

The hepatic microenvironment and immune cell recruitment in colorectal liver metastasis



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By

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Declaration

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Abbreviations

°C	Degrees Celsius
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CRC	Colorectal cancer
CTLA-4	T-lymphocyte-associated protein 4
LAG-3	Lymphocyte activation gene 3
CD-	Cluster of differentiation
CRLM	Colorectal liver metastases
CSF	Colony-stimulating factor
NIH	National Cancer Institute
APC	Antigen presenting cell
TCA	Tricarboxylic acid
ATP	Adenosine triphosphate
LPS	Lipopolysaccharides
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DAB	Diaminobenzidine
DC	Dendritic cell
cDC	Conventional dendritic cells
pDC	Plasmacytoid dendritic cells
DMSO	Dimethyl Sulfoxide
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FasL	FAS ligand
FFPE	Formalin fixed paraffin embedded
FCS	Foetal calf serum
FSC	Forward scatter
SSC	Side scatter
FSC-A	Forward scatter- area
FSC-H	Forward scatter- height
SSC-A	Side scatter- area
GF	Growth factor

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HMNC	Hepatic mononuclear cell
IFN	Interferon
KIR	killer immunoglobulin like receptor
PGE ₂	Prostaglandin E ₂
TIGIT	T-cell immunoglobulin and ITIM domain
IHC	Immunohistochemistry
IL-	Interleukin
IM	Invasive margin
iNKT-cell	Invariant natural killer T-cell
LSEC	Liver sinusoidal endothelial cell
MAIT cell	Mucosal-associated invariant T-cell
MDSC	Myeloid derived suppressor cell
M-MDSC	Monocytic myeloid derived suppressor cell
PMN-MDSC	Polymorphonuclear myeloid derived suppressor cell
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
MR1	MHC I-related molecule
MICA/B	MHC I polypeptide-related sequence A/B
HBV	Hepatitis B virus
NK-cell	Natural killer cell
NKG2D	Natural killer cell activating receptor
miRNA	Micro ribonucleic acid
NKT-cell	Natural killer T-cell
Pb	Peripheral blood
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PD-1	Programmed Death protein 1
PD-L1	Programmed Death-Ligand 1
PlGF	Placenta growth factor
ROS	Reactive oxygen species
IDO	Indoleamine 2,3-dioxygenase

SVUH	St. Vincent's University Hospital
TCR	T-Cell receptor
TME	Tumour microenvironment
TGF- β	Transforming growth factor beta
Th-cell	T helper cell
TLR	Toll like receptor
TNF-	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAIL	(TNF)-related apoptosis inducing ligand
Treg cell	Regulatory T-cell
VEGF	Vasculature endothelial factor
VEGFR	Vasculature endothelial factor receptor
$\gamma\delta$ T-cell	Gamma delta T-cell
LCM	Liver conditioned media
CM	Conditioned media
IM	Invasive margin
TIL	Tumour-infiltrated lymphocyte
TBSI	Trinity Biomedical Science Institute
FMO	Fluorescence minus one
SOP	Standard operating procedure
IMS	Industrial methylated spirits
IPP	Isopentenyl pyrophosphate
NO	Nitric oxide
H&E	Haematoxylin and eosin
SITC	Society for immunology of cancer
HBSS	Hanks balanced salt solution
RIPA	Radioimmunoprecipitation
PMSF	Phenylmethysulfonyl fluoride
HRP	Horseradish peroxide
SEM	Standard error mean
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
MIF	Migration inhibitory factor

Abstract

The liver is uniquely enriched with anti-tumour immune cells. However, liver metastasis is anticipated in over half of colorectal cancer patients. The value of the type and location of immune cells to prognosis has been well established in colorectal cancer, but is not clear in colorectal-liver metastasis (CRLM). We hypothesized that the immune repertoire is altered in CRLM, and that effector T-cells correlate to prognosis. To investigate this, paraffin embedded tumour and tumour adjacent tissues from patients (n=62) who underwent resection for CRLM, and donor (n=14) liver tissues collected during liver transplantation were recalled. CD45 (pan leukocyte marker), CD3 (T-cells), and CD8 (cytotoxic T-cells) were localised by immunohistochemistry and quantified. In donor liver tissue, leukocytes and T-cells were found to accumulate around portal triads compared to the parenchyma. In CRLM tissue, leukocytes and T-cells accumulated at the invasive margin separating tumour from morphologically normal adjacent tissue. These cells appeared to penetrate the tumour tissue through several defined penetration points at the invasive margin, an observation not previously described. Average absolute cell counts for all three markers were found to be significantly altered in CRLM tissue compared to donor liver (CD45 $P < 0.0001$, CD3 $P < 0.0001$, CD8 $P < 0.0001$) with significant interpatient variation. High vs low immune recruiters were defined by the median cell counts within tumours, and were found to significantly correlate with overall survival (CD3 $P = 0.04$, CD8 $P = 0.006$).

The numbers of tumour infiltrating immune cells affects survival, and cellular trafficking is dictated by chemokines which might provide novel immunotherapeutic targets. We hypothesized that dysregulation in chemokines compromises immune infiltration and may explain low survival in the group of CRLM patients with low T-cell infiltration. Fresh liver tissue was obtained from CRLM resections (n=15), and during liver transplantations (n=14) for flow cytometry, and protein analysis. Levels of CCL8, the monocyte chemoattractant, were significantly higher in the

tumour (155.4 ± 31.4 pg/mg of protein) compared to donor liver (39.6 ± 9.3 pg/mg of protein; $p=0.003$), with significant interpatient variation. Levels of CXCL9, the T-cell chemoattractant, were significantly higher in the tumour (282.3 ± 22.5 pg/mg of protein) compared to donor liver (172.9 ± 21 pg/mg of protein; $p=0.003$), with significant interpatient variation. Blocking CXCL9 significantly reduced chemotaxis of leukocytes ($P=0.04$) and CD8 cells ($P=0.04$) in response to tumour conditioned media.

In summary, we demonstrated a prognostic value for immune infiltration in CRLM patients. We highlighted the significance of CXCL9 in T-cell migration. Cancer therapy has been revolutionized by the possibilities of manipulating the immune system; CXCL9 may present a novel immunotherapeutic target for colorectal liver metastases.

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CHAPTER ONE:
GENERAL INTRODUCTION

1. Introduction

The immune system is capable of detecting abnormal cells, and is therefore essential in the defence against cancer growth. "Cancer is a dumb disease" (Sidow and Spies, 2015). Cancers or tumours are highly unorganised collection of cells which grow uncontrollably with high rates of random mutation. In contrast, the immune system is very smart. Immune populations have highly organised and sophisticated anti-tumour response and activities which are tightly regulated. Despite the efficiency of the immune system, random uncontrolled cancer growth is faster in some individuals. The accumulation of the right mutations at the right time then leads to the tumour successful establishment and successful immune evasion.

Cancer therapy has been revolutionised by the ability to manipulate the immune response. In 2018, Tasuku Honjo and James P. Allison received the Nobel Prize in Physiology or Medicine for their discovery of cancer therapy by inhibition of negative immune regulation, otherwise known as immunotherapy to checkpoint inhibitors. The understanding of different cellular and non-cellular elements of the tumour environment, and the role each component plays towards tumour suppression or progression is revealing exciting novel therapeutic targets. Immunotherapies have been approved for the treatment of many cancer types; designed to manipulate patient's own immune system and improve anti-tumour immune responses. As successful as these therapies have proven to be, responses to immunotherapies are still low (Havel et al., 2019). Immune infiltration in the tumour microenvironment has been proposed to be essential for the efficacy of immunotherapies, however very little has been done to investigate this hypothesis (Bonaventura et al., 2019, Havel et al., 2019). Here we explore different elements of the tumour environment in colorectal liver metastasis aiming to reveal new therapeutic targets.

1.1. The Tumour Environment

The tumour environment is made up of transformed cells, normal cells, immune cells, blood flow, oxygen, and nutrients. The interactions between these different elements affects secreted cytokine levels, immune cell function, and therefor tumour progression.

1.1.1. Tumour Blood Flow

Tumours need blood flow to supply oxygen and nutrients (De Palma et al., 2017). the process of angiogenesis is the formation of new blood vessels; triggered in the tumour environment by different signals including the metabolic stress of hypoxia and low pH, and the mechanical stress of pressure building as tumour cells proliferate (Carmeliet and Jain, 2000). Tumour angiogenesis is affected by an array of cytokines like vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF), C-X-C ligand 8 (CXCL8), CXCL12, and colony-stimulating factor (CSF) among many others. VEGF is a cytokine produced in response to angiogenic signals, and is a key cytokine regulator in the process of angiogenesis. These cytokines are secreted by all the cellular components of the tumour environment including tumour cells, and immune cells. As angiogenesis is an important determinant of tumour progression, it is also a possible target in cancer therapy.

1.1.2. Tumour Metabolism

The National Cancer Institute (NIH) defines cell metabolism as the sum of all chemical changes that take place in a cell through which energy and basic components are produced for essential processes. Metabolic pathways are regulated through positive and negative feedback loops as well as components of the microenvironment. Under normal conditions, oxygen levels and nutrient supply supports tricarboxylic acid (TCA) cycle and oxidative phosphorylation to produce energy in the form of adenosine triphosphate (ATP) (O'Neill et al., 2016). ATP is an energy molecule made

up of a nitrogen base, a sugar, and three phosphate groups (Jeffrey and Norman, 2013).

The Warburg effect has been used to describe the major shift in metabolic activity associated with tumour cell growth; defined as the tumour cells' preference to consume more glucose through less efficient ATP producing pathways with an associated increase in lactate production irrespective of oxygen availability (Potter et al., 2016, Warburg et al., 1927). Cancer cells were shown to favour aerobic glycolysis for the speed of energy turnover (Cairns et al., 2011). However, the production of lactate has since been linked to serious effects on immune cell populations; studies have shown that lowering the pH in the tumour microenvironment establishes cytotoxic T-cells anergy leading to reduced cytokine secretion and T-cell receptor (TCR) expression (Bellone et al., 2013). Previous work by our lab indicates that lactate leads to reduced NK-cell intracellular pH sequentially resulting in mitochondrial dysfunction and apoptosis (Harmon et al., 2019b).

1.1.3. Non-immune Cells of the Tumour Environment

The tumour microenvironment includes cells other than the malignant cells, and the immune cells. Those cells include and epithelial cells, fibroblasts, pericytes, and in some cases adipocytes (Balkwill et al., 2012). Fibroblasts are the most abundant cell type in the stroma. They are essential for production of extracellular matrix proteins, wound healing, and support of the tissue architecture (Alkasalias et al., 2018). The altered composition of the tumour environment recruits fibroblasts by activating them into myofibroblasts; sequentially leading to tissue fibrosis and promoting tumour progression (LeBleu and Kalluri, 2018, Balkwill et al., 2012). Pericytes provide structural support to blood vessels, and low vasculature pericyte composition has been linked to higher metastasis and low prognosis in some cancer (Balkwill et al., 2012). Adipose tissue in the tumour environment is mostly linked to increased tumour progression with a lot of research lately focusing on the paralyzing effect of adipose tissue on immune effector cell function (Michelet et al., 2018).

1.1.4. Immune Cells of the Tumour environment

Immune cells in the tumour environment are a mix of tissue-resident and recruited cells (Fan and Alexander, 2016). Recruited immune cells are circulating immune cells; they respond to a chemokine gradient that triggers their migration into the tumour bearing tissue (Agace, 2008). It is also known that tissues and organs have resident immune cell populations which are phenotypically and functionally unique (Norris et al., 1998, Gasteiger et al., 2015, Davies and Taylor, 2015). These immune cells do not circulate through blood but instead take-up residence within the tissue, and are mainly tailored to maintain local homeostasis (Chou and Li, 2018). therefore, the resident repertoire is highly specialised and dependent on each tissue and its requirements (Turner et al., 2018, Masopust and Soerens, 2019).

During tumour development, the mutations characterising the dysplastic and anaplastic stages of tumour development induce the expression of tumour-specific and tumour-associated antigens in the form of novel peptides from viruses, aberrant mRNA splicing, protein translational alterations, reactivation of normally silent genes, and overexpression of genes (Murphy and Weaver, 2016). These antigens, as well as several more tumour-associated lipids, and stress markers are recognised through an array of immune populations from the myeloid and lymphoid lineage. The anti-tumour immune response is very powerful, and so successful tumour progression is dependent on its ability to evade or inhibit the immune response, recruit and reprogramme suppressor immune populations, and prevent recruitment of effector immune populations.

1.1.4.1. Macrophages: anti-tumour mechanisms vs pro-tumour capacity

Macrophages have important roles in both the innate and adaptive immune responses. Macrophages reside in almost all tissues, where they arise from progenitors during embryonic development. Alternatively, bone marrow secreted immature monocytes migrate into tissues where they differentiate

into macrophages. They are potent anti-microbial phagocytes, important in inducing inflammation, and for the secretion of cytokines (Murphy and Weaver, 2016). On one hand, macrophages were shown to kill tumour cells in-vitro following stimulation with lipopolysaccharides (LPS) and interferon gamma (IFN- γ). Upon activation, macrophages kill tumour cells by secreting lytic factors into neoplastic cells. On the other hand, macrophages were also shown to aid tumour progression in response to interleukin (IL)-10 and IL-4. Tumour associated macrophages can directly inhibit cytotoxic T-cells by expressing inhibitory ligands such as programmed cell death 1 (PD-L1), cytokines like IL-10 and TGF- β , as well as ROS. Indirectly, macrophages can promote tumour progression by recruiting suppressive immune populations like regulatory T-cells (Treg cells), and by inhibiting dendritic cells (DCs) from activating other anti-tumour populations (Denardo and Ruffell, 2019).

