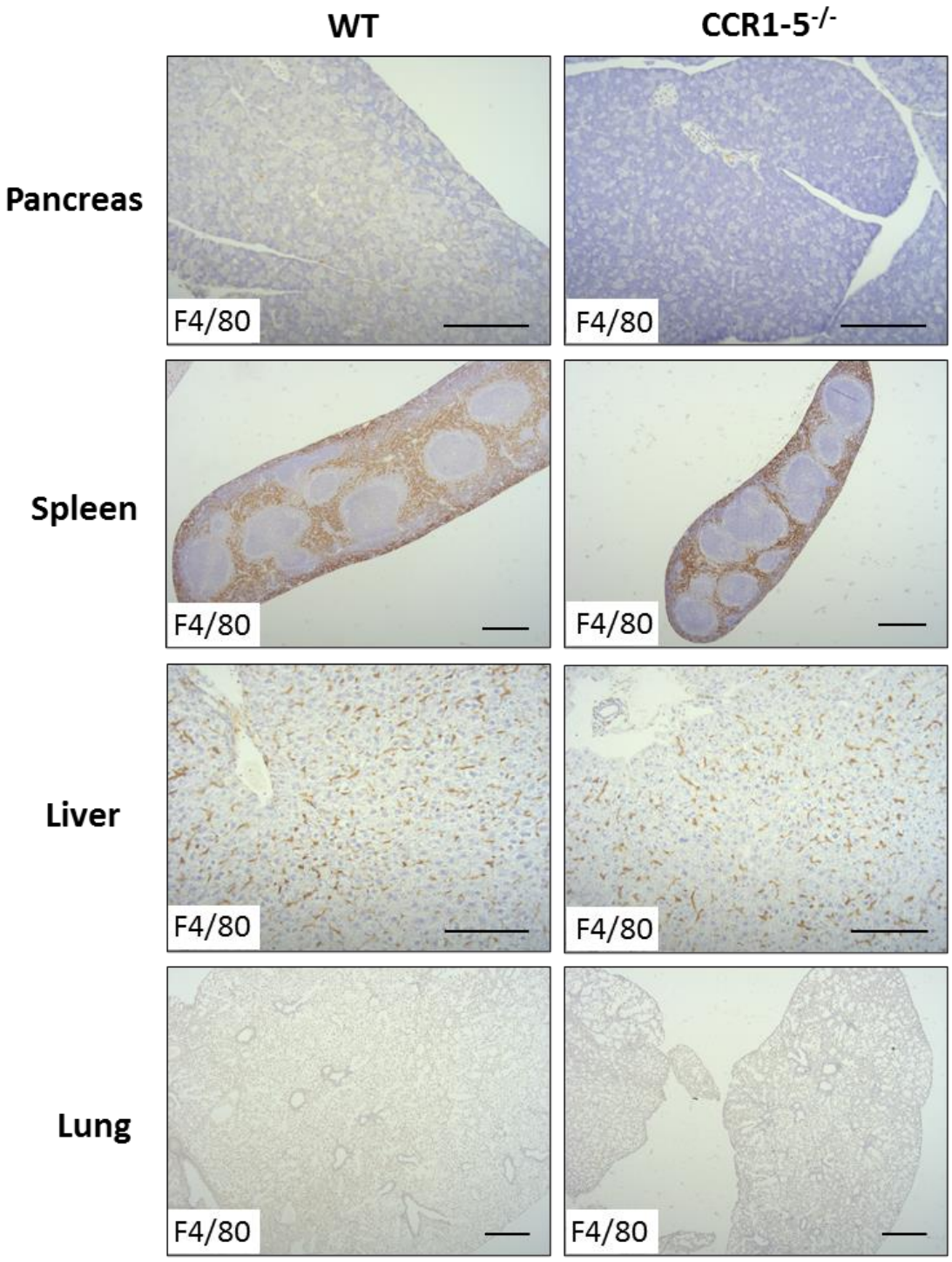

Figure 3.5 Comparison of macrophage presence in tissue from WT and CCR1-5$^{-/-}$ mice by F4/80 immunohistochemistry.

Representative images of F4/80 immunohistochemistry staining for macrophages in 6 week old WT (left) and CCR1-5$^{-/-}$ (right) mice.

Macrophages in liver tissue from 6 week old WT and CCR1-5$^{-/-}$ mice were quantified using the HALO image analysis platform (Figure 3.6). There did not appear to be any difference in macrophage presence between WT and CCR1-5$^{-/-}$ mice livers. This further suggests that while monocyte release into the circulation is reduced, it does not impact the numbers of macrophages in the tissues. It is likely that these cells in the liver are predominantly resident hepatic Kupffer cells.

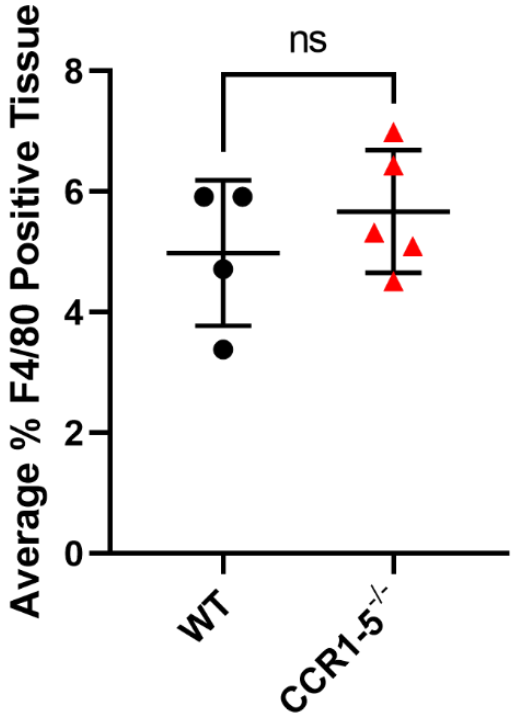

Figure 3.6 Macrophages are present in CCR1-5$^{-/-}$ mice livers at levels comparable to WT mice.

Quantitative analysis of immunohistochemistry staining in livers from the CCR1-5$^{-/-}$ mouse model compared with WT mice. P value non-significant (n.s.). N ≥ 4 , Mann Whitney. Each dot on the graph represents liver tissue scored from one mouse.

In order to investigate the effect of long-term loss of CCR1-5 receptors in otherwise WT mice, cohorts of CCR1-5$^{-/-}$ mice were aged up to 1.5 years old. Organs from these mice were collected, formalin fixed and paraffin embedded. Slides were obtained from each mouse, stained with haematoxylin and eosin (H&E) and representative images taken (Figure 3.7). No histological changes were observed between the groups. This suggests that CCR1-5$^{-/-}$ mice age completely normally and there are no long term effects from the loss of monocyte trafficking in healthy mice.

Finally, the levels of F4/80 positive macrophages in organs from the aged mice were assessed and representative images taken (Figure 3.8). Again, F4/80 positive cells were present in the spleen and liver of both genotypes of mice, whilst in the pancreata and lung, we could see very few macrophages. No obvious differences in macrophage numbers in these tissues were observed between WT and CCR1-5$^{-/-}$ mice.

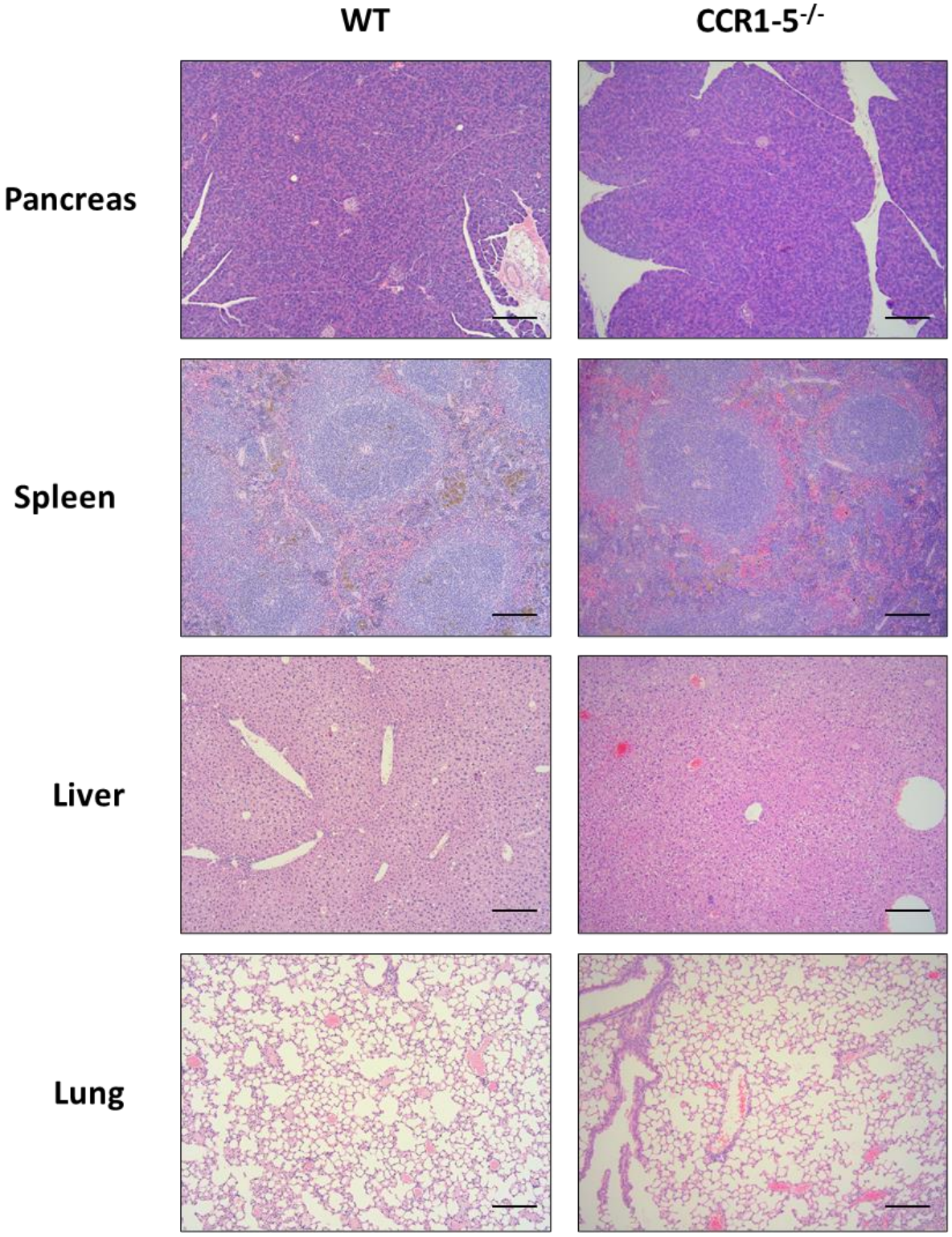

Figure 3.7 H&Es of organs from WT and CCR1-5$^{-/-}$ mice at 18 months show no changes in histology.

Representative images of H&E staining from 18 month old WT (left) and CCR1-5$^{-/-}$ (right) mice. Scale bar 100µm.

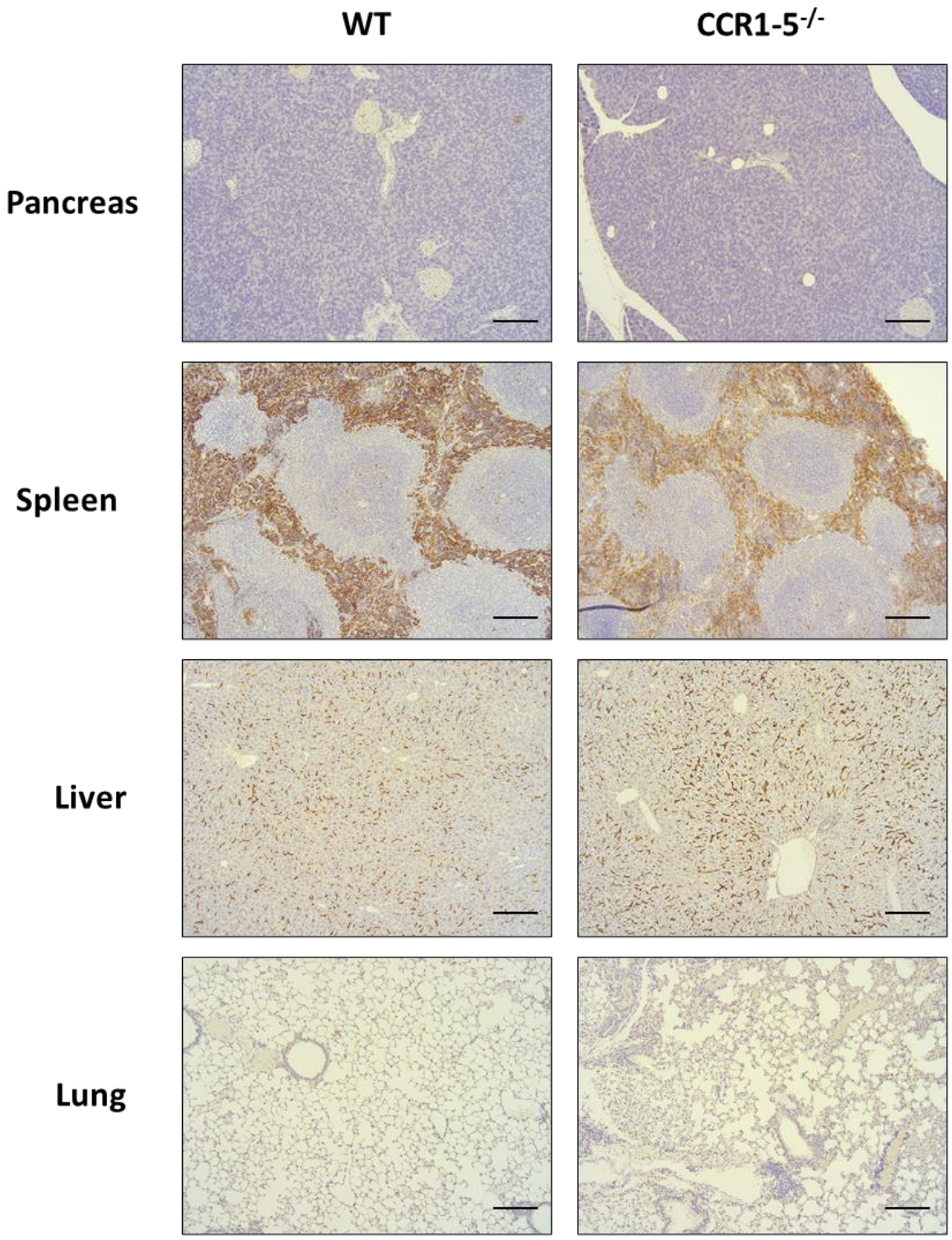

Figure 3.8 Comparison of macrophage infiltration in tissue from 18 month old WT and CCR1-5$^{-/-}$ mice by F4/80 immunohistochemistry.

Representative images of F4/80 immunohistochemistry staining for macrophages in 18 month old WT (left) and CCR1-5$^{-/-}$ (right) mice.

To confirm the deletion of CCR genes, I decided to assess the presence of CCR2, as a surrogate for the CCR1-5 locus. I aged KPC and KPC CCR1-5$^{-/-}$ mice until the clinical end point. Tumours were processed for flow cytometry for the presence of CCR2. Data obtained confirms that CCR2 is completely absent in tumour tissue from KPC CCR1-5$^{-/-}$ mice (Figure 3.9).

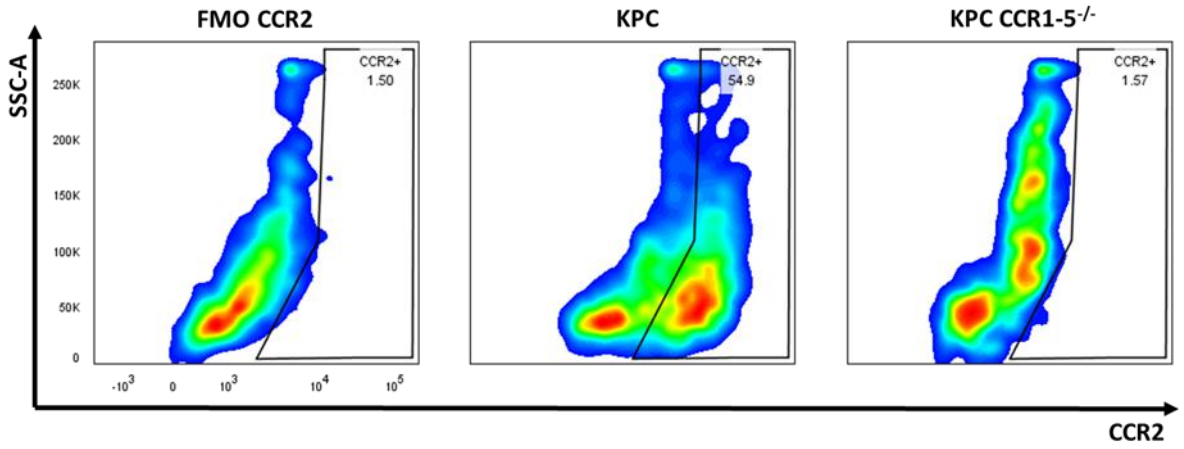

Figure 3.9 The absence of CCR2 in tumour tissue from KPC CCR1-5$^{-/-}$ mice

Flow cytometry analysis of tumour tissue from end point KPC and KPC CCR1-5$^{-/-}$ mice. Tumour tissue was stained with Brilliant Violet 650-conjugated monoclonal antibody against CCR2. Percentages refer to cells in F4/80+ CD11b+ positive gate.

3.3.2 Investigating the role of infiltrating macrophages in tumour initiation

3.3.2.1 *CCR1-5 homozygosity has no effect on tumour initiation in KPC mice*

I next wanted to assess the role of infiltrating macrophages on tumour initiation in KPC mice. I identified 6 weeks as a time-point at which the majority of mice have widespread PanIN. Therefore, I aged KPC and KPC CCR1-5$^{-/-}$ until 6 weeks of age. The pancreata from these mice were sampled, formalin fixed and paraffin embedded. Slides were obtained and stained with haematoxylin and eosin (H&E), and representative images taken (Figure 3.10). Histological analysis of the tissues revealed the formation of early precursor lesions also known as pancreatic intraepithelial neoplasia (PanIN), within the pancreas both in KPC and KPC CCR1-5$^{-/-}$, as well as areas of acinar to ductal metaplasia (ADM).

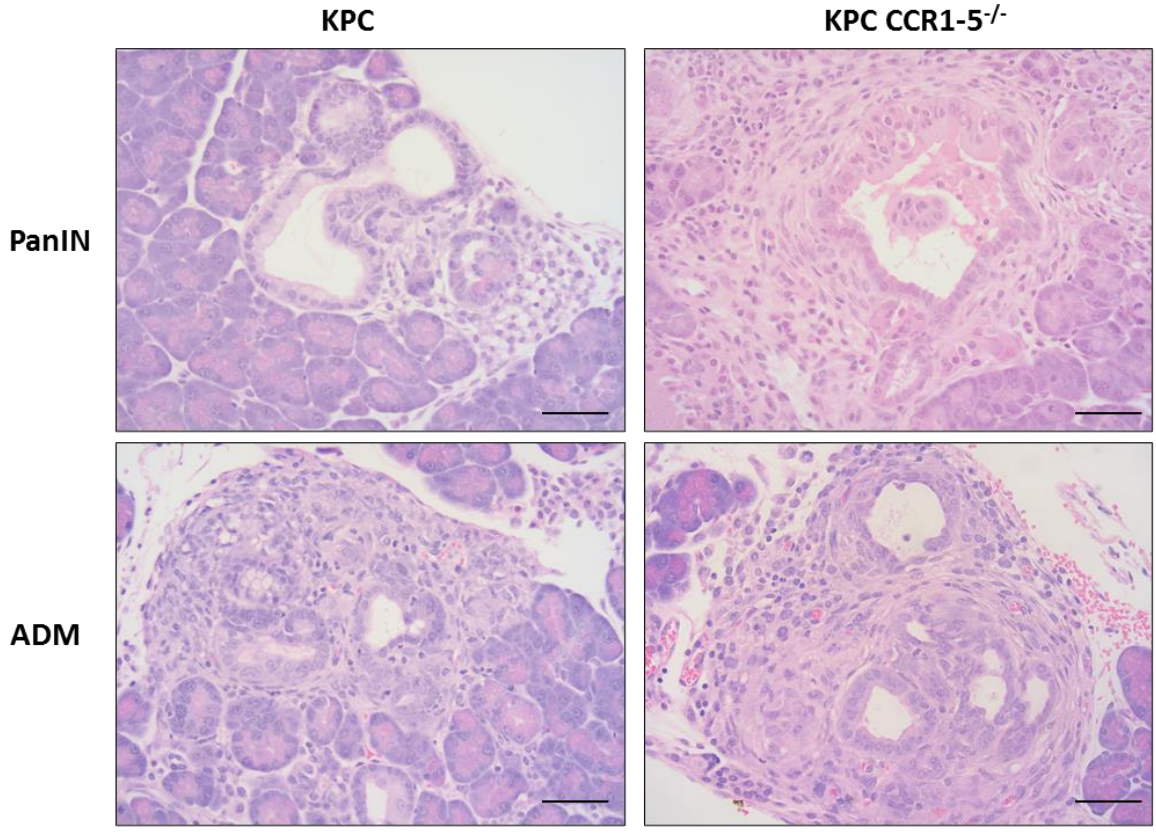

Figure 3.10 Formation of early precursor lesions in pancreata from KPC and KPC CCR1-5$^{-/-}$ mice at 6 weeks.

Representative images of H&E staining of the pancreas from 6 week old KPC (left) and KPC CCR1-5$^{-/-}$ (right) mice showing the presence of early PanIN and ADM lesions.

Next, I examined PanINs from each cohort to investigate if there is a difference in the abundance or grade of these PanIN lesions (Figure 3.11). No significant changes in the

number or grade of PanINs were observed between the cohorts. Overall, pancreata from 6 week old KPC CCR1-5$^{-/-}$ mice presented with a wider range of PanIN lesions, with a higher number of advanced PanINs. However, no evidence of PDAC was observed in the tissue analysed. On the other hand, two out of five KPC mice have developed areas of PDAC at 6 weeks of age.

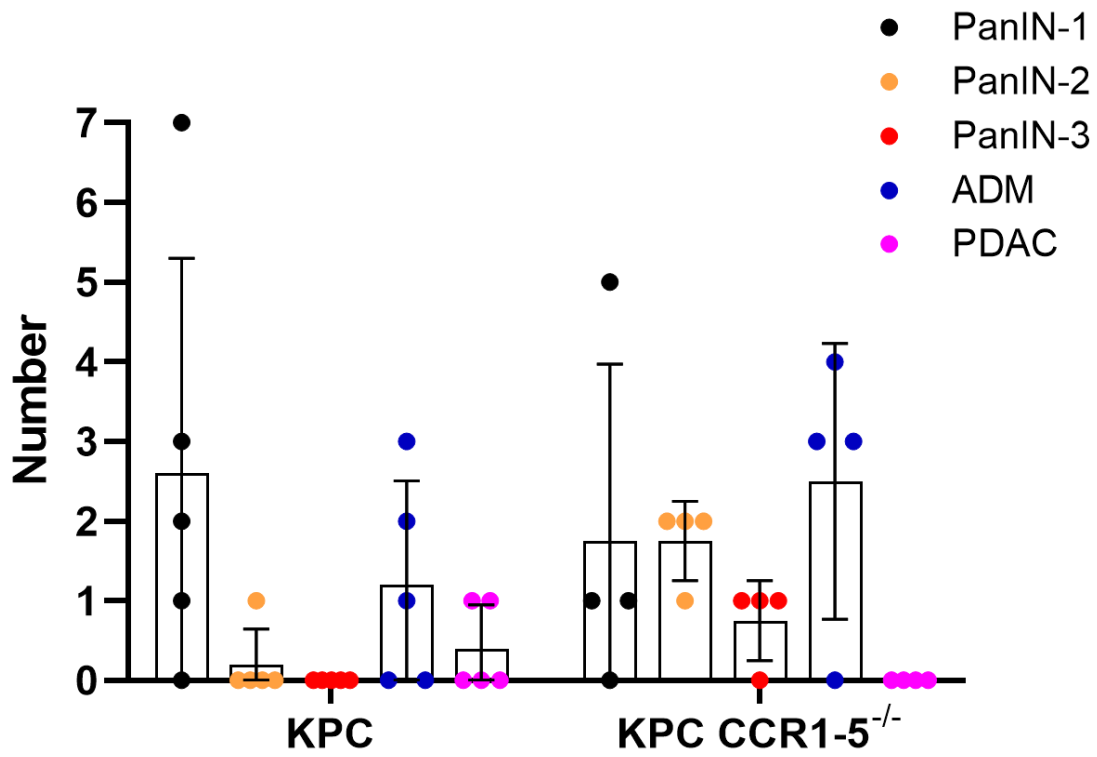

Figure 3.11 PanIN and ADM area scoring in 6 week old KPC and KPC CCR1-5$^{-/-}$ mice.

H&E stained tissue sections were analysed and scored for PanIN lesions and other pathologies. The graph indicates the numbers of PanINs present in mice from each cohort, and their grade. P value non-significant (n.s.), Mann Whitney.

Since it is known that PanIN lesions are mostly growth arrested/senescent (Morton, Timpson et al. 2010) I next assessed the proliferation status of PanINs present in these time-point mice to investigate if there is a difference between the two groups. Tissue sections were stained for the proliferation marker, Ki67, and representative images taken (Figure 3.12).

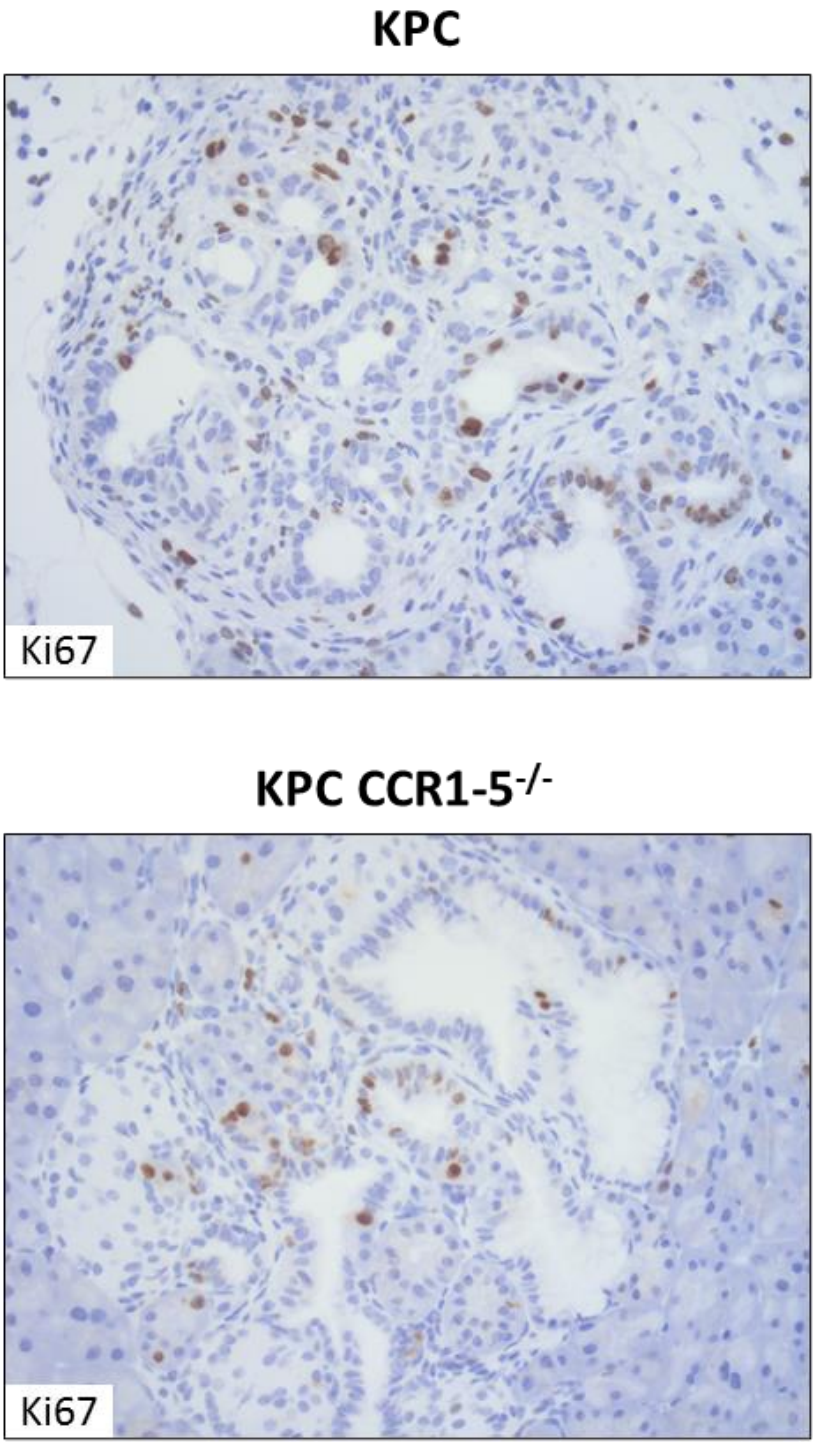

Figure 3.12 Expression of Ki67 in PanINs from 6 week old KPC and KPC CCR1-5$^{-/-}$ mice.

Cell proliferation shown by Ki67 immunohistochemistry in PanINs from 6 week old KPC (top) and KPC CCR1-5$^{-/-}$ (bottom) mice.

Ki67 immunohistochemical staining in PanINs was also scored manually (Figure 3.13). While some PanINs from KPC mice presented with a relatively high proliferation index, the overall results revealed no significant difference between KPC and KPC CCR1-5$^{-/-}$ mice.

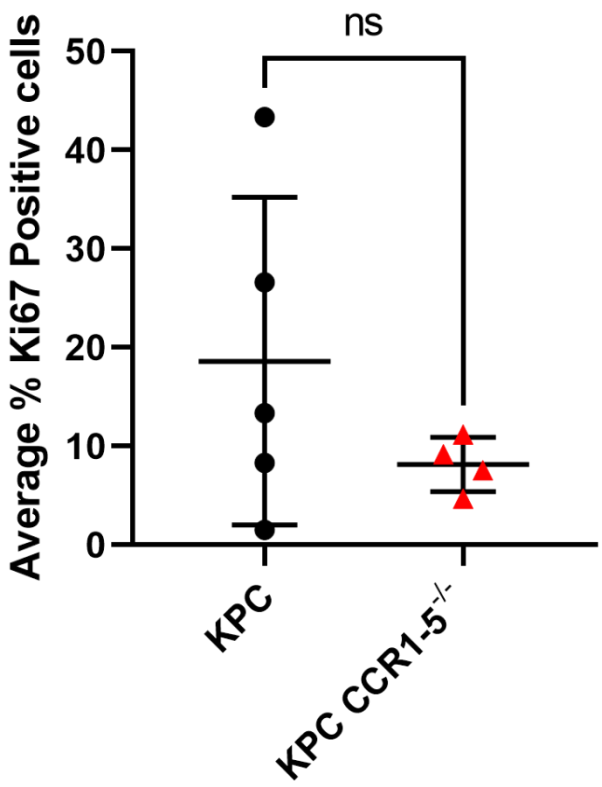

Figure 3.13 Quantification of Ki67 staining in PanINs from KPC and KPC CCR1-5$^{-/-}$ mice at 6 weeks of age.

Pancreatic tissue from 6 week old KPC and KPC CCR1-5$^{-/-}$ mice was stained for the proliferation marker, Ki67. The average percentage of Ki67 positive cells in PanINs was determined for each mouse. P value non-significant (n.s.). N ≥ 4 , Mann Whitney. Each dot on the graph represents the average percentage of Ki67 positive cells in PanINs in one mouse.

Next, I investigated whether loss of CCR1-5 has any effect on desmoplasia during the early stages of PanIN formation. Pancreatic tissue from 6 week old KPC and KPC CCR1-5$^{-/-}$ mice was stained for F4/80 and Sirius Red and representative pictures taken (Figure 3.14). The data indicate that the formation of stroma adjacent to PanINs occurs at an early stage both in KPC and KPC CCR1-5$^{-/-}$ mice. To investigate whether there is a difference in macrophage recruitment to the areas surrounding PanINs, I quantified immunohistochemistry staining for F4/80 using the HALO image analysis platform (Figure 3.15). Using annotation tools, I carefully selected PanIN areas and adjacent stroma. PDAC areas were excluded from the analysis. The results revealed no change in macrophage numbers surrounding PanINs in 6 week old KPC CCR1-5$^{-/-}$ mice as compared to KPC mice.

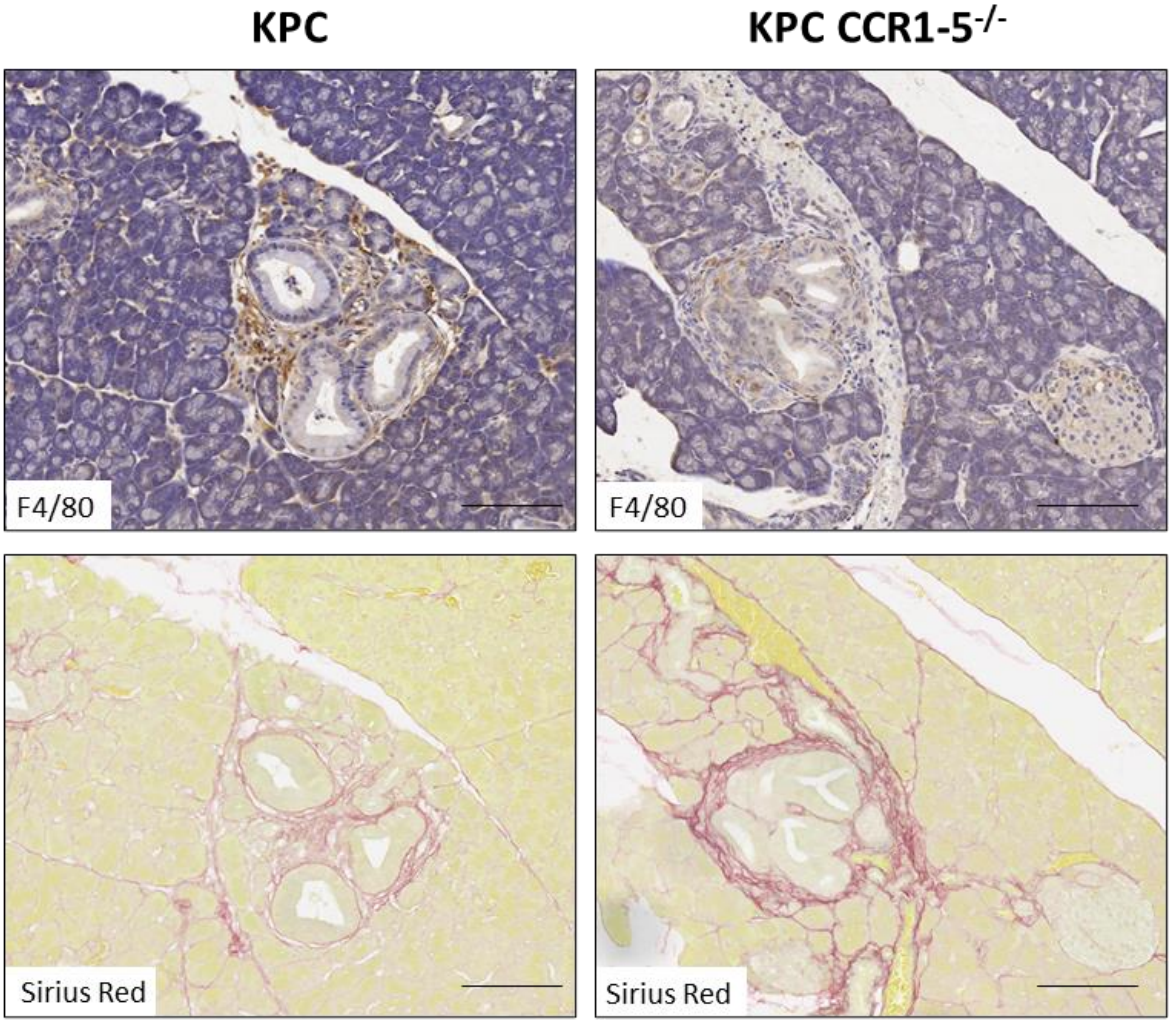

Figure 3.14 Desmoplasia adjacent to PanINs from KPC and KPC CCR1-5$^{-/-}$ mice at 6 weeks of age.

Representative immunohistochemistry images of pancreas with PanINs from 6 week old KPC and KPC CCR1-5$^{-/-}$ mice. Pancreas tissue was stained with F4/80 (macrophage marker) and Picrosirius red (collagen deposition). Scale bar 100µm.