1.1.4.2. Dendritic cells: normal function, and tumour associated influences

DCs are essential antigen presenting cells. They arise in the bone marrow and migrate to tissue through circulation. In circulation, there are two main types of DCs, conventional DCs (cDC) and plasmacytoid DCs (pDC). DCs continuously sample extracellular fluid including soluble molecules, nutrients, and antigens in a process known as micropinocytosis (Lim and Gleeson, 2011). cDCs process and present ingested microbes to activate T-cells, bridging the innate and adaptive immune responses. Similarly, DCs can take up tumour associated antigens and present them to T-cells to initiate an immune response. pDCs on the other hand are innate immune cells that produce cytokines which regulate immune responses (Murphy and Weaver, 2016).

Similar to macrophages, DCs can be recruited by tumour cells to support immune suppression. Cytokines like IL-6, IL-10 and VEGF in the tumour microenvironment can suppress DCs. The tumour microenvironment can also lead to stress in the endoplasmic reticulum leading to lipid accumulation in DCs, and their suppression. The tumour microenvironment can also cause DCs to induce formation of suppressive immune populations (Steinman and Banchereau, 2007). As well as arrest T-cell proliferation by

driving Indoleamine 2,3-dioxygenase (IDO) expression (Schupp et al., 2019). IDO is an enzyme that catabolizes the essential amino acid tryptophan, and was found to correlate with reduced T-cell responses (Mellor and Munn, 2004).

1.1.4.3. Neutrophils and the MDSC controversy

Neutrophils are short-lived cells that mature in the bone marrow. They are powerful phagocytes with degradative enzymes and antimicrobial substances stored in their cytoplasmic granules, which aid in destroying microorganisms. In the tumour environment, neutrophils can be both pro and anti-tumour (Powell and Huttenlocher, 2016). Anti-tumour neutrophils limit tumour growth by direct cytotoxicity, and antibody dependent cytotoxicity. They can also drive activation of other immune cells including T-cells and DCs. However, neutrophils can also be recruited by the tumour environment. Tumour-associated neutrophils can promote tumour growth by secreting cytokines like epidermal growth factor (EGF) and VEGF, which we will cover more later. They are also involved in tumour metastasis, and limit effector T-cell function (Coffelt et al., 2016).

Myeloid-derived suppressor cells (MDSCs) are a myeloid population with highly suppressive activity. High tumour infiltration of MDSCs mostly correlates to bad prognosis across many tumour types. There are two types of MDSCs, monocytic MDSC (M-MDSC) and polymorphonuclear MDSC (PMN-MDSC). They are both identified by a collection of markers none of which is exclusive to MDSCs. Both types of MDSCs are cluster of differentiation (CD)33⁺, CD11b⁺, Human leukocyte antigen (HLA)-DR⁻ Lin⁻. However, M-MDSC is also CD14⁺, while PMN-MDSC is CD15⁺ (Ostrand-Rosenberg and Fenselau, 2018). Although an extensive amount of work has been done of MDSCs, some immunologists still believe that PMN-MDSCs are tumour associated neutrophils because are also identified as CD11b⁺CD15⁺ cells with a multi-lobed nuclear morphology (Moses and Brandau, 2016).

1.1.4.4. Activation of classical T-cells against tumour cells

CD8 T-cells can directly recognise and kill tumour cells. Their activation physiologically requires two signals; the first being the presentation of peptides through MHC, and the second being the binding of CD28 on the T-cell membrane to the B7 (CD80) protein which is expressed by tumour cells or antigen presenting cells. upon activation, CD8 T-cells can rapidly secrete high levels of TNF- α and IFN- γ , perforin, granzymes, and Fas ligand (FasL) (Hadrup et al., 2013). Cytotoxic T-cells adopt a memory phenotype that accelerates their response upon subsequent antigen exposure; the chronic antigen presentation in the TME upregulates receptors including PD-1, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), and T-cell immunoglobulin and ITIM domain (TIGIT) which then lead to cytotoxic T-cell exhaustion and inhibition (Reiser and Banerjee, 2016).

CD4 T-cells on the other hand are either directly activated by MHC II expressing tumour cells or indirectly through MHC II presentation of tumour associated lipids on the surface of antigen presenting cells (APCs). CD4 T-cells in return are able to induce direct killing of MHC II expressing tumour cells or indirectly eliminate tumour cells by activating macrophages and NK-cells (Haabeth et al., 2014). CD4 T-cells also contribute to cytotoxic T-cell activation. Expression of the CD40 ligand on activated CD4 T-cells binds CD40 on DCs, thereby increasing their antigen-presentation ability (Borst et al., 2018).

1.1.4.5. The anti-tumour activity of NK-cells

Natural killer (NK)-cells are subcategorised as innate lymphocytes with important roles in the anti-viral immune responses, homeostasis, and anti-tumour surveillance (Bassani et al., 2019). NK-cells can be further categorised by their surface expression of CD56 and CD16 into the cytotoxic CD56^{dim}CD16⁺ cells characterised by high secretion of perforin and granzyme, and the predominantly cytokine producing CD56^{bright}CD16⁻ cells which produce high amounts of IFN- γ , TNF- β , and IL-10 (Cooper et al., 2001). NK-cells are widely known for their direct cytotoxicity against virally infected cells. However, they also have potent anti-tumour capacity. They

are activated in response to the presence of an activation signal with the simultaneous absence of an inhibitory signal (See Fig.1.1). Normal cells express MHC I that acts as an NK-cell inhibitory ligand leading to the induction of tolerance upon interaction with NK-inhibitory receptors such as the killer immunoglobulin like receptor (KIRs). Upon activation, NK-cells exert direct cytotoxicity mediated via perforin and granzymes, FasL, TNF-related apoptosis-inducing ligand (TRAIL), and IFN- γ (Murphy and Weaver, 2016, Parham, 2014).

As NK-cells are a powerful neutraliser of transformed and abnormal cells, the tumour microenvironment is filled with components derived by the tumour to evade NK-cells' anti-tumour responses. Transforming growth factor beta (TGF- β) and prostaglandin E₂ (PGE₂) downregulate natural killer cell activating receptor (NKG2D) expression, a key activation receptor of NK-cells against tumour cells (Hayakawa and Smyth, 2006). Increased adenosine levels in the TME reduces cytokine secretion by NK-cells. Also, secretion of vesicles containing microRNAs (miRNAs) by tumour cells into the tumour microenvironment (TME) have been linked to downregulation of NKG2D, perforin, granzyme, and other pathways impairing NK-cell function (Bassani et al., 2019).

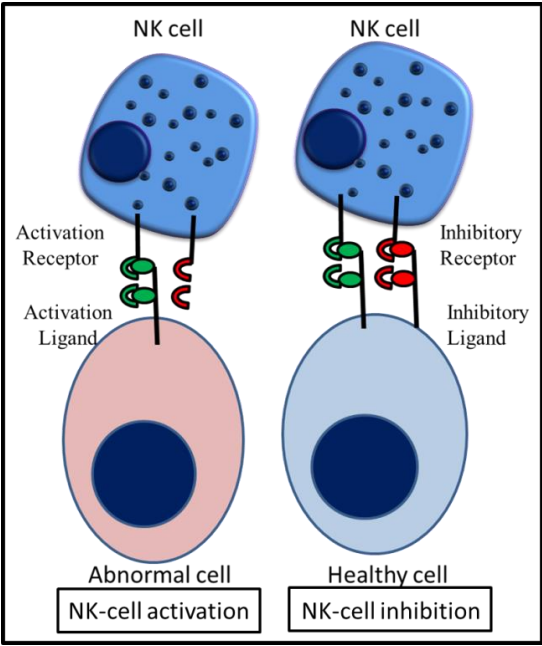


Figure 1.1: Mechanism of NK-cell tolerance and activation.

NK-cells express activation receptors that recognise novel antigens, and inhibitory receptors that recognise normal major histocompatibility complex (MHC) I expression.

1.1.4.6. iNKT-cells anti-tumour capacity

Invariant NKT (iNKT) cells are CD1d restricted T-cells that express NK markers including CD56 and CD161 (Krovi and Gapin, 2018). CD1d is a transmembrane antigen presentation molecule formed of heterodimers with beta-2-globulin and a hydrophobic antigen groove (Brutkiewicz, 2006, Paduraru et al., 2006). iNKT-cells recognise endogenous and exogenous lipids presented through CD1d, and their anti-tumour activity is through recognition of tumour-associated glycolipids presented on CD1d. iNKT-cells can also be indirectly activated through several CD1d upregulation pathways including IL-12 expression of DCs, and by the effect of IFN- β on DCs and macrophages. Activation of iNKT-cells promotes the activation of NK and CD8 T-cells through their secretion of IFN- γ , IL-2, and IL-4, and their upregulation of T-cell costimulatory molecules (Woo et al., 2015).

1.1.4.7. $\gamma\delta$ T-cell recognition of tumour cells

Gamma delta T-cells ($\gamma\delta$ T-cells) are distinguished from classical T-cells by the structure of their TCR. Classical T-cells express TCRs made up of α and β protein chains. Alternatively, $\gamma\delta$ T-cells express TCRs made up of γ and δ protein chains (Bluestone et al., 1995). There are three $\gamma\delta$ T subsets in humans categorised by their V δ chains into V δ 1, V δ 2, V δ 3 subsets. They are not restricted by MHC-antigen presentation and do not require co-stimulation (Lo Presti et al., 2018). $\gamma\delta$ T-cell recognise tumour-associated phosphoantigens, mitochondrial F1-ATPase, apolipoprotein A-1 expression on tumour cells, as well as stress signals including MHC I polypeptide-related sequence A (MICA), MICB, and NKG2D (Woo et al., 2015). $\gamma\delta$ T-cell knockout mice were found more susceptible to tumour development suggesting they are essential for part of the anti-tumour response (Girardi et al., 2001). Upon activation, $\gamma\delta$ T-cells can induce direct cytotoxicity through secretion of perforin and granzymes, IFN- γ , TNF- α , FasL, and

TRAIL, and also mediate indirect anti-tumour effects through activating DCs, which in return activate classical T-cells (Paul et al., 2014).

1.1.4.8. MAIT cells in tumour immunity

Mucosal associated invariant T (MAIT) cells are MHC I-related molecule (MR1) restricted population that expresses a semi-invariant TCR, Va7.2-Ja33 (Sundstrom et al., 2015). MAIT cells are identified by the combination of Va7.2 expression and CD161 expression; they are abundant in the intestine and liver, and are mostly known for their recognition of conserved microbial antigens (Gapin, 2014). Upon activation, MAIT cells produce IFN- γ , TNF- α , and IL-17A suggesting a role for them in the tumour microenvironment. Numbers of MAIT cells in different cancer types presents controversial correlations to outcome (Zabijak et al., 2015, Ling et al., 2016).

1.1.5. Tumour cytokine microenvironment

Cytokines include different groups of proteins that mediate communication between cells to enable migration, activation, and inhibition (Landskron et al., 2014). Divided into subfamilies depending on their principle known function, they include growth factors, interleukins, interferons, and chemokines. Hijacking immune mechanisms and evading the immune response requires tumour cells to modulate the microenvironment to support tumour promoting pathways.

1.1.5.1. Growth factors

Growth factors (GF) are small polypeptides that bind transmembrane receptors to stimulate growth specific intracellular signalling pathways (Witsch et al., 2010a). Growth factors are integral in the tumour microenvironment and through several phases of cancer development including clonal expansion, invasion, and angiogenesis (Witsch et al., 2010b).

Vascular endothelial growth factors (VEGF)

The family of VEGF is made up of five ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D Placenta growth factor (PlGF)) and three receptors, with VEGF receptor (VEGFR)-2 being the main transmitter of VEGF signalling. VEGF has been reported to be variably upregulated in cancer and linked to *RAS*, *Wnt*, and *von Hippel-Lindau* mutations (Ferrara et al., 2004). Several VEGF neutralisation drugs including bevacizumab, aflibercept, and ramucirumab are used to treat a number of cancers (Yang et al., 2016); studies showed that interrupting uptake of these drugs yields unfavourable outcomes due to increased tumour revascularisation (Zuniga et al., 2010) and increased chances of metastasis (Yang et al., 2016).

Epidermal growth factor (EGF)

The family of EGF receptors includes four members; EGF receptor HER1, HER2, HER3, and HER4. These receptors are overexpressed in many cancer types and are accompanied by aggressive tumour behaviour (Mendelsohn and Baselga, 2000). Trastuzumab, is a therapy targeting HER2 and has been approved for breast cancer and metastatic gastric cancer therapy (Mohan et al., 2018).

Transforming growth factor β (TGF- β)

TGF- β is involved in many different pathways affecting proliferation, differentiation, regeneration, and several more depending on the cell type and surrounding microenvironment (Dahmani and Delisle, 2018). The role of TGF- β in cancer is difficult to define because it has several contradictory effects. On one hand, studies showed that increased combined TGF- β and activin (a member of TGF- β family) was associated with shorter survival (Staudacher et al., 2017). On the other hand, TGF- β acts as an important anti-tumour agent through triggering apoptosis in cells with oncogenic mutations (Massagué, 2012). Targeting TGF- β in cancer therapy has been a challenge due to its dual contribution towards tumour progression and tumour suppression. Although TGF- β inhibitors have not yet been approved

in cancer therapy, several inhibitors have been ruled safe and are enrolled in clinical trials across many malignancies (Löffek, 2018).

1.1.5.2. Interleukins

Interleukins are factors secreted by immune and non-immune cells, and have the ability to control inflammatory responses. They are largely conserved between mice and humans, and include 17 described families with different structures and functions (Yao et al., 2016).

Interleukin-6 (IL-6) family

The IL-6 family are glycoproteins with four alpha helices in their secondary structure; they are involved in many functions including B and T-cells growth and differentiation (Jones and Vignali, 2011). Increased IL-6 expression has been shown in several types of malignancies at both protein and RNA level (Naugler et al., 2007, Karczewska et al., 2000, Rutkowski et al., 2003). Main contributors of IL-6 in the tumour microenvironment include tumour cells, tumour associated macrophages, and CD4⁺T-cells; although IL-6 has known anti-tumour functions by promoting cytotoxic T-cell trafficking and T-cell proliferation and survival, it also can promote tumour cell proliferation and survival, and promote metastasis (Fisher et al., 2014).

IL-10 family

The IL-10 family includes nine members with known integral roles in preserving tissue epithelial layers (Ouyang et al., 2011). IL-10 is produced by the majority of immune populations including T-cells, monocytes, and NK-cells (Ouyang and O'Garra, 2019). It suppresses immune responses through downregulation of MHC II expression, and inhibiting production of IL-1 β , and TNF- α (Sabat, 2010). Increased levels of IL-10 have been reported in melanoma and lymphoma patients with a correlation to poor prognosis (Sato et al., 2011). Despite the known immunosuppressive

nature of IL-10, no therapies to date have been approved in cancer (Ouyang and O'Garra, 2019).

1.1.5.3. Interferons

Interferons are immunomodulatory cytokines with major implications in anti-viral responses (Randall and Goodbourn, 2008). The family is subdivided into three subgroups; type I, type II, and type III (Donnelly and Kotenko, 2010). Type I IFNs are produced by the majority of cells in response to pattern recognition receptors or TLRs (Colonna, 2007). Besides their antiviral capacity, type I IFNs are involved in proliferation, differentiation, and trafficking of immune populations. They can directly inhibit IL-4 production and induce the production of IL-27, subsequently affecting differentiation of T-helper (Th)1-cells (producers of IL-2, TNF- β , and IFN- γ), and Th17-cells (producers of IL-17) respectively. Type I IFNs can induce immune suppression through production of IL-10, and promote T-cell inhibitory ligands, as well as downregulate IFN- γ receptor expression. Type I IFNs can also promote antigen presentation by DCs, and regulate CD8 T cell production of IFN- γ , perforin, and granzymes (McNab et al., 2015). Their involvement in immune trafficking is mediated through their induction of chemokines including CXCL10 and CXCL11, and their involvement in tumour immune surveillance is through inhibition of tumour cell proliferation, down-regulation of oncogenes, and up-regulation of MHC I expression (Ludigs et al., 2012, Stetson and Medzhitov, 2006). Tumour progression is promoted by downregulation of type I and II IFN, or the downregulation of their receptors as they can reduce tumour cell proliferation and drive anti-tumour immune responses (Di Franco et al., 2017). IFNs represent another target for cancer therapy. Although the use of IFN- γ is still limited and is largely still confined to clinical trials with good prospects, IFN- α has been approved for clinical use against several different malignancies including melanoma, leukaemia and lymphoma (Asmana Ningrum, 2014, Ni and Lu, 2018).

1.1.5.4. Chemokines

Chemokines are a large family of small proteins including 50 members subgrouped structurally into four subfamilies including CXC, CC, CX3C, and C, where "X" represents the number of conserved amino-acids (Balkwill, 2004). These peptides were first described for their ability to induce cell motility but have been involved in many more functions since (Gerard and Rollins, 2001, Mollica Poeta et al., 2019). The role of chemokines in promoting tumour immunity is through recruitment of immune populations to the site of tumour. Secreted chemokines and the expression of their corresponding receptors determine the density and type of recruited cells (Balkwill, 2004).

Different immune cells respond to different chemokines depending on their expression of the different chemokine receptors. CD8 T-cells and NK-cells express CXC-chemokine receptor 3 (CXCR3) which binds to CXC-chemokine ligand (CXCL) 9 and CXCL10. Treg cells express CCR4, the receptor for CCL22, and CCR10, the receptor for CCL28. CCL22 is secreted mainly by macrophages and tumour cells, while CCL28 is found in hypoxic regions. This suggests an anti-tumour role for CXCR3, and in contrast suggests a role for CCR4 ligands in tumour promotion. The majority of NKT-cells express CCR2, CCR5, and respond to CCL2, however NKT-cell trafficking in the tumour microenvironment is poorly understood. MDSCs also respond to CCL2 recruitment into the tumour microenvironment, and CXCL8 which affects its trafficking and degranulation (Nagarsheth et al., 2017). In many cases, tumour cells are able to suppress chemokine expression by deoxyribonucleic acid (DNA) methylation to downregulate T-cell recruitment and facilitate immune evasion. On the other hand, chemokine expression supports tumour cell growth and invasion. Chemokines like CXCL17, CXCL12, and CXCL8 increase angiogenesis and promote tumour growth. CCR9 interactions with its ligands were shown to increase resistance to chemotherapy. CCL2, CCL3, CCL5, and CCL18 promote tumour invasion and metastases (Johnson et al., 2010). Targeting chemokines in cancer therapy is still in infancy with few used in clinical settings. Examples include CXCR4 antagonist, and CCR4 monoclonal antibody (Mollica Poeta et al., 2019).

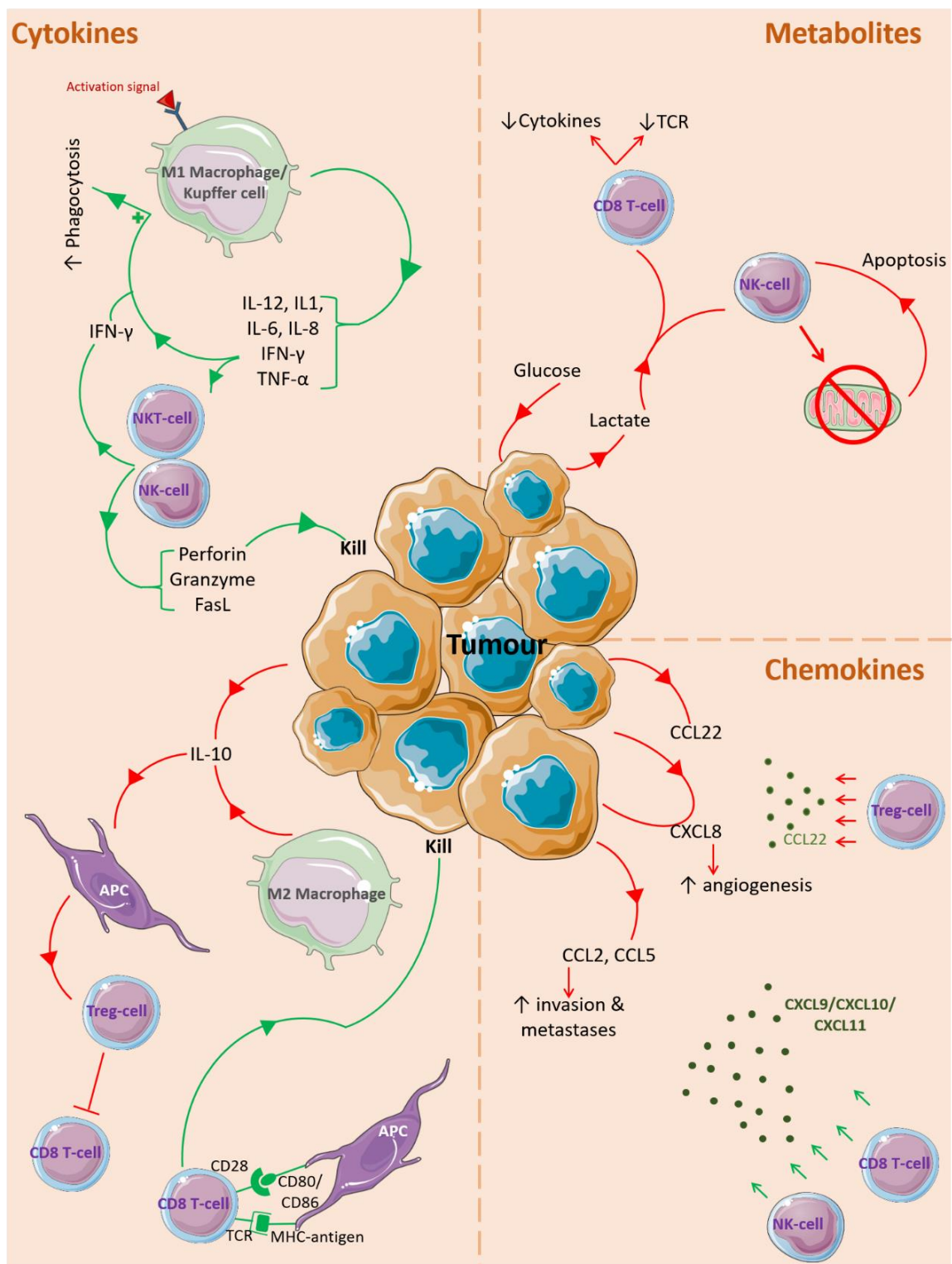


Figure 1.2: The tumour microenvironment.

Representation of some of the interactions between tumour cells and their microenvironment. Red arrows represent unfavourable (tumour promoting) pathways, and green arrows represent favourable (tumour suppressing) pathways.

1.2. Immune evasion

Immune evasion is the term given to the processes by which transformed cells avoid detection and elimination by the immune system. One central role of the immune system is to recognise transformed cells and eliminate them before they are established as a tumour (Swann and Smyth, 2007). Different members of the immune system can recognise and eliminate tumour cells through different activation pathways that include both the expression of abnormal or non-self-antigens, the absence of self-antigens, and the upregulation of stress signals (Dunn et al., 2002, Kather and Halama, 2019). Transformed cells can evade the immune system through several mechanisms including hiding from immune cells, and inactivating immune cells (Rabinovich et al., 2007).

1.2.1. Escaping T-cell recognition

As mutations build up in transformed cells, certain mutations can lead to reduced MHC expression. This can either be due to disrupted normal trafficking of MHC molecules to the cell surface, or through secreting autocrine proteases that cleave MHC molecules from the cell surface (See Fig.2) (Murphy and Weaver, 2016). As a result, transformed cells escape T-cell recognition and activation since MHC antigen presentation is a main activation stimulus (Muenst et al., 2016).

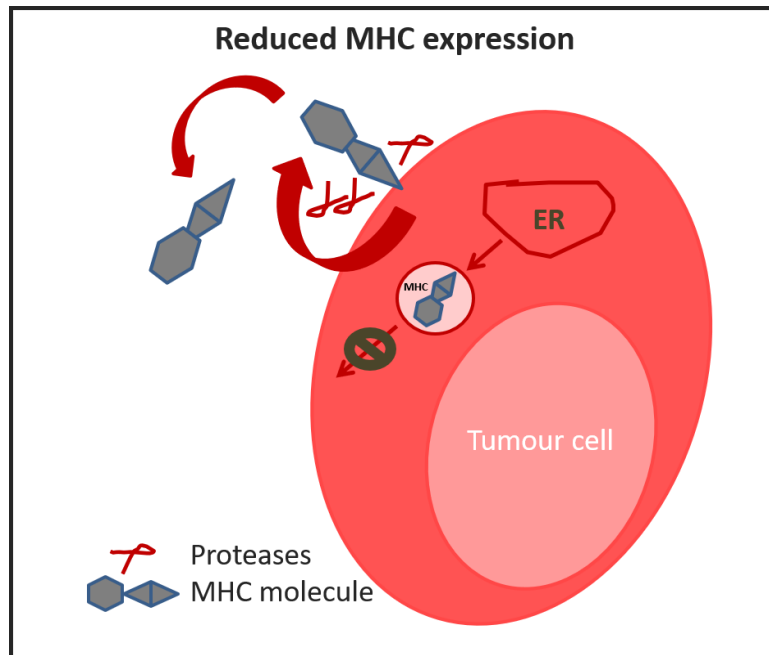


Figure 1.3: downregulation of MHC expression by tumour cells.

Tumour cells can escape T-cell recognition by downregulating MHC expression by interrupting normal MHC transfer to the cell surface, and/or by producing proteases that cleave off MHC molecules from the cell surface.

1.2.2. Escaping non-MHC facilitated immune recognition

Transformed cells can also alter the production of proteins that block activation receptors, or over activate immune cells into physiological exhaustion. This includes the upregulation of soluble NKG2D ligands, IL-10 and TGF- β , and also the downregulation of TRAIL, and FAS, which has been shown to negatively affect the activity of $\gamma\delta$ T-cells, NKT-cells, NK-cells, and T-cells (Groh et al., 2002).

1.2.3. Tumour exploitation of immune checkpoints

Both lymphoid and myeloid immune populations express checkpoint inhibitors; a group of membrane-bound molecules which primarily function to prevent continuous activation of effector populations to prevent tissue damage and autoimmunity (Greten and Sangro, 2018). CTLA-4 is a co-

inhibitory receptor expressed on the T-cell membrane to induce its suppression when binding to B7. PD-1 is a co-stimulatory protein which in return activates regulatory T-cells and exerts suppression of other immune populations (Lam et al., 2017). LAG-3 is a membrane-bound receptor, homologous to CD4 structure, and was found to downregulate T-cell activation and IFN- γ , TNF- α , IL-2, and IL-4 production through its interactions with MHC II. Tumour cells are able to use these checkpoints to inhibit the immune response through their production of PD-L1, B7 and MHC II (See Fig.1.3) (Ostrand-Rosenberg et al., 2014, Goldberg and Drake, 2011).

1.3. Anti-cancer immunotherapy

Once tumour cells reach the stage of detectable disease, a major determinant of prognosis is the immune response (Gonzalez et al., 2018). The main aim of immunotherapies is to boost the immune response through blocking immune suppression, or providing T-cell co-stimulation to fight against cancer. they include monoclonal antibodies with specific targets, non-specific immunotherapies including interferons and interleukins, viral vaccines and oncolytic virus therapy (Lesterhuis et al., 2011). Therefore, massive resources have been invested in the development of immunotherapies, and many have been approved for clinical use.

1.3.1. Checkpoint inhibitors targeted immunotherapies

Immune checkpoints are fundamental regulatory molecules that maintain immune cell activity within an acceptable range. They protect the host from autoimmunity (Wei et al., 2018). A big group of immunotherapies is based on the understanding and recruitment of immune checkpoint inhibitors. Ipilimumab is a human anti-CTLA-4 monoclonal antibody that enhances T-cell activation by blocking CTLA-4 from binding to B7 and promoting CD28 co-stimulation of T-cell (Sansom, 2000, Hodi et al., 2010). Nivolumab and pembrolizumab are both human anti-PD-1 monoclonal antibodies that block PD-L1 from inducing CD8 T-cell apoptosis, CD4 T-cell differentiation into

regulatory T (Treg)-cells, and NK-cell impairment (Fessas et al., 2017, Parham, 2014). Anti-LAG-3 antibodies have reached human clinical trials with promising results but have not yet been approved (See Fig.1.4) (He et al., 2016, Andrews et al., 2017).