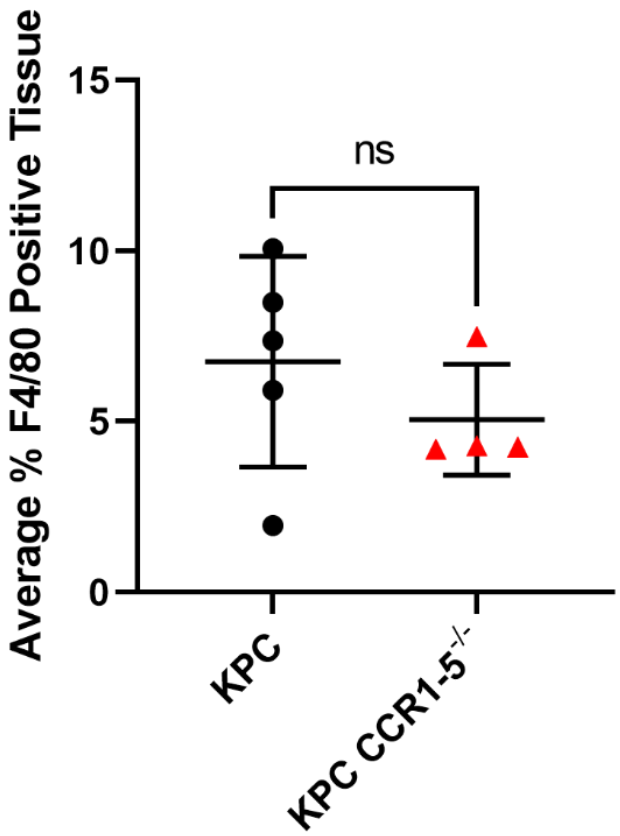

Figure 3.15 Quantification of macrophages surrounding PanINs in 6 week old KPC and KPC CCR1-5$^{-/-}$ mice.

Quantitative analysis of immunohistochemistry staining for F4/80 in the KPC CCR1-5$^{-/-}$ mouse model compared with KPC mice. P value non-significant (n.s.), Mann Whitney. Each dot on the graph represents a whole tumour from one mouse. N ≥ 4

Together, my data show no evidence that the absence of CCR1-5 has any effect on early PanIN lesion formation or proliferative status at the 6 week time point. The analysis of the stromal compartment also suggest that at 6 weeks, KPC and KPC CCR1-5$^{-/-}$ mice do not exhibit any significant differences in numbers of macrophages residing in close proximity to PanINs. As shown previously, pancreatic tissue from otherwise wild-type CCR1-5$^{-/-}$ mice at 6 weeks displays very low numbers of F4/80 positive macrophages. The data presented here imply that as PanINs arise, tissue resident macrophages are able to expand and populate the stromal compartment.

3.3.3 Investigating the role of infiltrating macrophages in PDAC

3.3.3.1 *Pdx1-Cre; LSL-Kras$^{G12D/+,}$ LSL-Trp53$^{R172H/+}$ (KPC) mice recapitulate the stromal microenvironment of human PDAC and has high infiltration of macrophages*

The *Pdx1-Cre; LSL-Kras$^{G12D/+}$, LSL-Trp53$^{R172H/+}$* mouse model (KPC) harbours conditional point mutations in *Kras* and *Trp53* genes that lead to the generation of constitutively active KRAS and mutant p53 within the pancreas. Mutations are induced upon the activation by cre recombinase under the control of transcription factor, *Pdx1* (pancreatic and duodenal homeobox 1). The KPC mouse model of PDAC recapitulates the human disease both phenotypically and histologically. KPC mice develop invasive and metastatic tumours. This model holds a great value for the tumour immune microenvironment studies because it reproduces many of the key features of the immune microenvironment observed in the human condition. This includes high levels of fibrosis with high collagen content, high infiltration of tumour-associated macrophages, abundant levels of fibroblasts and exclusion of effector T cells (Figure 3.16).

What is more, the KPC mouse model develops tumours that metastasize to distant organs. Metastases are most common in the liver, lungs and diaphragm (Figure 3.17). More rarely metastases are observed in spleen. The tendency of KPC tumours to metastasize as well as the distribution of metastases in the distant organs mirrors tumour metastases in human pancreatic cancer. Therefore, this model is a valuable tool for the investigation of PDAC dissemination.

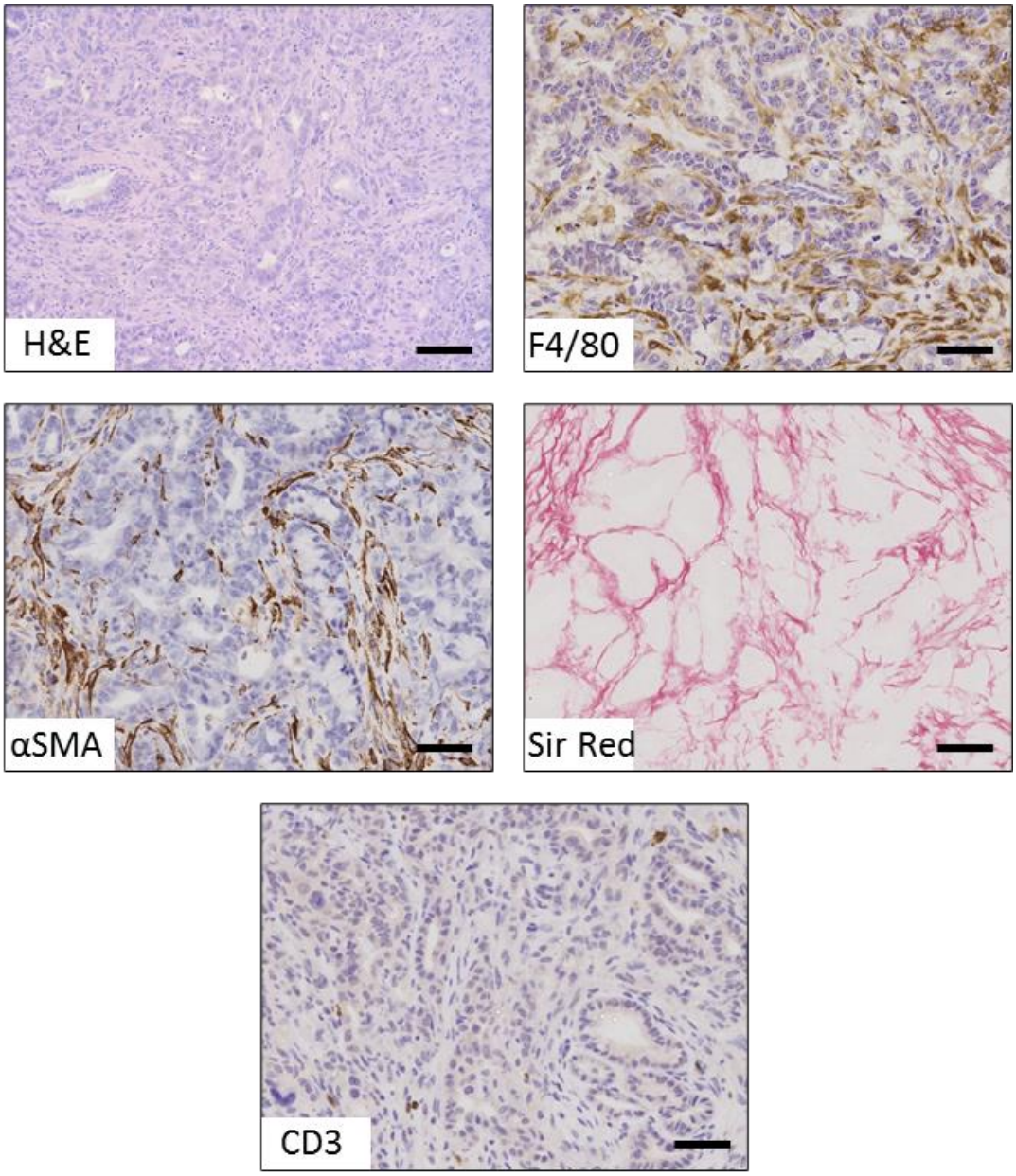

Figure 3.16 *Pdx1-Cre; Kras$^{G12D/+}$, p53$^{R172H/+}$* (KPC) mice develop invasive tumours that represent the histology and tumour immune microenvironment of human disease.

Representative images of KPC tumour tissue showing Haematoxylin and Eosin (H&E) staining, or immunohistochemistry to detect macrophages (F4/80), activated stellate cells (αSMA), collagen I (Sir Red) and T cells (CD3). Scale bar 50µm.

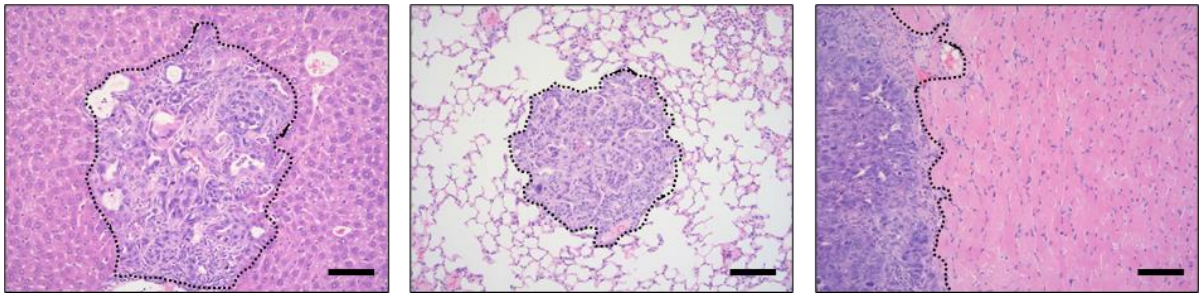

Figure 3.17 KPC tumours metastasize to distant organs.

Representative images of Haematoxylin and Eosin (H&E) staining of pancreatic tumour metastases in the liver (left), lung (middle) and diaphragm (right) in KPC mice. Scale bar 20µm.

I was keen to exploit a model that recapitulated human tumours in terms of high macrophage infiltration and the lack of response to the current immunotherapies. I wanted to explore the option of depleting infiltrating macrophages and monitor the effect it has on pancreatic tumour growth. As described in section 1.4, the recruitment of monocytes to the tissues is largely controlled through the chemotactic signalling through C-C chemokine receptors. Therefore, I hypothesised that genetic knock-out of CCR genes would prevent macrophage infiltration to the pancreas and subsequently to the PDAC in KPC model.

To begin with, I wanted to confirm that pancreatic cancer cells are secreting inflammatory cytokines responsible for chemotactic migration and infiltration of monocytes into the tissues. Therefore, I analysed the data from a study previously performed in our lab in which conditioned media from pancreatic cancer cell lines was used to perform cytokine array analysis. I focused my attention primarily on the C-C chemokine receptor ligands.

Cytokine array confirmed production of chemotactic proteins such as the monocyte chemoattractant protein-1 (MCP-1; CCL2) which binds directly to CCR2 and is a potent chemotactic factor for monocytes (Figure 3.18) What is more, expression of macrophage inflammatory protein 1-alpha (MIP-1 α; CCL3) was observed along with other proteins responsible for monocyte chemotaxis. Cytokine array confirmed that pancreatic cancer cells are secreting C-C chemokines. Therefore, genetic ablation of C-C chemokine receptors in KPC mice should result in the decrease of monocyte infiltration in tissues, including pancreas.

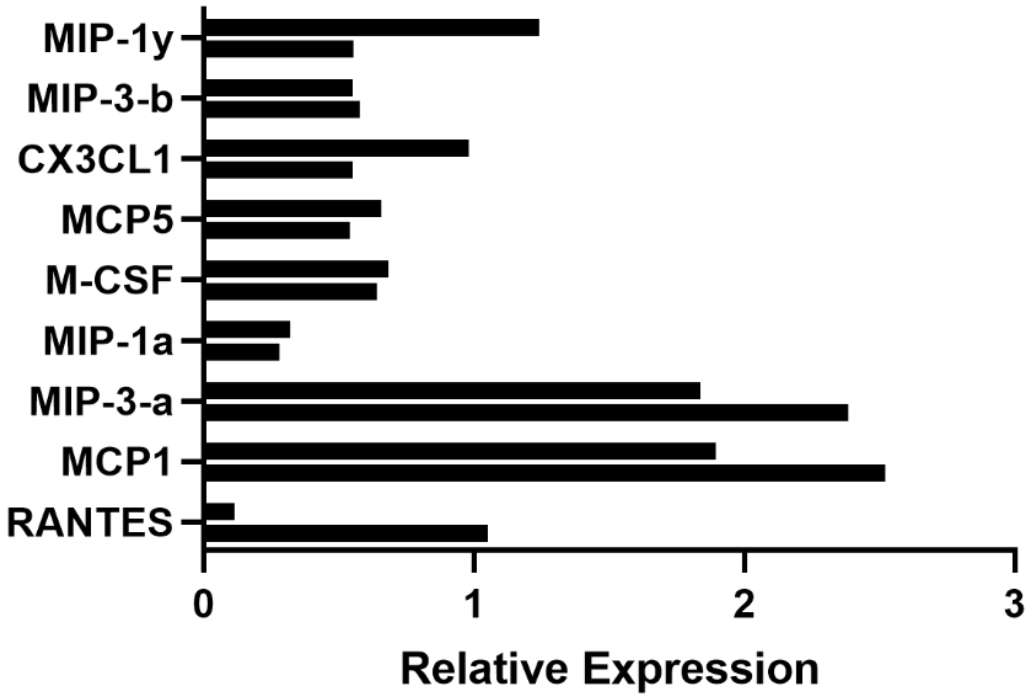

Figure 3.18 KPC tumour cells secrete chemokines responsible for monocyte chemotaxis.

Graph illustrating relative expression of chemokines from the conditioned media of two KPC cell lines, n=2. Relative expression, as normalised to the positive control spots provided by biotin-conjugated IgG printed directly onto the cytokine array membrane. (with thanks to Karen Pickering for the raw data).

3.3.3.2 *CCR1-5 homozygosity fails to alter PDAC-specific survival and phenotype in KPC mice*

To assess the role of infiltrating macrophages in pancreatic tumour development and progression, I aged and monitored KPC and KPC CCR1-5$^{-/-}$ mice until the clinical end point. However, Kaplan-Meier survival analysis showed that there was no significant difference in survival in KPC CCR1-5$^{-/-}$ mice (or KPC CCR1-5$^{+/-}$ mice) compared with KPC controls (Figure 3.19).

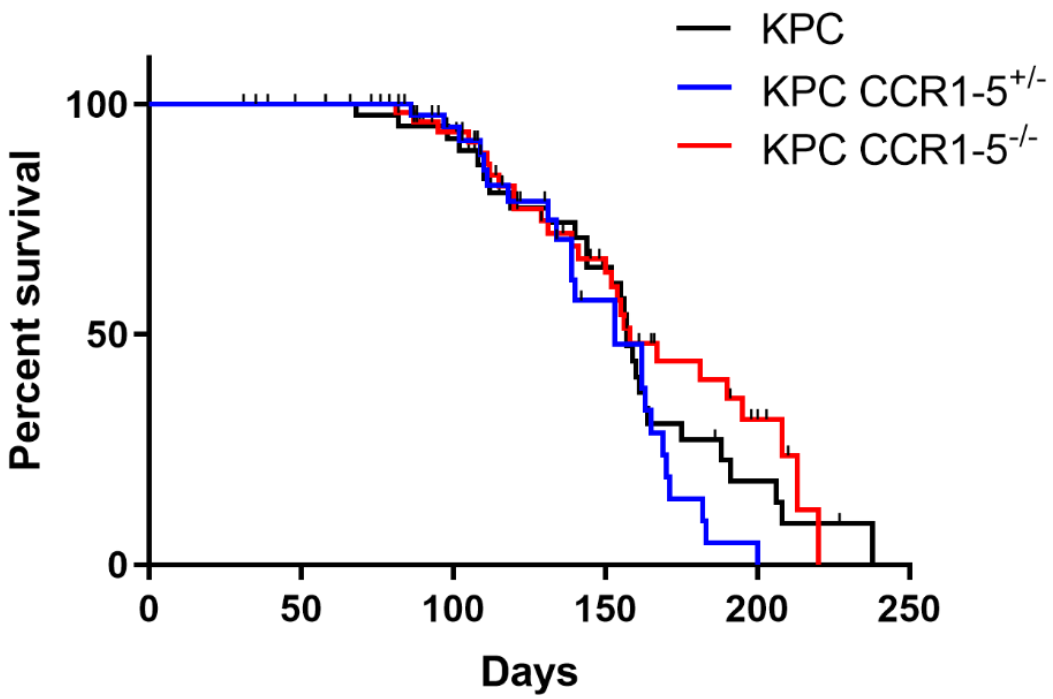

Figure 3.19 CCR1-5$^{-/-}$ homozygosity fails to alter PDAC-specific survival in KPC mice.

The Kaplan-Meier survival curve of *Kras$^{LSL.G12D/+;}$ Trp53$^{R172H/+;}$ Pdx1Cre; CCR1-5$^{-/-}$* mice. Deaths due to non-PDAC causes are censored out and appear as vertical lines on survival curves. WT (N=42); KPC CCR1-5$^{+/-}$ (N=46); KPC CCR1-5$^{-/-}$ (N=60) Log-rank (Mantel-Cox) test, non-significant.

At endpoint, all mice were dissected, their gross pathology assessed, and organs collected for further analysis. Pancreas, spleen, liver and lungs were fixed in formalin and paraffin embedded. Representative images were taken of slides stained with haematoxylin and eosin (H&E) (Figure 3.20).

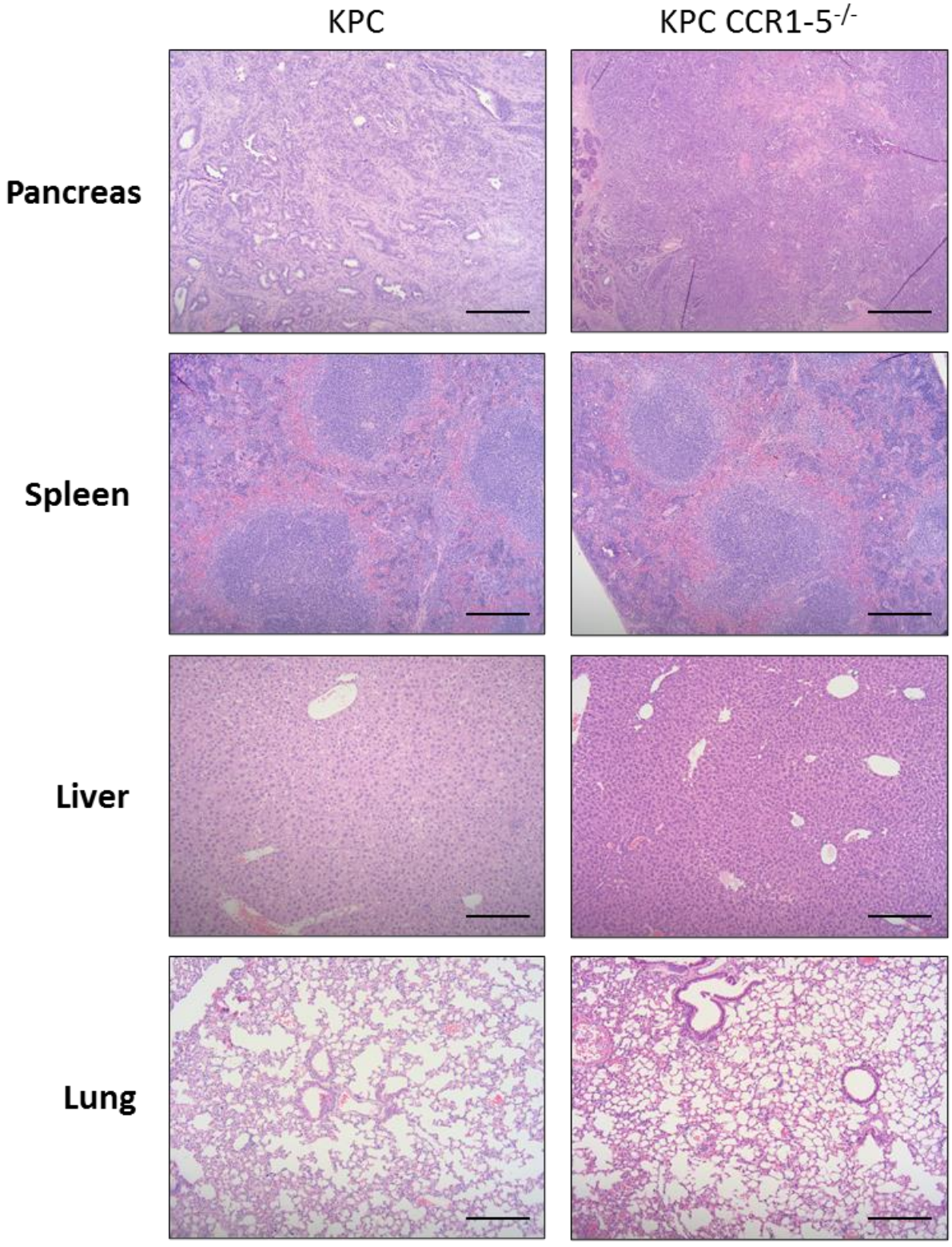

Figure 3.20 CCR1-5 deficiency in KPC mice has no effect on tumour and organ histology in mice with end stage disease.

Representative images of H&E staining of tumours and organs from end point KPC and KPC CCR1-5$^{-/-}$ mice demonstrating no significant changes in histology between the two groups. Scale bar 100µm.

No significant differences in tumour histology were observed. What is more, CCR1-5 deficiency did not have any visible effect on the histology of spleen, liver and lungs. No abnormalities, other than pancreatic tumours and metastases, or the extra-pancreatic pathologies previously observed in KPC mice (e.g. papilloma, lymphoma), were observed in KPC CCR1-5$^{-/-}$ mice during dissections. Further, there was no difference in the incidence of PDAC, or the number of mice censored due to other pathologies, between genotypes. Together, these data suggest that preventing monocyte trafficking to the tumour has no effect on tumour progression.

The end point tumour microenvironment was studied by immunohistochemistry. To begin with, tumours from KPC and KPC CCR1-5$^{-/-}$ mice were stained for macrophages. The immunohistochemical analysis revealed high macrophage abundance in KPC CCR1-5$^{-/-}$ mice, similar to that observed in KPC control mice (Figure 3.21). Pancreatic ductal adenocarcinoma (PDAC) is characterized by high levels of fibrosis with high collagen content that is believed to play a role in treatment response. What is more, tumour associated macrophages have been shown to play a role in collagen deposition, cross-linking and linearization during tumour development. To study if the loss of CCR1-5 in KPC tumours has an effect on extracellular matrix (ECM) structures, sections of PDAC were stained for collagen (Sirius Red) and α-smooth muscle actin (α-SMA) **(**Figure 3.21).

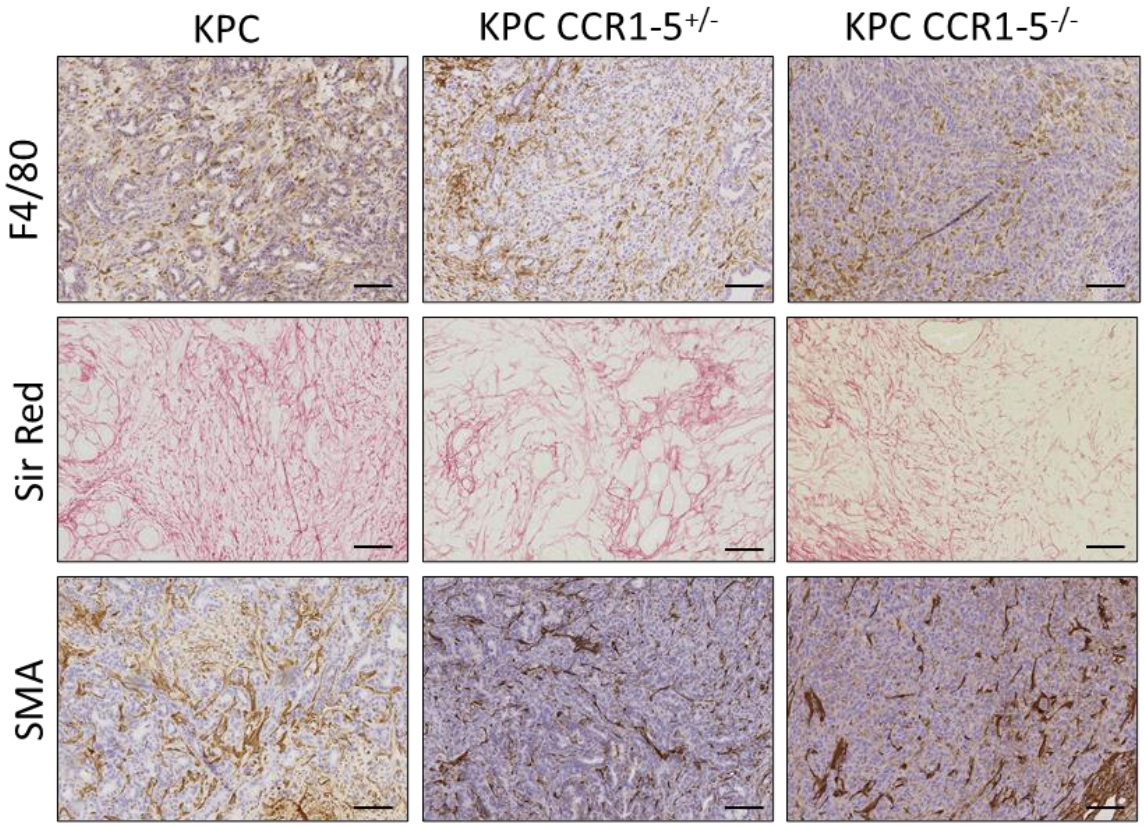

Figure 3.21 Evaluation of desmoplasia in end point tumours.

Representative immunohistochemistry images of PDAC sections from end point KPC and KPC CCR1-5$^{-/-}$ mice. Tumours were stained with F4/80 (macrophage marker), Sirius red (collagen deposition) and α-smooth muscle actin (fibroblast marker). Scale bar 100µm.

Stained sections were also quantified using the HALO image analysis platform (Figure 3.22). Interestingly, no significant changes in macrophage numbers or tumour desmoplasia between KPC and KPC CCR1-5$^{-/-}$ mice were detected. These data suggest that the CCR receptors are not required to maintain the intra-tumoural macrophage population.

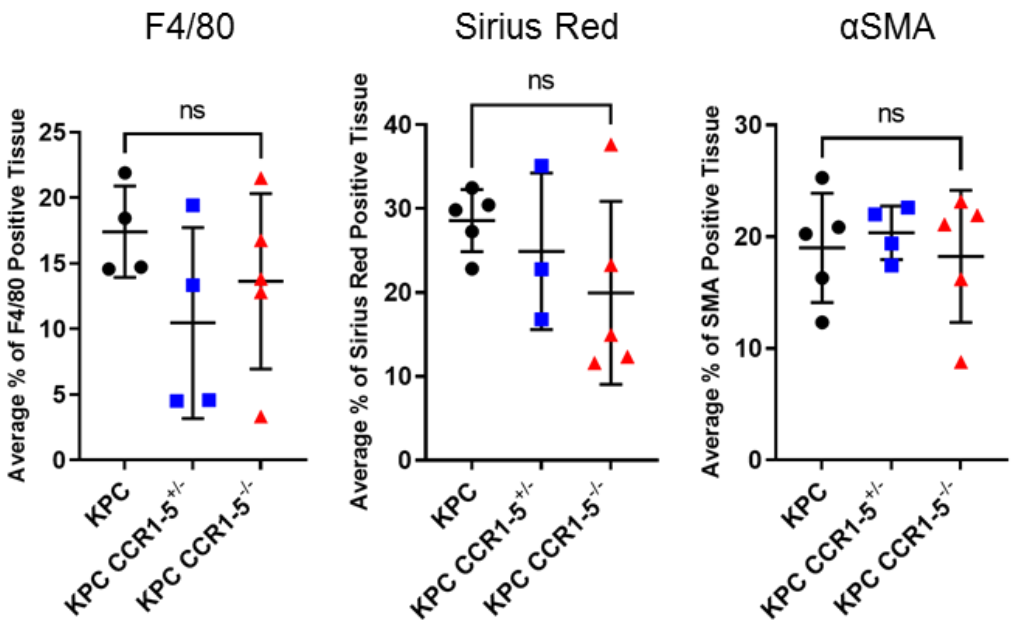

Figure 3.22 Quantification of desmoplasia markers in KPC CCR1-5$^{-/-}$ tumours.

Quantitative analysis of immunohistochemistry staining in tumours from the KPC CCR1-5$^{-/-}$ mouse model compared with KPC CCR1-5$^{+/-}$ and KPC mice. P value non-significant (n.s.)., Mann Whitney. Each dot on the graphs represents a whole tumour from one mouse. N ≥ 4

Finally, flow cytometry analysis was performed to investigate the status of macrophage polarization in tumours from KPC CCR1-5$^{-/-}$ mice (Figure 3.23). End point tumours were dissected and single cell suspensions obtained. Cells were stained for the well-established macrophage polarization markers: MHCII, CD80, CD86, CD206 and Arg1.

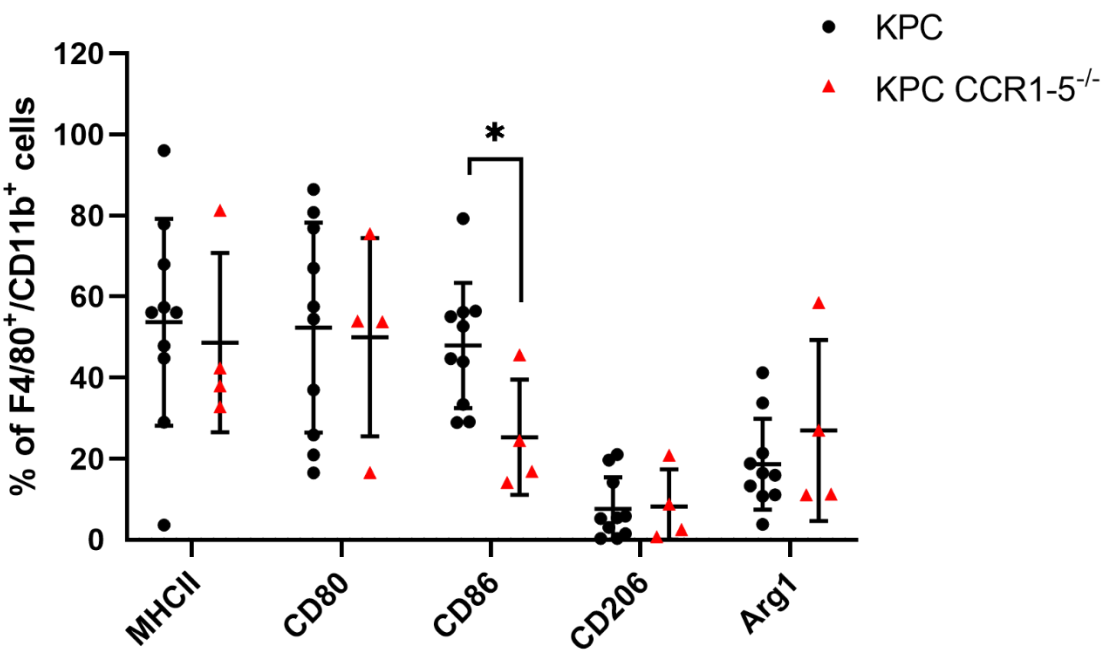

Figure 3.23 Evaluation of macrophage polarization status in PDAC from KPC CCR1-5$^{-/-}$ mice.

Flow cytometry analysis of tumour tissue from end point KPC and KPC CCR1-5$^{-/-}$ mice. N≥4,Mann Whitney, *p=0.0265

As emphasized in the literature, I observed an array of differential expression of selected macrophage polarization markers, rather than a uniform shift towards a pro- or anti-inflammatory phenotype. Tumours exhibited high heterogeneity within the the same genotypes. Out of the five different polarization markers tested, I only observed a significantly reduced expression of the co-stimulatory molecule, CD86, in tumours from KPC CCR1-5$^{-/-}$ mice. Although, the expression of CD86 on macrophages is generally associated with the M1-like phenotype of TAMs, given the absence of change in the expression of other markers I concluded that TAMs in KPC and KPC CCR1-5$^{-/-}$ mice do not exhibit vast phenotypic differences. Overall, these results imply that environmental cues, rather than the cell ontogeny, are dictating macrophage functional phenotype.

Finally, I assessed peripheral blood leukocyte populations in end point mice (Figure 3.24). However, as seen previously (3.2.2), ProCyte Dx Haematology Analyser fails to detect a decrease in circulating monocyte levels in KPC CCR1-5$^{-/-}$ mice. All in all, no significant changes were observed in leukocyte populations across these genotypes.

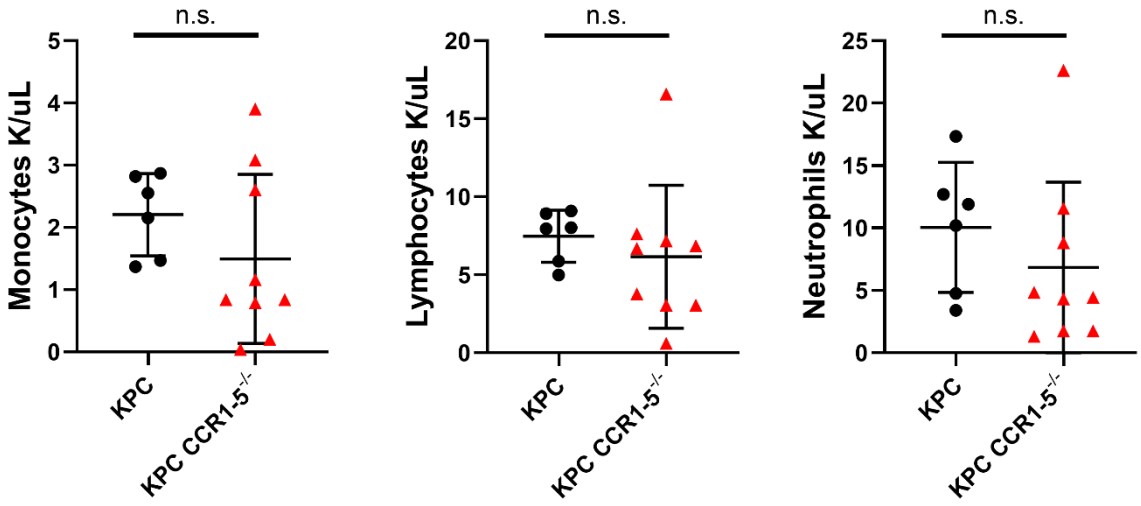

Figure 3.24 Analysis of peripheral blood from KPC and KPC CCR1-5$^{-/-}$ mice.

Graphs illustrate peripheral blood leukocyte counts. Blood was taken and stored in EDTA tubes until processed with ProCyte Dx Haematology Analyser. KPC (N=6) vs KPC CCR1-5$^{-/-}$ (N=9), Mann Whitney.

In order to measure monocyte levels in peripheral blood with greater precision, I performed flow cytometry analysis. Flow cytometry data clearly shows that CCR receptor-lacking mice have significantly reduced numbers of circulating Ly6C^{hi} monocytes when compared with KPC mice (Figure 3.25) This data further support the observation that was made in peripheral blood from CCR1-5$^{-/-}$ mice at 6 weeks of age demonstrating significantly reduced levels of Ly6C^{hi} inflammatory monocytes. Despite the lack of effect on tumourigenesis, the genetic knockout of CCR1,2,3 and 5 clearly causes the reduction of circulating Ly6C^{hi} monocytes.

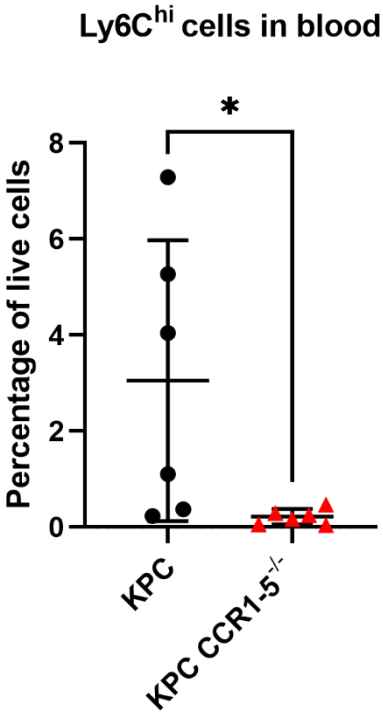

Figure 3.25 End point tumour-bearing KPC CCR1-5$^{-/-}$ mice display reduced numbers of circulating Ly6C^{hi} inflammatory monocytes.

Flow cytometry analysis of peripheral blood from mice with end stage tumours. Blood samples were stained with a PECy7-conjugated monoclonal antibody against Ly6C and a FITC conjugated CD11b monoclonal antibody. KPC N=3 vs KPC CCR1-5$^{-/-}$ N=5 , p= 0.0260, Mann Whitney.

3.3.4 Investigating the role of infiltrating macrophages on metastasis

3.3.4.1 *CCR1-5 homozygosity fails to alter metastasis burden in KPC mice*

To assess the role of infiltrating macrophages on the formation of metastasis, I assessed the KPC and KPC CCR1-5$^{-/-}$ aged until the clinical end point and sacrificed when exhibiting clinical sign of the disease. Organs were grossly inspected during the dissection for any visible metastasis. Collected tissue was formalin fixed and paraffin embedded. In order to assess the presence of micro metastasis, tissues were cut and stained for H&E. Slides were analysed using light microscopy (Figure 3.26). Representative images were taken. Images reveal that CCR1-5 homozygosity does not prevent the formation of tumour metastasis in KPC mouse model. Metastasis were observed at the typical locations, such as liver, lungs, and diaphragm.