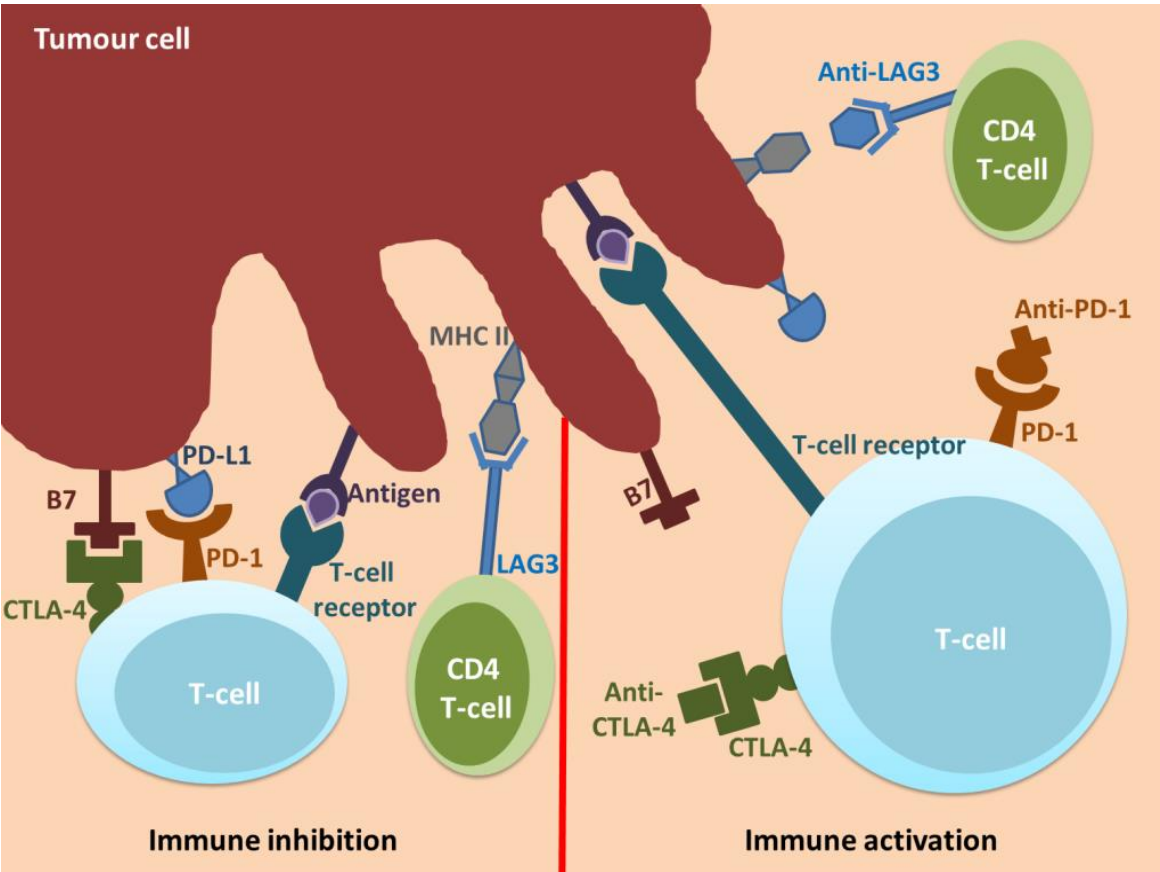


Figure 1.4: Immune checkpoint-inhibitors in the tumour microenvironment.

Immunotherapy counteracts immune suppression through blocking inhibitory molecules to maximise immune activation.

1.3.1.1. Cytokine immunotherapies

Immunostimulatory cytokines including IL-2 and IFN- α are non-specific immunotherapies that boost the immune response (Galluzzi et al., 2014). IL-2 was the first successful immunotherapy used in the clinical setting as a driver of T-cell expansion and was approved for the treatment of renal cell carcinoma in 1994 and metastatic melanoma in 1998 (Sim and Radvanyi,

2014). IL-2 can stimulate CD8 T-cell growth and differentiation into memory cells, as well as maintain regulatory T-cell functionality and controls the differentiation of CD4 T-cells into different subsets including Th1, and Th2 (Rosenberg, 2014). The main challenge in utilising IL-2 as an immunotherapy is its higher affinity binding to Tregs enabling them to consume IL-2 more than other immune cells (Jiang et al., 2016).

IFN- α is a type I interferon. As mentioned earlier type I IFNs are involved in proliferation, differentiation, and trafficking of immune populations. IFN- α has been used since the late 1990s for the treatment of over 14 types of cancer including both solid tumours and haematological malignancies (Belardelli et al., 2002). IFN- α immunotherapy is believed to suppress tumour cell growth, simultaneously it increases MHC expression which subsequently aids in immune recognition, and is involved in the activation of immune cells including DCs, NK-cells, B-cells, and T-cells (Di Franco et al., 2017). Among the main factors affecting IFN- α immunotherapy efficacy is the tumour cell downregulation of IFN receptors (Di Franco et al., 2017).

1.4. Liver malignancy

Liver malignancies can be either primary or secondary. The latest global rates of cancer incidence show liver cancer as the sixth most common site of cancer growth. What is interesting to note is that despite the advancements in the fields of medical sciences, mortality rates in liver malignancy have not changed for decades, and liver cancer is the second highest contributor to cancer related mortalities at a global level (IARC, 2014). Primary hepatic malignancies are neoplasms that originate in the liver. They are divided into four types including hepatoblastomas, angiosarcomas, cholangiocarcinomas, and hepatocellular carcinomas (HCC), the most common type. HCC originates from hepatocytes and is mostly associated with chronic liver disease in response to hepatitis B (HBV) and hepatitis C (HCV) viral infections, autoimmune disease, or alcohol abuse, leading to cirrhosis and developing then into a neoplasm over time (Dragani, 2010). Secondary liver malignancies are neoplasms that originate in other sites in the body and metastasise into the liver.

1.4.1. Liver metastases

Primary cancers from almost any organ can metastasise into the liver, with the most common metastases stemming from the colon, breast, lung, and pancreas. The liver is one of the main sites of metastases in humans. Over 50% of primary colorectal cancer (CRC) (Coimbra et al., 2015), 25% of breast cancer patients (Wang et al., 2017), over 50% of lung cancer patients (Ren et al., 2016), and 30% of stage III pancreatic cancer patients develop liver metastasis (Dong et al., 2017).

1.4.1.1. Development of liver metastases

The liver is the first organ to encounter metastatic cells originating in the gut. Blood from the gut and the heart drain from circulation into the liver sinusoids through the portal triad then drain out of the liver through the central vein (See Fig.1.5) (Abdel-Misih and Bloomston, 2010). As blood from the gut transits in the liver for nutrient metabolism and detoxification before being fed to the hepatic vein, the liver encounters circulating tumour cells originating from the gut and is therefore more susceptible to metastasis establishment (Vidal-Vanaclocha, 2008). The arrest of tumour cells into hepatic sinusoids presents the first stage of metastases progression. If successful, metastasis progresses to the second stage of host stromal cell recruitment into avascular micro-metastases. This is then followed by the angiogenic phase of endothelial cell recruitment, which then progresses into the growth phase and the establishment of clinical metastases (Van Den Eynden et al., 2013).

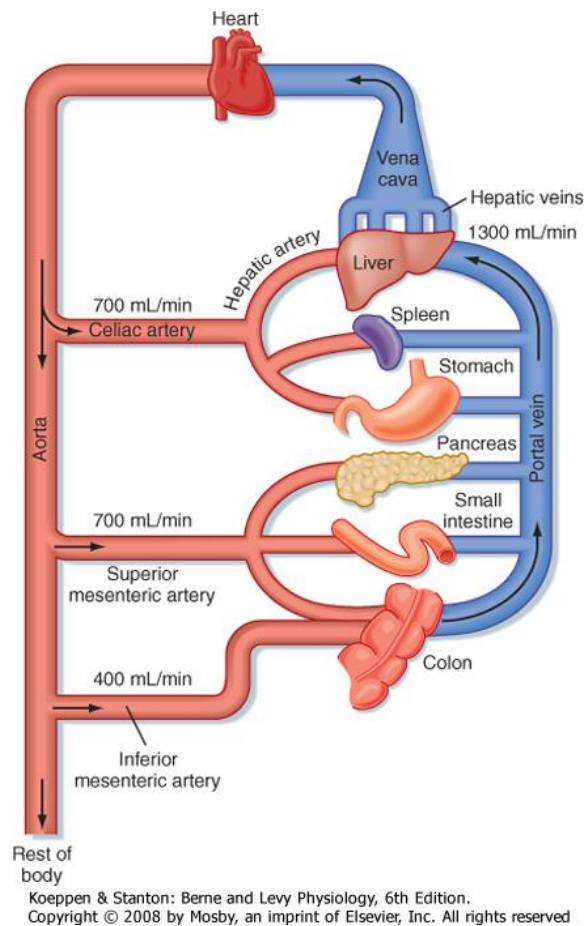


Figure 1.5: Blood flow to and from the liver (Berne et al., 2010).

1.4.1.2. Colorectal liver metastases

CRC is the third most prevalent and fourth highest cause of mortality compared to all other cancer sites (See Fig.1.6). Liver metastases secondary to CRC is the chief cause of mortality in that cohort, a serious health problem as 50% of primary colorectal cancer patients are anticipated to develop liver metastases (Geoghegan and Scheele, 1999, Vatandoust et al., 2015, Riihimäki et al., 2016).

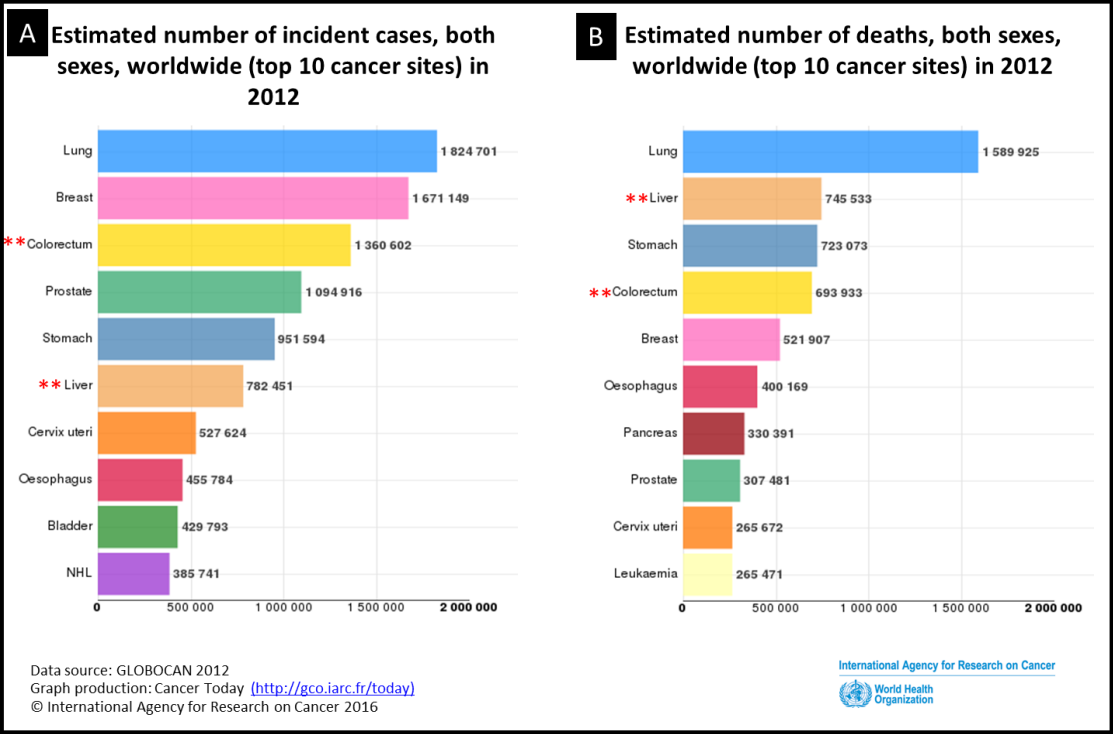


Figure 1.6: Global prevalence of cancer incidence, and mortality rate for both sexes in the top 10 cancer sites.

A) Global prevalence of cancer incidence for both sexes in the top 10 cancer sites. B) Global cancer related mortality rate for both sexes in the top 10 cancer sites.

**colorectal and liver related estimates.

1.5. Hepatic anti-tumour immunity

The liver’s anatomy reflects various aspects of immunological support for its functions. The liver is the largest internal organ, responsible for an array of metabolic functions including nutrient processing and storage, filtration and detoxification of blood draining in through the portal vein from the gut, and is also the body’s main producer of immune regulators including acute phase proteins, complement, clotting factors, and many other cytokines. With 30% of the total volume of blood in the body passing through the liver every minute, and its wide range of functions; the liver is under tremendous pressure to keep its immune microenvironment under strict control and maximise its functional and immunological capacity. The parenchymal portion of the liver is made up of hepatocytes which represent 80% of all liver cells (Kmiec, 2001); even though they are predominantly involved in

the metabolic functions of the liver, hepatocytes have also been shown to be linked to the innate and adaptive immune responses through their expression of MHC I, their ability to activate T-cells, and their production of acute phase proteins and complement (Crispe, 2016). The non-parenchymal portion of the liver consists of endothelial cells, Kupffer cells, biliary cells, stellate cells, and lymphocytes (Racanelli and Rehermann, 2006). Together, these populations demonstrate a very powerful immune microenvironment capable of tipping the scale towards immune tolerance or activation, and are very efficient at making the right decision (Robinson et al., 2016). This careful balance is evident in the 100 fold drop of pathogen derived molecules between portal and peripheral venous blood and the general low prevalence of allergies towards dietary molecules (Jenne and Kubes, 2013).

1.5.1. Liver Endothelial cells

To supports its functions, the liver's vasculature consists of a sinusoidal honeycomb designed to considerably slow down the blood flow. Those sinusoids are lined by endothelial cells which are highly efficient in detecting and presenting antigens to the immune system. Liver sinusoidal endothelial cells (LSECs) represent about half of all the liver's non-parenchymal cells. They mediate their immune functions through their expression of pattern recognition receptors like toll-like receptor (TLR) 3 which recognises viral components, TLR4 which is a signalling receptor for LPS, TLR7 being another viral component detector, and TLR9 which recognises bacterial and viral components (O'Neill et al., 2013). LSECs also connect to the immune system through their expression of MHC I and MHC II, which are important for antigen presentation, and CD80 and CD86, which are costimulatory molecules essential for T-cell activation (Jenne and Kubes, 2013, Racanelli and Rehermann, 2006). Other than protecting the liver against pathogenic threats, LSECs also protect the liver against risk of tumour metastasis, especially from primary gut malignancies as the ability to escape immune activation and pass through the endothelium to reach hepatocytes is a very important stage in establishing metastases (Poisson et al., 2017).

1.5.2. Liver Stellate cells

Hepatic stellate cells reside within the space of Disse; a gap created by the lack of organised basement membrane of LSECs found within sinusoids to separate hepatocytes from the blood flow. Representing a small 1% fraction of non-parenchymal cells, Stellate cells are known for their lipid storage functions but also express antigen presentation molecules including MHC I, MHC II, CD80, CD86, and CD1d. Stellate cells are known to produce monocyte and neutrophil chemoattractants when activated. Stellate cells have also been shown to secrete cytokines including IL-6, and TGF- β as well as inducing cytokine production by innate lymphoid populations (Friedman, 2008, Crispe, 2009). Hepatic stellate cells have an important anti-tumour immune surveillance role; they can polarise macrophages to an anti-tumour M1 phenotype, and upregulate NK-cell recruitment (Raj and Attardi, 2013).

1.5.3. Myeloid cells

1.5.3.1. Kupffer cells

The liver possesses the largest human tissue resident macrophage population known as Kupffer cells. Unique for their stationary status, and their ability to phagocytose mobile bacteria, these cells regenerate and spend their cycles within the liver. They can drive immune tolerance by IL-10, and PD-L1 secretion, and can produce both pro and anti-inflammatory cytokines. Kupffer cells are known to have very weak T-cell activation in response to commensal bacteria, but can also become potent antigen presenting cells in response to viral components and other pathogen derived antigens, reflecting the complexity of their immune recognition capacity (Heymann et al., 2015). Macrophages are influenced by the cytokine microenvironment into a pro-tumour tolerant phenotype by IL-4 and IL-10, or into an anti-tumour phenotype through IFN- γ exposure (Mantovani and Sica, 2010).

1.5.3.2. Dendritic cells

DCs are also important in immune activation and tolerance. They are phenotypically divided into myeloid DCs, important antigen presenting cells, and plasmacytoid DCs that are important in sensing and reacting to viral components through their expression of TLR7 and TLR9. Liver resident DCs are typically at resting state under healthy conditions, and they promote tolerance through CTLA-4 and PD-1 inhibition of activated lymphocytes (Kelly et al., 2014). Activated DCs can downregulate their inhibitory signals and upregulate their migratory capacity through the space of Disse to extrahepatic lymph nodes and activate T-cells (Racanelli and Rehermann, 2006).

1.5.3.3. Hepatic MDSCs

Myeloid-derived suppressor cells (MDSC) are also found in the healthy liver, and are expanded in pathology (Bernsmeier et al., 2017, Burga et al., 2015, Zhang et al., 2018). Phenotypically, they are CD33⁺, CD11b⁺, HLA-DR⁺, arginase 1 (Arg-1)⁺, indoleamine 2,3-dioxygenase 1 (IDO1)⁺, and are divided into monocytic (CD14⁺) or polymorphonuclear (CD15⁺) MDSCs (Elliott et al., 2017). As the name suggests, MDSCs are known for their immune suppression capacity and are therefore an important contributor to tumour progression. Tumour induced hepatic MDSCs have been shown to suppress T-cell proliferation, and also manipulate liver metabolism to reach T-cell dysfunction by depleting local L-arginine which is needed for lipid metabolism (Elliott et al., 2017, Hoechst et al., 2008). MDSCs have also been shown to inhibit NK-cell cytotoxicity through their NKp30 receptor (Hoechst et al., 2009).

1.5.4. Lymphocytes

The hepatic lymphocyte repertoire is unique in its proportions, phenotypes and functions compared to peripheral blood (Pb). They collectively participate in detoxification of venous blood, and maintain liver homeostasis (Crispe, 2009).

1.5.4.1. MHC-I and MHC-II restricted classical T-cells

Conventional T-cells are characterised by single positivity for CD4 or CD8 and express $\alpha\beta$ TCR. CD4⁺T-cells are activated through MHC II presentation of antigens, while CD8⁺T-cells are activated through MHC I molecules, and both require co-stimulatory signals through CD80 or CD86 for activation (Koretzky, 2010). CD4⁺T-cells undergo clonal expansion upon activation and drive B cell differentiation into the antibody-secreting plasma cell, while CD8⁺T-cells directly kill target cells. These cells account for two thirds of peripheral blood CD3⁺cells with the majority of them being CD4⁺T-cells. In comparison, conventional T-cells represent only 40% of hepatic CD3⁺cells with a reversed CD4 to CD8 cell ratio and three times more CD8 than CD4 cells. The liver also contains a significantly higher population of double negative (CD4⁻CD8⁻) T-cells (Norris et al., 1998). Most hepatic T-cells produce IFN- γ , TNF- α , and IL-2. A subset of hepatic T-cells produces IL-4 with IFN- γ , a characteristic of Th0-cells which are thought to be either undifferentiated or regulatory cells (Doherty and O'Farrelly, 2000a).

1.5.4.2. Hepatic MAIT cells

MAIT cells are particularly enriched in the liver. The enrichment of MAIT cells in the liver suggests that they have a major role in defence and maintaining homeostasis. They are identified by their expression of CD56, CD161, and Va7.2 TCR (Tang et al., 2013), with the majority of human MAIT cells being CD8⁺ (Reantragoon et al., 2013). They also express high levels of IL-18R, enabling them to release IFN- γ and TNF- α in response to IL-12 and IL-18 (Kurioka et al., 2016). MAIT cells have not been investigated in colorectal liver metastasis (CRLM), and very little is known about the role of MAIT cells in cancer. However, their expression of IFN- γ and TNF- α suggests a role for them in anti-tumour responses.

1.5.4.3. Hepatic NKT-cells

Human NKT-cells are a lot rarer compared to murine tissue, but they are still significantly expanded within the human liver compared to peripheral blood. NKT-cells express CD3 along with CD4, CD8, CD56, CD161, and CD69. The human Va24Vβ11⁺ TCR NKT-cells were found to be significantly lower in hepatic CRLM (Kenna et al., 2003a). Hepatic NKT-cells predominantly produce Th1 cytokines (IFN-γ, TNF-α), and they do not produce IL-2 or IL-4. They are subcategorised as type I, the pro-inflammatory subset, and type II, the anti-inflammatory subset. Type I NKT-cells secrete different cytokines depending on their activation pathway; in response to ischemia, IL-18 and IL-12, NKT-cells secrete IFN-γ, while in response to α-galactosylceramide (α-GalCer), they secrete IFN-γ, IL-4, and IL-17 which then leads to further lymphocyte stimulations and the recruitment of neutrophils and myeloid cells. Type II NKT-cells are the more abundant subset, they can suppress pro-inflammatory cytokines produced by type I to protect the liver against damage, and have been linked to several immune regulatory functions (Bandyopadhyay et al., 2016).

1.5.4.4. Hepatic γδT-cell

A relatively small subset of hepatic CD3⁺ T-cells express the γδ TCR, and are expanded in the liver six fold compared to peripheral blood (Kenna et al., 2004b). Not all γδT-cells are restricted by MHC presentation of antigens; they can be activated by an array of stimuli including their recognition of the endogenous upregulation of isopentenyl pyrophosphate (IPP), a characteristic of tumour cells that allows them to initiate a response specific to tumour cells without affecting healthy tissue. In response, γδT-cells can upregulate the expression of CD36 on tumour cells, rendering them susceptible to scavenging by DCs and macrophages (Latha et al., 2014). Sequentially, γδT-cells would then serve an APC role initiating a specific CD8⁺T-cell response, or activate their own cytotoxic capacity through the upregulation of perforin/granzymes, IFN-γ, TNF-α, and IL-17, and mediates cell death through FasL, and TRAIL. Immune therapies based on γδT-cell activation and enrichment has been associated with improved survival in prostate cancer, and renal cell carcinoma (Zou et al., 2017, Zhao et al., 2018).

1.5.4.5. Hepatic NK-cells

NK-cells are integral players in the liver microenvironment. Accounting for over half of hepatic lymphocytes, NK-cells represent a potent innate anti-viral and anti-tumour immune population. Identified as CD45⁺CD3⁻CD56⁺, these cells have been subcategorised into a CD56^{bright} population which is known for a high production of immunoregulatory cytokines, and CD56^{dim} populations which exhibits potent cytotoxicity and less cytokine production. NK cells have been described to be phenotypically and functionally distinct in the liver compared to Pb. Hepatic CD56^{bright} and CD56^{dim} populations don't exhibit any difference in IFN- γ secretion, a feature distinct from Pb, and was shown to be regulated through the liver's cytokine microenvironment (Harmon et al., 2016). NK-cells are involved in hepatic tolerance through their production of suppressive cytokines including TGF- β , and IL-10, and they have been described as the liver's first line of defence against tumour cells directly through killing target cells or indirectly through inducing hepatocytes and LSECs to produce chemokines for T-cell recruitment (Racanelli and Rehermann, 2006, Peng et al., 2016).

1.6. Cancer immune infiltration and prognosis

1.6.1. Linking immune cells to prognosis

Many studies have shown that immune cells appear to promote tumour growth which led most non-immunologists to condemn the presence of any immune populations in the tumour microenvironment (Smith and Kang, 2013, Grivennikov et al., 2010). Neutrophils have been linked to metastatic cell arrest in the liver, and Kupffer cells have been shown to increase cellular adhesion molecules suggesting they can facilitate tumour cell adhesion and establishment (Van Den Eynden et al., 2013). The presence of Tregs, and MDSCs in several types of malignancies correlate with poor prognosis (Mourmouras et al., 2007, Mougiakakos et al., 2010, Gonda et al., 2017). Regulatory cells have been shown to suppress the anti-tumour immune responses through their secretion of suppressive molecules like IL-10 and TGF- β , killing effector T-cells through granzyme B and TRAIL,

manipulating normal T-cell function by depleting essential metabolic modulators like IL-2, and recruiting DCs with indoleamine 2,3-dioxygenase (IDO) to inhibit antigen-specific T-cell proliferation leading to non-direct suppression (Facciabene et al., 2012, Hwang et al., 2005). MDSCs on the other hand can interfere with T-cell proliferation through depleting amino acids by enzymes, producing nitric oxide (NO) and reactive oxygen species (ROS) that suppress both T-cells and NK-cells by inducing cellular damage, and subsequently inducing suppression of cytotoxic immune cells by secreting IL-10 and TGF- β similar to Tregs, upregulate checkpoint inhibitors, and activate T-reg (Umansky et al., 2016).

However, immunologists have also shown the anti-tumour capacity of the immune system evident now by the expansion of immunotherapies (Zitvogel et al., 2008). It is important therefore to understand that the type and the location of immune cells, as well as many other factors within the microenvironment including the cytokine milieu, and the metabolic state, all contribute to the metastatic tumour progression or limitation (Galon et al., 2006, Pitt et al., 2016).

1.6.2. History of tumour infiltrating lymphocytes' correlation to survival

Despite the narrative around the negative effect of the immune system in the tumour microenvironment, there is certainly something to be said about numbers of immune cells that have major potency against malignant and metastatic cells (Grivennikov et al., 2010). However, the major breakthrough in understanding how tumour cells and their products interfere with anti-tumour immunity has stimulated the revolution in cancer treatment via immunotherapies (Kamta et al., 2017). Therefore, enumerating the numbers of these cells has become a major prognostic tool.

For over a century, the presence of immune cells in the tumour microenvironment has been described, and for decades, the presence of lymphocytes in the tumour microenvironment has been linked to prognosis (Underwood, 1974). Tumour infiltrating lymphocytes (TILs) were identified

and localised based on morphology alone in haematoxylin and eosin (H&E) stained histology sections. The link between the number of lymphocytes within the tumour and good prognosis was observed across many malignancies as early as the 1970s (Riesco, 1970a, Watt and House, 1978a, House and Watt, 1979a). With the development of specific antibody-based immunohistochemistry, researchers started visualising leukocytes and T-cells by targeting specific markers like CD45 and CD3, enabling a far more specific and more reliable measure of these populations in tumour tissue.

It was shown that absolute cell counts of cytotoxic T-cells indicated as CD8⁺ cells, CD3⁺ T-cells, and memory T-cells as CD8⁺CD45RO⁺ cells almost always correlate with good prognosis across many cancer types including HCC (Ding et al., 2018), pancreatic cancer (Miksch et al., 2019), lung carcinoma (Soo et al., 2018), melanoma (Fu et al., 2019), and colorectal carcinoma (Galon et al., 2016), among many others (Denkert et al., 2010, Loi et al., 2013, Hwang et al., 2012). Specific Immune populations were quantified based on their histological location, and many groups found positive correlation between absolute numbers of cytotoxic T-cells within the tumour as well as the tumour invasive margin, and prognosis as well as response to therapy.