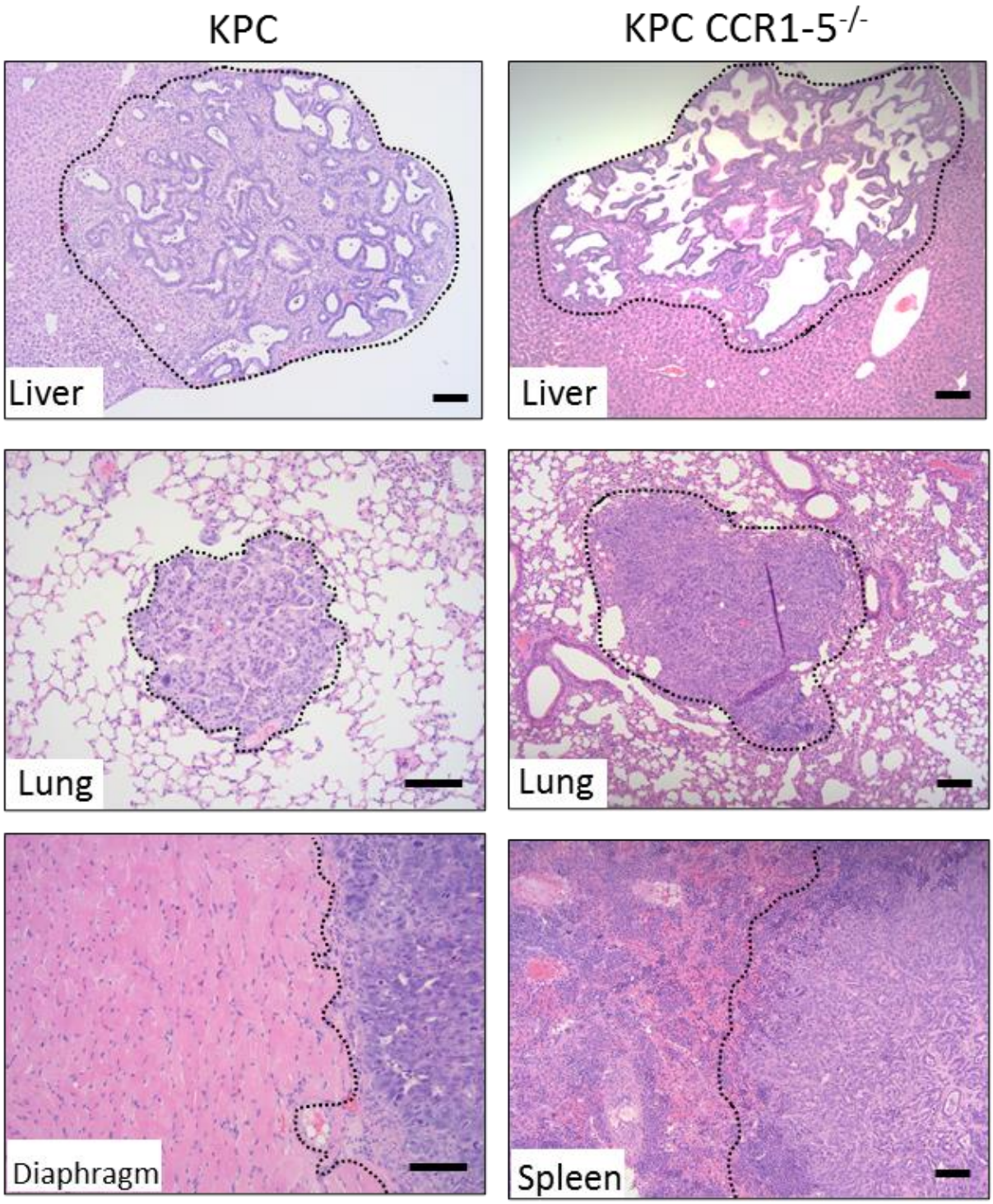

Figure 3.26 KPC CCR1-5$^{-/-}$ tumours metastasize to distant organs.

Representative images of haematoxylin and eosin (H&E) staining of tumour metastases in KPC CCR1-5$^{-/-}$ with end point tumours as compared to KPC mice. Scale bar 100µm.

Next, I wanted to assess the burden of metastasis in KPC CCR1-5$^{-/-}$ mice as compared with KPC mice. Quantitative analysis of metastasis did not reveal any significant difference in the presence of metastases between the two cohorts (Table 3.1). These data suggest that deletion of the CCR receptors and subsequent lack of monocyte trafficking does not affect the formation of metastasis at least in our model.

	KPC	KPC CCR1-5 $^{-/-}$
Metastasis	10	7
No Metastasis	18	23
Percentage	35%	23.3%

Table 3.1 Table comparing levels of metastasis between KPC CCR1-5$^{-/-}$ and KPC mice.

The internal organs were inspected for evidence of metastases during mice dissection. Histological examination of liver, lungs and diaphragm was carried out to check for microscopic presence of metastases. Metastatic incidence is displayed in the table above. KPC (Control) vs KPC CCR1-5$^{-/-}$

Having seen no impact on the occurrence of metastases in KPC CCR1-5$^{-/-}$ mice, I sought to investigate whether the levels of macrophages at metastatic sites are different in KPC CCR1-5$^{-/-}$ mice as compared with KPC mice. Liver sections with confirmed metastases were stained for F4/80. Metastases were annotated and F4/80 staining was quantified (Figure 3.27). However, I observed no change in macrophage numbers at metastatic sites between the cohorts analysed. Collectively, these data suggest that tissue resident macrophages are not only capable to sustain primary tumour growth in KPC CCR1-5$^{-/-}$ mice, but also play an important role at distant organs too.

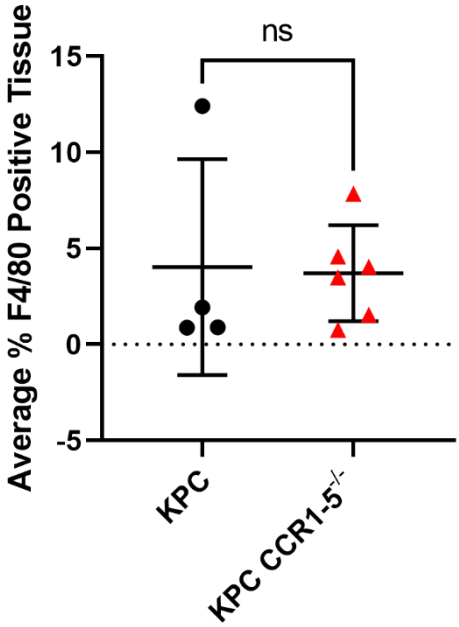

Figure 3.27 Quantification of macrophages in liver metastases from KPC CCR1-5$^{-/-}$ mice.

Quantitative analysis of immunohistochemical staining for F4/80 in liver metastases from the KPC CCR1-5$^{-/-}$ mouse model compared with those from KPC mice. P value non-significant (n.s.), N ≥ 4 Mann Whitney. Each dot on the graph represents metastases from one mouse.

3.4 Discussion

Macrophages are critical components of the tumour microenvironment, and their recruitment to pancreatic precursor lesions is regarded as one of the earliest immune responses during tumour initiation. Here we crossed the KPC mouse model of pancreatic cancer with mice lacking C-C chemokine receptors 1, 2, 3 and 5, to investigate the role of infiltrating macrophages on tumour initiation, development and progression. It is well established that monocytes rely on CCR2 to exit the bone marrow, as well as for trafficking into the tissues (Serbina and Pamer 2006).

Literature suggests that Ly6C^{hi} inflammatory monocytes represent approximately 2-5% of circulating white blood cells in mice under normal conditions and have the potential to be rapidly recruited to the sites of inflammation, including malignant and pre-malignant lesions (Serbina, Jia et al. 2008) During peritonitis, the recruitment of inflammatory monocytes to the pancreas is mediated via CCR2 (Si, Tsou et al. 2010).

In accordance with previously published studies investigating the role of CCR2, I found that in the absence of CCR1-5 receptors, mice have significantly reduced numbers of circulating Ly6C^{hi} inflammatory monocytes. On the other hand, the frequency of circulating Ly6C^{lo} monocytes was minimally affected, as demonstrated by data in this thesis as well as previous publications (Serbina and Pamer 2006) (Dyer, Medina-Ruiz et al. 2019). Together, these data indicate that CCR2 is less important for the trafficking of Ly6C^{lo} monocytes from the bone marrow.

It appears from my experiments that CCR1-5 receptors are playing a role in monocyte egress from the bone marrow. This observation is further supported by the previous study (Dyer, Medina-Ruiz et al. 2019). Interestingly, no accumulation of monocytes was observed in the bone marrow under normal conditions. However, a previous study indicates that activated monocytes accumulate in the bone marrow after infection with *Listeria monocytogenes* (Serbina and Pamer 2006). Whether activated monocytes accumulate in bone marrow during the development of PDAC is yet to be determined.

Intriguingly, pancreata from CCR1-5$^{-/-}$ mice sampled at a 6 week time point presented with macrophages. However, when I looked at the overall presence of CCR2 in the end point tumours from KPC CCR1-5$^{-/-}$ mice I found that CCR2 was absent, thus validating the model. Therefore, I hypothesised that macrophages observed in tissues from CCR1-5$^{-/-}$ mice are resident macrophages. As emerging data suggests, tissue-resident macrophages are established during embryonic development from yolk sac-derived precursors around

embryonic day 8. (Ginhoux, Greter et al. 2010). Fate mapping studies indicate that these macrophages persist in adult mice independently of haematopoietic stem cells (HSCs) (Schulz, Perdiguero et al. 2012). Recent studies in pancreatic cancer have demonstrated that tumour-associated macrophages (TAMs) originate from both embryonic development and the bone marrow. It was observed that macrophages originating from the yolk sac make up a significant proportion of TAMs and, unlike monocyte derived TAMs, exhibit a profibrotic transcriptional profile. Tissue resident macrophages were demonstrated to undergo a significant expansion during PDAC progression (Zhu, Herndon et al. 2017). It is, therefore, likely that macrophages observed in the end stage tumours from KPC CCR1-5$^{-/-}$ mice have populated tumours through *in situ* proliferation rather than being replenished from recruited monocytes. However, resident macrophages in pancreas do not present with a unique marker whose expression could be readily assessed in our mouse model to test this hypothesis. They have been reported to express low levels of MHC-II, however, there was no significant difference in MHC-II expression on macrophages from endpoint tumours from KPC CCR1-5$^{-/-}$ mice. If time permitted I would like to conduct fate-mapping studies to further test the hypothesis.

In contrast to the reported profibrotic role in PDAC, there is evidence to suggest that tissue-resident macrophages exhibit anti-inflammatory properties during the early stages of tissue damage. A study led by Uderhardt et al, demonstrated that tissue-resident macrophages rapidly sense cell damage and 'cloak' the damage site by extending their membrane processes. By doing so, macrophages prevent neutrophil-mediated inflammatory damage and maintain tissue homeostasis (Uderhardt, Martins et al. 2019). In order to study the role of infiltrating macrophages at the stage of tumour initiation, I studied the pancreata of 6 week old KPC and KPC CCR1-5$^{-/-}$ mice. However, no significant differences were observed in the numbers or grade of PanIN lesions in KPC CCR1-5$^{-/-}$ mice as compared with KPC mice. From the data collected, I concluded that infiltrating macrophages do not play a significant role in tumour initiation. Previous findings suggest that tissue-resident macrophages suppress inflammatory damage and play a tissue-protective role in response to acute disruptions of stromal integrity (Uderhardt, Martins et al. 2019). However, I demonstrated that ablation of infiltrating macrophages does not induce an anti-tumourigenic effect during the PanIN formation stage. My findings indicate that tissue-resident macrophages are sufficient to maintain the immune-microenvironmental cues that can promote early lesion formation and progression.

We previously demonstrated that inhibition of macrophages, via CSF1R inhibition, in KPC tumour-bearing mice leads to tumour shrinkage and improved overall survival (Candido,

Morton et al. 2018). What is more, tumours from the treated cohort exhibited a significantly reduced desmoplastic reaction and appeared 'squishy' upon inspection. Therefore, I assessed tumourigenesis in KPC CCR1-5$^{-/-}$ mice lacking infiltrating macrophages. However, I observed that KPC CCR1-5$^{-/-}$ mice have no survival benefit when compared with KPC mice, and no indication of reduced desmoplasia in tumours from KPC CCR1-5$^{-/-}$ mice was observed. This is likely explained by the fact that immunohistochemical analysis of end point tumours revealed high macrophage abundance in KPC CCR1-5$^{-/-}$ mice. This indicated that resident macrophages are sufficient to sustain tumour growth and maintain the fibrotic tumour microenvironment observed in KPC mice.

Having demonstrated no significant effect on the initiation and progression of the primary tumour, in the setting of infiltrating macrophage ablation, I sought to investigate the secondary metastatic site. Pancreatic tumours most often metastasize to liver, which was also the case for KPC and KPC CCR1-5$^{-/-}$ mice. In liver, macrophages are the most abundant immune cells and can be broadly classed into two groups: resident hepatic macrophages, also known as Kupffer cells, or monocyte-derived macrophages that originate from the bone marrow. Compelling evidence from bone marrow chimeras demonstrated that macrophages surrounding metastatic sites are exclusively bone marrow derived. It was shown that mice with genetic knockout of *Pik3cg*, which leads to a defect in monocyte recruitment under inflammatory conditions, had significantly reduced macrophage numbers in metastasis-bearing livers after intrasplenic implantation with KPC cells. What is more, PI3Kγ depletion reduced metastatic frequency and size (Nielsen, Quaranta et al. 2016). Interestingly, impairment in circulating monocytes by genetic knockout of *Ccr2* or *Nur77* had no effect on tumour growth in orthotopic PDAC models (Zhu, Herndon et al. 2017). Together, these findings suggest that infiltrating, bone marrow derived macrophages might play different roles in pancreatic cancer metastasis versus primary tumours. However, the analysis of the metastatic sites in KPC CCR1-5$^{-/-}$ mice implies that genetic ablation of CCR1-5 has no effect on macrophage numbers at metastatic sites or the frequency of metastasis at least in our model. Nevertheless, it is necessary to mention that the KPC and KPC CCR1-5$^{-/-}$ mice used in my study are of purebred C57BL/6 strain, and our lab has found that these mice exhibit lower rates of metastasis than mice of mixed background.

In this study, I aimed to gain a better understanding of the role of infiltrating macrophages at the stages of tumour initiation, progression and, eventually, tumour dissemination at the distant site, with the hope that this knowledge would help to guide new therapeutic approaches for pancreatic cancer. My data, along with other studies investigating the role of macrophages, indicate that clinical approaches targeting monocyte and macrophage

trafficking and infiltration are likely to be hampered by the multiple functions of macrophages, some of which may be tumour suppressive. What is more, systemic inactivation or depletion of macrophages could potentially have toxicity implications in patients. However, several therapeutic applications to impair TAM recruitment or viability are either being tested in clinical trials or have already reported no clinical activity. This includes a clinical trial with trabectedin, non-specific myeloid cell depleting chemotherapeutic agent, that demonstrated no activity as a single agent for metastatic pancreatic cancer (Belli, Piemonti et al. 2016). CSF1R inhibitors have also shown very limited antitumor effects in refractory pancreatic cancer (Papadopoulos, Gluck et al. 2017).

My results further raises the question whether macrophage origin or environmental cues dictate macrophage functional phenotype. The data I present imply that targeting specific signals that promote tumorigenesis rather than specific cell populations might be more beneficial in fighting tumourigenesis. While tumour cell induced metabolic reprogramming of stromal cells is widely studied, the extrinsic factors regulating macrophage functional responses are not well established. Further experiments are needed to determine what external signals prime infiltrating and tissue-resident macrophages for different functional responses during tumour progression. Thus, I am in the process of performing a large scale phenotypic analysis of TAMs using mass cytometry, CyTOF. The panel of 46 extracellular and intracellular markers will be used to perform TAM characterization with the hope that this will yield some targets that could be further validated and serve as therapeutic targets.

4 Pharmacological inhibition of macrophage expansion

4.1 Introduction

Previously, we reported that macrophage depletion by inhibiting CSF1R in KPC mice with established tumours resulted in the shrinkage of the tumours as well as improved overall survival. What is more, we observed changes in the desmoplastic stroma. Tumours treated with CSF1R inhibitor had decreased collagen I abundance and displayed less prominent alpha SMA expression (Candido, Morton et al. 2018). To our surprise, the absence of chemokine receptors responsible for monocytic recruitment to the tumours in KPC CCR1-5$^{-/-}$ mice had no effect on overall survival. Moreover, we did not observe any changes in the desmoplastic stroma. However, we observed that intra-tumoural macrophage abundance was unaffected in these mice, suggesting that tissue-resident macrophages were sufficient to populate the tumour. Interestingly, tissue resident macrophages are reported to be able to proliferate *in situ* during tumor progression (Zhu, Herndon et al. 2017). However, during the early stages of tumorigenesis, there is evidence to suggest that tissue-resident macrophages have tumor suppressive properties (Uderhardt, Martins et al. 2019) These findings led us to more closely investigate the role different macrophage populations might play in early tumour development and hypothesise that depletion of macrophages during early tumourigenesis might differentially affect survival in KPC vs KPC CCR1-5$^{-/-}$ mice.

4.2 Experimental aims

To investigate the role of both infiltrating and tissue resident macrophages in early tumour development, we set up experimental studies where we pharmacologically deplete macrophages using CSF1R inhibitor.

4.3 Results

4.3.1 Macrophage inhibition at an early time point

At 6 weeks KPC mice start developing PanIN lesions that later lead to the development of a tumour. Therefore, the 6 week timepoint seemed to be the best time to target macrophages and observe their role in tumour development. Thus, in the first instance, we started treating KPC and KPC CCR1-5$^{-/-}$ mice with CSF1R small molecule inhibitor (AZD7507) from 6 weeks of age until the clinical end point. As shown by the Kaplan-Meier curve, neither KPC nor KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from 6 weeks survived longer than

untreated controls (Figure 4.1). In fact, survival appeared to be significantly reduced in both KPC and KPC CCR1-5$^{-/-}$ mice compared with the untreated controls of the same genotypes. These results were surprising, given our previous findings that inhibition of CSF1R in KPC mice with established tumours leads to the shrinkage of tumour and significantly improved overall survival. Indeed, these data suggest that during the early stages of tumourigenesis, macrophages may play a tumour suppressive role. Moreover, the reduced survival was seen in both genotypes of mice, whereas the survival of untreated KPC CCR1-5$^{-/-}$ mice was unchanged compared to KPC mice, indicating that infiltrating macrophages may be tumour suppressive in early lesions. However, the lack of markers for these macrophage subsets makes it difficult to determine how much each contributes to the tumour immune microenvironment in the KPC mice compared with the KPC CCR1-5$^{-/-}$ mice.

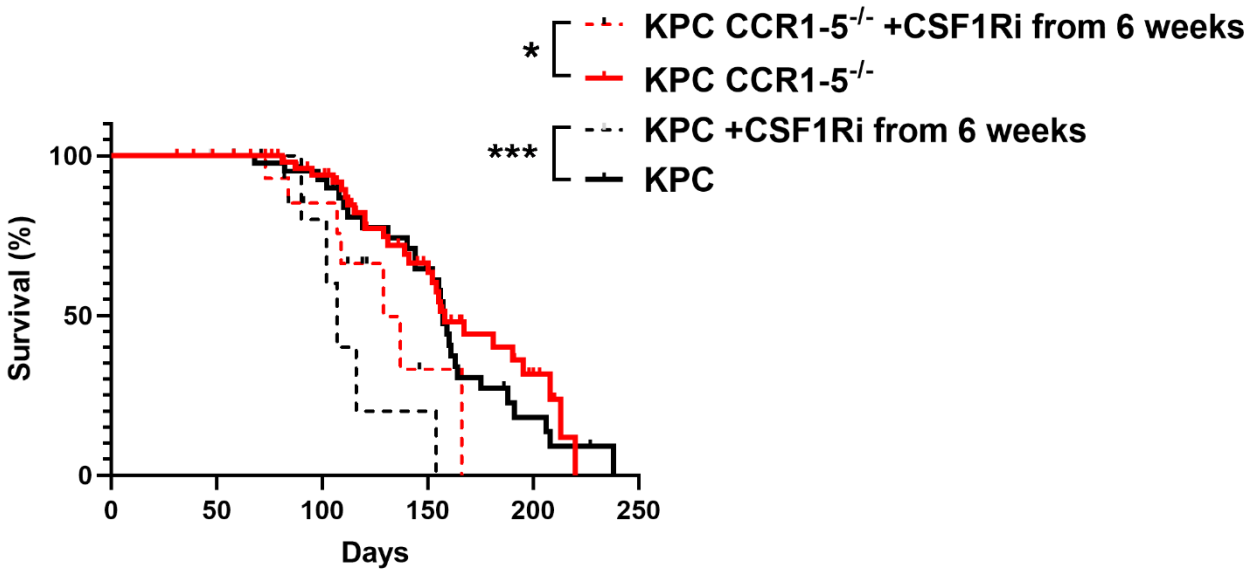

Figure 4.1 Macrophage depletion from an early time point fails to extend overall survival of KPC mice.

The Kaplan-Meier survival curves of untreated *LSL-Kras*$^{G12D/+;}$ *LSL-Trp53*$^{R172H/+;}$ *Pdx1-Cre* (KPC) and KPC CCR1-5$^{-/-}$ mice and KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1Ri from 6 weeks of age. Deaths due to non-PDAC causes are censored out and appear as vertical lines on survival curves. KPC (N=42); KPC CCR1-5$^{-/-}$ (N=60); KPC CCR1-5$^{-/-}$ +CSF1Ri from 6 weeks (N=16); KPC +CSF1Ri from 6 weeks (N=6); Log-rank (Mantel-Cox) test *P=0.0283, ***P=0.0008

I wanted to investigate whether there were any effects on the histological phenotype of tumours in these mice. Thus, end point tumours were collected and representative H&E pictures taken (Figure 4.2). No noticeable differences in tumour histology in terms of differentiation status, necrosis or inflammatory phenotype between the cohorts were

observed, suggesting that macrophage depletion prior to tumour development has no impact on tumour histology.

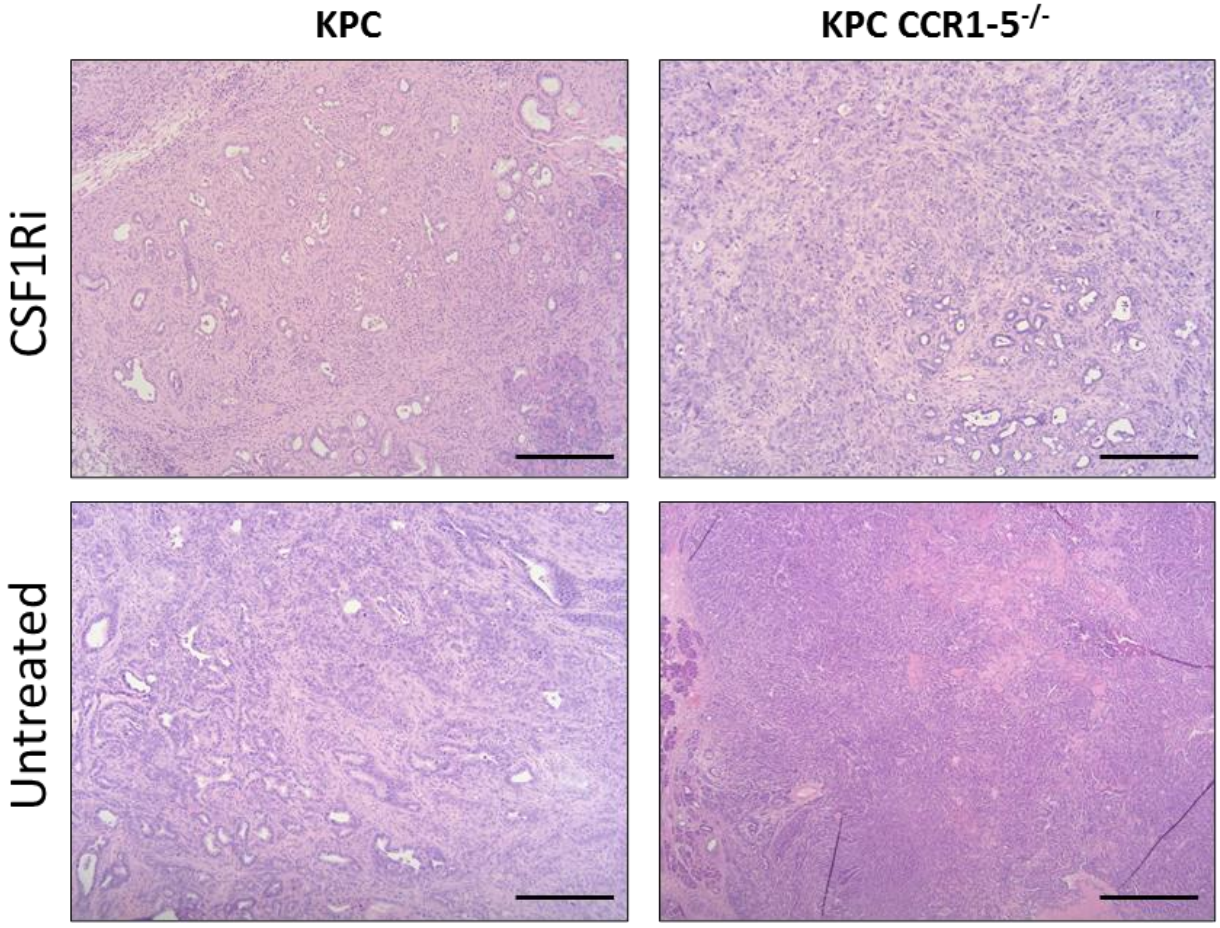

Figure 4.2 Tumour histology from mice treated with CSF1R inhibitor from 6 weeks of age.

Representative images of haematoxylin and eosin (H&E) staining of end point tumours from KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor (AZD7507) (top) as compared with untreated controls (bottom). Scale bar 100µm.

To investigate the effects of CSF1R inhibition on the number of macrophages within the tumours at endpoint, tumour sections were stained for the macrophage marker, F4/80, and representative pictures taken (Figure 4.3).

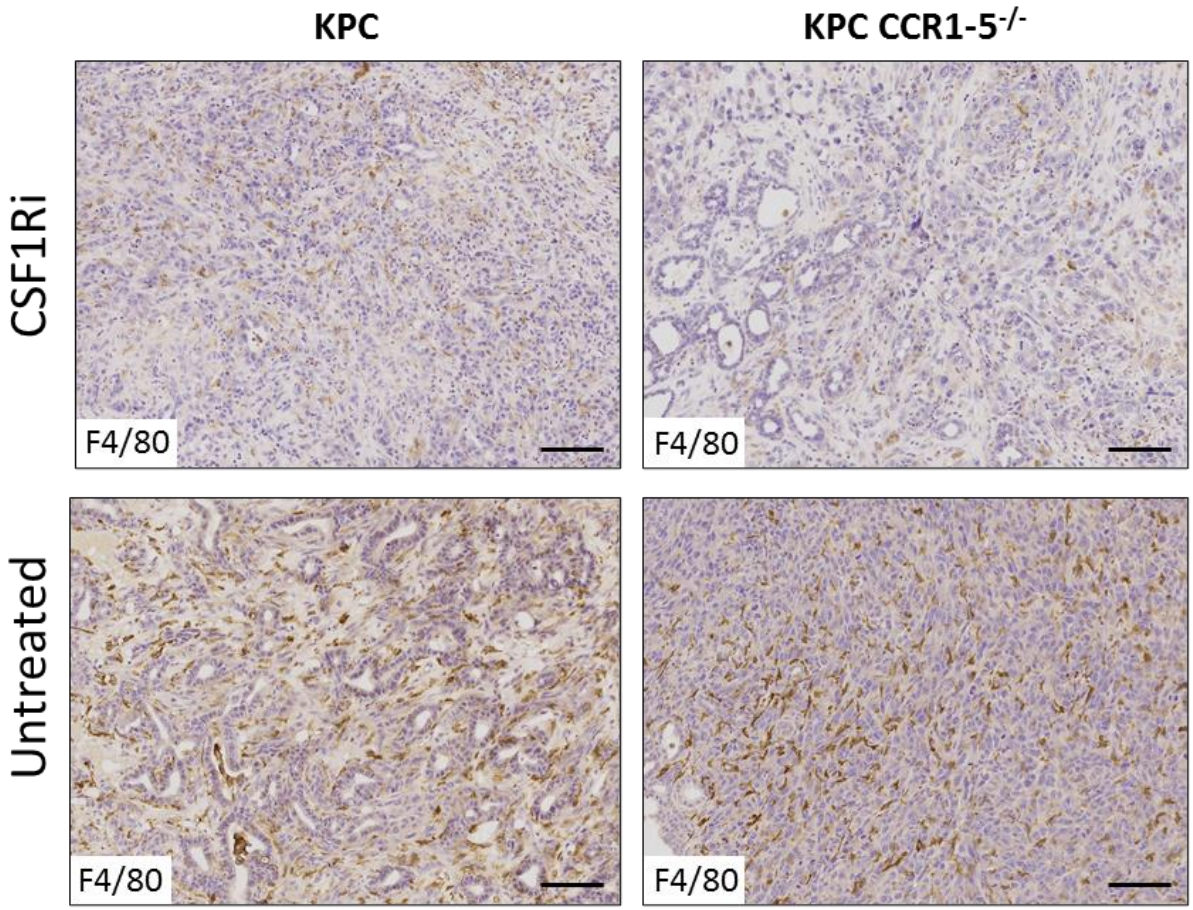

Figure 4.3 Macrophage depletion with CSF1R inhibitor in KPC mice from 6 weeks of age.

Representative immunohistochemistry images of staining for the macrophage marker, F4/80, in end point tumours from KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor (AZD7507) (top) as compared with untreated controls (bottom). Scale bar 100µm.

Stained sections were examined and quantified using the image analysis platform HALO. We saw a consistent depletion of tumour associated macrophages in mice treated with CSF1R inhibitor as compared with controls (Figure 4.4). These data verify that macrophages were successfully depleted by treatment with the CSF1R inhibitor and thus confirm that macrophage depletion prior to tumour development does not restrict tumourigenesis, but rather promotes tumourigenesis.

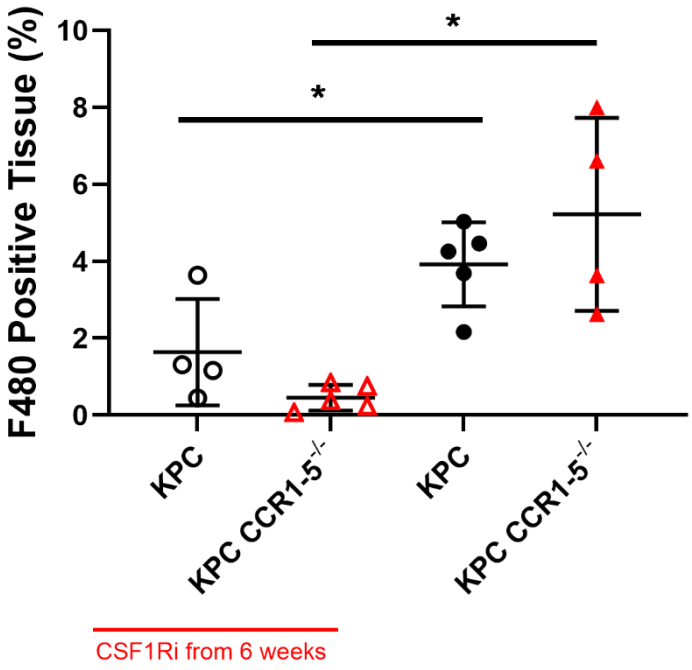

Figure 4.4 CSF1R inhibition reduces macrophage infiltration in tumour models.

Quantitative analysis of immunohistochemistry staining in tumours from the KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from 6 weeks of age as compared with untreated controls. KPC (N=5); KPC CCR1-5$^{-/-}$ (N=4); KPC +CSF1Ri from 6 weeks (N=4); KPC CCR1-5$^{-/-}$ +CSF1Ri from 6 weeks (N=5); P value KPC +CSF1R from 6 weeks vs KPC 0.0317., KPC CCR1-5$^{-/-}$ +CSF1Ri from 6 weeks vs KPC CCR1-5$^{-/-}$ 0.0159 Mann Whitney. Each dot on the graph represents a tumour from one mouse. * denotes P < 0.05

Previously we found that CSF1R inhibition in KPC mice with established tumours leads to a complete macrophage depletion and increased infiltration of CD3+ T cells (Candido, Morton et al. 2018). Therefore, I wanted to assess tumours from mice treated with CSF1R inhibitor from an early time point for the infiltration of CD3+ T cells. Tumour sections were stained for CD3+ T cells. Representative pictures taken were taken (Figure 4.5) and staining was quantified using HALO (Figure 4.6).

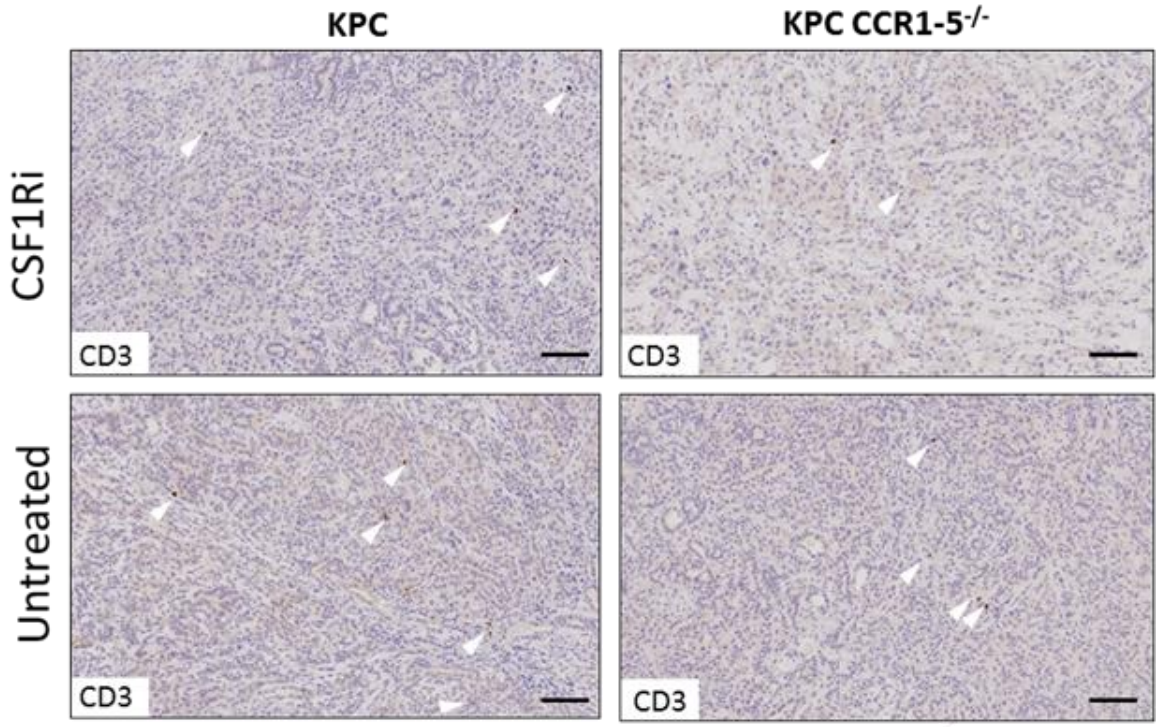

Figure 4.5 CD3+ T cell infiltration in tumours from mice treated with CSF1R inhibitor from 6 weeks of age.

Representative immunohistochemistry images of staining for CD3+ T cells in end point tumours from KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor (top) as compared with untreated controls (bottom). Scale bar 100µm.

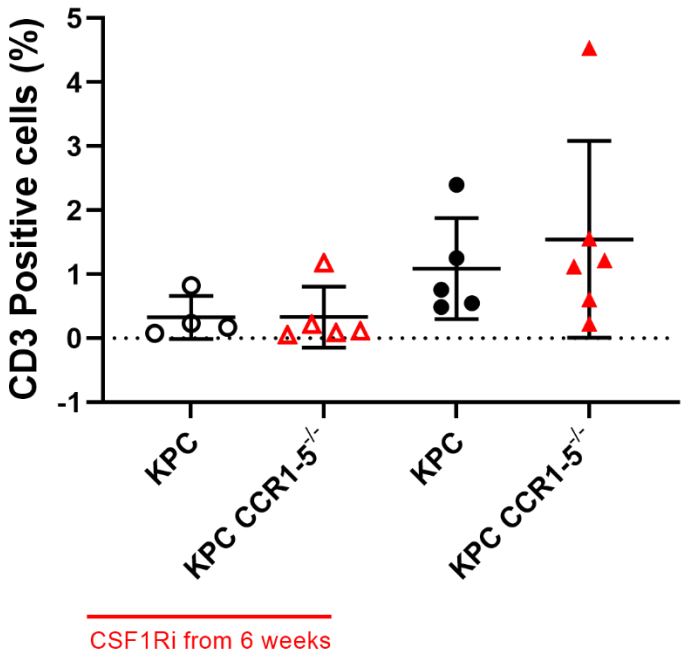

Figure 4.6 CSF1R inhibition has no effect on CD3+ T cell infiltration in tumour models.

Quantitative analysis of immunohistochemistry staining for CD3+ T cells in tumours from the KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from 6 weeks of age as compared with untreated controls. KPC (N=5); KPC CCR1-5$^{-/-}$ (N=6); KPC +CSF1Ri from 6 weeks (N=4); KPC CCR1-5$^{-/-}$ +CSF1Ri from 6 weeks (N=5), Mann Whitney. Each dot on the graph represents a tumour from one mouse.

Overall, no significant changes were observed in the infiltration of CD3^{+} T cells in tumours in either KPC or KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from 6 weeks of age. There were very few infiltrating T cells in any of the tumours, and if anything, there was a trend towards reduced T cell infiltration in mice treated with CSF1Ri prior to tumour development. These results suggest that tumours can adapt during progression to create an immunosuppressive environment even in the absence of macrophages.

To establish whether neutrophils are playing a role in supporting tumour immunosuppressive environment in the setting of macrophage depletion with CSF1R inhibitor, I assessed neutrophil infiltration in tumours from these cohorts (Figure 4.7). Overall, no significant changes in neutrophil infiltration were observed between the cohorts.

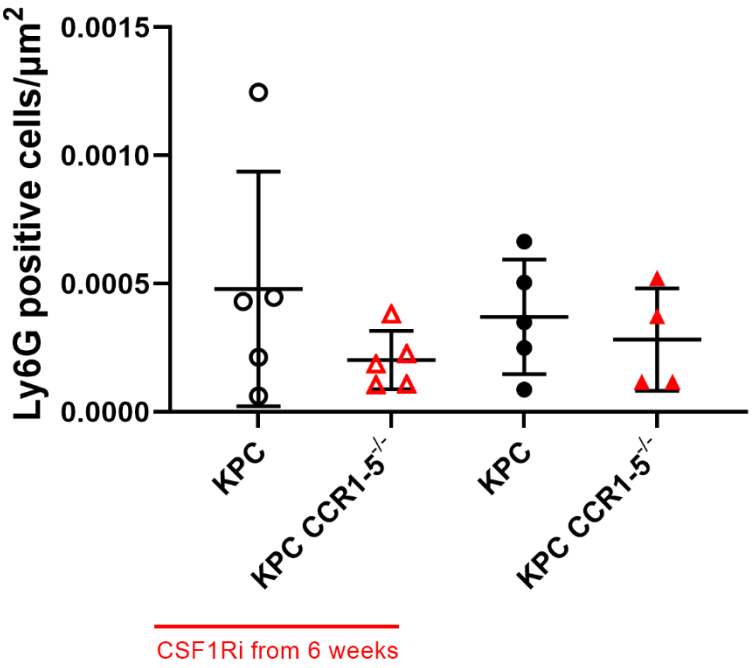

Figure 4.7 CSF1R inhibition has no effect on neutrophil infiltration in tumour models.

Quantitative analysis of immunohistochemistry staining for Ly6G positive cells in tumours from the KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from 6 weeks of age as compared with untreated controls. KPC (N=5); KPC CCR1-5$^{-/-}$ (N=4); KPC +CSF1Ri from 6 weeks (N=5); KPC CCR1-5$^{-/-}$ +CSF1Ri from 6 weeks (N=5), Mann Whitney. Each dot on the graph represents a tumour from one mouse.

Since we previously found that macrophages in PDAC arising in the KPC model expressed PD-L1, and that depletion of macrophages in established tumours resulted in reduced levels of PD-L1 in tumours I wanted next, to look at the presence of PDL-1 in the control and CSF1Ri-treated tumours in the KPC and or KPC CCR1-5$^{-/-}$ mice. RNA *in situ* hybridization, also known as RNAScope, was performed on tumours that were formalin-fixed and paraffin-embedded. Representative pictures were taken (Figure 4.8).

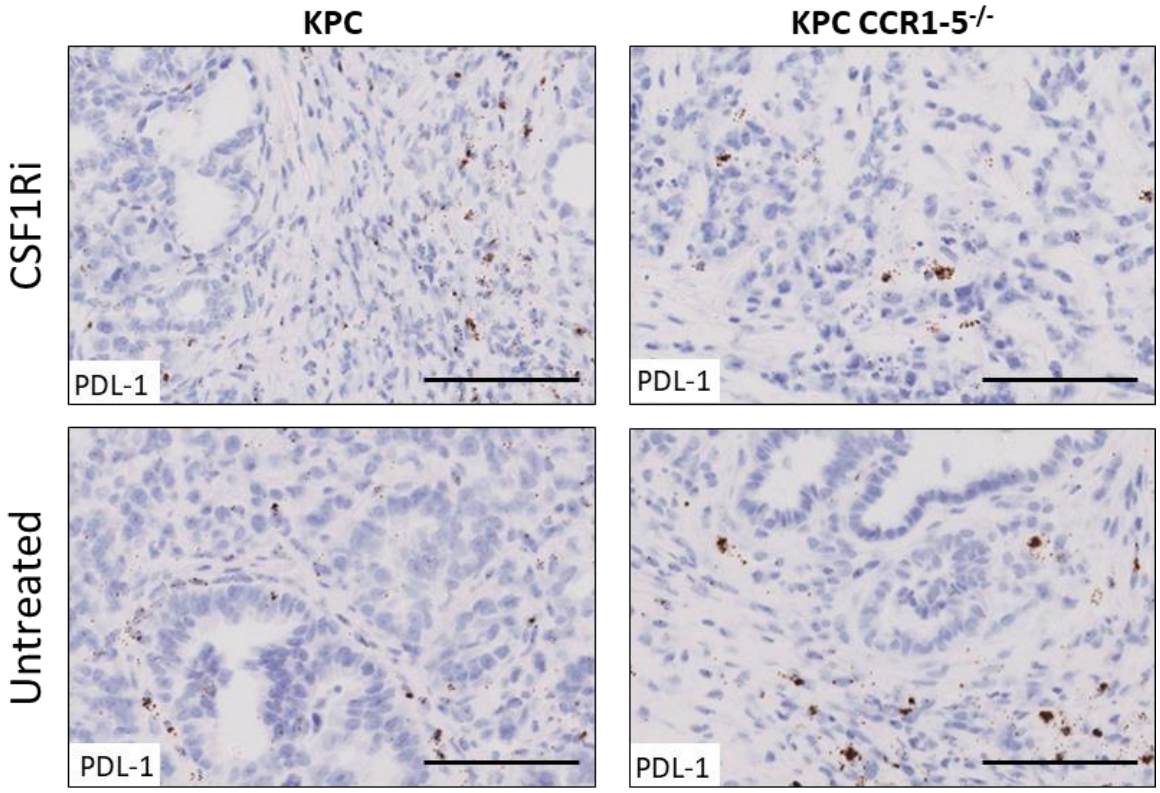

Figure 4.8 PD-L1 expression in tumours from mice treated with CSF1R inhibitor from 6 weeks of age.

Representative images of RNAScope for PD-L1 in tumours from KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from 6 weeks (top) as compared with untreated controls (bottom). Scale bar 100µm.

Stained sections were examined and quantified using image analysis platform HALO. Interestingly, no significant changes were observed in the expression of PD-L1 between the genotypes in the presence or absence of CSF1R inhibition (Figure 4.9), suggesting that other cell types within the tumour are capable of expressing PD-L1. Together, my data show that depletion of macrophages, including tissue resident macrophages, prior to tumour development does not restrict PDAC development and progression. It is tempting to speculate that in the absence of macrophages during tumour development, other cells within the tumour microenvironment are sufficient to restrain the adaptive immune response and may even be reprogrammed to upregulate PD-L1.

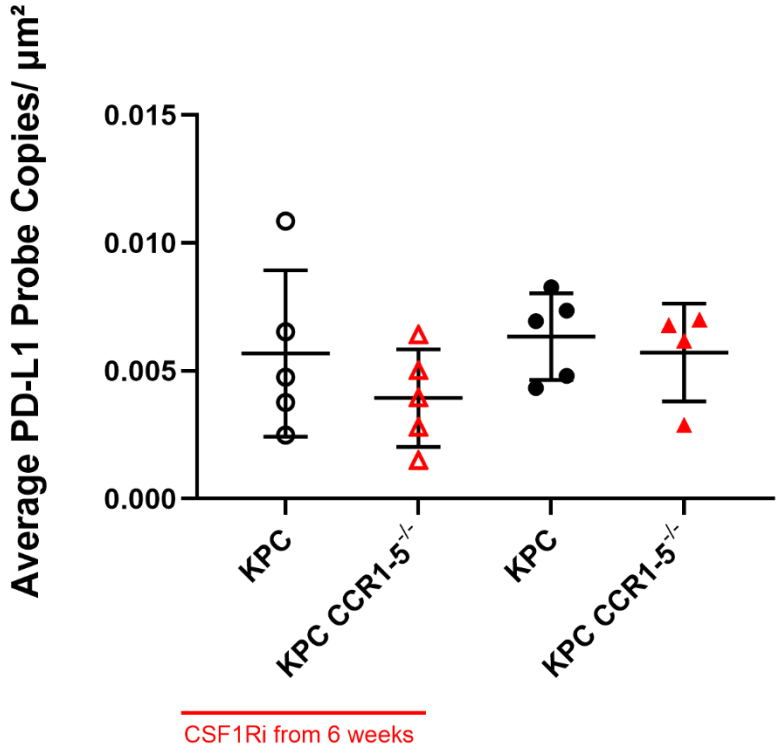

Figure 4.9 CSF1R inhibition has no effect on PD-L1 expression in tumour models.

Quantitative analysis of RNAScope for PD-L1 in tumours from the KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from 6 weeks of age as compared with untreated controls. KPC (N=5); KPC CCR1-5$^{-/-}$ (N=4); KPC +CSF1Ri from 6 weeks (N=5); KPC CCR1-5$^{-/-}$ +CSF1Ri from 6 weeks (N=5); Mann Whitney. Each dot on the graph represents a tumour from one mouse.

To assess whether neutrophils could be accounting for the PD-L1 expression in tumours from mice treated with CSF1R inhibitor from 6 weeks of age, I have compared pictures of Ly6G immunohistochemistry staining and RNA *in situ* hybridisation for PD-L1. Pictures were taken in the same tumour region (Figure 4.10). Although more sophisticated analysis is needed to confirm this observation, the images imply that, indeed, neutrophils are expressing PD-L1 in tumours from mice treated with CSF1 receptor inhibitor.

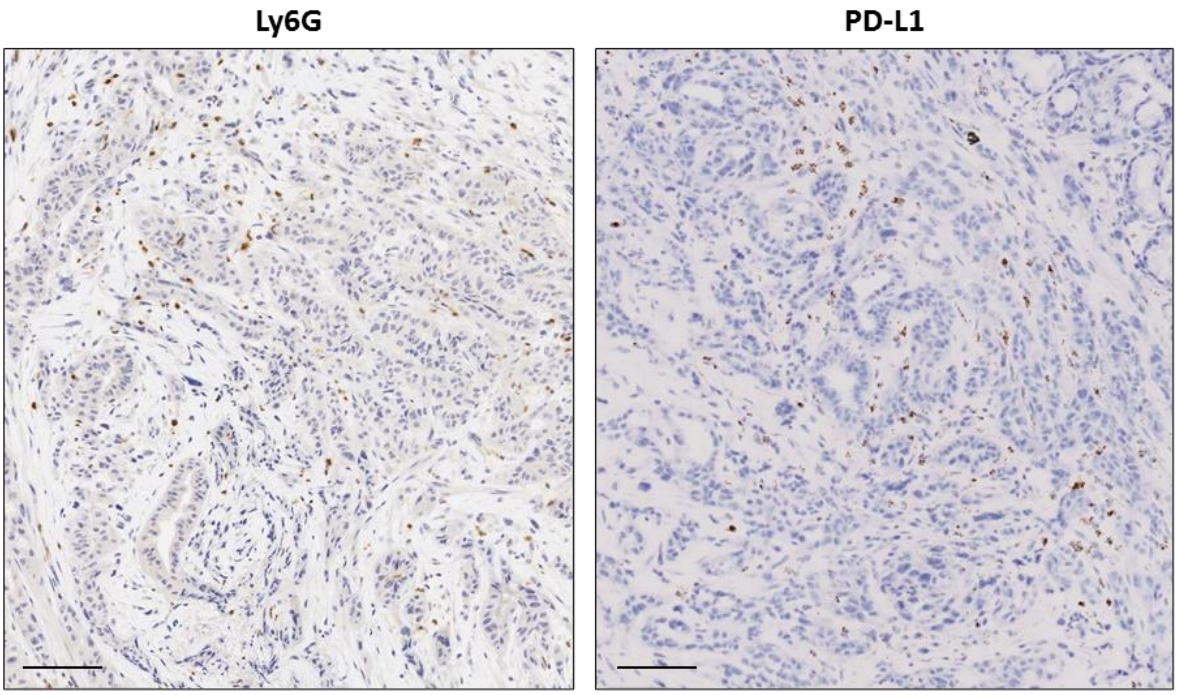

Figure 4.10 Neutrophils may express PD-L1 in PDAC in KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from 6 weeks of age.

Left panel: Image of Ly6G immunohistochemical staining on tumour tissue from a KPC CCR1-5$^{-/-}$ mouse treated with CSF1R inhibitor from 6 weeks of age. Right panel: Image of PD-L1 RNA *in situ* hybridization on tumour tissue from the same KPC CCR1-5$^{-/-}$ mousee treated with CSF1R inhibitor from 6 weeks of age. Images represent the same region in the tumour. Scale bar 100µm.

To assess whether CSF1R inhibition from early time point leads to any systematic changes in peripheral blood composition, I collected peripheral blood and analysed it using the ProCyte Dx Haematology Analyser (Figure 4.11).

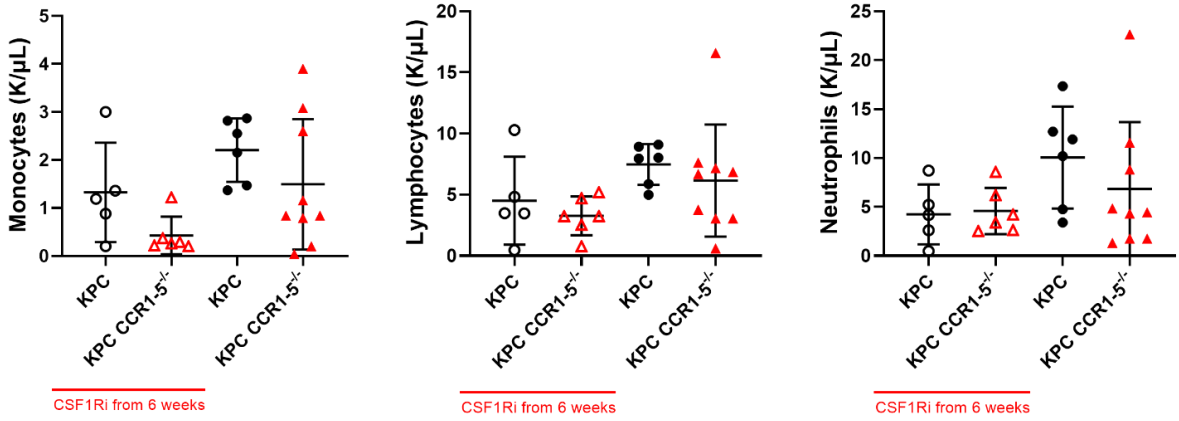

Figure 4.11 Analysis of peripheral blood from KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from 6 weeks.

Graphs illustrate peripheral blood counts from mice with end point tumours. Blood was taken and stored in EDTA tubes until processed with ProCyte Dx Haematology Analyser. KPC +CSF1Ri from 6 weeks (N=5), KPC CCR1-5$^{-/-}$ +CSF1Ri from 6 weeks (N=6), KPC (N=6), KPC CCR1-5$^{-/-}$ (N=9), Mann Whitney.

I did not observe any significant effect on blood monocyte levels following inhibition of CSF1R from 6 weeks of age, although a non-significant reduction was observed between treated and untreated KPC CCR1-5$^{-/-}$ mice. This is perhaps not surprising since blocking CSF1R has previously been shown to have no effect on blood monocyte counts (MacDonald, Palmer et al. 2010). While lymphocyte levels in peripheral blood seem to be unchanged between the cohorts, a trend towards a decrease in neutrophil numbers in mice treated with CSF1R inhibitor was observed. However, this does not seem to affect neutrophil numbers in end point tumours. Overall, the data demonstrates no significant effect on the composition of the peripheral blood between CSF1Ri treated and untreated KPC and KPC CCR1-5$^{-/-}$ mice.

Having observed unexpectedly reduced survival in KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from an early timepoint, as compared with untreated controls, I hypothesise that macrophages might be playing opposing roles during tumour initiation versus tumour progression. As previously described, macrophage ablation using a CSF1R inhibitor in mice with established tumours significantly enhanced their survival (Candido, Morton et al. 2018). In contrast, treatment initiation at the stage of PanIN formation led to decreased survival as compared with untreated mice. This observation could be indicative of an anti-tumourigenic role of macrophages in the early stages of tumour formation or possible rewiring of metabolic cues in the tumour microenvironment as a result of macrophage depletion.

To gain a better understanding of what effect macrophage depletion has on PanIN formation at an early time point, I treated mice of 6 weeks of age with CSF1R inhibitor for 7 days. Mice were culled and sampled after 1 week of treatment. PanIN lesions in the sections of pancreata were scored manually (Figure 4.12)

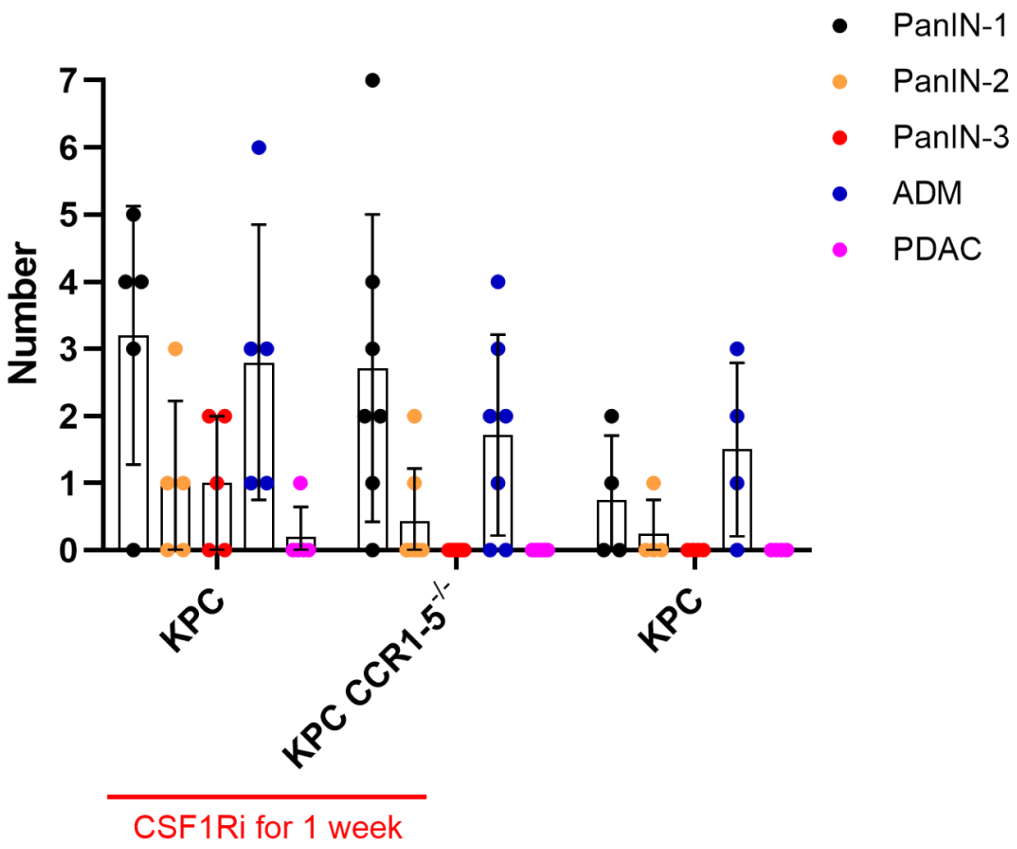

Figure 4.12 PanIN and ADM area scoring in 7 week old KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1Ri for 1 week

H&E stained tissue sections were analysed and scored for PanIN lesions and other pathologies. The graph indicates the numbers of PanINs present in mice from each cohort, and their grade. P value non-significant (n.s.), Mann Whitney

Although the presence of PanIN lesions were assessed in relatively small cohorts of mice, a trend towards an increase in the number of PanIN lesions can be observed in mice treated with CSF1Ri for 1 week prior to sampling as compared with untreated KPC mice at 7 weeks. This further supports the observation that treatment with CS1Ri from 6 weeks of age in KPC mice results in significantly reduced survival. However, the molecular mechanism behind it remains unknown and, thus, further study, including assessment of the proliferative status of these lesions, is needed.

4.3.2 Pharmacological inhibition of CSF1R in established tumours using anti-CSF1R antibody

AZD7507 is not currently a viable candidate for clinical trials so to confirm our findings in the established tumour setting and test a more clinically relevant agent, I decided to use Cabiralizumab (an anti-CSF1 receptor antibody developed by BMS). Cabiralizumab is an investigational antibody that inhibits the CSF1 receptor. Cabiralizumab has been previously shown to block the activation and survival of monocytes and macrophages in preclinical models (Bellovin, Wondyfraw et al. 2017). What is more, Cabiralizumab entered Phase II clinical trial in combination with nivolumab (anti-PD-1 antibody) with and without chemotherapy in patients with advanced pancreatic cancer. However, early last year it was announced that it failed to meet its primary endpoint.

In order to perform a preclinical trial using Cabiralizumab, I aged KPC mice until they presented with palpable tumour, but prior to the onset of symptoms. The presence of tumour was confirmed using the Vevo 3100 Ultra High Frequency ultrasound preclinical imaging system. Mice were randomly assigned to the two treatment groups: isotype control or anti-CSF1 receptor antibody and treated until the clinical end point. Mice were monitored for symptoms throughout and by weekly ultrasound imaging to measure changes in tumour volume. The Kaplan-Meier survival curve was generated (Figure 4.13). Interestingly, unlike our study that showed improved overall survival in mice treated with the small molecule inhibitor for CSF1R, we did not observe a change in overall survival in this comparable study using antibody against CSF1R.

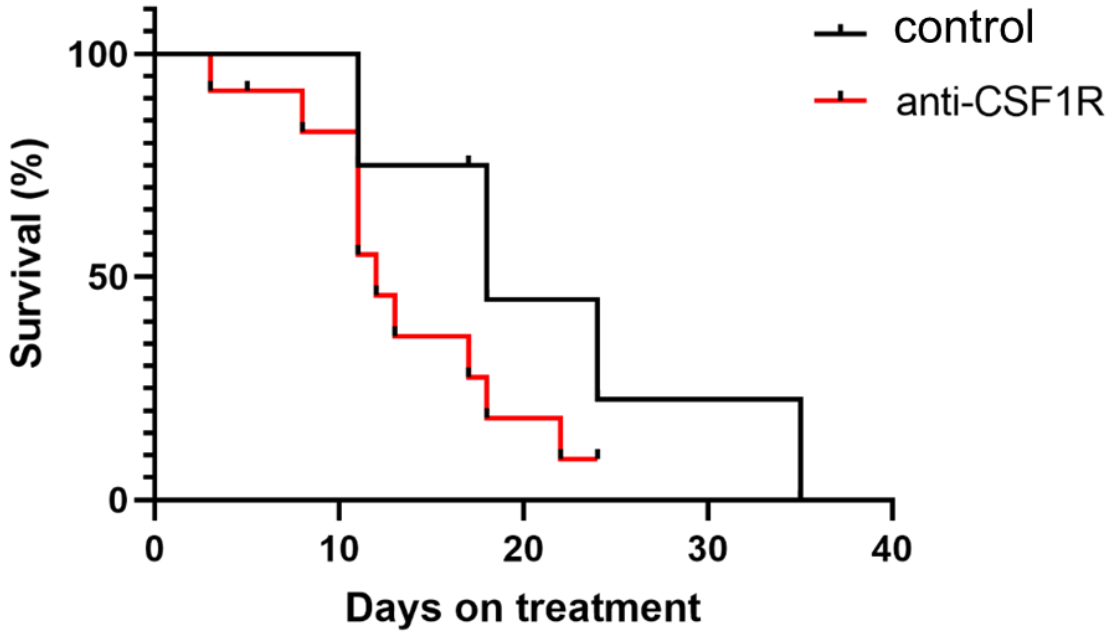

Figure 4.13 Macrophage depletion in KPC mice with established tumours using anti-CSF1 receptor antibody (Cabiralizumab) fails to extend overall survival.

The Kaplan-Meier survival curve of *LSL-Kras*$^{G12D/+}$; *LSL-Trp53*$^{R172H/+}$; *Pdx1-Cre* mice with established pancreatic tumours treated with anti-CSF1 receptor antibody or isotype control. Deaths due to non-PDAC causes are censored out and appear as vertical lines on survival curves. Vehicle (N=8); anti-CSF1 receptor antibody (N=12) Log-rank (Mantel-Cox) test, non-significant.

Although, Cabiralizumab had no effect on overall survival, I further investigated tumour growth rate between mice treated with Cabiralizumab or isotype control. Tumour volume was monitored weekly using Vevo 3100 Ultra High Frequency ultrasound. The graph below illustrates changes in tumour volume over time in individual KPC mice treated with Cabiralizumab or isotype control (Figure 4.14).

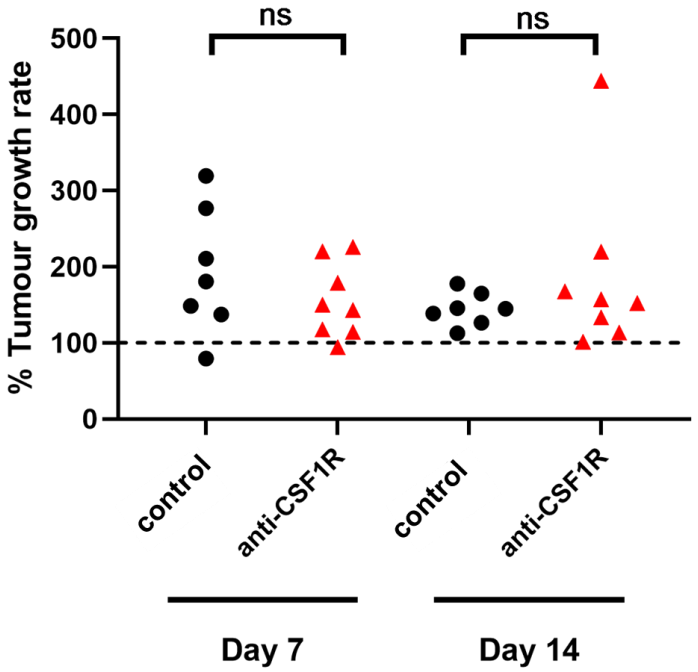

Figure 4.14 Tumour growth in KPC mice treated with anti-CSF1 receptor antibody (Cabiralizumab)

Graph illustrating the % tumour volume change in KPC mice treated with anti-CSF1 receptor antibody (Cabiralizumab) or isotype control. Isotype control n=7, anti-CSF1 receptor antibody n=8, Mann Whitney. Tumour growth rate at 7 days post treatment start reflects the growth from Day 0 to Day 7. Tumour growth rate at 14 days reflects the growth from Day 7 to Day 14.

The data demonstrate no significant difference in tumour growth rate between Cabiralizumab or vehicle treated mice. However, it could be thought that tumour growth rate is slightly reduced during the first week of treatment with Cabiralizumab. To investigate this on a cellular level, tumours sections were stained for the proliferation marker, Ki67, and staining was quantified (Figure 4.15).

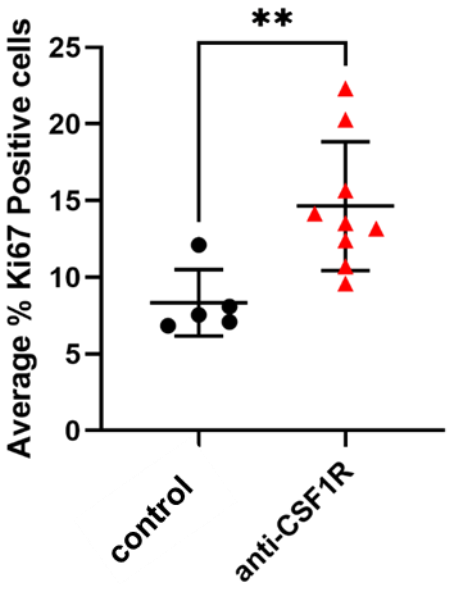

Figure 4.15 Cabiralizumab treatment increases proliferation in established KPC tumours.

Quantitative analysis of immunohistochemistry staining for Ki67 positive cells in tumours from KPC mice treated with anti-CSF1 receptor antibody (Cabiralizumab), N=9 or isotype control, N=5, p= 0.0040, Mann Whitney. Each dot on the graph represents a tumour from one mouse.

Interestingly, quantification of Ki67 revealed that tumours treated with anti-CSF1R antibody had increased proliferation status. Overall, the lack of effect on overall mouse survival and tumour growth rate is in contrast with our previously published results that were obtained using a small molecule inhibitor of CSF1R (Candido, Morton et al. 2018). This observation raises the question whether anti-CSF1R antibody is able to induce the anticipated effect on macrophages. Thus, tumour sections were stained for the macrophage marker, F4/80, and representative pictures were taken (Figure 4.16). Immunohistochemistry staining demonstrated that tumours from mice treated with Cabiralizumab presented with a high abundance of TAMs. Staining was further quantified to investigate whether any reduction in overall macrophage numbers could be observed in the treated mice (Figure 4.17).

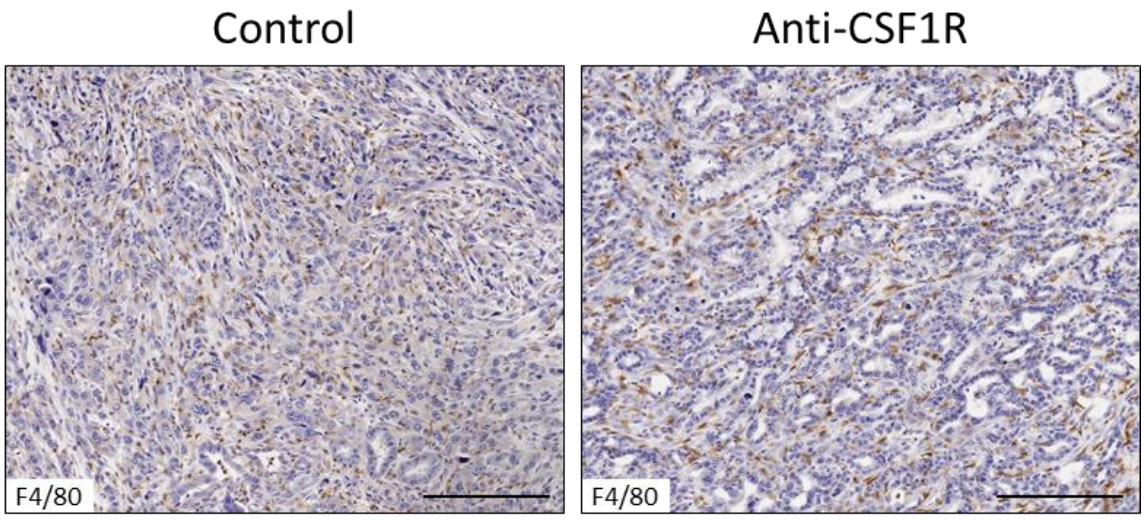

Figure 4.16 Macrophages in tumours from mice treated with anti-CSF1R antibody.

Representative immunohistochemistry images of staining for F4/80 positive macrophages in end point tumours from KPC mice treated with anti-CSF1R antibody (Cabiralizumab) as compared with isotype control treated. Scale bar 200µm.

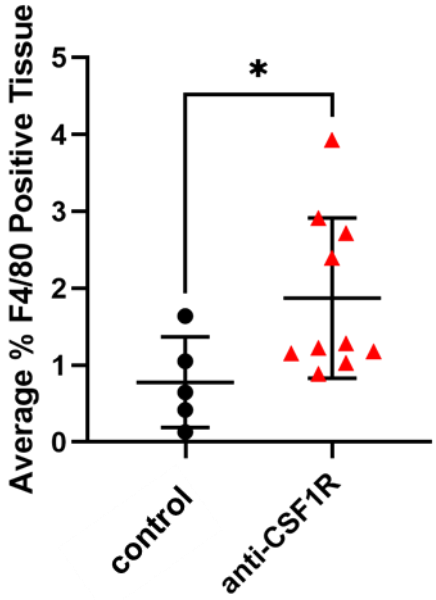

Figure 4.17 Cabiralizumab fails to deplete macrophages in KPC tumours.

Quantitative analysis of immunohistochemistry staining in tumours from KPC mice treated with anti-CSF1R antibody as compared with isotype control treated controls. Isotype control (N=5); anti-CSF1R antibody (N=10); P=0.0400, Mann Whitney. Each dot on the graph represents a tumour from one mouse.

The results demonstrated here provide evidence that, unlike CSF1R inhibitor, anti-CSF1R antibody fails to deplete macrophages in established KPC tumours. In fact, we observed that treated tumours had significantly higher macrophage infiltration. However, it is worth stating the tumour that presented with the highest level of F4/80 macrophages, was from a mouse only treated with antibody for three days. Mouse exhibited clinical signs of the disease and had to be sampled. Hence, it could be speculated that this time was not sufficient to observe the full effect of Cabiralizumab. If we were to exclude that value, the difference in macrophage presence between vehicle and antibody treated mice loses significance, although there is still a trend towards increased macrophage abundance. Overall, it can be concluded that anti-CSF1R antibody fails to induce the anticipated effect on macrophages and presumably therefore on overall survival.

I set out to investigate whether the lack of survival benefit in Cabiralizumab treated mice could be a result of overall toxicity in treated mice. I assessed whether Cabiralizumab treatment induced any systematic changes in leukocyte populations in peripheral blood that could indicate potential toxicity issues. Peripheral blood was collected from the end point mice and analysed using the ProCyte Dx Haematology Analyser (Figure 4.18).

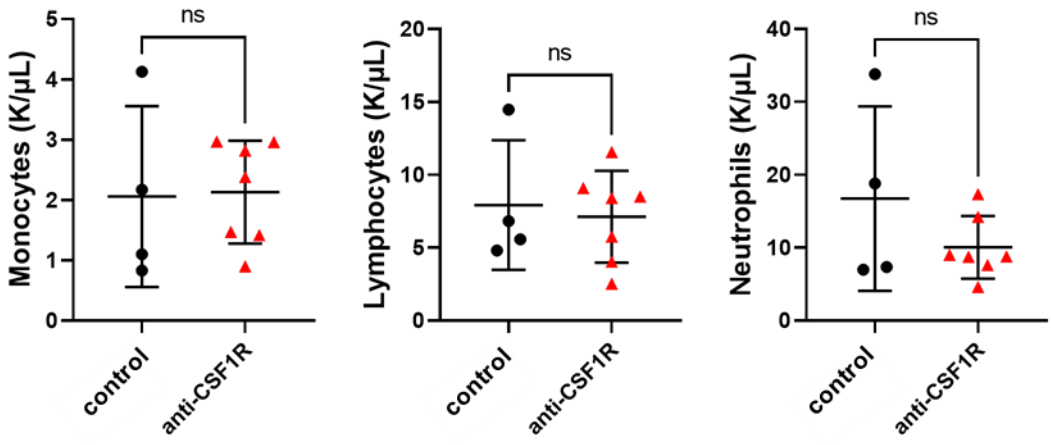

Figure 4.18 Analysis of peripheral blood from KPC mice treated with anti-CSF1 receptor antibody (Cabiralizumab).

The graphs illustrate peripheral blood leukocyte counts from mice with end point tumours treated with anti-CSF1 receptor antibody or isotype control. Blood was taken and stored in EDTA tubes until processing using the ProCyte Dx Haematology Analyser. Vehicle (N=4) vs anti-CSF1R (N=7). Mann Whitney.

Interestingly, the results from this analysis did not reveal any changes in leukocyte populations in the peripheral blood as a result of treatment. As seen in previous sections, Procyte Heamatology Analyser may not be sensitive enough to detect small changes in

peripheral blood cell populations. Therefore, additional analysis is required to determine any systematic changes in peripheral blood from mice treated with Cabiralizumab. Further work is also needed to understand why the anti-CSF1R antibody fails to deplete tumour associated macrophages in tumour-bearing KPC mice, in comparison with the small molecule inhibitor, AZD7507, which we previously demonstrated is able to deplete TAMs, inhibit tumour growth and extend mice survival.

4.4 Discussion

Failure of immunotherapy to induce an anti-tumourigenic effect is often attributed to the immunosuppressive nature of the tumour microenvironment. Tumour associated macrophages are critical drivers of immune escape. Thus, strategies to inhibit the effects of macrophages could have therapeutic potential. We have previously reported that ablation of macrophages with CSF1R inhibitor, AZD7507, significantly extended survival in KPC mice and resulted in tumour volume reduction (Candido, Morton et al. 2018). I wanted to establish the role of macrophages in early tumour formation. Therefore, I initiated treatment with CSF1R inhibitor in KPC and KPC CCR1-5$^{-/-}$ mice from 6 weeks of age. In contrast to our previous findings, early initiation of CSF1R treatment resulted in reduced overall survival in treated mice when compared with untreated controls. It was confirmed, using immunohistochemistry, that macrophage depletion with CSF1R inhibitor from an early time point was successful and sustained. Therefore, the lack of survival benefit in the treated cohort implies that TAMs can be substituted by different stromal components when depleted during tumour formation. The data also suggested that macrophages may have an anti-tumourigenic effect in early tumour initiation.