A little over a decade ago, Jerome Galon developed an immunohistochemistry based method and highlighted the importance of immune infiltration and its correlation to survival in colorectal cancer patients (Galon et al., 2006). Galon assessed the number of immune cells in two locations, the centre of tumours and in adjacent tissue surrounding the tumour with immunohistochemistry staining of the main immune effector populations; he found CD3 T-cells, CD8 T-cells, and CD45RO memory T-cells counts in these different locations were correlated to survival data. Galon's results demonstrated that the density and location of key effector immune populations was a better prognostic tool compared to the standard UICC-TNM malignancy classification method, which is based on tumour burden, cancer infiltration to lymph nodes, and metastasis. Studies done on some T-cell subsets including Th2-cells, Th17-cells, and Treg yielded controversial and inconsistent findings between different types of malignancies when correlated to survival (Galon et al., 2016, Kirilovsky et al., 2016). On the other hand, studies on CD8 T-cells and Th1-cells proved

a universal diagnostic value with very few exceptions (Brudvik et al., 2012). Galon recently lead the society for immunology of cancer (SITC) immunescore validation project, an international collaboration across 23 centres in 17 countries to correlate immune infiltration with survival and response to therapy following a standardised method of immune density and location analysis in all stages of colon cancer patients. Upon completion in 2016, the immunescore was shown to be a strong predictor of prognosis regardless of age, sex, and tumour stage in colorectal cancer patients with indications on correlation of CD3 and CD8 cell expression with response to chemotherapy, radiotherapy, and immunotherapy in several cancer types including breast cancer, and colon cancer (Kirilovsky et al., 2016, Galon et al., 2016).

1.6.3. Tumour infiltrating lymphocytes and prognosis in liver-metastasis

Focusing on liver-metastasis and investigations done with immunohistochemistry, TILs in colorectal-liver metastases were found to have a positive correlation between survival and CD8⁺ T-cells (Mlecnik et al., 2018), negative correlation of T-regs to survival, and controversial correlation with CD4⁺ T-cells (Katz et al., 2009a, Katz et al., 2013). In cholangiocarcinoma-liver metastases, IL-17⁺ TILs had a negative correlation with survival (Gu et al., 2012). In pancreatic neuroendocrine-liver metastases, low levels of T-regs in the tumour were a predictor of good survival (Katz et al., 2010).

1.7. Project rationale, hypothesis, and aims

1.7.1. Rationale

The availability and functionality of immune cells within the tumour microenvironment are influenced by several elements including their interaction with tumour cells, cytokines including chemokines and growth factors, as well as physiological conditions including metabolite availability,

oxygen availability and pH. The tumour microenvironment can affect immune accumulation by limiting recruitment or by inducing immune cell death. The localisation and quantification analysis of TILs is limited by practical challenges. One of the main limitations in human liver is the lack of a true normal tissue to use for base line values, as well as the interpatient variability.

Our lab had been interested in liver immunology for over two decades. The liver's immune repertoire is unique and reflects a potent anti-tumour network. Despite the liver's capacity to detect and fight tumour cells, over 50% of colorectal cancer patients develop liver metastases suggesting exceptionally powerful mechanisms of immune evasion. The liver's anti-tumour populations are regulated and controlled by a cytokine microenvironment; therefore, it is important to study the microenvironment of tumour bearing livers.

The microenvironment in CRLM has been compared to donor liver, and our group reported the significant increases of IL-12, IL- 18, IL-10, and the significant decrease of IL-15 in total protein samples extracted from CRLM biopsies and donor liver biopsies (Kelly et al., 2004, Kelly et al., 2006b). We also looked into the expression of growth factors and chemokines in secreted protein samples prepared by incubating tumour and donor biopsies in ex-vivo media for 72 hours and found that the levels of CXCL10, VEGF, and CCL5 were significantly higher in the tumour bearing liver compared to donor liver (Hand et al., 2018b).

As most studies in liver metastases and also in other cancer types agree on the benefit of cytotoxic T-cells within the tumour, and as other studies demonstrate an altered inflammatory signature in the tumour bearing liver, it is important to investigate the factors affecting the recruitment and enrichment of CD8 cells in the tumour microenvironment. Understanding the changes in cytokine expression and linking them to changes in immune regulation and recruitment will contribute to identifying better prognostic markers, and will define more reliable approaches to combined treatment strategies.

1.7.2. Hypothesis

We hypothesise that in colorectal liver metastases, the cytokine microenvironment is an important contributing factor to immune recruitment and paralysis. We believe that identifying key regulators of immune trafficking will present new targets for immunotherapy to boost immune infiltration, enhance response to therapy, and improve overall survival.

1.7.3. Aims

In this body of work, our overall aims were to:

- Investigate the density and location of key immune populations in healthy liver donor, and CRLM tissue.
- Investigate the inflammatory milieu of healthy liver donor vs CRLM tissue.
- Link immune infiltration to chemokine signature of CRLM tissue

2. MATERIALS AND METHODS

2.1. List of Reagents, materials and instruments used:

Table 2.1: General reagents used with manufacturer details

Reagent	Company	location
Cell Isolation and culture		
Bovine Serum Albumin	Sigma-Aldrich	Poole, UK
Collagenase IV	Sigma-Aldrich	Poole, UK
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	Poole, UK
Distilled, sterile, nuclease-free water	Gibco (Thermo Fisher Scientific)	Paisley, UK
EasySep™ Buffer	STEMCELL	Cambridge, UK
Ethanol	Sigma-Aldrich	Poole, UK
Fetal bovine serum (FBS)	Gibco (Thermo Fisher Scientific)	Paisley, UK
Ficoll-Paque	VWR (GE healthcare)	Chicago, IL, USA
Hank's balanced saline solution	Gibco (Thermo Fisher Scientific)	Paisley, UK
Industrial methylated spirits (IMS)	Lennox	Dublin, Ireland
Penicillin-Streptomycin	Invitrogen (Biosciences)	
Phosphate buffered saline	Gibco (Thermo Fisher Scientific)	Paisley, UK
Red blood cell lysis buffer	Sigma-Aldrich	Poole, UK

Reagent	Company	location
RPMI 1640 + L-glutamine	Gibco (Thermo Fisher Scientific)	Paisley, UK
Sterile phosphate buffered saline (PBS)	Gibco (Thermo Fisher Scientific)	Paisley, UK
Trypan blue	Sigma-Aldrich	Poole, UK
X-VIVO 15 media by Lonza	VWR (Nunc Inc.)	Radnor, PA, USA
Protein extraction		
Aprotinin	VWR (Nunc Inc.)	Leicestershire, UK
Leupeptin	Fisher Scientific	Leicestershire, UK
Phenylmethylsulphonyl fluoride	Fisher Scientific	Leicestershire, UK
Sodium orthovanadate	VWR (Nunc Inc.)	Leicestershire, UK
Flow cytometry		
CountBright™ absolute counting beads	Thermo Fisher Scientific	Poole, UK
Sodium azide	Sigma-Aldrich	Poole, UK
Histology processing, and immunohistochemical staining		
4% paraformaldehyde	Sigma-Aldrich	Poole, UK
Casein	Vector Lab Supplies	Burlingame, CA, USA
Citric acid	Sigma-Aldrich	Poole, UK
DPX	Sigma-Aldrich	Poole, UK
EnVision IHC detection kit	Dako (Agilent Technologies)	Santa Clara, CA, USA

Reagent	Company	location
Eosin	Merck	Darmstadt, Germany
Haematoxylin	Dako (Agilent Technologies)	Santa Clara, CA, USA
Histoclear	National Diagnostics	Atlanta, GA, USA
Hydrogen peroxide	Sigma-Aldrich	Poole, UK
Magnesium sulfate	Sigma-Aldrich	Poole, UK
Sodium bicarbonate	Sigma-Aldrich	Poole, UK
Sodium citrate	Sigma-Aldrich	Poole, UK
ELISA		
TMB	KPL	MD, USA
TMP stop reagent	Sigma-Aldrich	Poole, UK

Table 2.2: Cell culture treatments

Stimulant/antibody	Company	Location
Anti-CXCL9	Fisher Scientific	Leicestershire, UK
IFN-γ	Thermo Fisher Scientific	Paisley, UK
CD3	Miltenyi Biotec Ltd.	Surrey, UK
CD28	Miltenyi Biotec Ltd.	Surrey, UK

Table 2.3: Tissue culture materials

Item	Company	Location
1.5/2.0 ml nuclease-free, sterile microcentrifuge tubes	Thermo Fisher Scientific	Paisley, UK
10 ml plastic pipettes	Cruinn Diagnostics (Grenier)	Dublin, Ireland
12-well flat bottom tissue culture plates	Cruinn Diagnostics (Cellstar)	Dublin, Ireland
24-well flat bottom tissue culture plates	Cruinn Diagnostics (Cellstar)	Dublin, Ireland
24 well transwell inserts 5.0um	Thermo Fisher Scientific	Paisley, UK
25 ml plastic pipettes	Cruinn Diagnostics (Grenier)	Dublin, Ireland
2mL cryovials	Thermo Fisher Scientific	Paisley, UK
5 ml plastic pipettes	Cruinn Diagnostics (Grenier)	Dublin, Ireland
6-well flat bottom tissue culture plates	Cruinn Diagnostics (Cellstar)	Dublin, Ireland
70µm filters	Falcon	NY, USA
96 well ELISA plates	Greiner Bio-One	Kremsmünster, Austria
96-well flat bottom tissue culture plates	Cruinn Diagnostics (Cellstar)	Dublin, Ireland
96-well round-bottom TC plates	Corning Life Sciences	MA, USA

Item	Company	Location
Plastic base moulds	VWR (Nunc Inc.)	Radnor, PA, USA
Cryovials	VWR (Nunc Inc.)	Radnor, PA, USA
Dissection Scissors	Thermo Fisher Scientific	Paisley, UK
Flow tubes	Thermo Fisher Scientific	Paisley, UK
Forceps with curved teeth	Thermo Fisher Scientific	Paisley, UK
Glass slides	Thermo Fisher Scientific	Paisley, UK
Microtome blade	Feather	Japan
Microwaveable pressure cooker	Nordic Ware	St. Louis park, MN, USA
Petri dishes	Greiner Bio-One	Kremsmünster, Austria
Scalpels	Swann Morton	Sheffield, UK
Stainless steel beads 5mm	Qiagen	Crawley, UK
Surgipath cassettes	Leica Biosystems	Wetzlar, Germany
T75 Tissue culture flask	Cruinn Diagnostics (Cellstar)	Dublin, Ireland
Vacutainers containing heparin anticoagulant	Greiner Bio-One	Kremsmünster, Austria

Table 2.4: List of antibodies used for Immunohistochemistry

Antibody	Catalogue number	Company
CD3	M725429-2	Dako
CD45	M070129-2	Dako
CD56	M730429-2	Dako
CD8	17953	Stemcell
CXCL9	GTX108422S	Source BioScience
CXCR3	MAB160-SP	R&D
TCR Va7.2	351702	Biolegend

Table 2.5: List of antibodies used for flow cytometry

Antibody	conjugate	Catalogue number	Company
CD161	BV421	339913	BioLegend
CD3	FITC	344804	BioLegend
CD3	Alexa-Flour 700	344821	BioLegend
CD4	FITC	130-098-206	Miltenyi Biotec
CD45	BV510	304035	BioLegend
CD45	FITC	304054	BioLegend
CD8	APC	561952	BD Biosciences
CXCR3	PE	130-120-592	Miltenyi Biotec
CXCR3	BV711	353732	BioLegend
TCR Va7.2	APC-Cy7	130-100-179	Miltenyi Biotec
Va7.2	APC-Cy7	130-100-179	Miltenyi Biotec

Antibody	conjugate	Catalogue number	Company
Zombie Aqua	NA		Biolegend
Zombie Yellow	NA	423103	Biolegend

Table 2.6: List of kits

Kit	Supplier	Location
EasySep™ Human CD8+ T Cell Isolation kit	STEMCELL	Cambridge, UK
Human CCL8 ELISA kit	Peprotech	London, UK
Human CXCL10 ELISA kit	Peprotech	London, UK
Human CXCL9 ELISA kit	Peprotech	London, UK
Human cytokine array	Abcam	Cambridge, UK
Human IFN-γ ELISA kit	Peprotech	London, UK
Human IL-10 ELISA kit	Peprotech	London, UK
Human IL-6 ELISA kit	Peprotech	London, UK
Pierce BCA protein assay	BioSciences	Dublin, Ireland
REAL™ EnVision™ detection system, rabbit/mouse	Dako (Agilent Technologies)	Santa Clara, CA, USA

Table 2.7: Equipment and software used with manufacturer details

Software/Equipment	Company	Location
Aperio Imagescope	Leica Biosystems	Newcastle, UK
Aperio slide scanner	Leica Biosystems	Newcastle, UK
Embedding instrument	Leica Biosystems	Newcastle, UK
FlowJo v.10 software	FlowJo LLC	Oregon, USA
Graphpad Prism v8.0	Graphpad Software Inc.	San Diego, CA, USA
Haemocytometer	ThermoFisher Scientific	Paisley, UK
ImageLab	Biorad	Hertfordshire, UK
LSR Fortessa	BD Biosciences	Allschwil, Switzerland
FACS Canto	BD Biosciences	Allschwil, Switzerland
Microtome	Leica Biosystems	Newcastle, UK
Microwave	Panasonic	
Olympus BX51 upright microscope	Leica Biosystems	Newcastle, UK
Olympus IX81 inverted microscope	Leica Biosystems	Newcastle, UK
ELISA plate reader	ThermoFisher Scientific	Paisley, UK
TMARKER	Nexus	Zurich, Switzerland

2.2. Cell culture

Cell culture and handling of blood samples and tissue biopsies were carried out according to strict standard operating procedures (SOPs). Cell culture was carried out in sterile conditions using laminar flow hoods and sterile or autoclaved materials and reagents. Blood and liver samples were treated as potentially infected and were processed and handled with caution.