Evidence from the literature support roles for macrophages in tumour promotion, initiation, but also tumour suppression. It is well established that macrophages are able to elicit an effect in other immune cells by secreting various immunomodulatory factors. Examples of tumour promoting factors include VEGF, EGF, TGF-β and ornithine, whereas nitric oxide produced by macrophages was shown to inhibit tumour growth (Mills, Shearer et al. 1992). Back in 1992, the 'double-edged sword' nature of macrophages was attributed to dual pathways of arginine metabolism in TAMs. It was demonstrated that tumour rejection or growth is associated with macrophage intrinsic arginine metabolism. Data demonstrated that arginine metabolism to the NO synthase pathway, yielding NO, favours tumour inhibition, whereas, if the predominant pathway of arginine metabolism is via arginase this could lead to increased tumour growth because ornithine, a precursor for molecules required for cell replication, is produced (Mills, Shearer et al. 1992). Other study groups have also reported that the lack of macrophage cytotoxicity in mammary tumour bearing mice is due to dowregulated NO production in these cells (Sotomayor, DiNapoli et al. 1995). From the data presented in this thesis, it can only be speculated that macrophages at the stage of PanIN formation can exhibit cytotoxic activity which is impeded by macrophage ablation via CSF1R inhibition. However, in depth analysis of macrophage metabolic changes at various stages of tumour initiation and progression are required to confirm this hypothesis.

To gain a better understanding of histological changes that occur at the PanIN formation stage in the setting of macrophage depletion, I treated 6 week old KPC and KPC CCR1-5$^{-/-}$ mice for 1 week and sampled pancreata once treatment was terminated. Indeed, PanIN scoring results indicated that there is a trend towards a decrease in the overall number of PanINs present in untreated, age matched mice. This further implies that macrophage ablation from an early stage promotes tumourigenesis. It suggests that the tumour microenvironment can be rewired to promote tumourigenesis eventually resulting in decreased overall survival. In established tumours, the stroma, of which TAMs represent the major population of inflammatory cells, can constitute the majority of the tumour. Thus, it could be hypothesised that macrophage depletion with CSF1R inhibitor in established tumours induces systemic changes in the tumour microenvironment which can not be overcome by tumour cells. Although the perception that macrophage depletion elicits opposing effects based on the stage of tumourigenesis is thought-provoking, further studies are required to determine the molecular cues behind this observation.

Finally, I demonstrated that CSF1R inhibition with Cabiralizumab (an anti-CSF1 receptor antibody developed by BMS) fails to elicit the anticipated effect on macrophages and tumourigenesis in KPC mice with established tumours. However, Cabiralizumab has been previously shown to block the activation and survival of monocytes and macrophages in preclinical models (Bellovin, Wondyfraw et al. 2017). What is more, Cabiralizumab entered Phase II clinical trial in combination with nivolumab (anti-PD-1 antibody) with and without chemotherapy in patients with advanced pancreatic cancer. However, early last year it was announced that it failed to meet its primary endpoint. It could be speculated that the lack of effect by the anti-CSF1R antibody is caused by overall immunogenicity. In the clinic, immunogenicity is assessed by the detection of anti-drug antibodies (ADA). Aside from severe responses to therapeutic proteins, immunogenicity can result in the generation of neutralizing antibodies that are able to inhibit functional activity of therapeutic antibodies. Whether this is the case in KPC mice treated with Cabiralizumab remains to be determined.

Overall, these data demonstrate that depletion of macropahages can elicit opposing effects depending on the stage of tumourigenesis. From the data presented, it could be speculated that macrophages exhibit anti-tumourigenic functions in the early stage of PanIN formation. Hence, macrophage depletion from an early stage of tumour initiation results in reduced overall survival in KPC mice. Whether the reduced survival is the result of loss of tumour-suppressive macrophage activities, rewiring of microenvironmental factors that support tumour growth, or due to the metabolic interplay between macrophages and tumour cells, remains to be understood.

5 Studying the potential role of B lymphocytes in Pancreatic Cancer

5.1 Introduction

The significance of anti-tumourigenic effects of T lymphocytes is well established. In contrast, the role of B cells in tumour development and progression is not widely understood, though, B cells, just like other immune cells such as macrophages, do infiltrate human tumours (Chang, Jiang et al. 2016). Mirroring human cancer, tumours from pancreatic cancer mouse models were also shown to be infiltrated with B cells. We previously showed in tumour bearing KPC mice that inhibition of macrophages through CSF1R receptor induces tumour shrinkage and results in the upregulation of molecules associated with immune activation. What is more, we observed an increase in infiltration of $CD19^+$ B cells in the treated tumours. Together, these data indicated an increase in local adaptive immunity (Candido, Morton et al. 2018). In contrast, recently published papers focusing on the role of B cells in pancreatic cancer reported a pro-tumourigenic role of B cells (Gunderson, Kaneda et al. 2016, Lee, Spata et al. 2016, Pylayeva-Gupta, Das et al. 2016). It was shown that transplanted pancreatic ductal epithelial cells harbouring activating Kras mutation exhibit reduced growth in B cell deficient mice when compared with wild-type mice (Pylayeva-Gupta, Das et al. 2016). Lee et al. studied the role of hypoxia-inducible factor 1α in pancreatic cancer and showed that its deletion leads to increased B cell infiltration in early PanIN lesions in GEM models, and accelerated tumourigenesis. When compared with wild-type mice, *p48-cre; Kras*$^{G12D/+}$ mice had reduced number of conventional B2 cells ($CD19^+CD43^-CD5^-$) and increased numbers of B1 ($CD19^+CD43^+IgM^{hi}$) cells (Lee, Spata et al. 2016). B1 are innate immune cells that are considered to produce the majority of natural antibodies against a broad spectrum of infections, however, the role of B1 cells in cancer setting is not yet defined. Currently, the data on the role of B cells in pancreatic cancer are conflicting. We believe that data gathered from studies that uses orthotopically engrafted tumours may be affected by the invasive techniques used in the process, and by the rewiring of signalling pathways of cells in culture before transplant. Therefore, our aim was to assess the effect of B cells on pancreatic cancer in a more clinically relevant model.

5.2 Experimental aims

To investigate the role of B cells in PDAC development and progression we developed a B cell deficient GEM model of pancreatic cancer. *Ighm*$^{-/-}$ mice (also known as muMt^{-}) were obtained from The Jackson Laboratory. In house, we crossed *Pdx1-Cre LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$ and *Ighm*$^{-/-}$ mice and bred them to homozygosity. This mouse model has no expression of membrane-bound IgM (Immunoglobulin Heavy Constant Mu (IgHM)) which is essential for B cell maturation. Thus, homozygous mice lack mature B cells. I aimed to establish the role of B cells in PDAC development and progression using this mouse model.

5.3 Results

5.3.1 Characterization of *Ighm* $^{-/-}$ mice

Firstly, I characterized the *Ighm*$^{-/-}$ model in otherwise wild-type mice. *Ighm*$^{-/-}$ and wild-type control mice were aged until 6 weeks of age and culled. The pancreata together with spleen, lobe of liver and lungs were sampled, formalin fixed, and paraffin embedded. Slides were obtained and stained with haematoxylin and eosin (H&E), and representative images taken (Figure 5.1). I observed that spleens from *Ighm*$^{-/-}$ mice are generally smaller in size. It is reported that B cells represent 44-58% of total cells in WT mouse spleen. Thus, the difference in gross spleen size observed between WT and B cell deficient *Ighm*$^{-/-}$ is consistent with the model. No other histological differences between the cohorts were observed. Thus, B cell deficiency causes no overt phenotype in these mice.

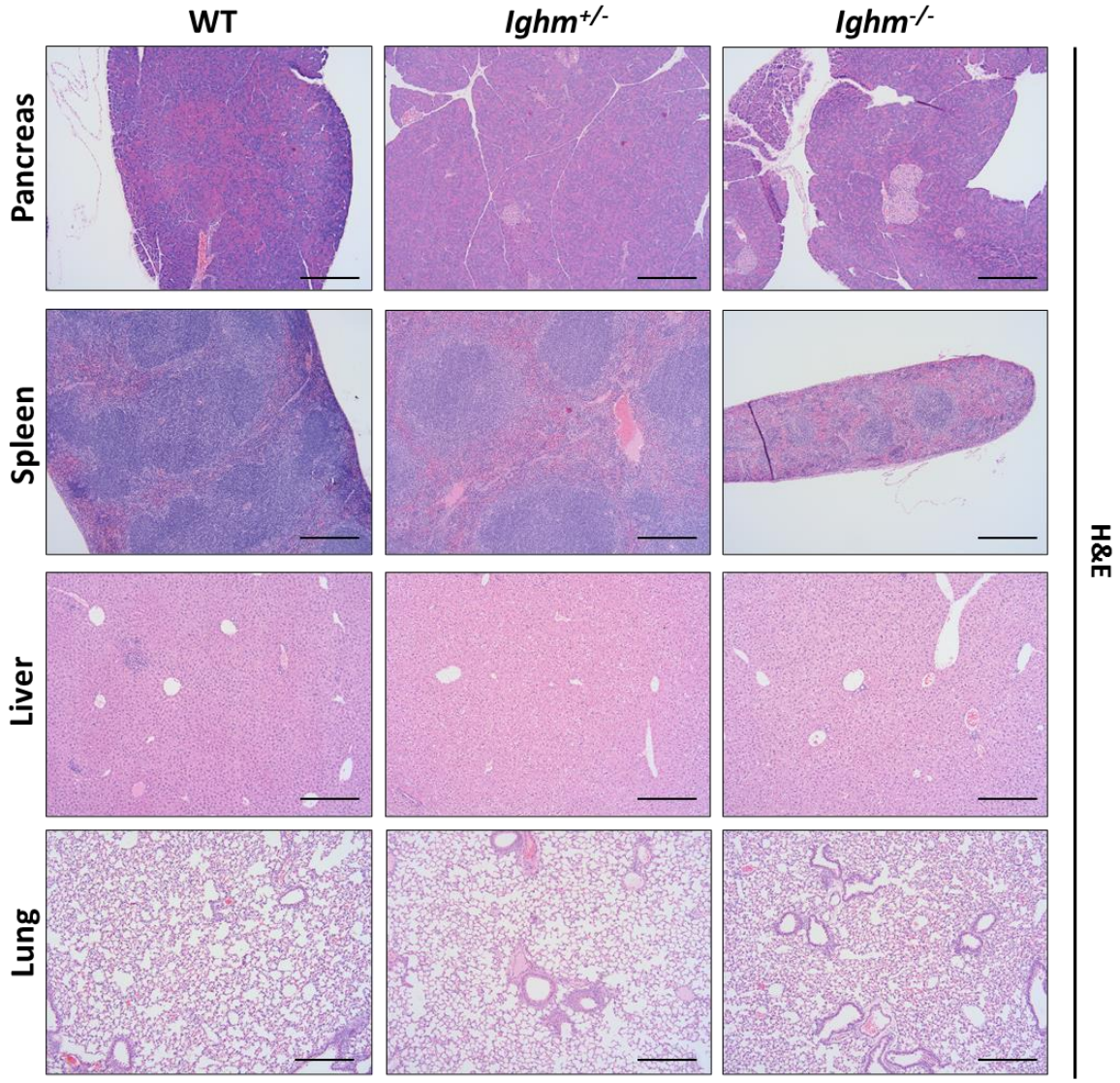

Figure 5.1 Histological comparison of WT and *Ighm*$^{-/-}$ mice.

Representative haematoxylin and eosin (H&E) staining of WT (left), *Ighm*$^{+/-}$ (middle) and *Ighm*$^{-/-}$ (right) pancreata, spleen, liver, and lung. Scale bar 100µm.

I also assessed peripheral blood leukocyte populations in these mice using the ProCyte Dx Haematology Analyser (Figure 5.2).

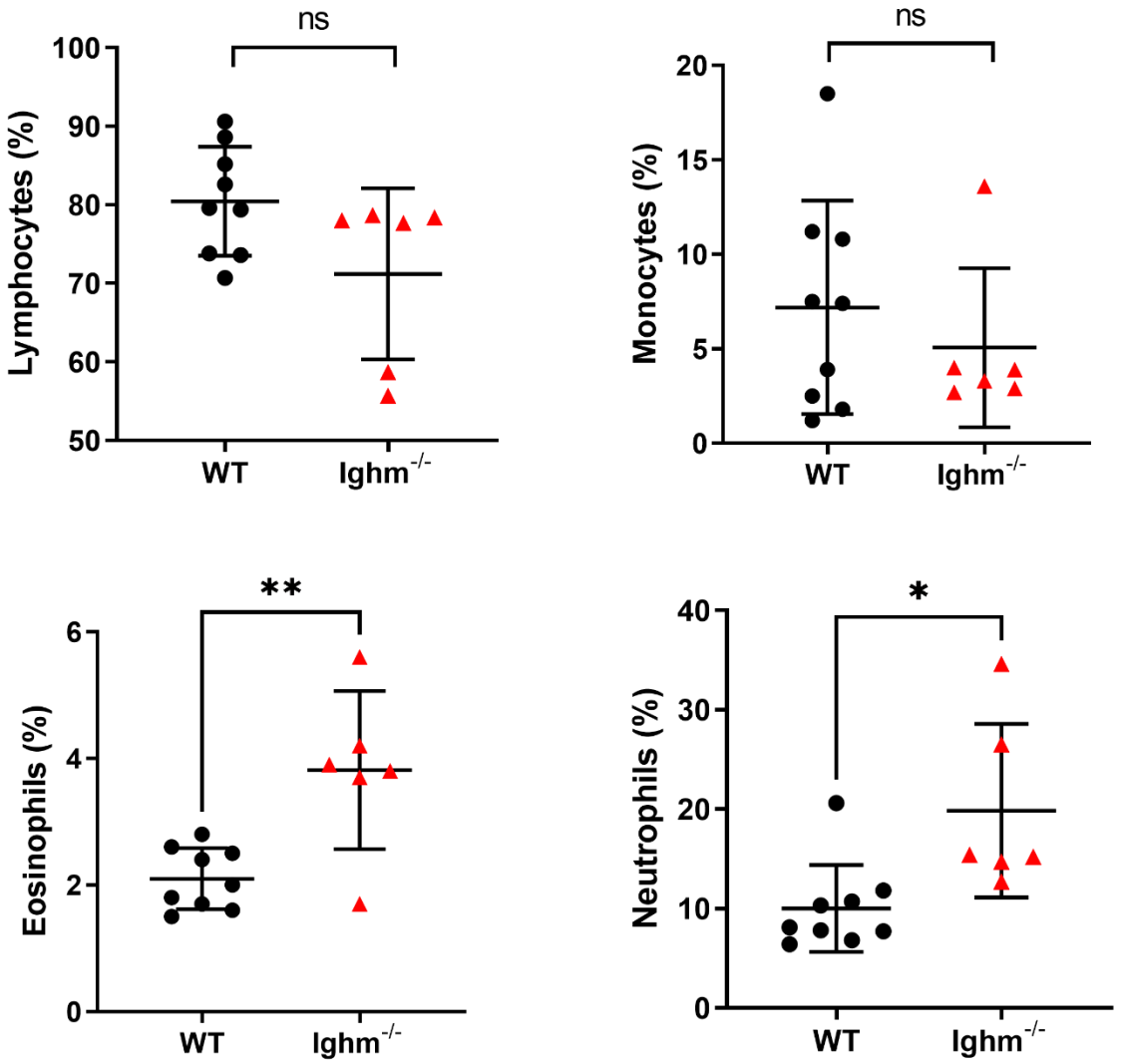

Figure 5.2 Analysis of peripheral blood from WT and *Ighm*$^{-/-}$ mice.

Graphs illustrate peripheral blood leukocyte counts. Blood was taken and stored in EDTA tubes until processed with ProCyte Dx Haematology Analyser. WT (N=9) vs *Ighm*$^{-/-}$ (N=6), Mann Whitney.

I observed that eosinophils and neutrophils are significantly increased in the peripheral blood from *Ighm*$^{-/-}$ mice. This observation was not previously reported by the original study that developed and characterized the *Ighm*$^{-/-}$ model (Kitamura, Roes et al. 1991).

Next, I looked at B cell presence within tissues of 6 weeks old *Ighm*$^{-/-}$ mice. To assess this, I performed immunohistochemical staining for the B cell marker, PAX-5, a transcription factor that is expressed throughout B-cell maturation (Figure 5.3).

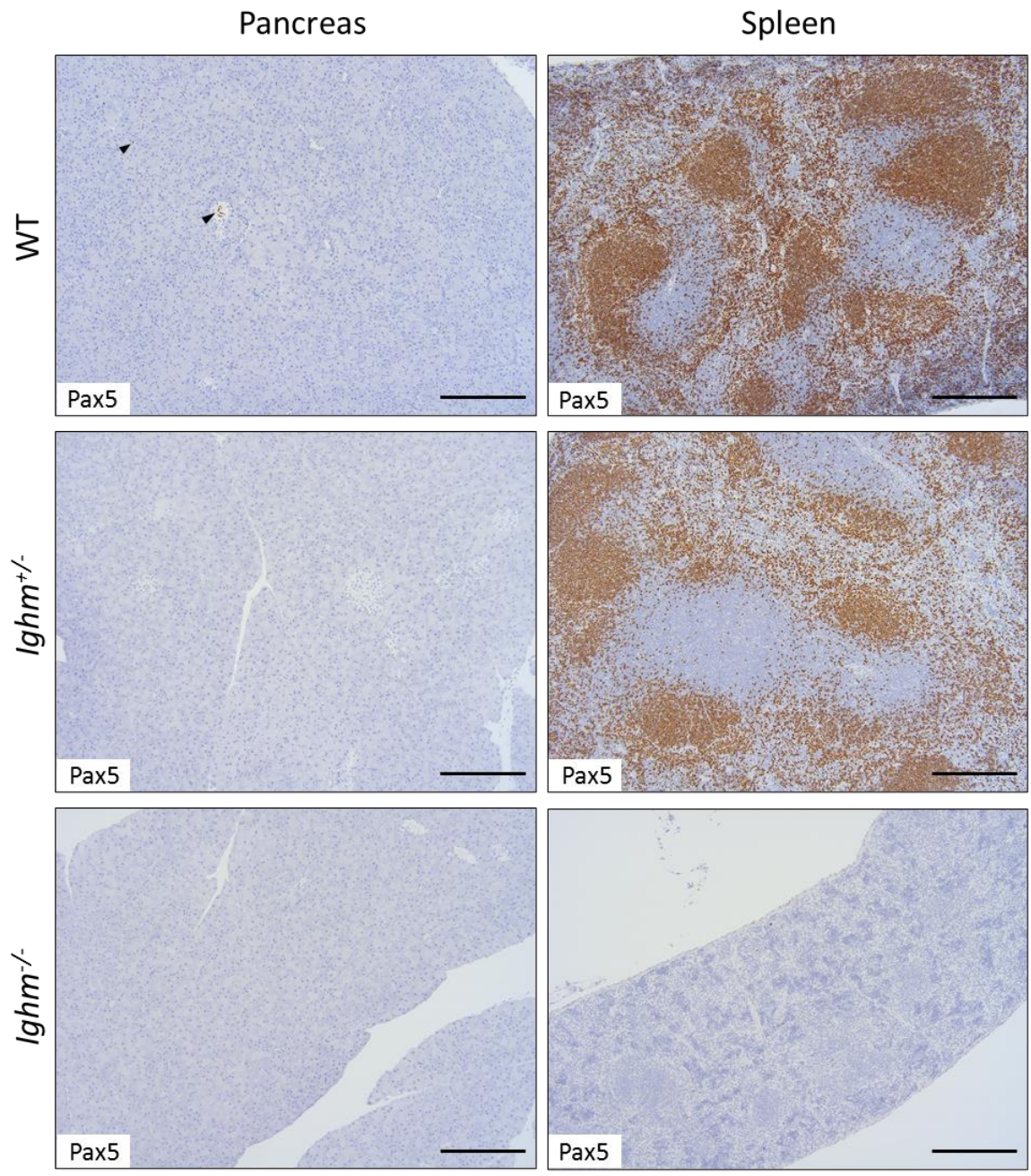

Figure 5.3 Pax5 immunohistochemistry.

Representative images of immunohistochemistry staining for Pax5 for B cells in 6 weeks old WT (top) *Ighm*$^{+/-}$ (middle) and *Ighm*$^{-/-}$ (bottom) mice. Scale bar 100µm.

It was clear that Pax5 positive cells are absent in tissues from *Ighm*$^{-/-}$ mice as compared to WT and *Ighm*$^{+/-}$ controls.

In order to investigate the effect of long-term loss of B cells in otherwise WT mice, a cohort of *Ighm*$^{-/-}$ mice was aged up to 1.5 years old. Organs from these mice were collected, formalin fixed, and paraffin embedded. Slides were prepared from each mouse, stained with haematoxylin and eosin (H&E), and representative images taken (Figure 5.4). These data show that there was no overt phenotype due to B cell deficiency, even in aged mice.

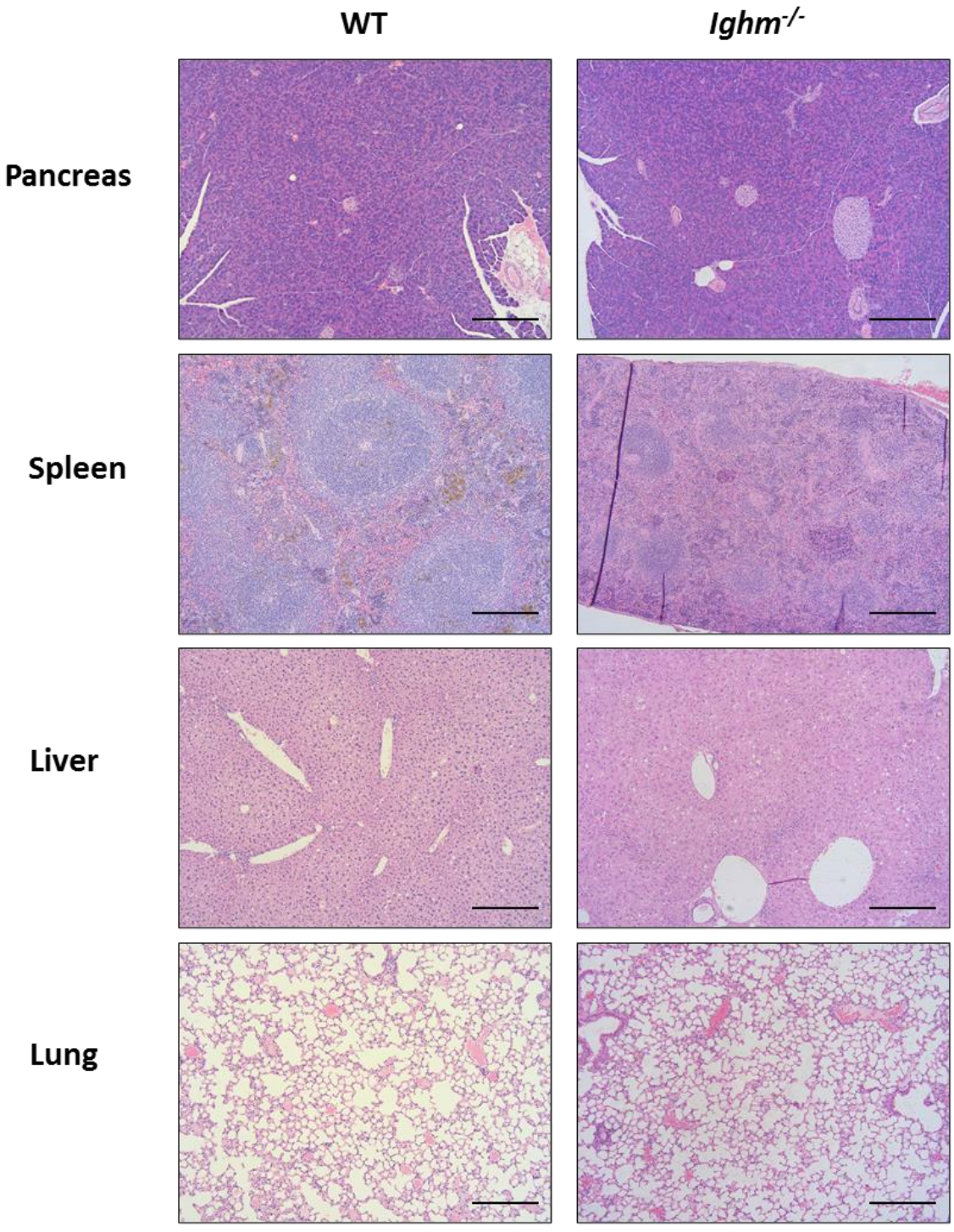

Figure 5.4 H&E of organs from WT and *Ighm*$^{-/-}$ mice at 18 months show no changes in histology.

Representative images of H&E staining of organs from 18-month-old WT (left) and *Ighm*$^{-/-}$ (right) mice. Scale bar 100µm.

5.3.2 The lack of B cells fails to alter PDAC-specific survival and phenotype in KPC mice

To assess the role of B cells in pancreatic tumour development and progression, I aged KPC and KPC *Ighm*$^{-/-}$ mice until the clinical end point and compared their survival using Kaplan-Meier survival analysis (Figure 5.5). However, I observed no difference in survival between KPC mice lacking B cells or proficient in B cells.

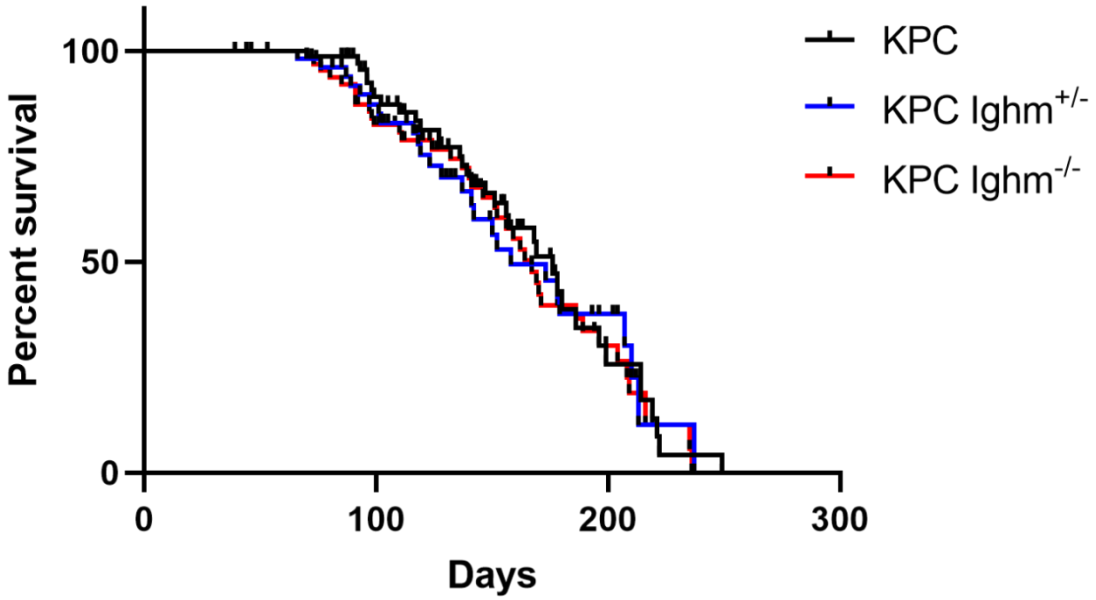

Figure 5.5 B cell deficiency fails to alter PDAC-free survival in KPC mice.

Kaplan-Meier survival analysis of *Kras*$^{LSL\text{-}G12D/+;}$ *Trp53*$^{R172H/+;}$ *Pdx1Cre* (KPC) *Ighm*$^{+/+}$, *Ighm*$^{+/-}$, and *Ighm*$^{-/-}$ mice. Deaths due to non-PDAC causes are censored out and appear as vertical lines on survival curves. KPC *Ighm*$^{+/+}$ (N=72); KPC *Ighm*$^{+/-}$ (N=51); KPC *Ighm*$^{-/-}$ (N=65) Log-rank (Mantel-Cox) test, non-significant.

Mice exhibiting disease symptoms, were dissected and organs collected. Pancreas, spleen, liver, and lungs were then fixed in formalin, and paraffin embedded. Representative images were taken of slides stained with H&E (Figure 5.6).

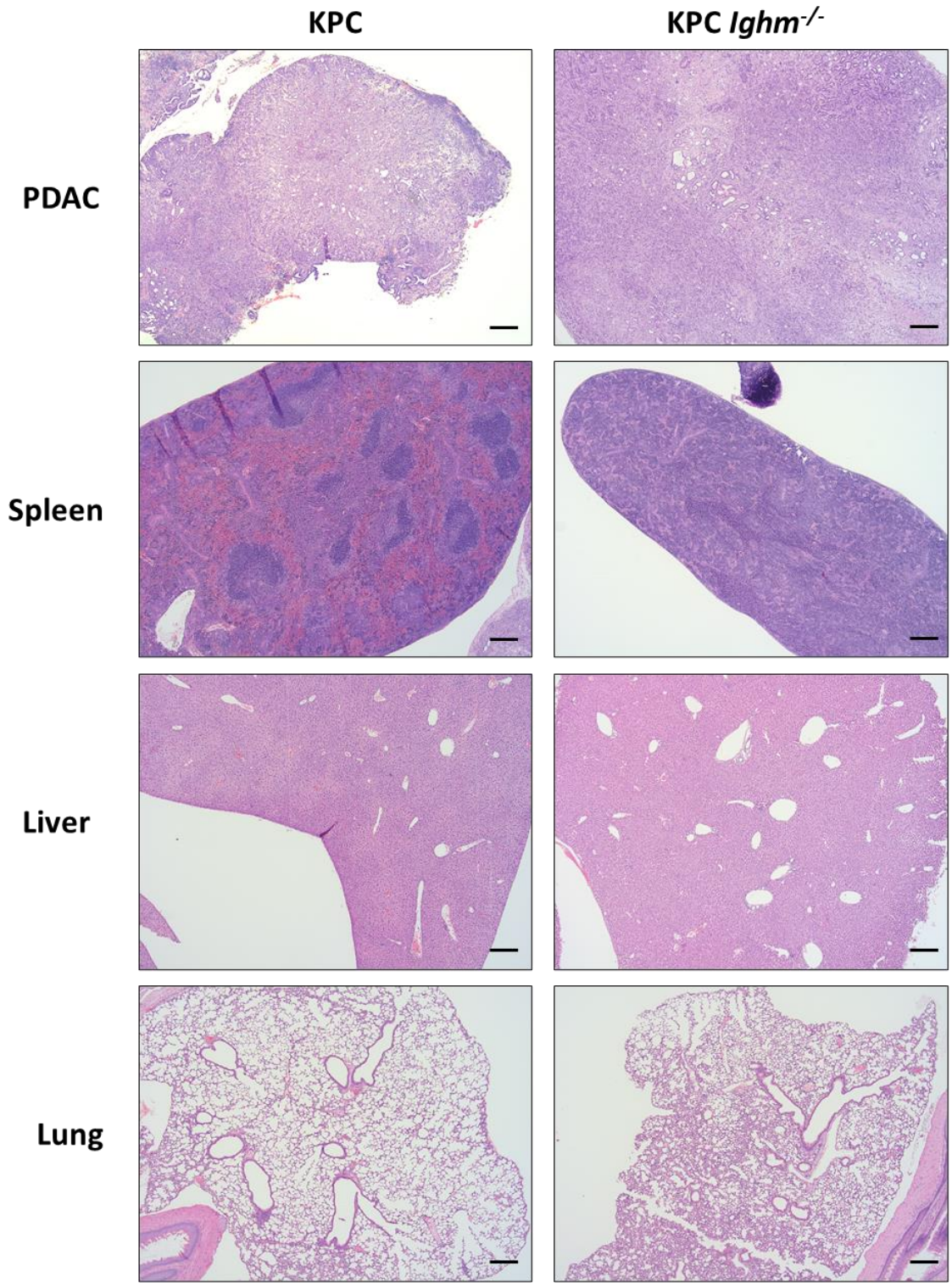

Figure 5.6 B cell deficiency in KPC mice has no effect on tumour and organ histology in mice with end stage disease.

Representative H&E images from end point KPC and KPC *Ighm*$^{-/-}$ mice demonstrating no significant changes in histology between the two groups. Scale bar 100µm.

As seen in *Ighm*$^{-/-}$ mice, spleens from KPC *Ighm*$^{-/-}$ mice differed histologically when compared with KPC WT controls. Spleens from KPC *Ighm*$^{-/-}$ mice lack white pulp B cells. No other histological differences between organs from KPC and KPC *Ighm*$^{-/-}$ mice were observed, and there was no difference in tumour histology between genotypes. Together, these data show that B cell deficiency has no impact on pancreatic tumour latency or phenotype in KPC mice.

Next, I confirmed the absence of B cells in KPC *Ighm*$^{-/-}$ mice by staining tumour and spleen sections for CD45R, an essential B cell receptor. Representative pictures were taken (Figure 5.7)

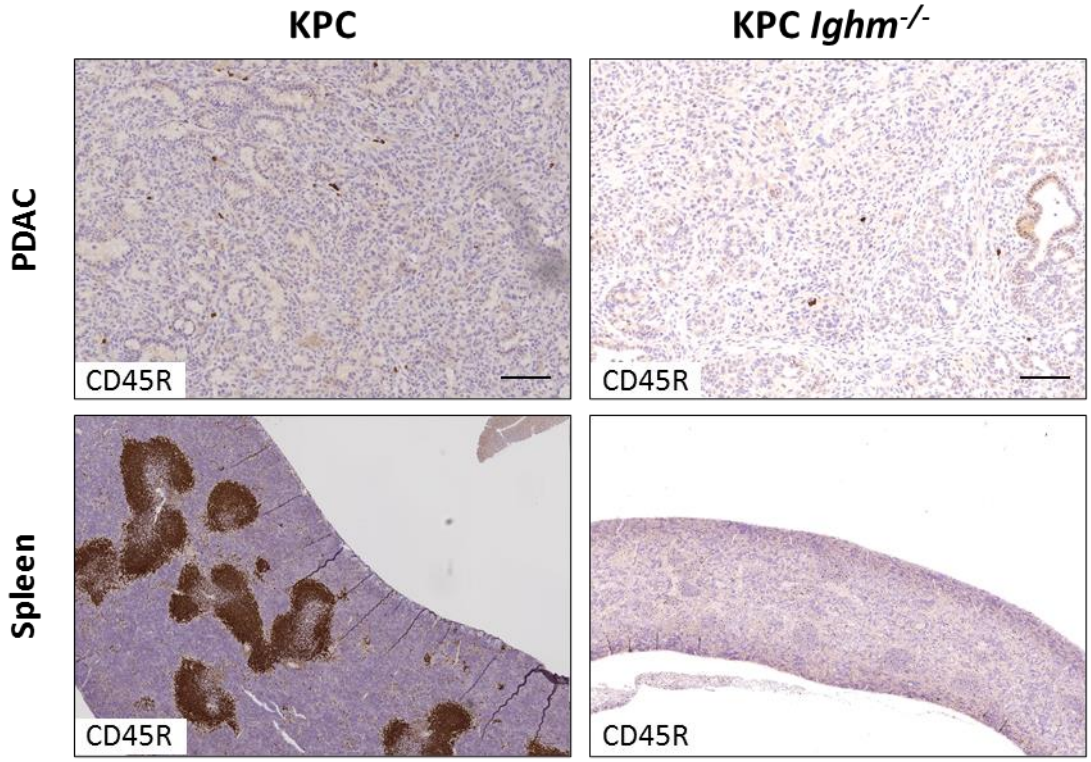

Figure 5.7 CD45R IHC staining confirms lack of B cells in KPC *Ighm*$^{-/-}$ mice.

Representative images of CD45R immunohistochemical staining on tumour and spleen from end point KPC and KPC *Ighm*$^{-/-}$ mice demonstrating lack of B cells. Scale bar 100µm.

This staining was quantified on the HALO image analysis platform (Figure 5.8).

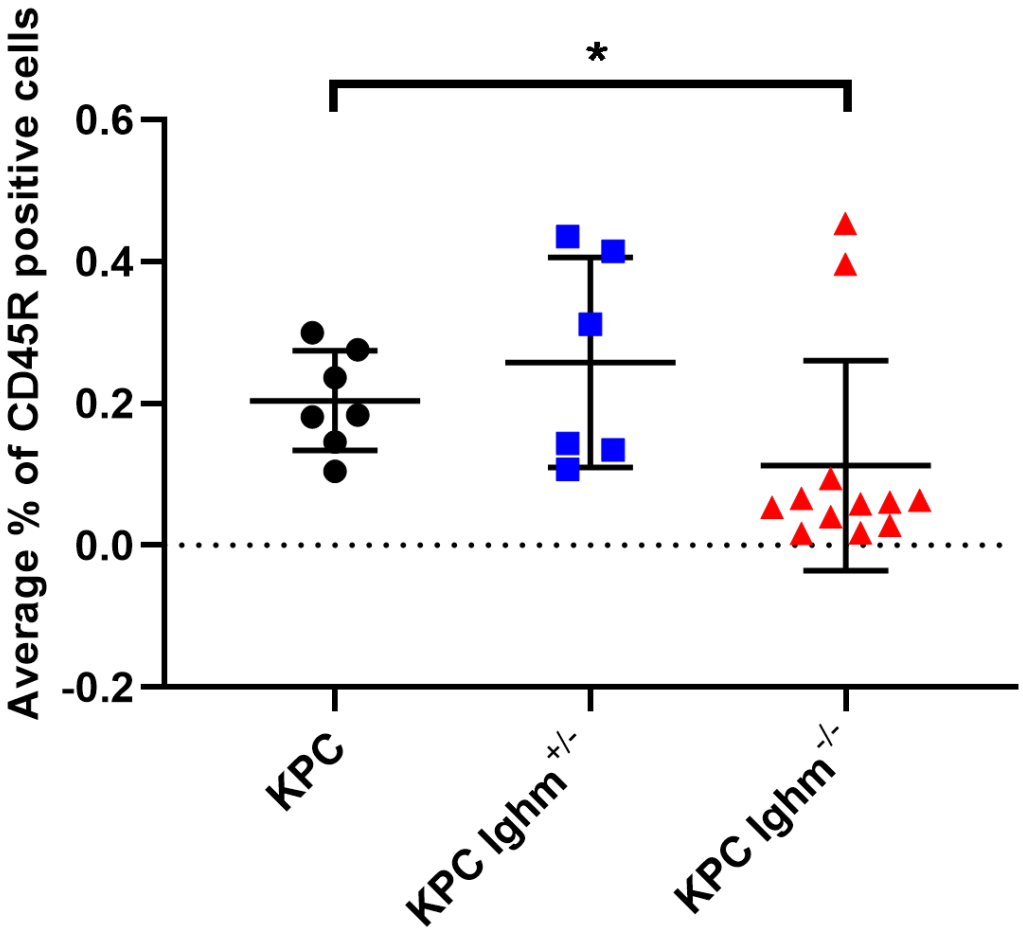

Figure 5.8 Quantification of CD45R positive B cells.

Quantitative analysis of immunohistochemistry staining for CD45R in tumours from the KPC *Ighm*$^{-/-}$ mouse model compared with KPC *Ighm*$^{+/-}$ and KPC. KPC n=7, KPC *Ighm*$^{+/-}$ n=6, KPC *Ighm*$^{-/-}$ n=12, P value = 0.0171, Mann Whitney. Each dot on the graph represents a whole tumour from one mouse.

The quantification confirmed that CD45R staining is significantly reduced in tumours from KPC *Ighm*$^{-/-}$ mice, however, two outliers in the KPC *Ighm*$^{-/-}$ cohort were observed. Representative pictures of CD45R staining in these mice, including an outlier with elevated CD45R^{+} cells, are shown in Figure 5.9.

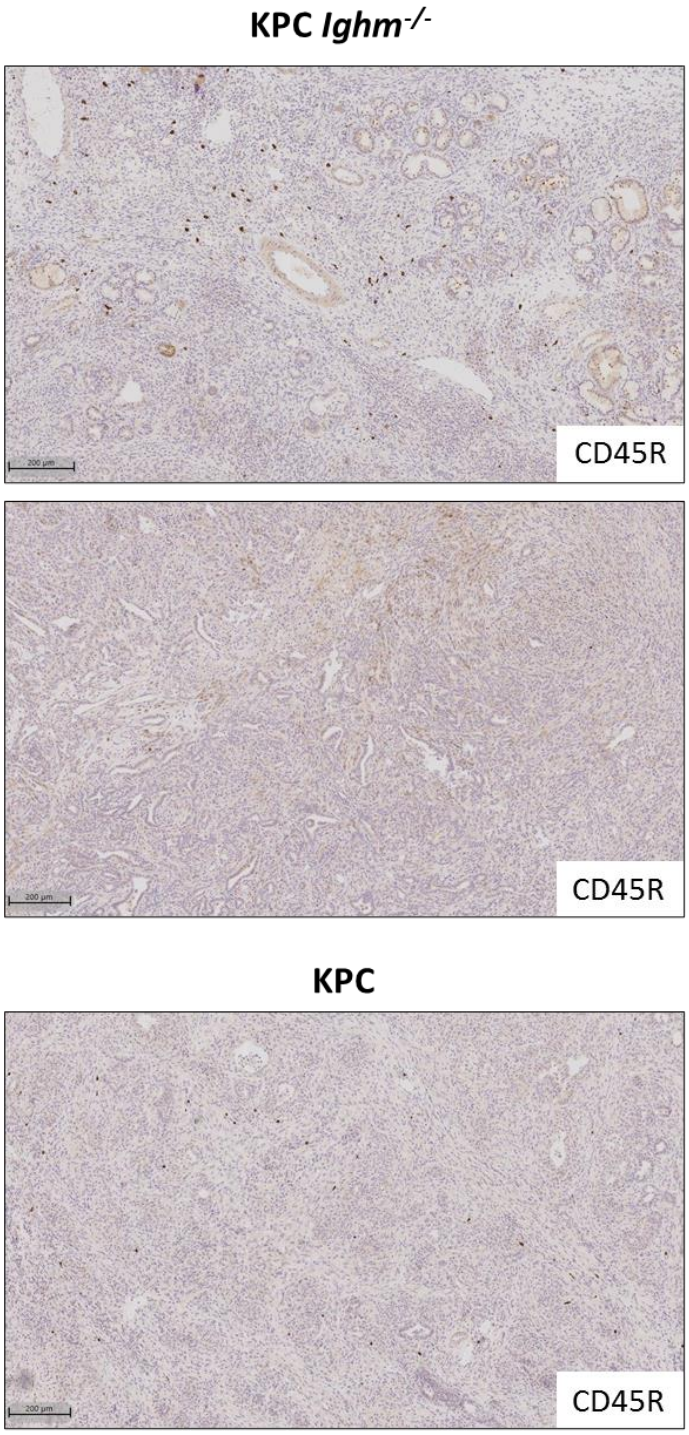

Figure 5.9 CD45R IHC staining in KPC and KPC *Ighm*$^{-/-}$ mice.

Top panel: Image of CD45R immunohistochemical staining on tumour from a KPC *Ighm*$^{-/-}$ 'outlier' mouse demonstrating increased tumour infiltration of CD45R+ cells. Middle and Bottom panels: Representative images of CD45R immunohistochemical staining in endpoint tumours from KPC *Ighm*$^{-/-}$ and KPC mice demonstrating a lack of positive cells in tumour from KPC *Ighm*$^{-/-}$ mice (middle) compared with KPC mice (bottom). Scale bar 200µm.

I observed that, although drastically reduced, the 2 outlier mice notwithstanding, some CD45R positive cells are still present in tissues from the B cell deficient KPC *Ighm*$^{-/-}$ mice. These were detected both in end point tumour tissue and spleen. CD45R is commonly used as a B cell marker. It is a transmembrane protein tyrosine phosphatase that is expressed on B cells at all developmental stages from pro-B cells through mature B cells and active B cells. However, it is also expressed on a small subset of T and NK cells. Therefore, I wanted to assess different B cell markers. Serial sections of end point tumours and spleens were stained for Pax5 and CD45R (Figure 5.10 and Figure 5.11). Representative images illustrate that although some CD45R positive cells are present in tumours and spleens from KPC *Ighm*$^{-/-}$ mice, these cells are negative for Pax5 staining. These data suggest that CD45R^{+} cells in KPC *Ighm*$^{-/-}$ mice are not B cells, but rather T or NK cells that express CD45R. The relevance of the differential infiltration of these cells in the 'outlier' tumours is not clear, since they did not affect tumour latency in these mice.

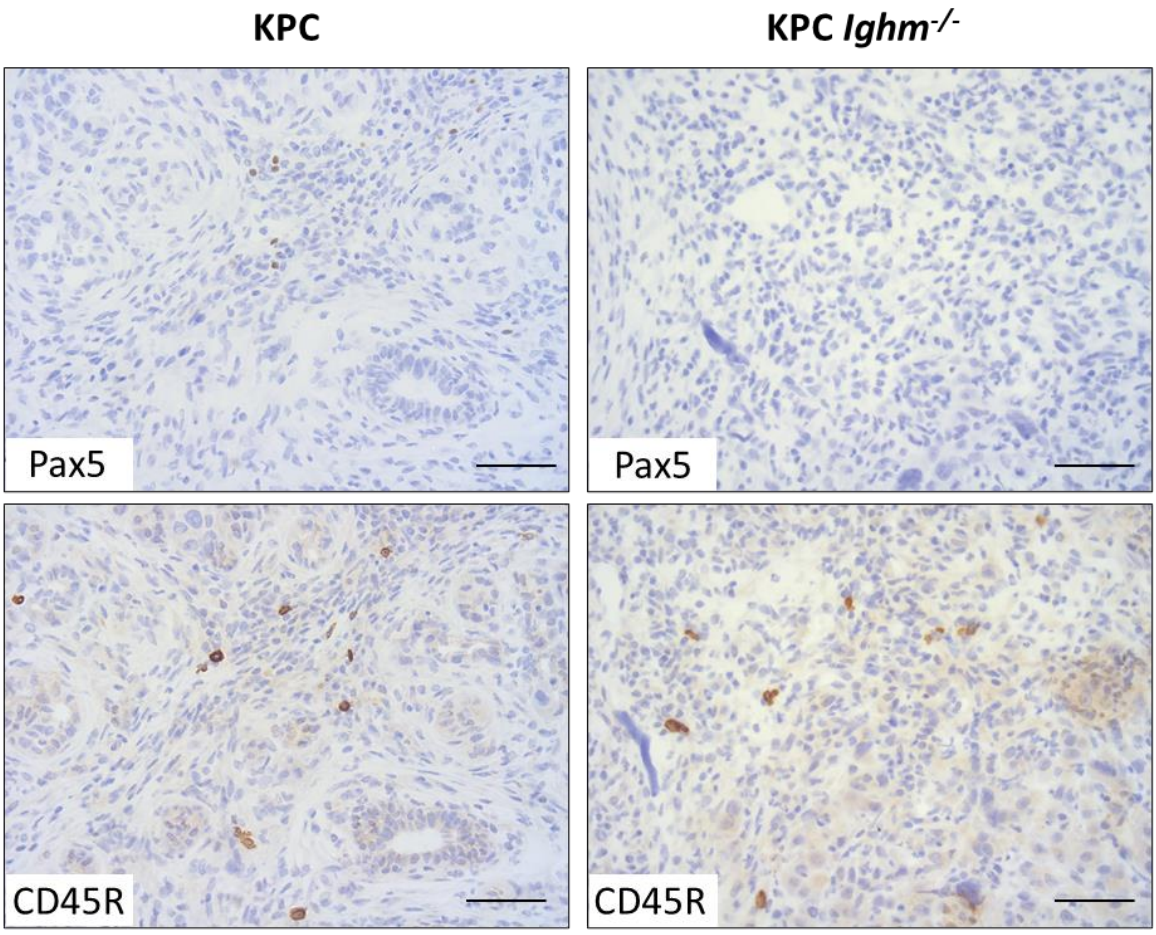

Figure 5.10 IHC for different B cell markers in serial sections of PDAC from KPC and KPC *Ighm*$^{-/-}$ mice.

Representative images of Pax5 and CD45R immunohistochemical staining on serial sections of tumours from end point KPC and KPC *Ighm*$^{-/-}$ mice demonstrating lack of B cells. Scale bar 20µm.

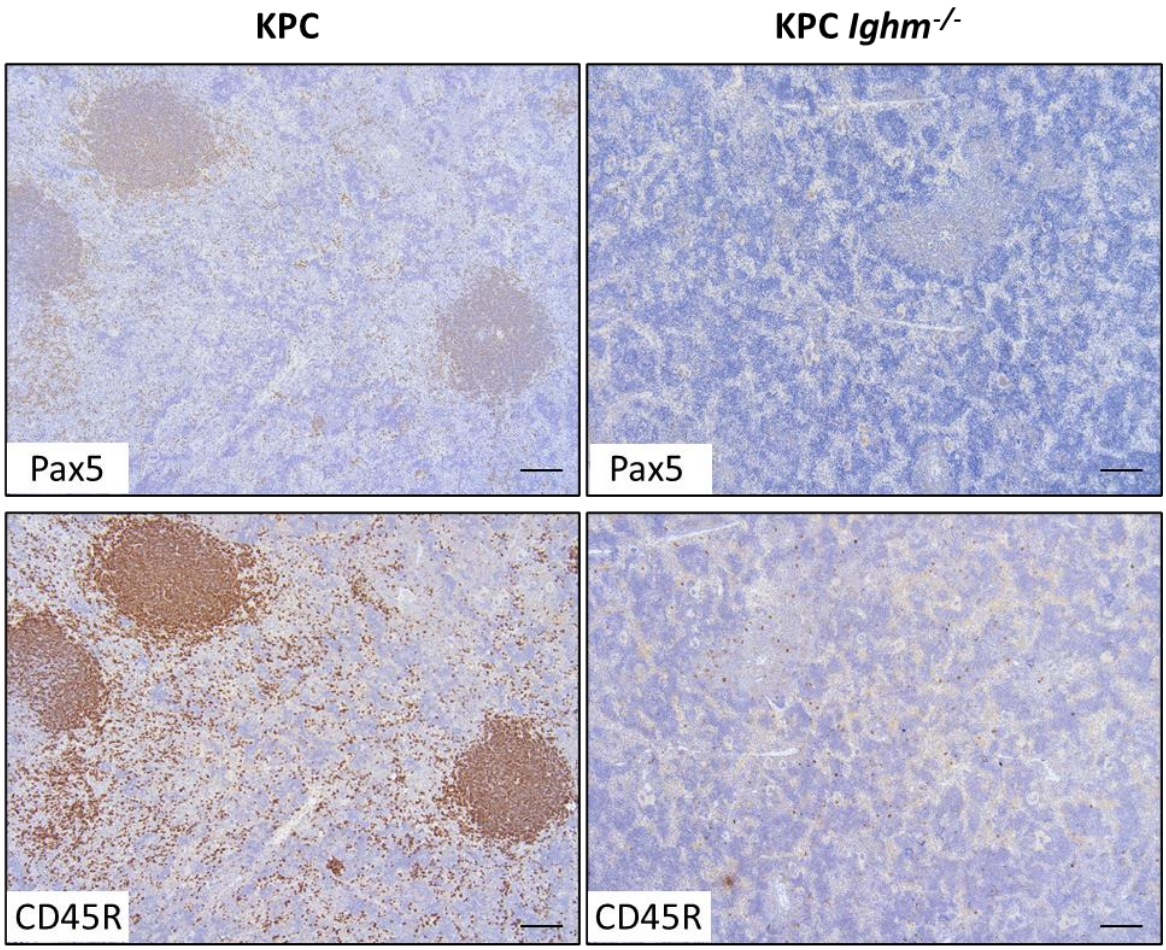

Figure 5.11 IHC for different B cell markers in serial sections of spleen from KPC and KPC *Ighm*$^{-/-}$ mice.

Representative images of Pax5 and CD45R immunohistochemical staining on serial sections of spleens from end point KPC and KPC *Ighm*$^{-/-}$ mice demonstrating lack of B cells. Scale bar 20µm.

End point tumours were also stained for markers of T cells, cytotoxic T cells, neutrophils and macrophages and staining was quantified (Figure 5.12). No significant changes were observed in immune infiltrate in end point tumours from KPC *Ighm*$^{-/-}$ mice, although if anything, there did appear to be a trend towards increased CD3^{+} T cells and increased neutrophils in some KPC *Ighm*$^{-/-}$ tumours.

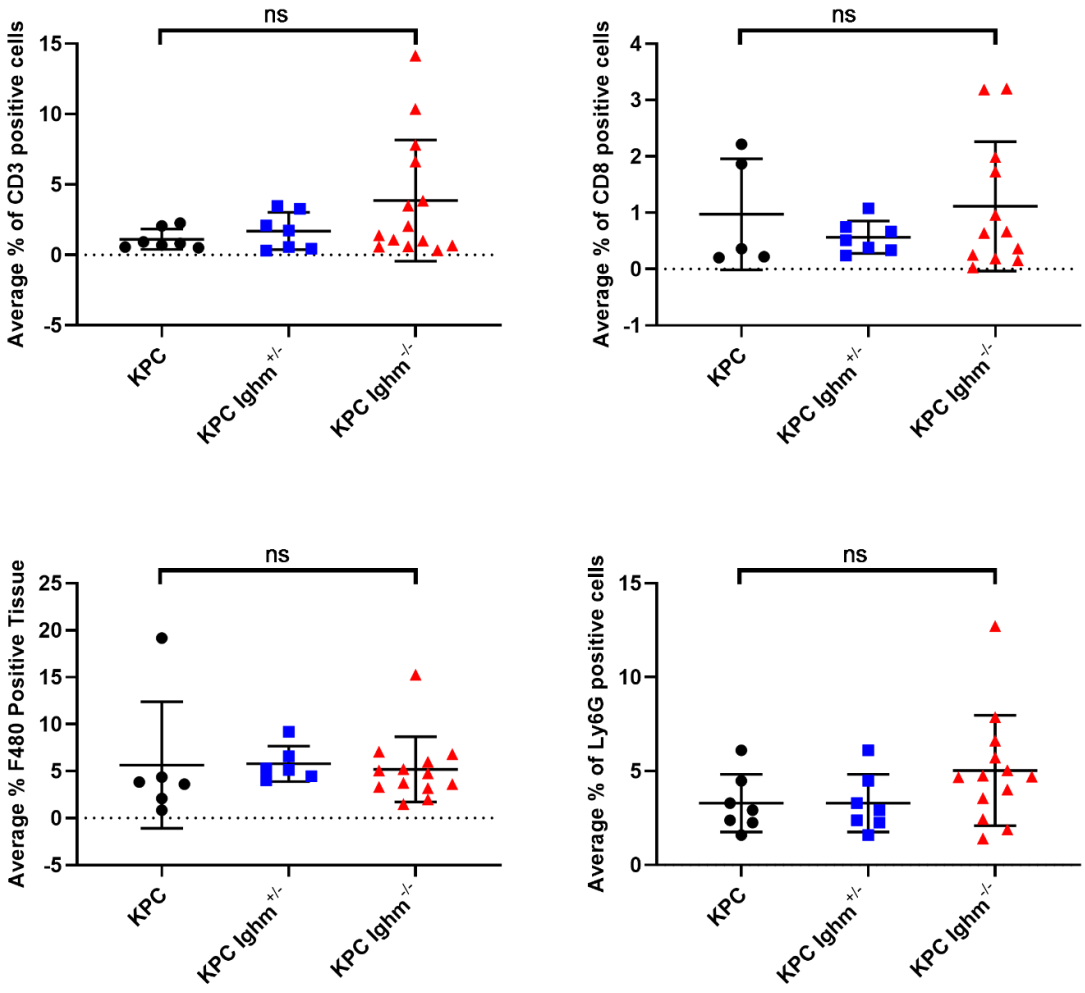

Figure 5.12 Quantification of immunohistochemistry staining on end point tumours from KPC *Ighm*$^{-/-}$ mice.

Quantitative analysis of immunohistochemistry staining for immune cell markers in tumours from the KPC *Ighm*$^{-/-}$ mouse model compared with tumours from KPC *Ighm*$^{+/-}$ and KPC mice. KPC n≥6, KPC *Ighm*$^{+/-}$ n≥6, KPC *Ighm*$^{-/-}$ n≥12, Mann Whitney. Each dot on the graphs represents a whole tumour from one mouse.

Since B cells are involved in T cell priming, I wanted to investigate whether the absence B cells in our KPC model is in any way effecting T cells therefore hindering the anti-tumourigenic effect of B cell depletion seen in the previously published papers. To

investigate this, I performed immune profiling of T lymphocytes from end point tumours from KPC and KPC *Ighm*$^{-/-}$ mice (Figure 5.13). Although, overall numbers of T lymphocytes trended towards an increase in KPC *Ighm*$^{-/-}$ tumours, the increase was not significant. No changes in cytotoxic, regulatory and helper T cell populations were observed in KPC B cell deficient mice versus KPC controls. The results obtained suggest that the absence of anti-tumourigenic effect in KPC *Ighm*$^{-/-}$ mice is not due to any T cell deficit.

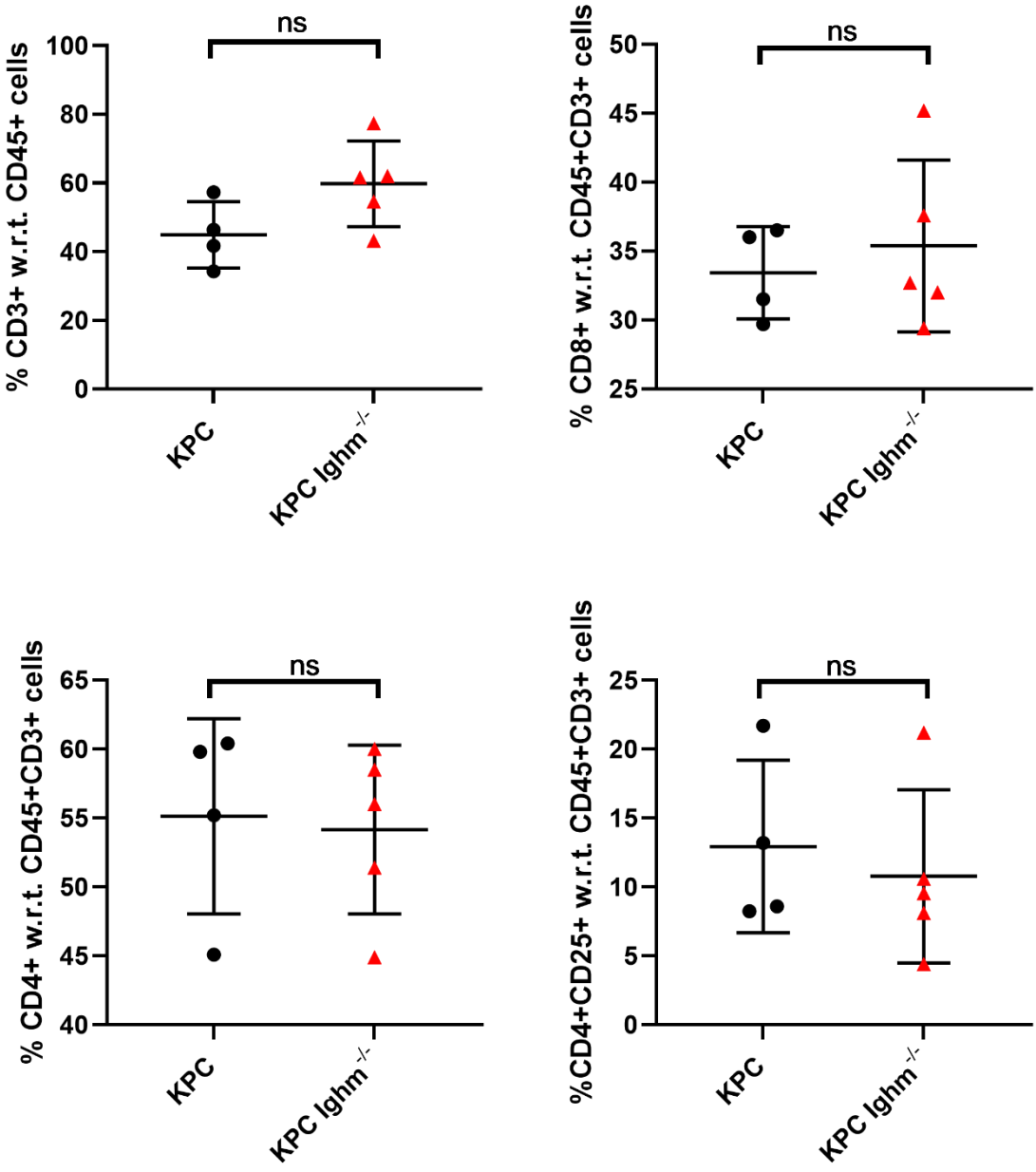

Figure 5.13 Quantification of T lymphocyte populations in KPC *Ighm*$^{-/-}$ end point tumours.

Graphs show quantification of T lymphocyte populations by flow cytometry in end point tumours (data shown are mean ± SD, n ≥4, unpaired t test) (w.r.t.- with respect to). Each dot on the graphs represents a whole tumour from one mouse.

Finally, I collected peripheral bloods from mice with end stage disease and analysed them using the ProCyte Dx Haematology Analyser (Figure 5.14).

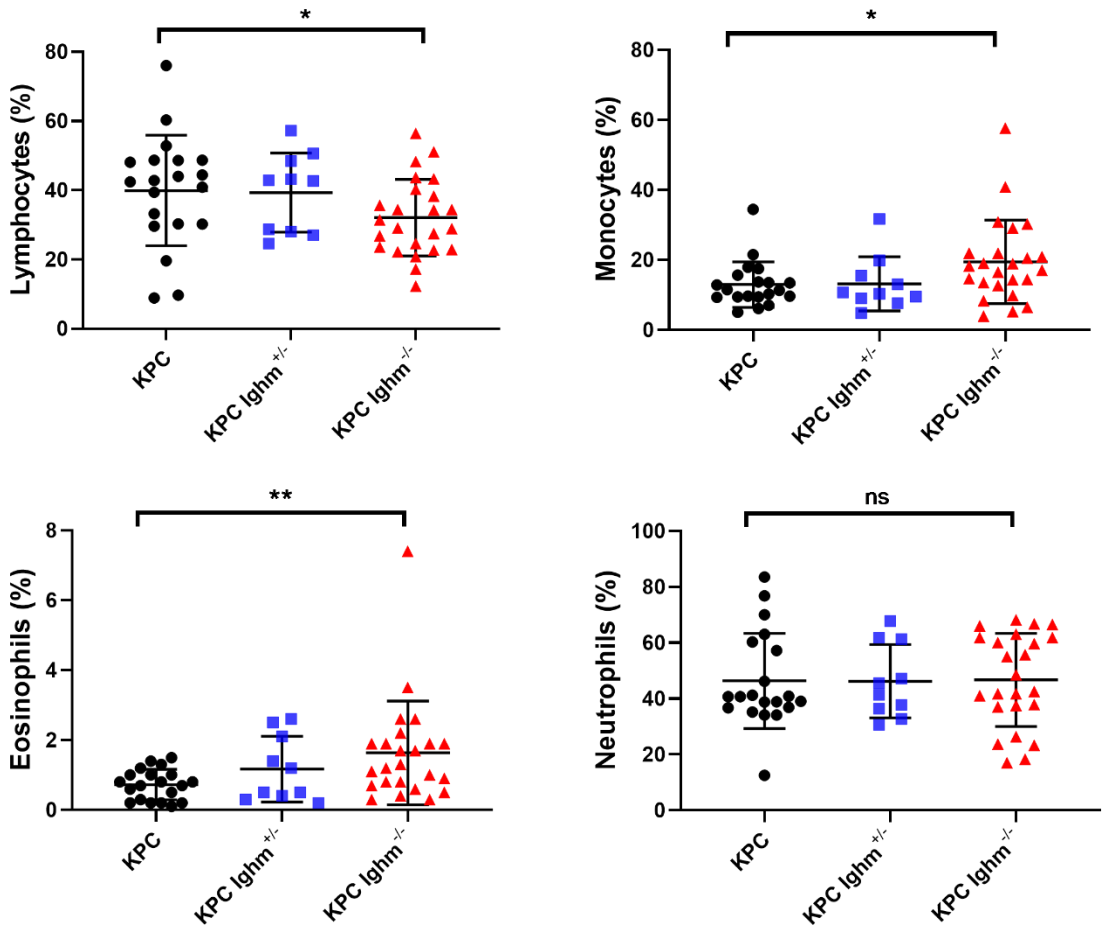

Figure 5.14 Analysis of peripheral blood from KPC and KPC *Ighm*$^{-/-}$ mice with end stage disease.

Graphs illustrate peripheral blood leukocyte counts. Blood was taken and stored in EDTA tubes until processed with ProCyte Dx Haematology Analyser. Data shown are mean ± SD, KPC (n=20), KPC *Ighm*$^{+/-}$ (n=10) and KPC *Ighm*$^{-/-}$ (n=24), Mann Whitney.

Similar to the data obtained from *Ighm*$^{-/-}$ mice at the 6 week time point, a significant increase in eosinophils was observed in KPC *Ighm*$^{-/-}$ mice as compared with KPC controls. What is more, monocyte numbers were significantly increased in peripheral blood from KPC *Ighm*$^{-/-}$ mice with end point tumours although this did not translate to higher numbers of macrophages in the tumours. Despite a trending increase in T lymphocyte numbers infiltrating KPC *Ighm*$^{-/-}$ tumours, overall lymphocyte count in peripheral blood is significantly decreased. The reason overall lymphocyte count is significantly lower in KPC *Ighm*$^{-/-}$ mice is due to the fact that these mice lack B lymphocytes. A limitation of the ProCyte Dx Haematology Analyser is that it does not differentiate between T and B lymphocytes. To

confirm the absence of B cells in peripheral blood from KPC *Ighm*$^{-/-}$ mice with end point tumours I used flow cytometry (Figure 5.15). The presence of CD45R was assessed and a significant decrease in expression of this marker was observed in KPC *Ighm*$^{-/-}$ mice. A small subset of CD45R^{+} cells remained, which I hypothesised were T or NK cells. To further validate this hypothesis, I also assessed peripheral blood from 6 week old *Ighm*$^{-/-}$ mice for the presence of the B cell markers B220 and CD19 (Figure 5.16). Flow cytometry data confirmed the complete absence of B cells in the *Ighm*$^{-/-}$ mouse model.

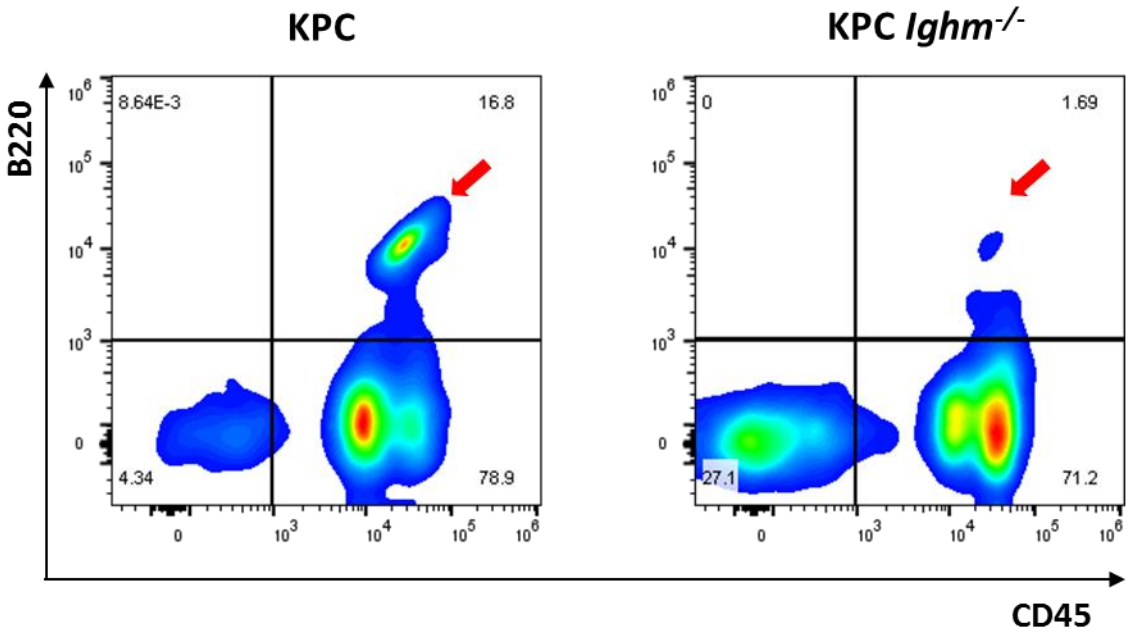

Figure 5.15 The absence of B cells in peripheral blood from KPC *Ighm*$^{-/-}$ mice.

Flow cytometry analysis of peripheral blood from end point KPC and KPC *Ighm*$^{-/-}$ mice. Peripheral blood cells were stained with APC-conjugated monoclonal antibody for B220 (CD45R) and FITC-conjugated antibody for CD45. Percentages refer to cells in 'Live' gate.

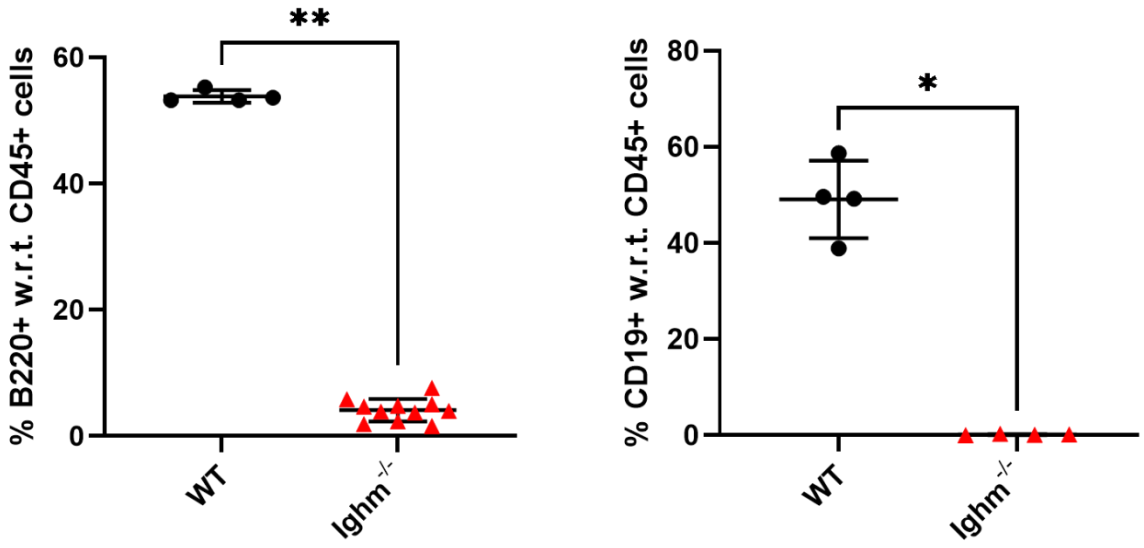

Figure 5.16 Quantification of flow cytometry data for B cells in peripheral blood from *Ighm*$^{-/-}$ mice.

Graphs illustrate data obtained from flow cytometry analysis of peripheral blood from 6 week old WT n=4 and *Ighm*$^{-/-}$ n≥4 mice., Mann Whitney (w.r.t.- with respect to). Peripheral blood cells were stained with APC-conjugated monoclonal antibody for B220, CD19 and FITC-conjugated antibody for CD45.

Here, I have characterised the B cell deficient KPC mouse model and confirmed the absence of B cells by various methods including flow cytometry and immunohistochemistry. KPC mice lacking B cells did not have any survival benefit (or deficit) over KPC mice. End point tumours were studied, and immune profiling of immune infiltrate performed, and no significant changes were observed in immune infiltrate in end point tumours. Neither did I observe any T cell deficit in KPC *Ighm*$^{-/-}$ mice which could potentially have impacted on the survival of these mice in the absence of B cells. Finally, there were no differences in the phenotype, histologically, of tumours. Overall, the data presented here imply that B cells do not play a significant role in tumour progression in KPC mice.

5.3.3 B cell depletion in established tumours

Because the KPC *Ighm*$^{-/-}$ mice have a B cell deficit prior to tumour initiation I wanted to rule out the possibility that any tumour-suppressive effects of B cell depletion in established tumours could be masked by tumour-promoting effects of B cells depletion during tumour initiation. To mimic the clinical settings and to rule out any effect of B cells on tumour initiation and early progression, I aimed to investigate whether B cell depletion in mice with established tumours, using an anti-CD20 B cell depleting antibody, would present a different outcome as compared with the KPC *Ighm*$^{-/-}$ mouse model which lacks B cells from an embryonic stage.

To perform B cell depletion in KPC mice with established tumours, mice were regularly palpated. The presence of tumour was confirmed using ultrasound before enrolling mice into treatment groups. Mice with confirmed tumours were enrolled either to the anti-CD20 treatment group or the isotype control group. Tumour growth was monitored weekly (Figure 5.17). As seen from the graph, no difference in tumour growth rate over the course of treatment with CD20 B cell depleting antibody was observed. What is more, the post-treatment survival analysis suggests that the depletion of B cells in mice with established tumours have no advantageous effect towards increased survival (Figure 5.18).

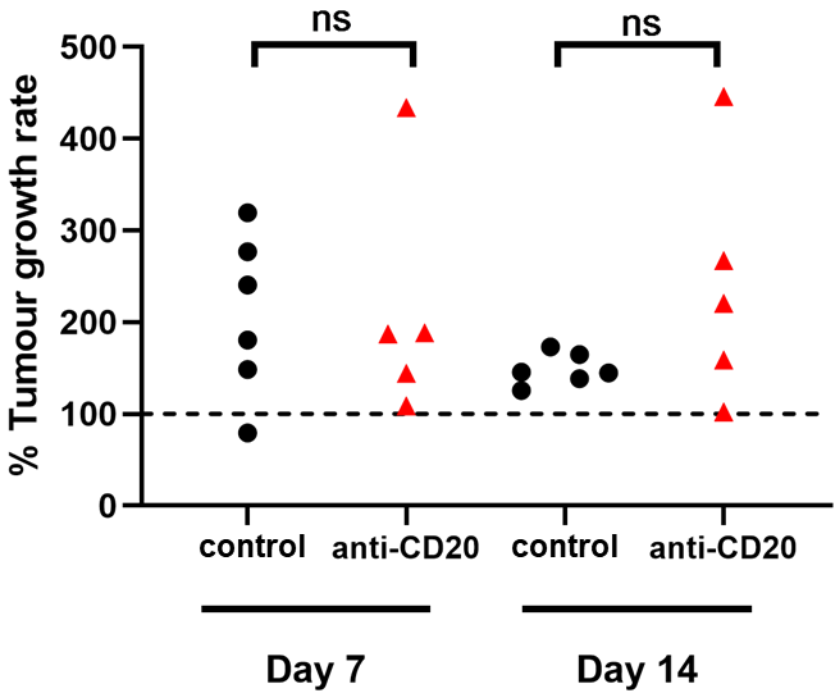

Figure 5.17 B cell depletion in KPC mice with established tumours has no effect on tumour growth rate.

Graph illustrates the % tumour volume change in KPC mice treated with anti-CD20 antibody or isotype control. Isotype control n=8, anti-CD20 n=5, Mann Whitney. Tumour growth rate at 7 days post treatment start reflects the growth from Day 0 to Day 7. Tumour growth rate at 14 days reflects the growth from Day 7 to Day 14.