2.3. Test samples

2.3.1. Human liver biopsies

Collection of liver samples was approved by the research ethics committee in St Vincent's University Hospital (SVUH). The National Liver Unit at SVUH in Dublin is the primary centre of hepatic metastasectomy in Ireland. Patients attending the National Liver Unit at SVUH for hepatic metastasectomy secondary for tumours of colorectal origin were eligible for inclusion in this study. Tissue biopsies from the outer rim of the resected tumour (referred to as "tumour"), from macroscopically normal tissue directly adjacent to the tumour (referred to as "adjacent"), and macroscopically normal tissue from the resection margin (referred to as "distal") were sampled by the pathologist on duty on the day of the resections (See Fig. 2.1A). These defined samples are used for our flow cytometry analysis, and protein analysis, and some immunohistochemistry analysis. All data relative to invasive margins (shown in chapter three) are based on identifying this region histologically, and only on recalled blocks from SVUH. These blocks were created following standard procedures at the pathology lab in SVUH. Qualified pathologists follow standardised sampling criteria producing samples of approximately 1.5cmX1.5cmX0.5cm of the outer rim of the tumour through to the adjacent tissue, and would therefore always include the junction between the tumour and adjacent, known as the invasive margin.

The National Liver Unit at SVUH in Dublin is also the only unit performing liver transplantations. Donor liver wedge biopsies are sampled by the

surgeons during orthotopic liver transplantation from donor livers (See Fig. 2.1B). All biopsies were transported in total RPMI media (RPMI + L-glutamine, 10% v/v Foetal Calf Serum (FCS), 100U/ml penicillin and 100µg/ml streptomycin) from SVUH to the lab in Trinity Biomedical Science Institute (TBSI).

Formalin fixed paraffin embedded (FFPE) CRLM and donor liver tissue were recalled from SVUH pathology archives. CRLM FFPE blocks were prepared at SVUH pathology laboratory following resections for CRLM between 2005-2007. Donor liver FFPE blocks were prepared at SVUH pathology laboratory following orthotopic liver transplantations between 2013-2017.

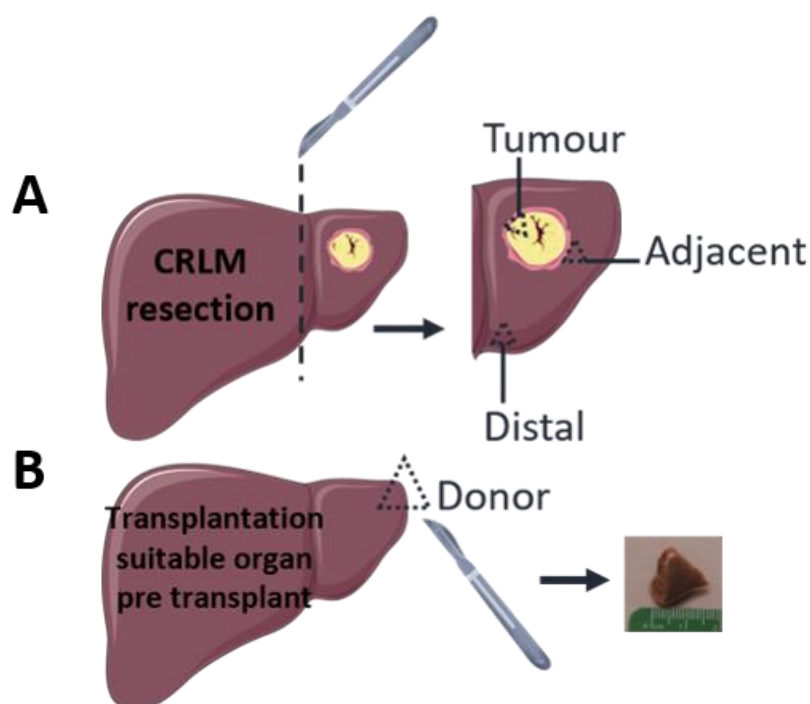


Figure 2.1: Schematic of obtained biopsies for experimentation.

A) Colorectal liver metastases tumour, adjacent, and distal biopsies

B) Liver donor biopsies

2.3.2. Human blood

Collection of peripheral blood samples was approved by the research ethics committee in Trinity Biomedical Science Institute. Fresh blood samples were

collected in heparin anticoagulated tubes at TBSI under the Trinity blood donor scheme. Buffy coat packs were collected from the National Blood Centre.

2.4. Liver tissue processing

Depending on sample size, biopsies were cut into 3-5mm³ pieces and divided to be processed or stored appropriately for immunohistochemistry, protein extraction, and to prepare tissue supernatants.

2.4.1. Tissue Fixation for immunohistochemistry

Liver biopsies obtained at the time of transplant or from surgical resection of hepatic malignancy were covered in 10% neutral buffered formalin solution (Sigma) within an hour of collection for 24 hours.

2.4.2. Tissue processing for protein extraction

Pieces of liver biopsies obtained at the time of transplant or from surgical resection of hepatic malignancy were placed into cryovials and then immersed in liquid nitrogen for one minute before being stored in liquid nitrogen tanks.

2.4.3. Preparation of liver conditioned media

Liver biopsies at 5 mm³ obtained at the time of transplant or from surgical resection of hepatic malignancy were placed into 24-well flat bottom tissue culture plates, and immersed in 500µL serum-free X-VIVO 15 media (Lonza). The plates were then incubated for 72 hours at 37°C with 5% CO₂. The media was then centrifuged at 300 rcf for five minutes, and the supernatant was transferred into a fresh eppendorf and frozen at -20°C for later experiments.

2.4.4. Isolation of hepatic mononuclear cells from liver tissue

To Isolate hepatic mononuclear cells (HMNCs) pieces from the biopsies are completely diced with a shaving motion using scalpels and transferred into a 50mL falcon tube containing:

A. First method: A mix solution of Collagenase Type IV 0.05% (Sigma), DNase I (10mg/mL) 0.002% (Sigma), 2% FCS (Gibco), 2% BSA at 30% w/v (GE Healthcare) diluted in hanks balanced salt solution (HBSS) (Gibco).

B. Second method: A mix of enzymes from Human Tumour Dissociation Kit (Miltenyi Biotec) following manufacturer's instructions.

Samples were then incubated at 37°C in a shaker with rotation at 180 rpm for 20 minutes to digest the liver biopsy tissue. Enzymatic reaction was then stopped by topping up the tube with ice cold HBSS. Tube contents were then filtered through 70 µm filters (BD Biosciences) to exclude debris. Hepatocytes were then pelleted by centrifugation at 30 rcf for three minutes at 4°C. HMNCs in the supernatant were then isolated by centrifugation at 300 rcf for 10 minutes at 4°C and collecting the pelleted cells. Cells were finally washed in 20mL of HBSS and centrifuged at 350 rcf for five minutes. Supernatant was discarded and final cell pellet was resuspended in total RPMI (containing L-glutamine, 10% v/v FCS, 100U/ml penicillin and 100µg/ml streptomycin), counted, and viability calculated using trypan blue exclusion assay.

2.5. Isolation of mononuclear cells from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque™ density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare). Buffy coats were diluted by injecting 20ml of 1X sterile phosphate buffered saline (PBS) into the bag then mixing it before emptying the bag into a sterile container. Blood was then carefully layered onto Ficoll at a 2:1 ratio

respectively in a 50mL falcon tube. Fresh blood was diluted by mixing with 1X PBS in a ratio of 2:1 respectively and layered onto 5mL Ficoll in 15mL falcon tubes. Falcon tubes were then centrifuged at 800 rcf for 25 minutes with break set to one. Buffy coat layers of PBMCs were then collected using a pasteur pipette and transferred into a fresh 50ml falcon to be washed twice by topping up the 50ml falcon with sterile 1x PBS and centrifuging at 300rcf for 5 minutes, discarding the supernatant and resuspending the pelleted cells by vortexing each time. Final cell pellets were resuspended in total RPMI (containing L-glutamine, 10% v/v FCS, 100U/ml penicillin and 100µg/ml streptomycin).

2.6. Cell viability and enumeration of cells

To count cells and calculate viability, cells were diluted as appropriate in Trypan blue depending on the cell pellet size. Neubauer Haemocytometer was covered with a glass cover slip and 10 µL of the cell/Trypan blue solution was pipetted to diffuse under the cover slip. Cells were visualised using a light microscope, and the number of cells in the four corner grids were counted. Cells touching the top and left borders of each grid section were not counted for better accuracy. The average from the counted corners is then calculated by dividing the total count by four, then that is multiplied by 10^4 then by the dilution factor for the final number of cells per mL. Dead cells allow Trypan blue to enter through their defective membranes making them appear blue and are excluded from cell counts.

2.7. Cryogenic storage of cells

Once counted, cells were pelleted by centrifugation at 400 rcf for five minutes at 4°C. Freezing mixture (50 % total RPMI, 40% FCS) was prepared and placed on ice for five minutes. Cell pellet was then resuspended in the freezing mixture at a concentration of $10\text{--}20 \times 10^6$ cells per mL. Cells were transferred to cryovials, and finally 10% DMSO was added and the cryovials were placed in a cryo 1°C freezing container

containing isopropanol and placed in a -80°C freezer overnight. Cryovials were then transferred to liquid nitrogen tanks for long term storage.

2.8. Flow cytometry

The technology of flow cytometry allows the investigation of individual cells and their expression of specific intracellular and extracellular markers. The complete list of antibodies used during the course of this work is listed in table 2.5. Antibodies were titrated in order to determine optimal concentrations for staining. Fluorescence-minus-one (FMO) controls were used to set gates where necessary.

2.8.1. Cell surface staining for flow cytometry

Number of cells stained for flow cytometry ranged from 5×10^5 to 1×10^6 . Staining was done in polystyrene FACS tubes. Cells were washed in PBA buffer (PBS containing 1.5% v/v BSA and 0.2% sodium azide (NaN_3)) by centrifugation at 350 rcf for five minutes and discarding supernatant. Cells were then resuspended in 100 μL PBA buffer containing the viability dye and incubated for 10-15 minutes, followed by a washing step. The cells are then resuspended in 100 μL PBA and the antibody mix required for extracellular markers of interest is added to the suspension. After an incubation of 15-30 minutes at 4°C in the dark, excess unbound antibodies were washed away by adding 3-4 mL of PBA followed by centrifugation at 350 rcf for five minutes and discarding supernatant. Compensation and cytometer settings were adjusted with unstained cells tube, live/dead cells tube, and compensation beads in separate tubes with each of the fluorochromes used in the panel.

2.8.2. Analysis of flow cytometry and gating strategy

Panels were analysed on either BD LSRFortessa, or FACS Canto. Cytometer used in each experiment type was consistent for all samples tested. Data files were exported as FSC files and all subsequent analysis was done on

FlowJo. For optimal gate setting, FMO controls were carried out with samples containing all but one of the antibodies in the panel.

2.8.3. Quantification of cells with count beads

Cell counts by flow cytometry was carried out with the use of CountBright™ absolute counting beads (Thermo Fisher Scientific). Beads were visualised on a plot of side scatter (SSC) vs forward scatter (FSC) and gated on. Identifying populations of interest was then carried out as normal. Absolute cell counts are then calculated using the cell count of gated population of interest, the beads count, the assigned bead count of the lot used, and the sample volume (See Fig. 2.2).

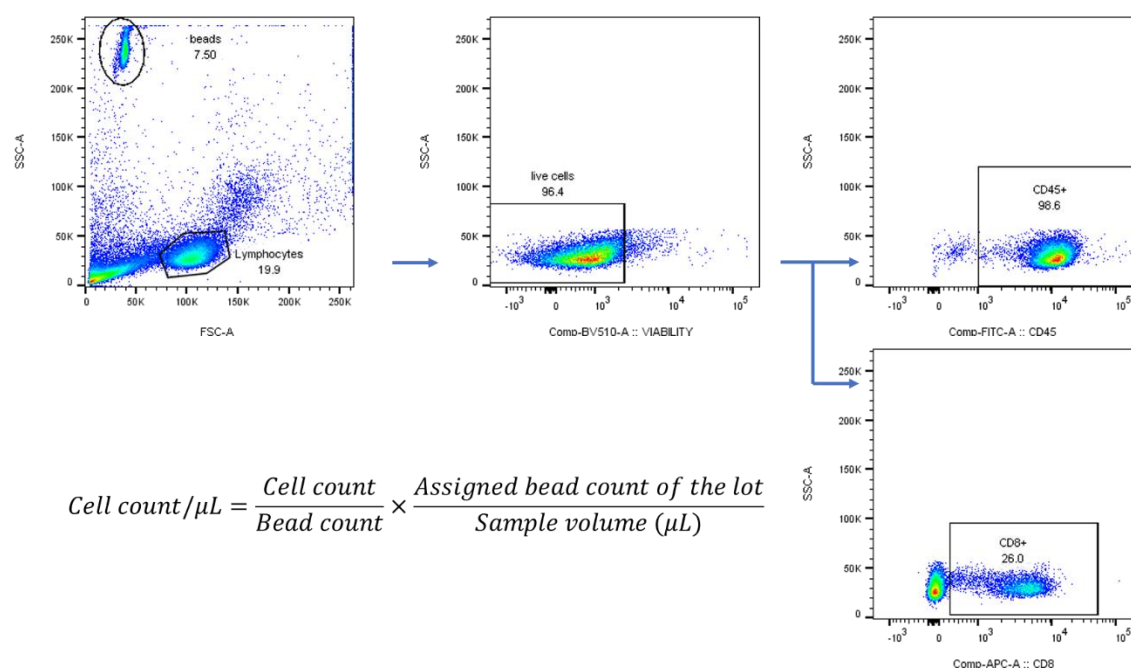


Figure 2.2: Gating strategy and cell count analysis for the chemotaxis assays.

Both count beads and lymphocytes were gated using the FSC-A vs SSC-A plot followed by gating on live cells using a viability dye. CD45+ and CD8+ were then gated and absolute number of cells were calculated using the formula set by the beads' manufacturer.

2.9. Immunohistochemistry

Immunohistochemistry (IHC) was utilised to localise and visualise targets of cellular populations and proteins in human liver tissue. All antibodies used during the course of this work are listed in table 2.4. Multiple concentrations of the antibodies were tested to determine optimal staining.

2.9.1. Tissue processing and paraffin embedding

After 24 hours in formalin, the tissue was transferred into labelled cassettes and placed into 70% ethanol until processed to allow paraffin infiltration. Processing protocol is 12 hours long, and involves dehydration through ethanol baths of increasing concentrations and clearing with xylene to displace water, then immersion in hot wax. Processed tissue was then placed into the appropriate mould size with the labelled cassette on top and covered with paraffin using the Leica paraffin dispenser instrument. Blocks were kept to solidify on a cold plate for a minimum of two hours.