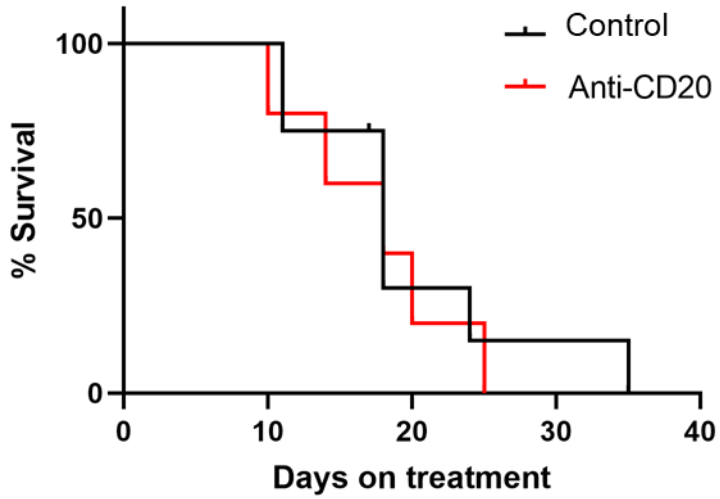

Figure 5.18 B cell depletion fails to extend survival of KPC mice with established tumours.

The Kaplan-Meier survival curve of *Kras*$^{LSL.G12D/+;}$ *Trp53*$^{R172H/+;}$ *Pdx1Cre*; mice. Isotype control (n=8); anti-CD20 (n=5), p=0.6247, Log-rank (Mantel-Cox) test, non-significant.

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Next, I wanted to confirm that B cell depletion with anti-CD20 antibody was effective. Mice from both treatment groups were sampled at the manifestation of clinical symptoms of disease and peripheral blood was collected. Peripheral blood was assessed for the presence of B cells using flow cytometry (Figure 5.19). According to manufacturer specifications, a single dose of anti-CD20 antibody successfully depletes B cells and the effect is sustained for over 20 days. Gradual return of the B cell population is reported after 20 days with full recovery around day 50. From the flow cytometry data collected, we observed that B cells were completely depleted in a mouse sampled after 14 days initial dose with anti-CD20 antibody. A small B cell population is present in peripheral blood from the mouse sampled at 20 days post treatment. This suggests that B cell population starts to gradually return at around 20 days post treatment. However, the tumour growth data (Figure 5.18) showed no indication that depletion of B cells in the first week of treatment has any effect on tumour shrinkage or growth rate. The same can be concluded from the survival data. Only one mouse survived 20 days after treatment with anti-CD20 and reached 25 days. Therefore, it was decided that there is no justification for repeating B cell depletion with an improved regime.

I also assessed tumours and spleens from mice treated with anti-CD20 antibody for the presence of B cells. Tissue sections were stained for CD45R, and representative pictures taken (Figure 5.20) and the staining quantified (Figure 5.21). These studies also showed that B cells were efficiently depleted in treated mice.

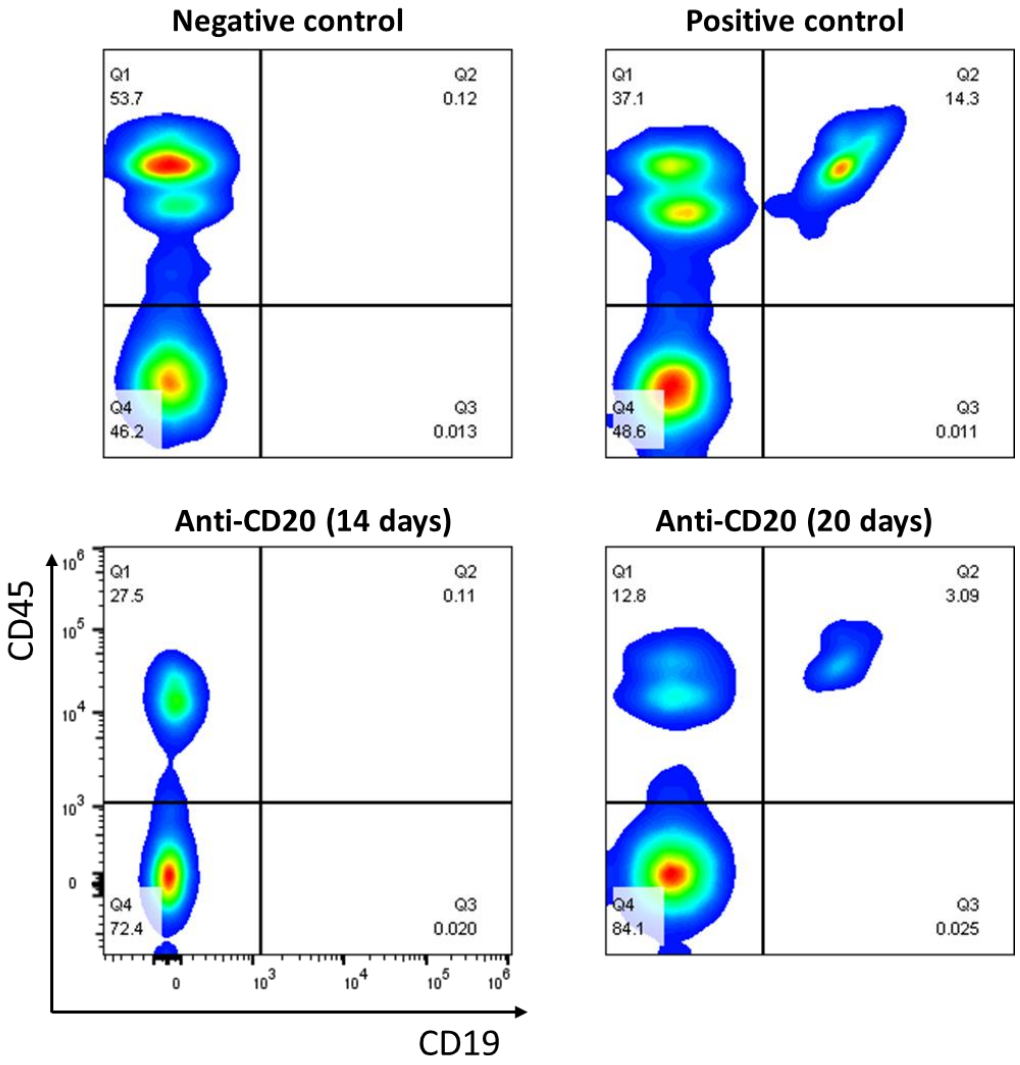

Figure 5.19 Peripheral blood B cell population is depleted with anti-CD20 antibody in KPC mice.

Flow cytometry analysis of the peripheral blood B cell population in KPC mice after anti-CD20 antibody treatment. Blood samples were stained with FITC-conjugated monoclonal antibody against CD45 and APC conjugated CD19 monoclonal antibody.

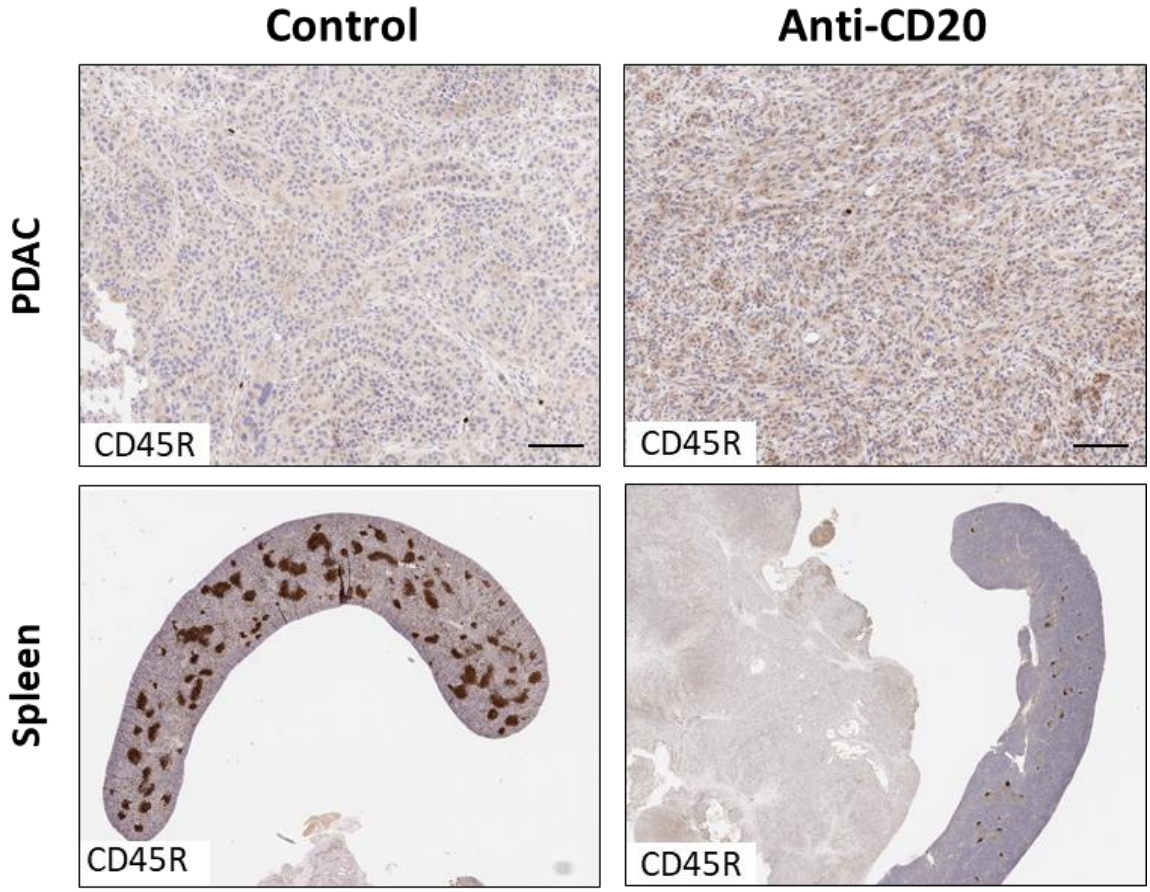

Figure 5.20 Images of CD45R staining on tissues from KPC mice treated with anti-CD20 antibody or vehicle.

Images of CD45R immunohistochemistry on tissues from KPC mice treated with B cell depleting anti-CD20 antibody (in this case for 18 days), or isotype control. Scale bar 100µm.

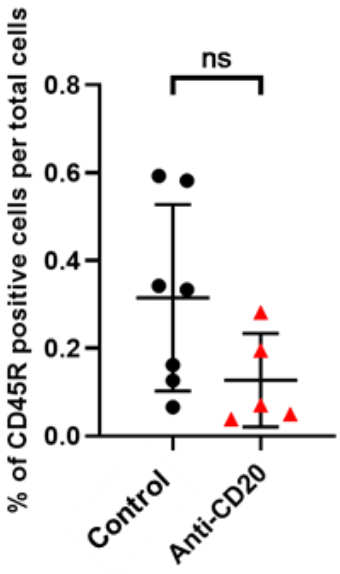

Figure 5.21 Quantification of B lymphocytes in tumours treated with anti-CD20.

Graph shows quantification of CD45R immunohistochemistry staining in end point tumours from KPC mice treated with B cell depleting anti-CD20 antibody or isotype control (data shown are mean ± SD, n ≥5, unpaired t test). Each dot on the graphs represents a whole tumour from one mouse.

To investigate whether there are any other changes in immune infiltrate in tumours treated with anti-CD20 antibody, tumours were assessed for T lymphocyte infiltration. Immunohistochemistry staining was quantified (Figure 5.22)

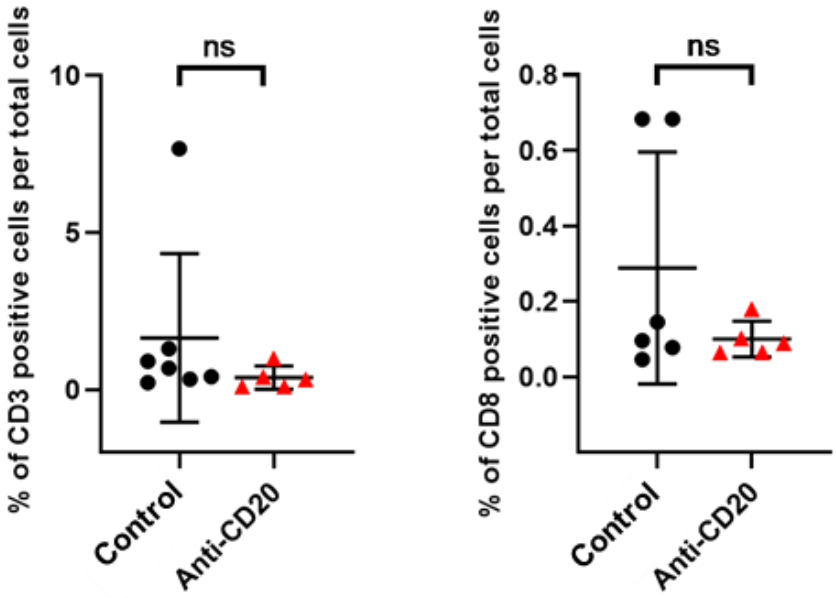

Figure 5.22 Quantification of T lymphocytes in tumours treated with anti-CD20 antibody.

Graph shows quantification of $CD3^+$ and $CD8^+$ T cells in end point tumours from KPC mice treated with B cell depleting anti-CD20 antibody or isotype control (data shown are mean ± SD, n ≥5, unpaired t test). Each dot on the graphs represents a whole tumour from one mouse.

Overall, the data collected from these experiments suggest that B cell depletion fails to induce any anti-tumorigenic effect in KPC mice with established tumours. What is more, the data presented here indicate that the absence of any anti-tumorigenic effect in KPC *Ighm*$^{-/-}$ mice is not masked by the complete knock out of B cells from early stage.

5.3.4 Allograft study in B cell deficient mice

Recently published articles focusing on the role of B cells in pancreatic cancer are at odds with the data presented here, and suggest that B cells might contribute to PDAC progression, depending on the biological context. Previous studies showing a pro-tumourigenic role of B cells used KC allograft or GEM models, rather than KPC models. In our hands, we have seen some evidence of different immune infiltration in KC versus KPC GEMM tumours. We observed that infiltration of CD45R positive cells is significantly higher in tumours from KC mice as compared with tumours from KPC mice (Figure 5.23 and Figure 5.24). Given the findings earlier in this chapter regarding CD45R expression, however, we cannot state with confidence that these are all B cells. Quantification of Ly6G and CD3 positive cells in these tumours did not reveal any other changes in terms of infiltration of either neutrophils or T cells (Figure 5.25).

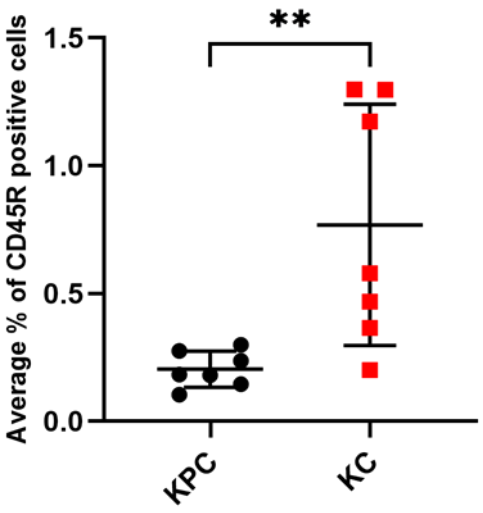

Figure 5.23 Comparison of B lymphocyte infiltration in KPC versus KC tumours.

Graph shows quantification of CD45R^{+} cells in end point tumours from KPC and KC mice (data shown are mean ± SD, n =7, p= 0.0041, unpaired t test). Each dot on the graphs represents a whole tumour from one mouse.

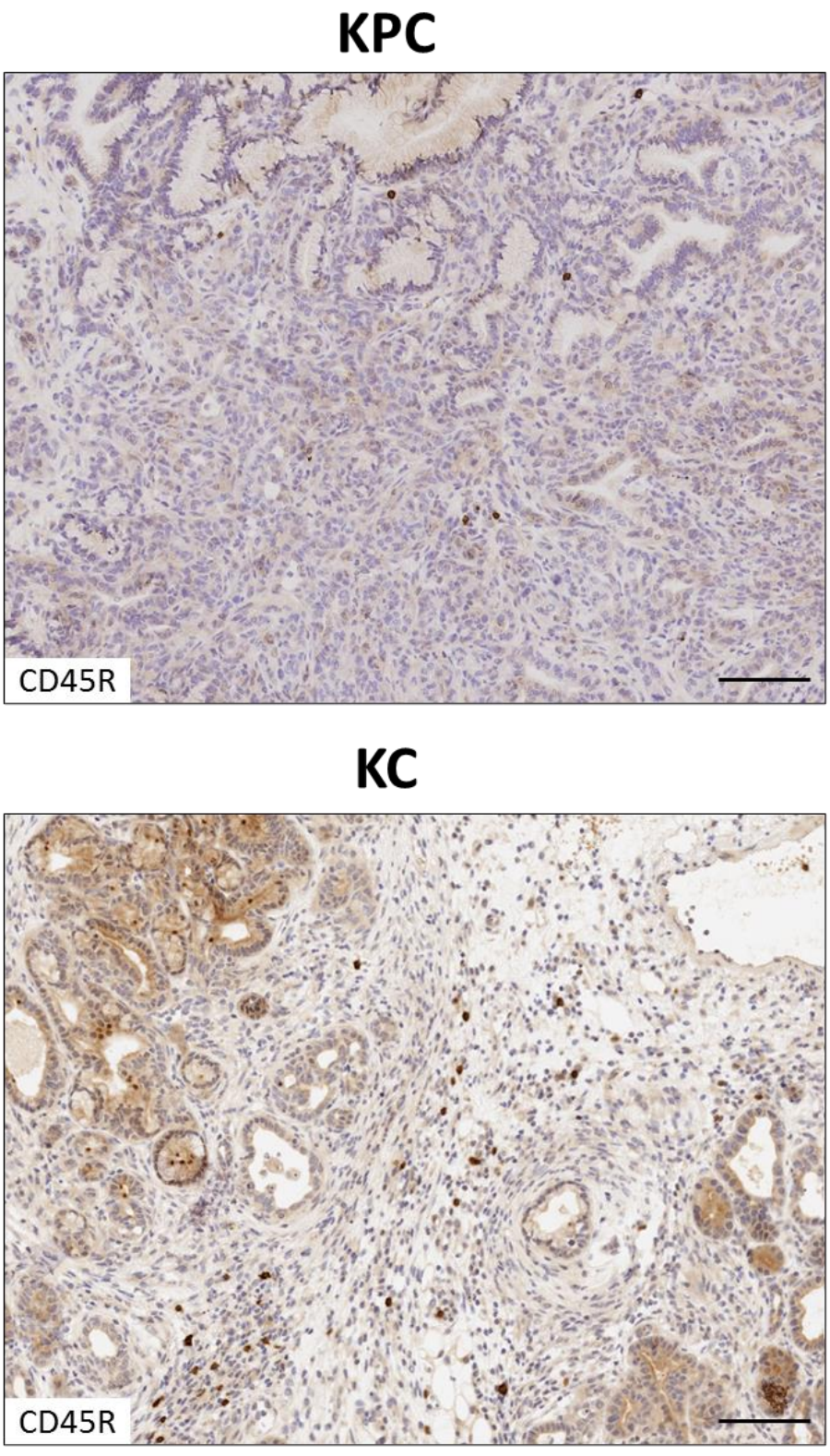

Figure 5.24 CD45R IHC staining on PDAC from KC and KPC mice.

Representative images of CD45R immunohistochemistry on PDAC from KC and KPC mice. Scale bar 100µm.

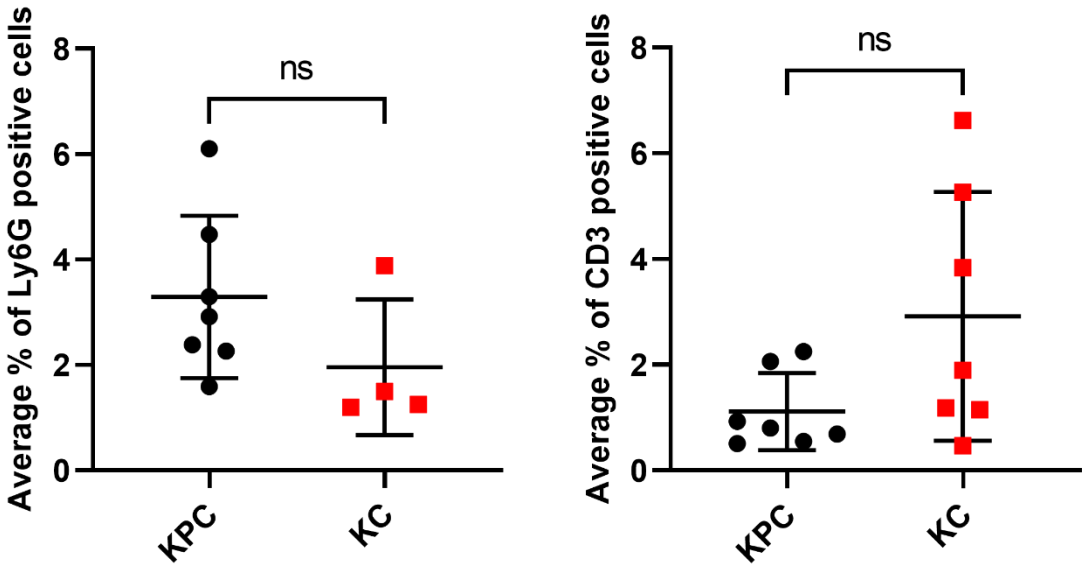

Figure 5.25 Immune cell infiltration in KPC versus KC tumours.

Graph shows quantification of Ly6G$^+$ and CD3$^+$ cells in end point tumours from KPC and KC mice (data shown are mean ± SD, KPC n≥7, KC n≥4, unpaired t test). Immunohistochemistry staining was scored using Halo software. Each dot on the graphs represents a whole tumour from one mouse.

Understanding the complete immune environment of these tumours and further dissecting the roles of B cells will be important for the development of effective immunotherapies for PDAC. Thus, I wanted to address the differences observed in the role of B cells seen between the published articles and the data I generated. Therefore, I aimed to investigate the effect of B cells in the syngeneic B cell-deficient mice using KC and KPC primary cell lines.

A syngeneic allograft experiment was performed using KC and KPC primary cell lines derived from end point tumours from C57BL/6 KC and KPC mice, respectively. Two distinct cell lines each of KC and KPC tumour origin were used to control the heterogeneous nature of primary cell lines. C57BL/6 wild-type or *Ighm*$^{-/-}$ mice were injected orthotopically in the pancreas with 1000 cells each and were sampled at the presentation of clinical sings of PDAC. Tumours were collected, formalin fixed, and paraffin embedded. Slides were obtained and stained with haematoxylin and eosin (H&E), and representative images taken (Figure 5.26). No histological differences between either KC and KPC tumours, or between WT or *Ighm*$^{-/-}$ hosts were observed.

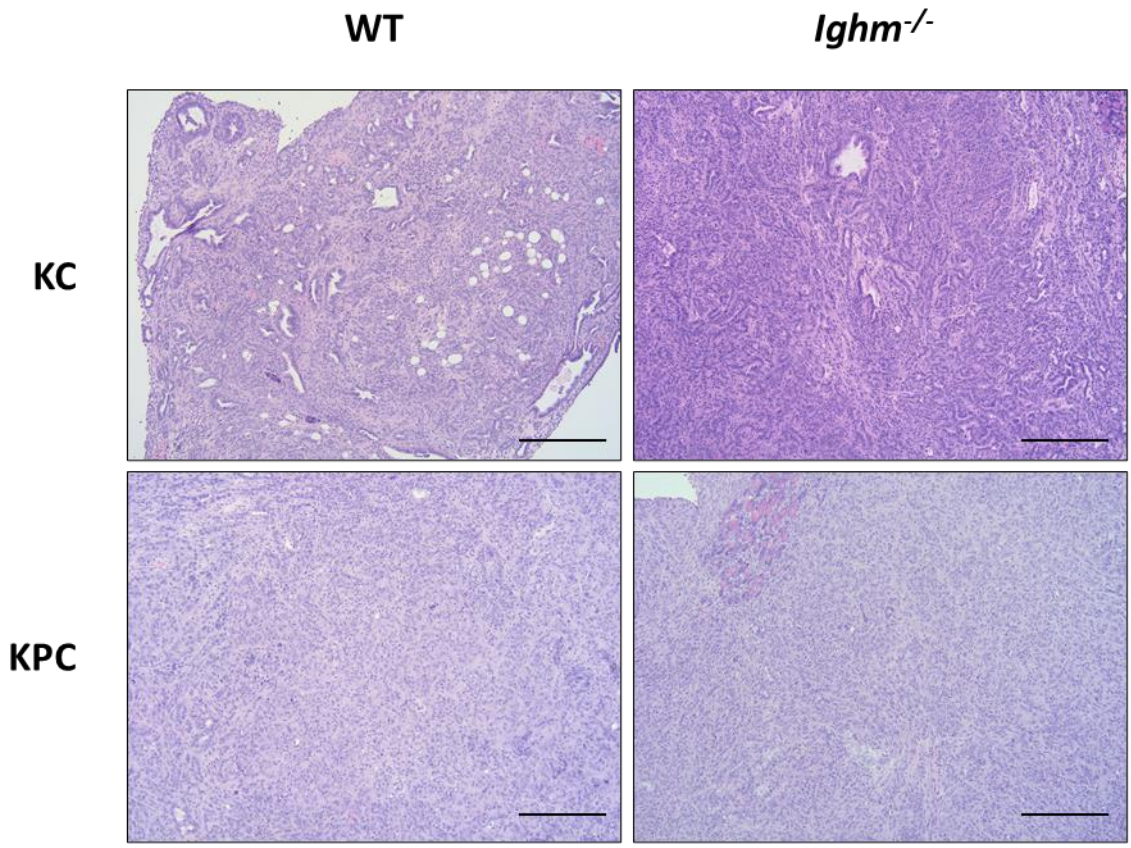

Figure 5.26 Histological comparison of orthotopic allograft tumours derived from KC and KPC cells in WT and *Ighm*$^{-/-}$ mice.

Representative images of haematoxylin and eosin (H&E) staining of KC (top) and KPC (bottom) cell derived tumours in WT (left) and *Ighm*$^{-/-}$ (right) mice. Scale bar 100µm.

As shown by the Kaplan-Meier curve, B cell deficient mice did not survive longer than WT controls both when implanted with either KC or KPC cell lines (Figure 5.27). I observed that mice implanted with KC cells survived consistently longer than mice implanted with KPC cells. This observation was made for WT and *Ighm*$^{-/-}$ mice. Overall, this indicates that B cells do not play a significant role in tumour development in syngeneic allograft models, at least in our hands. This further supports the results observed in the B cell deficient GEMM.

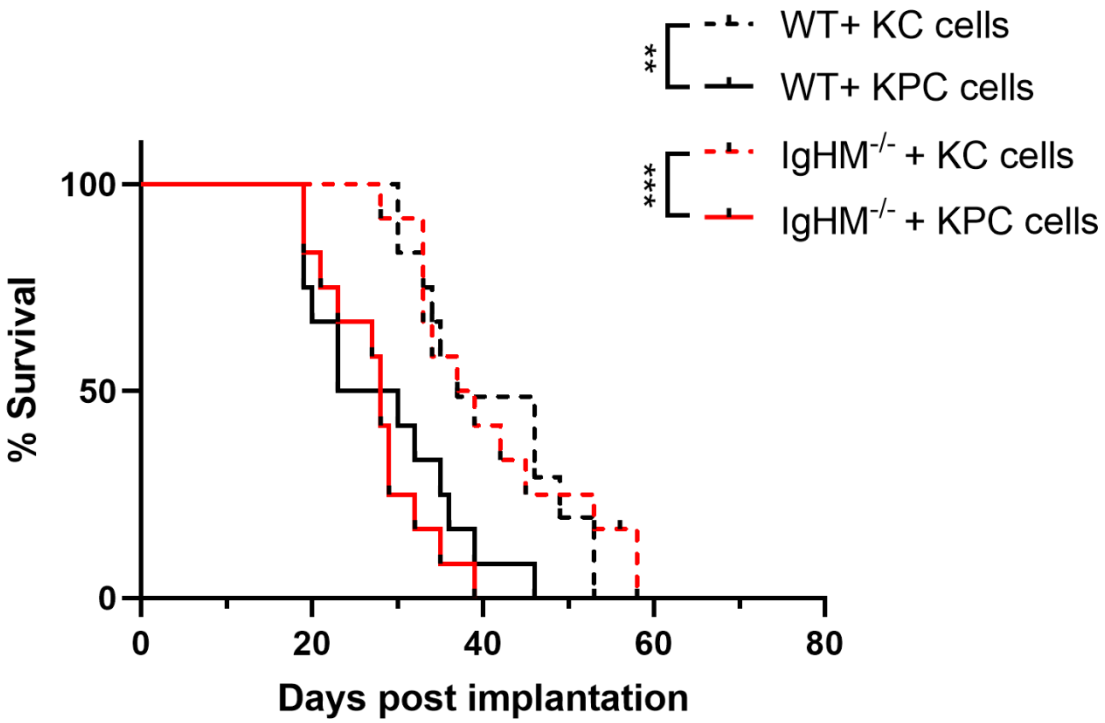

Figure 5.27 Absence of B cells fails to extend survival in PDAC allograft models.

Kaplan-Meier survival analysis of orthotopically-transplanted mice. KC (n=6+6) and KPC (n=6+6) cells were implanted in the pancreas of WT (n=6 per cell line) and *Ighm*$^{-/-}$ (n=12 per cell line) mice Log-rank (Mantel-Cox) test. I did not observe any differences between the 2 KC cell lines, or between the 2 KPC cell lines, so the data are combined here.

Next, I aimed to investigate whether the difference in B cell infiltration seen between KC and KPC mice would be present in the transplant models. The end point tumours from WT mice implanted with KC and KPC cells were collected and assessed for the presence of B cells. Flow cytometry data revealed no difference in $CD19^+$ B cell infiltration between KC and KPC cell line derived orthotopic allograft tumours (Figure 5.28). It is possible that the difference observed in the GEMM models is due to the marked difference in tumour latency between the genotypes, rather than the p53 status.

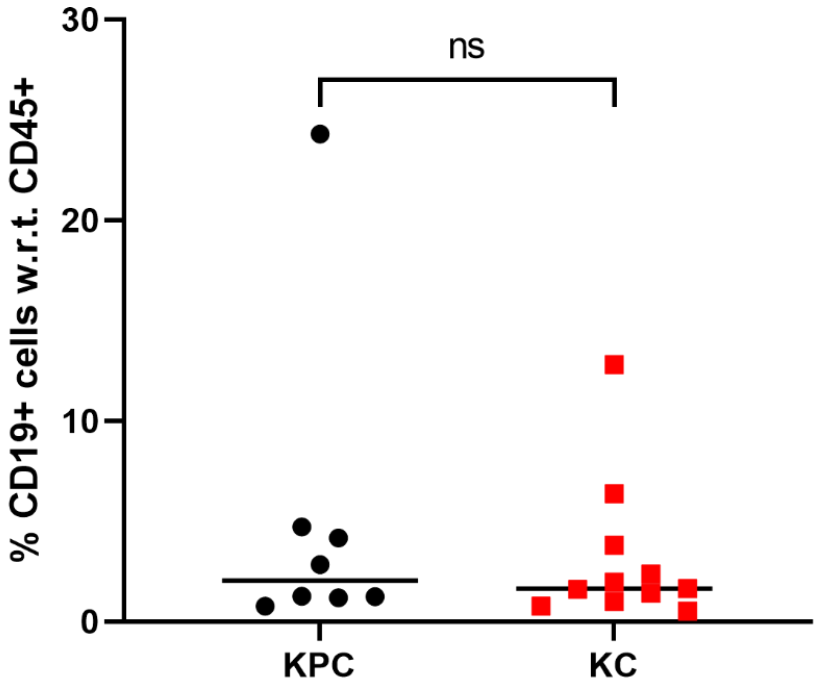

Figure 5.28 Quantification of flow cytometry data for $CD19^+$ B cells in KC and KPC cell line derived transplant tumours.

Graph illustrating data obtained from flow cytometry analysis of KC and KPC cell line derived allograft tumours implanted in WT mice. Single cell suspensions from tumours were stained with APC-conjugated monoclonal antibody for CD19 and FITC-conjugated antibody for CD45; KPC N=8 vs KC N=11, Mann Whitney.

Infiltration of T lymphocytes was also assessed in these tumours using flow cytometry (Figure 5.29). Interestingly, I observed that tumours derived from KPC implanted cells presented with significantly higher infiltration of $CD3^+$ T lymphocytes compared with KC cells. No changes were observed between tumours in WT versus *Ighm*$^{-/-}$ mice.

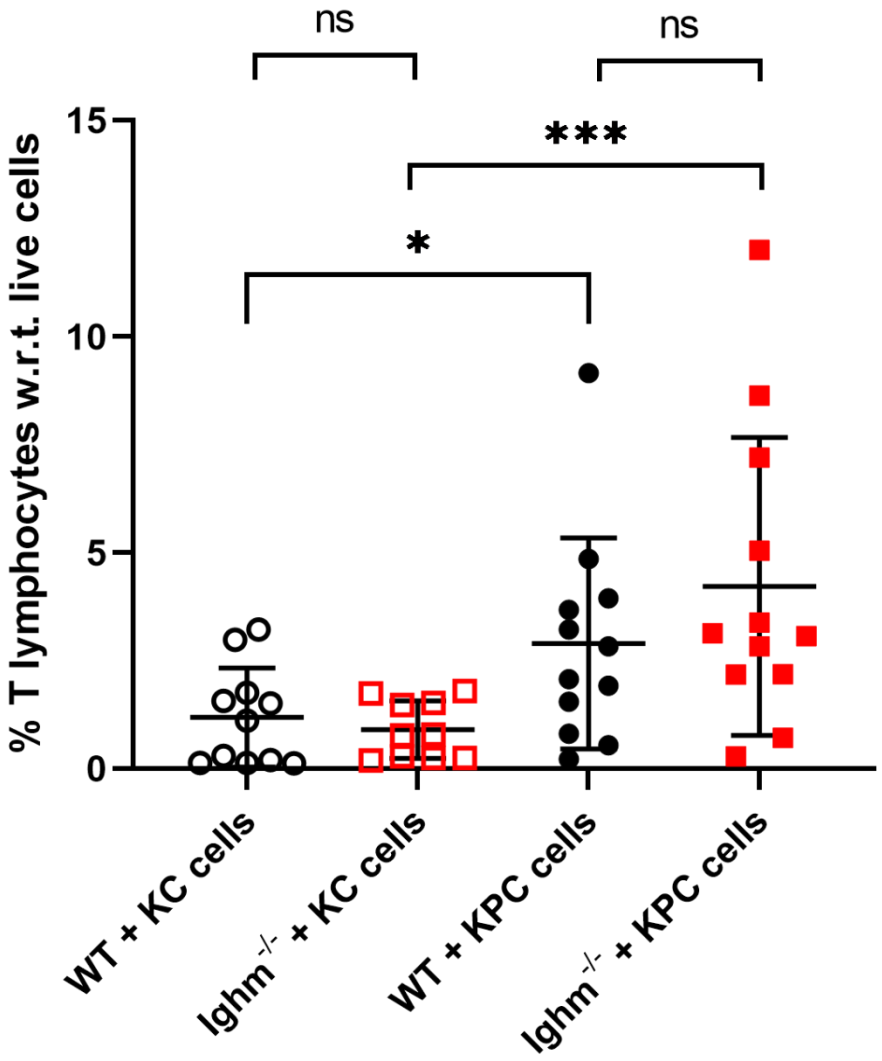

Figure 5.29 Quantification of flow cytometry data for $CD3^+$ T cells in KC and KPC cell line derived syngeneic allograft tumours from WT and *Ighm*$^{-/-}$ mice

Graph illustrating data obtained from flow cytometry analysis of KC and KPC cell line derived allograft tumours implanted in WT mice and *Ighm*$^{-/-}$ mice. Single cell suspensions from tumours were stained with Pacific Blue-conjugated monoclonal antibody for CD45 and FITC-conjugated antibody for CD3; N≥10, Mann Whitney. * denotes P<0.05; *** denotes P<0.0001 to 0.001

The clear difference in T lymphocyte infiltration between tumours of KC versus KPC origin observed by flow cytometry was also reflected in immunohistochemical staining for CD3 positive cells (Figure 5.30). Interestingly, this contrasts with the situation in KPC vs KC GEMMs where there was a trend towards decreased T cell infiltration. It could be speculated that in a transplant setting, mutant p53 can elicit an immune response, whereas in the GEMM model, the latency of KC tumours allows the accumulation of additional immunogenic mutations.

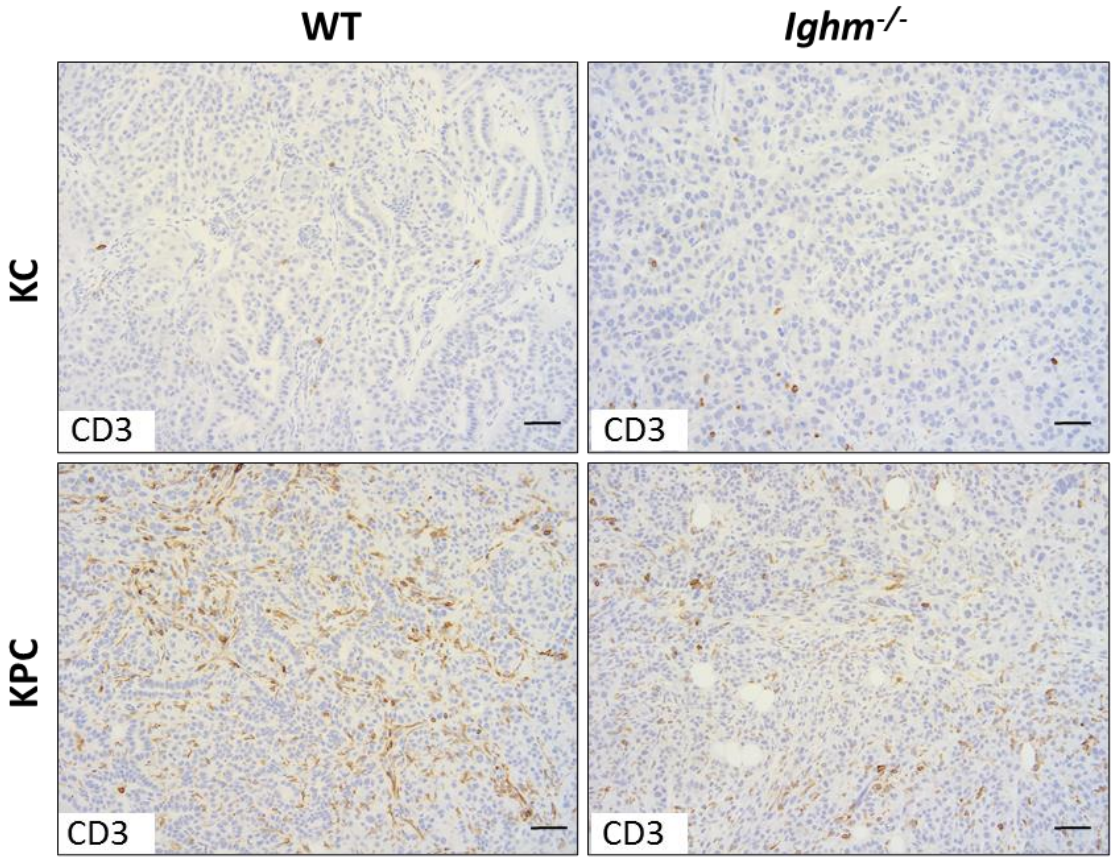

Figure 5.30 Increased infiltration of CD3 positive cells in orthotopic allograft tumours derived from KPC cells.

Representative images of CD3 immunohistochemistry staining of KC (top) and KPC (bottom) cell derived tumours in WT (left) and *Ighm*$^{-/-}$ (right) mice. Scale bar 20µm.

When specific T lymphocyte populations were analysed, I also observed reduced numbers of $CD69^+$ T lymphocytes levels in KC versus KPC origin tumours, but only in B cell deficient mice (Figure 5.31) CD69 is a well-known early activation maker for T lymphocytes. Previously, studies of acute pancreatitis demonstrated that degree of T cell activation, measured by the expression of cell surface marker CD69 correlated with the severity of pancreatitis (Glaubitz, Wilden et al. 2020). However, the reason behind the reduced presence of $CD69^+$ T cells in KC tumours from B cell deficient mice is unclear at the moment. It could be speculated that KC origin tumours are less immunogenic and in this setting, B cells could play a more important role in T cell activation. However, the survival data do not indicate that this has any effect on tumour growth. Between the same cohorts, we also observed a marked increase in T helper cells ($CD45^+CD3^+CD4^+$) in KC tumours from B cell deficient mice. Although KPC origin tumours had significantly increased infiltration of $CD3^+$ cells, the expression of CD8 and CD4 was not increased in these tumours. In contrast, there was a trend towards elevated $CD8^+$ cells in KC cell derived tumours. From the data collected, the relevance of the increased infiltration of $CD3^+$ lymphocytes in the transplanted KPC tumours is unclear, but the results imply that this is attributable to tumour, and not host genotype.

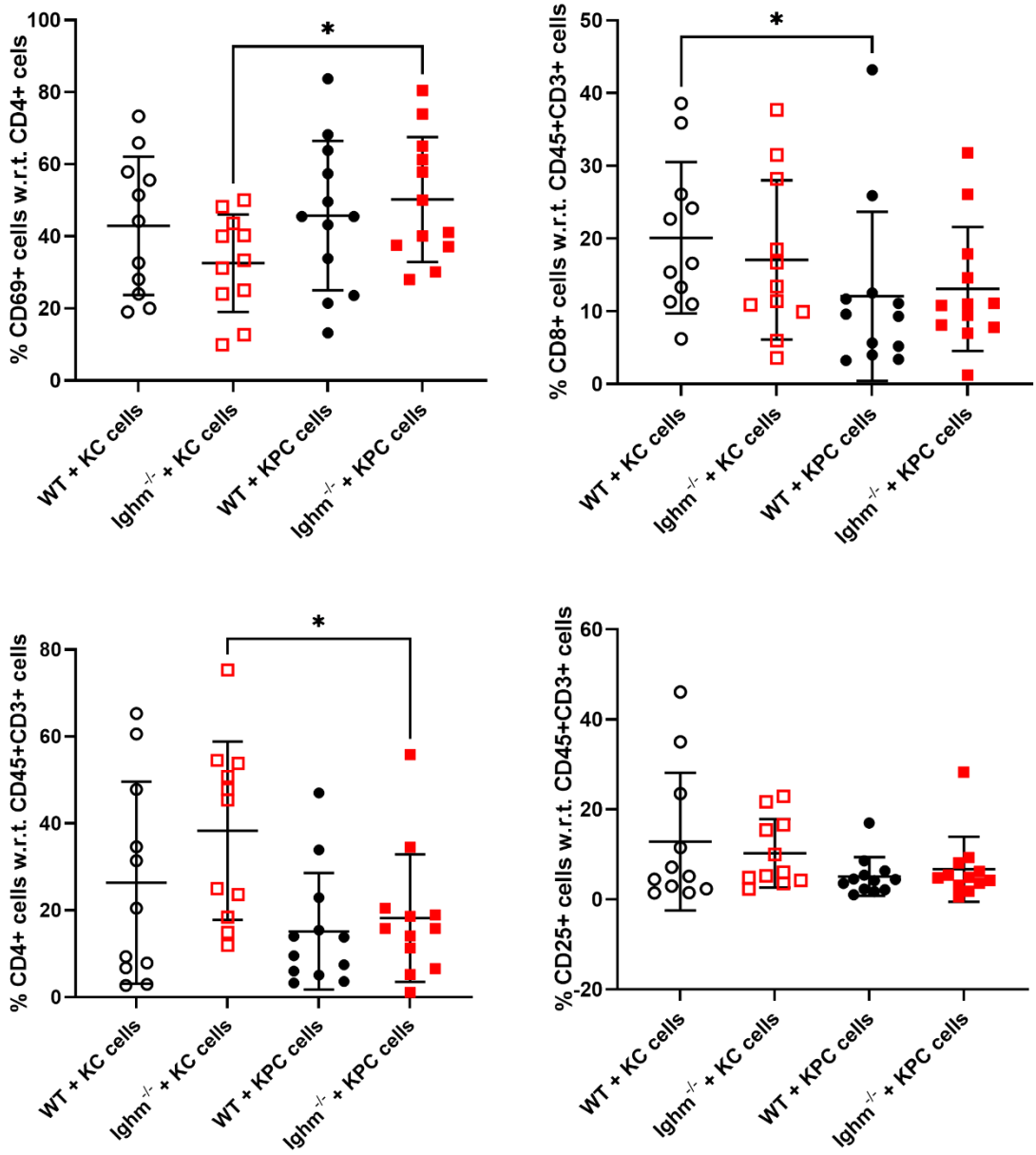

Figure 5.31 Quantification of flow cytometry data for T lymphocyte populations in KC and KPC cell line derived allograft tumours from WT and *Ighm*$^{-/-}$ mice.

Graphs illustrate data obtained from flow cytometry analysis of KC and KPC cell line derived allograft tumours implanted in WT mice and *Ighm*$^{-/-}$ mice.; N≥10, Mann Whitney.

Finally, peripheral blood cell populations from allograft mice with end point disease were analysed (Figure 5.32). Flow cytometry data confirmed the absence of $B220^+$ B cells in *Ighm*$^{-/-}$ mice. What is more, $Ly6C^+$ and $Ly6G^+$ cell populations were significantly increased in B cell deficient mice These observations are somewhat reflected in peripheral blood analysis from 6 week old *Ighm*$^{-/-}$ mice, which demonstrated an increase in neutrophils and, also, in analysis of peripheral blood samples from end point KPC *Ighm*$^{-/-}$ mice which demonstrated significantly increased monocytes. However, implantation of PDAC cells of the two genotypes did not have a differential systematic effect on peripheral blood cell populations. Interestingly, the data also suggest that the altered peripheral blood composition in B cell deficient mice has no impact on tumour growth.

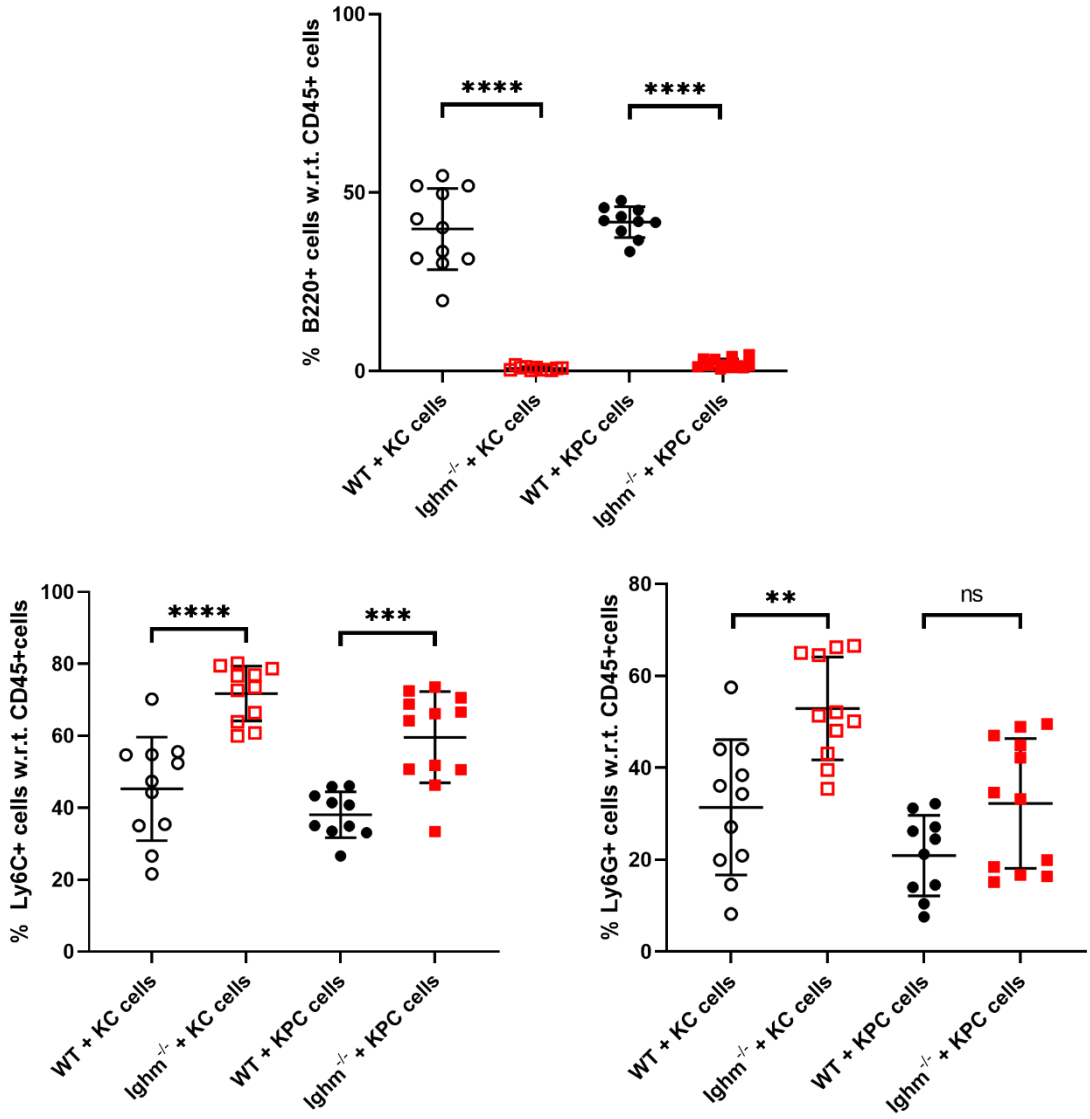

Figure 5.32 Analysis of peripheral blood cell populations from allograft mice.

Graphs illustrate data obtained from flow cytometry analysis of peripheral blood from WT and *Ighm*$^{-/-}$ mice implanted with KC or KPC cells; N≥10, Mann Whitney.

5.3.5 B cell infiltration in *Hif1a*-deficient mice

Lastly, we were also able to address the findings reported by Lee et.al, indicating that *p48-Cre*;LSL-*Kras*^{G12D} ;*Hif1a* $^{fl/fl}$ mice display enhanced development of PanIN lesions as a result of a significant increase in intrapancreatic B lymphocytes (Lee, Spata et al. 2016). This study demonstrated that the B cell–depleting anti-CD20 monoclonal antibody inhibited progression of PanINs in this model. In our lab, we have previously generated and aged KPC *Hif1a*$^{fl/fl}$ and KPC *Hif1a*$^{fl/fl}$ *Hif2*$^{fl/fl}$ mice. The survival data showed no indication that the deletion of hypoxia-inducible factor 1α (*Hif1α*) has any survival benefit in KPC mice (Figure 5.33). However, we wanted to assess the levels of B cell infiltration in the end point tumours from these mice and see how it relates to the published data. Tissue slides were stained for CD45R, and staining was quantified (Figure 5.34). Strikingly, we observed that KPC *Hif1a* $^{fl/fl}$ had significantly lower infiltration of B lymphocytes as compared with KPC mice, again confirming our previous findings that indicate the B cells are not a major influence on pancreatic tumourigenesis.

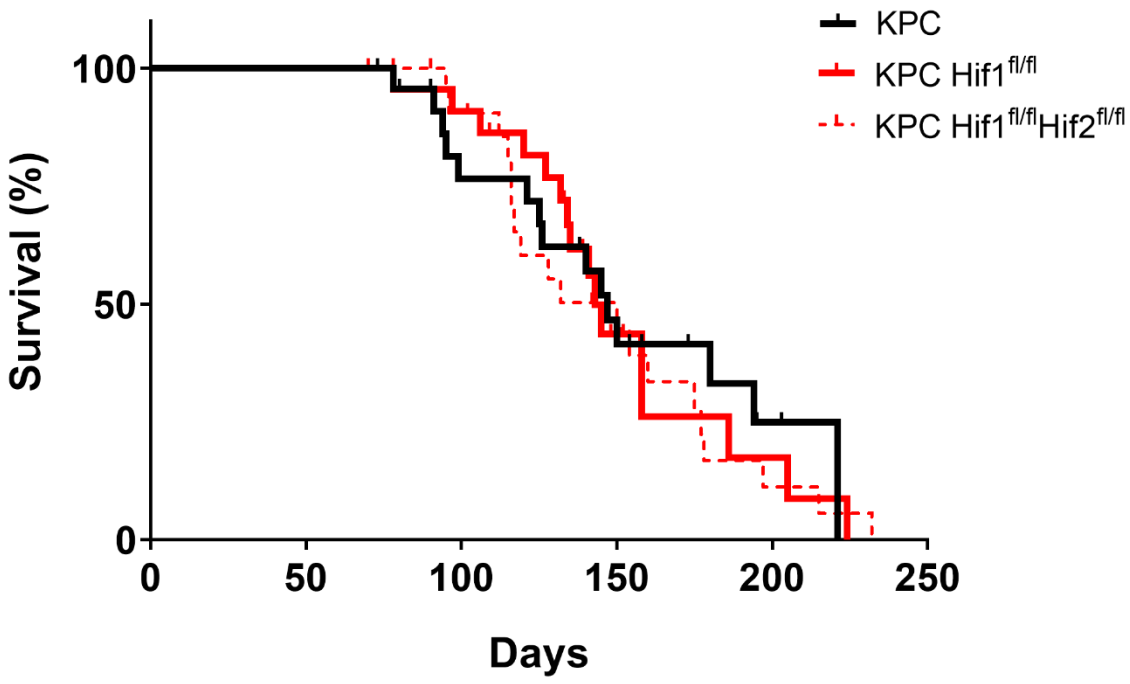

Figure 5.33 *Hif1α* and *Hif2* deletion fails to alter survival of KPC mice.

Kaplan-Meier survival analysis of *Kras*$^{LSL.G12D/+;}$ *Trp53*$^{LSL-R172H/+;}$ *Pdx1Cre* (KPC); KPC *Hif1a*$^{fl/fl}$ and KPC *Hif1a*$^{fl/fl}$; *Hif2*$^{fl/fl}$ mice. KPC N=24, KPC *Hif1a*$^{fl/fl}$ N=22; KPC *Hif1a*$^{fl/fl}$; *Hif2*$^{fl/fl}$ N=47, Log-rank (Mantel-Cox) test.

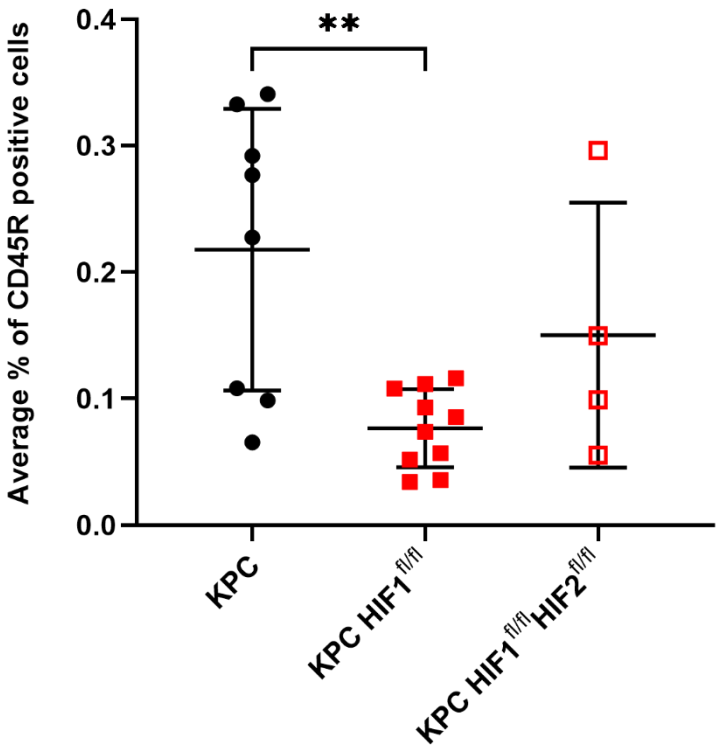

Figure 5.34 B lymphocyte infiltration in *Hif1a*$^{fl/fl}$ KPC mice.

Graph showing quantification of immunohistochemistry staining for CD45R^{+} cells in end point tumours from KPC, KPC *Hif1a*$^{fl/fl}$ and KPC *Hif1a*$^{fl/fl}$; *Hif2*$^{fl/fl}$ mice (data shown are mean ± SD, n≥4, p= 0.0085, unpaired t test). Each dot on the graphs represents a whole tumour from one mouse.

5.4 Discussion

There have been efforts to elucidate the role of B cells in pancreatic cancer. Despite preclinical studies, as well as clinical trials, targeting B cells, their role in pancreatic cancer remains controversial. Studies of tumour global gene expression profiles have identified B-cell gene signatures to be associated with improved metastasis-free survival for several cancer types such as ovarian and breast (Iglesia, Vincent et al. 2014) (Schmidt, Böhm et al. 2008). In pancreatic cancer, infiltration of B cells was linked to either favourable or worse prognosis depending on the spatial distribution of B cells (Castino, Cortese et al. 2016). A retrospective study on 104 PDAC patients revealed that B cells occupy two histologically distinct compartments. It was shown that B cells are either scattered throughout tumour or organised in tertiary lymphoid tissue. The retention of B cells within the tertiary lymphoid tissue correlated with increased infiltration of CD8 T cells and favourable prognosis. On the other hand, B cells scattered at the tumour-stroma interface correlated with worse prognosis. Interestingly, *in vivo* data from our lab demonstrated that expression of immunogenic programs associated with B cells were significantly upregulated in mice with improved survival that resulted from macrophage depletion with CSF1R inhibitor (Candido, Morton et al. 2018).

The role of T cells in antitumour response is widely studied and novel therapies harnessing the anti-tumoural activity of T cells have been established. In contrast, the role of B cells in the immune response to tumour development and progression is not yet established. Previously published literature suggested a pro-tumourigenic role of B cells in mouse allograft models of PDAC (Gunderson, Kaneda et al. 2016, Pylayeva-Gupta, Das et al. 2016). Based on these findings, several clinical trials were initiated with the aim to target B cells via the inhibition of Bruton tyrosine kinase (BTK) (Hong, Rasco et al. 2019, Overman, Javle et al. 2020, Tempero, Oh et al. 2021). BTK is a non-receptor enzyme member of the Tec kinase family that is a critical mediator of B cell receptor signalling in the development and functioning of adaptive immunity. In mature B cells, BTK is essential for pre-B and B cell receptor-mediated proliferation and survival (Middendorp, Dingjan et al. 2003). Clinical trials were designed to test BTK inhibitors as a single agent or in combination with anti-PD1/PDL-1 and chemotherapy. Although the treatments were well tolerated overall, low clinical activity was reported.

To investigate the discrepancies observed between the published literature and the absence of efficacy in clinical trials, I performed orthotopic allograft experiments using a B cell-deficient syngeneic mouse model. In contrast to the published literature, I observed that B

cell deficiency has no survival benefit for mice implanted with either KC or KPC primary cell lines. It is possible that the reduced tumour growth observed by Gunderson et al. could be explained by the use of cell lines of a different genetic background (*Pdx-Cre; LSL-KrasG12D* cells harbouring null mutations in *p16Ink4a* or *Trp53* (*p53 2.1*), or by the use of a different B cell-deficient mouse model ($JH^{-/-}$ mice that possess a deletion in the J segment of the Ig heavy chain locus and thus do not express IgM or IgG). However, one could argue that the platform of evidence to support clinical trials should extend across multiple models unless a mechanistic reason can be found to explain why efficacy is only achieved in certain systems. Pylayeva-Gupta et al. also used an alternative cell line model, employing KrasG12D expressing, or KPC-derived pancreatic duct epithelial cells in transplant experiments to demonstrate a tumour-promoting role for B cells. However, they also demonstrated reduced growth of KPC cell line-derived tumours in B cell deficient hosts in contrast to my data (Gunderson, Kaneda et al. 2016, Pylayeva-Gupta, Das et al. 2016). In agreement with these previous observations by Gunderson et al and Pylayeva-Gupta et al, Spear et al. also performed a study in which they observed a reduction in final tumour weight in µMT mice implanted with KPC cells. However, the observed change was minimal considering the large cohort numbers (n=~17-25)(Spear, Candido et al. 2019). At the moment it is difficult to explain these discrepancies and further work is required to better understand the differences in the systems. However, our work does seem to explain why clinical trials in this space have been unsuccessful.

Our allograft experiment also demonstrated that mice implanted with KPC primary cells had consistently shorter survival in comparison with mice implanted with KC primary cells. The survival data of these mice is in agreement with the GEMM studies as it is well-established that p53 loss of function significantly enhances tumour development. However, KC cell lines were generated from endpoint tumours that likely accumulated further tumour-promoting mutations, which does suggest that mutation of p53, specifically, drives tumourigenic behaviour. This is in line with previous findings in our lab (Morton et al., 2010). Interestingly, although there was no difference in T cell infiltration in tumours transplanted into WT versus *Ighm*$^{-/-}$ mice, I observed that tumours derived from KPC implanted cells had significantly higher T lymphocyte infiltration. What is more, we also observed a slight increase in $CD69^{+}$ T cells in KPC tumours which suggests that T cells are activated. However, there was no difference in $CD19^{+}$ B cell infiltration between KC and KPC cell line derived orthotopic allograft tumours. It could be speculated that mutant p53 elicits an adaptive immune response. However, survival data did not indicate any advantageous effect of increased infiltration of T lymphocytes. In contrast, previously reported data demonstrated

that unlike p53 wild-type tumours, p53-deficient tumours can reorchestrate the innate immune response through suppression of effector $CD4^+$ and $CD8^+$ T cells (Blagih, Zani et al. 2020). In this study p53-null pancreatic cancer cells were shown to alter the cytokine profile of suppressive myeloid cells, which in turn suppressed cytotoxic T lymphocytes and Th1 cell differentiation. Results collected from various studies imply that different tumour promoting drivers interact to regulate immune tolerance in cancer and that further investigations are required to fully understand how best to utilize these findings.

It is well understood that development of tumours in the GEM and in the orthotopic model is vastly different. GEMMs of PDAC follow a well-established model of disease progression that involves formation of PanIN lesions and their progression into invasive tumours that are marked with pronounced stromal infiltration. In contrast, orthotopic tumour models exhibit fast disease progression with an average survival of ~20 days. While orthotopic models might seem to be a more attractive way for the studies of pancreatic cancer due to the fast disease progression, allograft tumours do not fully recapitulate tumour histology observed in human cancer.

To address the differences between the models further, I have investigated B cells in GEMM of PDAC. Notably, I observed that tumours from KC mice had higher infiltration with B cells as compared with KPC tumours. However, this trend was not observed in the KC and KPC cell line derived orthotopic allograft tumours. It could be speculated that the differences observed in the GEM but not in the allograft models are due to the tumour latency, as the KC mouse takes significantly longer to develop tumours. Also, it is unclear whether p53 status could have an effect on the infiltration of B cells. Previously it was shown that mutant p53 blocks the activity of innate immune signalling and infiltration in tumour microenvironment through the downregulation of TBK1, IRF3, and STING (Ghosh, Saha et al. 2021). However, data on the effects of mutant p53 on the adaptive immune system is lacking. To investigate this further, the status of p53 in primary KC and KPC cell lines used for allograft experiments would have to be assessed.

To further examine the role of B cells in the GEMM, *Ighm*$^{-/-}$ (also known as *mu*Mt$^-$ or *μMT*$^{-/-}$*)* mice were crossed with KPC mice. Overall, no survival benefit was observed for mice deficient for B cells. The tumour immune microenvironment from these mice was investigated. No significant changes were observed in immune infiltrate in end point tumours from KPC *Ighm*$^{-/-}$ mice, although if anything, there did appear to be a trend towards increased $CD3^+$ T cells and increased neutrophils in some KPC *Ighm*$^{-/-}$ tumours. It is known that B cells play a role in immune regulation and can influence the function of other immune

components such as T cells by presenting antigens, secreting cytokines, and participating in co-stimulation. Therefore, the trend towards increased $CD3^+$ T cells and neutrophils in B cell deficient mice was not anticipated, and again in contrast to the findings of Gunderson et al and Pylayeva-Gupta et al. However, these results suggest, at least, that the absence of any anti-tumourigenic effect in KPC *Ighm*$^{-/-}$ mice is not due to T cell deficit. From the survival data of these mice we can conclude that although KPC *Ighm*$^{-/-}$ tumours have slightly increased numbers of $CD3^+$ cells this does not have a beneficial effect on survival. It is possible that $CD3^+$ T cells are not activated in these tumours. However, to further assess this, the status of T cell activation markers has to be investigated. Interestingly, monocyte numbers were also significantly increased in peripheral blood from KPC *Ighm*$^{-/-}$ mice with end point tumours, however, this did not translate to higher numbers of TAMs within the tumours. Clearly the loss of B cells does elicit systemic effects on other immune populations, however, we observed no impact on tumourigenesis in any of our models.

Genetic ablation of B cells in these models showed no effect on the development and progression of PDAC in KPC model. However, we understand that this study design has limitations and neither model type fully represents the clinical trial setting, where tumours are established in the presence of B cells before being subjected to B cell targeting therapies. To replicate the clinical approach, KPC mice with confirmed tumours were enrolled on treatment with B cell depleting anti-CD20 antibody. Tumour growth from treated and untreated mice was monitored over time, however, no changes in tumour growth rate or overall survival were observed in the anti-CD20 treated cohort as compared with vehicle controls.

Previously, several attempts were made to pharmacologically inhibit B cells using anti-CD20 antibody in allograft settings. A study led by Castino et al administered anti-mouse CD20 antibody targeting B cells 3 days after the implantation with Panc02 cells. Mice treated with depleting antibody and vehicle control were sampled at day 21. The authors found that mice treated with anti-CD20 had tumours of reduced size as compared with vehicle controls (Castino, Cortese et al. 2016). However, the results could not be replicated by another study group that used KPC primary cell line (Spear, Candido et al. 2019). In this study, the authors treated KPC mice with palpable tumours with anti-CD20 antibody and did not observe any changes in tumour weight (Spear, Candido et al. 2019). All in all, the data described here provide further evidence that the selection of study models often lead to discrepancies in the studies of tumour immune microenvironment. Together, however, my data overwhelmingly suggest that B cells do not play a significant role in pancreatic tumour progression.

6 Concluding Remarks and Future Directions

The results described in this thesis, combined with previously published data, provide clear evidence that targeting certain immune cell subtypes can elicit a broad range of effects depending on model system use, but also the stage of tumourigenesis. Despite clear evidence from the literature showing macrophage functions in immune suppression, tumour cell invasion and resistance to chemotherapy, I demonstrate that ablation of chemotactic signalling through CCR1, 2, 3 and 5 has no effect on overall KPC mouse survival. However, I demonstrate high macrophage abundance in tumours from KPC CCR1-5$^{-/-}$ mice. These data suggest that the CCR receptors, and likely monocyte chemotaxis, are not required to maintain the intra-tumoural macrophage population in pancreatic cancer. Further, I also observe no change in desmoplastic stroma by examining the presence of α-smooth muscle actin and sirius red staining. These results demonstrate the ability of resident macrophages to sustain tumour growth in the absence of infiltrating macrophages. However, macrophage lineage tracing studies were not performed, therefore we can only speculate that genetic knockout of CCR1,2,3 and 5 completely abolished the infiltration of monocytes into the tissues from the circulation. Given the time and resources, performing lineage tracing studies in KPC CCR1-5$^{-/-}$ mice would allow us to confirm the origin of macrophages present in end point tumours from these mice. At present, this could not be adequately achieved by other methods, as resident macrophages in pancreas do not present with a unique marker whose expression could be readily assessed.

Having demonstrated no appreciable changes in desmoplastic stroma in tumours, in the setting of infiltrating macrophage ablation, I set out to identify phenotypic changes in TAMs present in the end point tumours from KPC CCR1-5$^{-/-}$ mice. The flow cytometry data I present in this thesis show no significant changes in expression of various pro- and anti-inflammatory markers of macrophages. However, there is an increasing appreciation that macrophage polarization is a multifaceted process that occurs over a continuum. One pitfall of using discrete flow cytometry marker panels to define macrophage subpopulations is that this approach may oversimplify the range of phenotypes present within the tumour microenvironment. Although my results provide a foundation for further efforts, deeper analysis of the complexity of TAMs within the pancreatic tumour microenvironment, using high throughput approaches is needed. Therefore, I set out to perform mass cytometry (Cytometry by time of flight, or CyTOF) for in-depth analysis of TAMs present in untreated KPC CCR1-5$^{-/-}$ and KPC tumours, and following macrophage ablation. Single cell suspensions of these tumours were stained using a panel of 46 extracellular and intracellular

myeloid cell markers. Due to time constraints, the data from this experiment could not be collected in timely fashion for presentation in this thesis, but I hope that this approach will provide exceptionally detailed information on macrophage polarisation, patterns of activity, and education of infiltrating vs resident TAMs.

In addition to potentially differing origins, TAM subpopulations may also exhibit phenotypic changes based on their spatial organisation. As is evident from previously published studies, different CAF populations reside in different locations and exhibit different functions based on spatial distribution (Öhlund, Handly-Santana et al. 2017). However, it is unclear so far whether different TAM populations have a specific location within tumours. The flow cytometry approach employed in my study does not support spatial studies of macrophages within tumours. Thus, I propose that spatial transcriptomics could be used in the future to further enhance our understanding of different TAM populations. Spatial transcriptomics is a ground-breaking molecular profiling technique that allows highly multiplexed spatial resolution of mRNAs in individual tissue sections. It allows researchers to spatially determine gene expression profiles on tissue sections of up to 800 targets and enables localisation of features at a cellular level and in heterogenous tumour regions. Thus, it would allow examination of macrophage functional subsets in the tumour microenvironment, in contrast to flow cytometry where spatial information is lost. What is more, this approach could be further enhanced by overlaying *in situ* images of gene expression with multiplex IF for markers of specific cell types such as CAFs to study how ablation of infiltrating macrophages affects CAF subtypes. This approach could allow us to identify the signals (and their cellular sources) regulating macrophage education during tumourigenesis.

Data gathered from pharmacological inhibition of macrophages using CSF1R inhibitor from 6 weeks of age in KPC and KPC CCR1-5$^{-/-}$ mice clearly demonstrates that the tumour microenvironment is capable of rewiring its molecular and metabolomic cues to sustain tumourigenesis in the absence of macrophages. The striking observation of reduced survival in KPC and KPC CCR1-5$^{-/-}$ mice treated with CSF1R inhibitor from early time point clearly demonstrates that depletion of certain immune cell subtypes can elicit opposing effects depending on the stage of tumourigenesis. From the data presented, it could be speculated that macrophages exhibit anti-tumourigenic functions in the early stage of PanIN formation. Hence, macrophage depletion from an early stage of tumour initiation results in reduced overall survival in KPC mice. Whether the reduced survival is the result of loss of tumour-suppressive macrophage activities, rewiring of microenvironmental factors that support tumour growth, or due to the metabolic interplay between macrophages and tumour cells, remains to be understood. It is appreciated, but little understood, that microenvironmental

factors can influence tumour metabolism. We previously demonstrated that manipulating myeloid cell signalling can have significant effects on the tumour microenvironment but also that the metabolic landscape could be significantly altered (Candido, Morton et al. 2018). Investigation of the metabolic features attributable to macrophages at any given point of tumourigenesis could uncover metabolic dependencies that can be exploited to target their pro-tumorigenic/ immunosuppressive signals, rather than completely depleting cells that may retain tumour-suppressive functions. However, the spatial context is generally lost when using conventional metabolomic analyses. Using mass spectrometry imaging (MSI) would allow the visualisation of molecules in 2D and 3D. MSI techniques, such as matrix-assisted laser desorption/ionisation (MALDI), could be used to visualise metabolite profiles in TAMs and elucidate the metabolic relationships between TAMs and tumour cells.

For the future studies, I would also propose examining the crosstalk between myeloid cells and fibroblasts. Metabolite exchange between tumour cells and fibroblasts has been described, however, exchange between immune cells and fibroblasts is less well-studied, despite macrophages facilitating fibrosis (Gentric and Mechta-Grigoriou 2021). The phenotypic or metabolic changes in CAFs induced by macrophage ablation have not been studied in this thesis. However, it could be hypothesised that ablation of macrophages can also cause a metabolic rewiring in fibroblasts that further enhances the fibrotic TME. It is becoming evident that CAFs in PDAC can have differing properties, some tumour suppressive (Öhlund, Handly-Santana et al. 2017). Thus, identifying and targeting the specific metabolic links between fibroblasts and macrophages could help us in tackling inflammatory fibrosis and tumorigenesis. The data I present further support the idea that targeting specific signals that promote tumorigenesis rather than specific cell populations might be more beneficial in fighting tumourigenesis.

Further work is also needed to decipher the molecular mechanisms responsible for contrasting results in the setting of B cell ablation in pancreatic cancer. As previously discussed, the results presented in this thesis are in contrast with previously published data suggesting a pro-tumourigenic role of B cells (Gunderson, Kaneda et al. 2016, Lee, Spata et al. 2016, Pylayeva-Gupta, Das et al. 2016). My data from GEMMs of pancreatic cancer lacking B cells show no evidence of B cell involvement in tumour progression. What is more, I took a further step to investigate the role of B cells in an allograft model where either KC or KPC primary cell lines were used. In agreement with findings in the KPC *Ighm*$^{-/-}$ mice, allograft studies demonstrated no increased survival for mice lacking B cells. Having observed these inconsistencies in the studies on the role of B cells in pancreatic cancer, I

question what molecular mechanisms are responsible for these differing results. In order to further dissect the mechanism involved in the variable response to B cell ablation, future studies should focus on studying different B cell subsets. Study models used in this thesis did not allow the investigation of effects of different B cell subsets. Therefore, the question whether ablation of different B cell subsets is able to elicit different effects on tumourigenesis in KPC mice remains to be answered. The information gained from such studies could further prove that ablation of entire immune cell populations in tumours has its limitations. It is possible that similar to CAF populations, B cells also exhibit different functions based on their spatial distribution. Literature suggests that the spatial distribution of B cells may predict the prognosis of human pancreatic cancer (Castino, Cortese et al. 2016). This further indicates that the ablation of B cells both in GEMM and human cancer has been oversimplified and that additional studies are needed to fully appreciate the dynamic B cell distribution and possibly diverse function.

Finally, B cells' role in immune regulation and the effect they have on the function of other immune components such as T cells, by presenting antigens, secreting cytokines, and participating in co-stimulation, should not be ignored. Here I presented data that demonstrates a trending increase in levels of T cells present in KPC *Ighm*$^{-/-}$ tumours, thus proving that the lack of any pro-survival effect in the B cell deficient model is not due to T cell deficit. However, additional studies are required to determine T cell activation status. It would additionally be interesting to investigate whether the increase in T cells in these tumours could lead to improved response to immunotherapy.

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