2.9.2. Paraffin blocks sectioning

Sections were cut from paraffin blocks of human liver tissue at 5µm thickness with a microtome. Ribbons of sequential sections were transferred to cold water and separated using an uncharged slide then transferred into a 50°C water bath before transferring them onto electrostatically charged glass slides. Optimal adherence of sections onto slides is then insured through a minimum incubation of two hours at 60°C.

2.9.3. IHC staining protocol

Slides were incubated at 60°C for at least 30 minutes before the start of protocol. Slides were then deparaffinised in three changes of HistoClear (National Diagnostics), an alternative to xylene, at 5 minutes each, and rehydrated in a sequence of decreasing concentrations of Industrial

Methylated Spirits (IMS) at 100%, 70%, and 50% for 5 minutes each, followed by immersion in deionised water for 5 minutes. Antigen retrieval was then carried out in 0.01M Sodium citrate at pH 6.0 in a pressure cooker for four minutes from the point it reached boiling temperature. The slides were then cooled under running water and incubated in PBS for five minutes. First blocking step was then carried out by covering sections in casein (1X) (Vectorlabs) for 20 minutes followed by two, five minutes washes in PBS. Primary antibody was then prepared to the desired concentration and covered the tissue sections for one hour followed by two, five minutes washes in PBS. Second blocking step was then carried out with 3% hydrogen peroxide for seven minutes followed by two, five minutes washes in PBS. Secondary antibody from the Dako REAL EnVision Detection System kit was pipetted onto the sections to cover them for a 30 minutes incubation followed by two, five minutes washes in PBS. The kit provides a dextran backbone coupled to numerous horseradish peroxidase and secondary antibodies and it reacts sufficiently with mouse and rabbit immunoglobulins. The positivity is then visualised with a mix of concentrated diaminobenzidine (DAB) solution and hydrogen peroxide substrate buffer covering sections until brown staining is evident or for a maximum incubation of 10 minutes followed by two, five minutes washes in PBS. The sections are then counter stained with haematoxylin for one to two minutes followed by one wash in PBS, and one in deionised water at five minutes each. Finally, sections are dehydrated in increasing concentrations of IMS (50%, 70%, 100%), cleared in three changes of histoclear, and mounted with a glass cover slip and mounting medium.

2.9.4. Qualitative and quantitative analysis of IHC slides

Stained slides were scanned with the Aperio slide scanner (Leica) and saved as ImageScope compatible files. Results were viewed in ImageScope and representative images of specific areas of qualitative observations were exported. For our quantitative analysis, scanned slides were viewed at low magnification on the ImageScope software, and 8-11 randomly selected areas were exported into an automated cell count software (TMARKER,

Nexus). The number of areas analysed for cell count depended on the frequency of cells in each section (See Fig 2.3). Cell counts were then manually verified for every section and fed into a formula to calculate absolute number of cells/mm². The used formula was as follows:

$$\text{Cells/mm}^2 = \frac{\text{total cells counted}}{\text{total area analysed (mm}^2\text{)}}$$

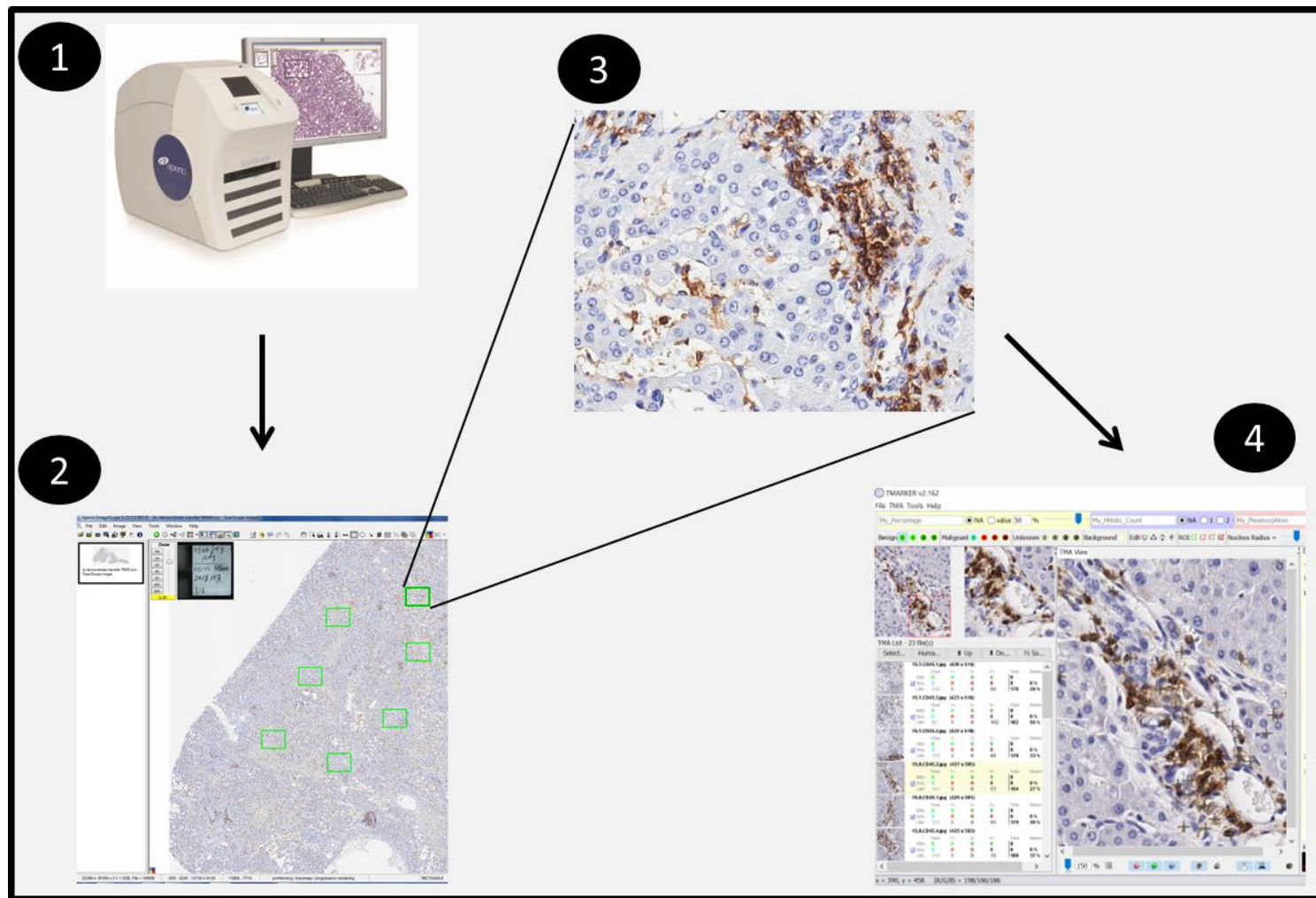


Figure 2.3: Steps of IHC analysis. 1) Stained slides are scanned with the Aperio slide scanner. 2) Scans are viewed in ImageScope and random areas of standardised size are marked at low magnification. 3) The images for each chosen area exported. 4) Images are uploaded into TMAKER for the optimised cell count algorithm.

2.10. Protein analysis

2.10.1. Protein extraction

Small sections of liver biopsies obtained at the time of transplant or from surgical resection of hepatic malignancy were homogenised for total protein extraction. Biopsies (average of 3-5mm²) were weighed and then incubated in 300 µL mix of radioimmunoprecipitation (RIPA) lysis buffer and protease inhibitors (6.5µL/mL aprotinin, 2 µL/mL sodium orthovanadate, 1 µL/mL leupeptin, and 10 µL/mL phenylmethylsulfonyl fluoride (PMSF)). Steel beads were then added to the biopsies and lysis buffer in eppendorfs and homogenised for 45 seconds at 25 revolutions/sec in a homogeniser. Eppendorfs were then immediately centrifuged at 12,000 rpm for five minutes at 4°C. Supernatants containing total protein were stored at -20°C until needed.

2.10.2. Total protein assay

Total amount of protein was measured with BCA Protein Assay Reagents (Pierce Biotech). Reagent B and A were mixed in a ratio of 1 in 50 respectively and 100 µL was added to each well in a 96 well plate. Samples were diluted as required and 3 µL of each of the samples and standards were then added to each well. Each sample/standard was tested in triplicate. The plate was incubated for 30 minutes at 37°C, and read on a spectrophotometer at a wavelength of 562nm. Standard curves were generated each time and the slope's equation was used to calculate protein concentrations taking into account any dilution factors.

2.10.3. Protein array

The cytokine microenvironment was investigated in total protein of homogenate samples of liver donor and liver CRLM biopsies. The Human Cytokine Antibody Array kit (Abcam) was used to test total protein extracted from three metastatic livers including separate tumour and tumour adjacent samples and three liver donors following manufacturer's

instructions. The array membranes had 80 cytokine targets attached to them and were analysed with chemiluminescence imaging system producing a semi-quantitative result where the amount of each cytokine corresponded to the intensity of its signal. The array's membrane images were uploaded onto Image Lab™ software (Bio-rad) which allowed the measurement of volume intensity and mean background for each target point (See Fig 2.4). The mean background for each point was subtracted from its volume intensity and then the average negative controls' intensity was further subtracted from the volume intensity. This was then normalised by defining one array as a reference and applying the following formula: $X(Ny)=X(y) \times P1/P(y)$; Where P1 is the average signal density of positive control spots on the reference array, P(y) is the average signal density of positive control spots on array "y", X(y) is the mean signal density of spot "X" on array for sample "y", and X(Ny) is the normalised signal intensity for spot "X" on Array "y".

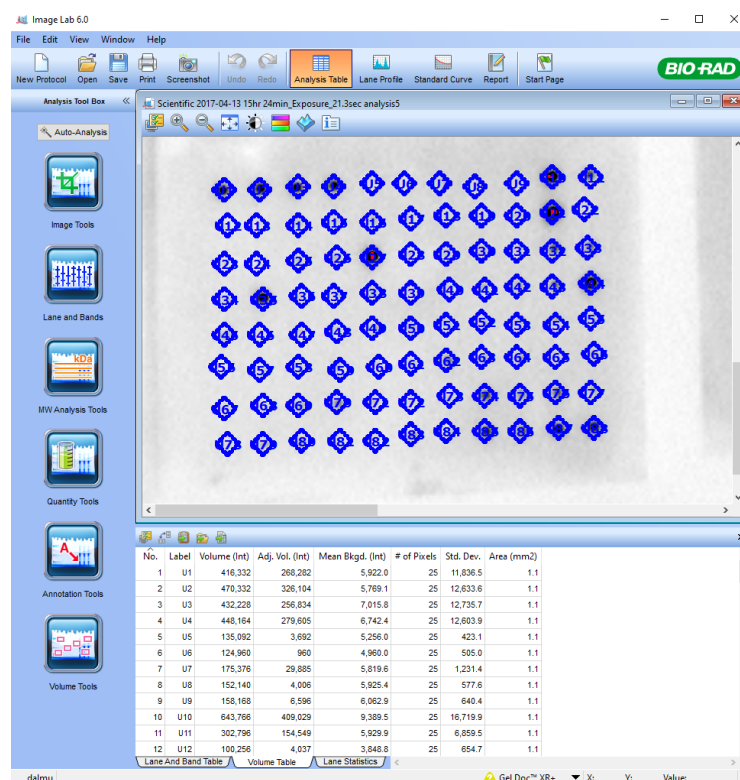


Figure 2.4: Image Lab™ volume analysis of chemiluminescence images.

2.10.4. Enzyme-linked immunosorbent assay (ELISA)

Concentration of IL-10, and IL-6, CXCL9, CXCL10, CCL8, and IFN- γ were quantified with their respective Human ELISA Kits according to the manufacturer's instructions (Peprotech). Samples were assayed in triplicate. The substrate used to visualise result was TMB with sulfuric acid as a stop solution after colour development, and measured at 450 nm with wavelength correction at 550 nm. Standard curves were generated each time and the slope's equation was used to calculate target concentrations taking into account any dilution factors. Results shown were normalised to weight of homogenised tissue, or to total amount of protein in each sample as indicated.

2.11. Purification of CD8+ T-cells

CD8+ T-cells were isolated with the EasySep™ Human CD8+ T-Cell Isolation Kit (STEMCELL) by immunomagnetic negative selection. The kit targets non-CD8+ T-cells for removal with antibodies and magnetic particles leaving desired cells behind. PBMCs isolated from buffy coats were resuspended at 5×10^7 cells/mL in EasySep™ buffer (STEMCELL). Up to 2 mL of sample was added to a 5 mL polystyrene round-bottom tube, and 50 μ L/mL of isolation cocktail was added to each sample and incubated at room temperature for 5 minutes. RapidSpheres™ were then vortexed for 30 seconds and 50 μ L/mL of it was added to each sample tube, and topped up to 2.5 mL with EasySep™ buffer, then incubated at room temperature for 3 minutes in the magnet. The desired cells were then poured out into a fresh tube, washed once, counted, and used in experiments.

2.12. Examining expression of CXCR3 in-vitro

To test CXCR3 expression on both activated and un-activated CD8+ T-cells. Isolated CD8+ T-cells were resuspended in total RPMI at 1×10^6 cells/mL. rounded-bottom 96-well plate was pre-coated with 1 μ g/mL anti-CD3 overnight at 4°C. 200 μ L of cells were aspirated into each well of the 96-well plate with 3 μ g/mL of anti-CD28. Wells either had both CD3 and CD28

for T-cell activation, or neither. Plate was incubated in 5% CO₂ in air, in 37°C for 72 hours. Samples were collected, washed once, and stained with viability dye, CD8 antibody, and CXCR3 antibody for flow cytometry analysis (See section 2.8 for details).

2.13. Cell culture of SW620 cell line

Cell culture and handling of blood samples and tissue biopsies were carried out according to strict SOPs. Cell culture was carried out in sterile conditions using laminar flow hoods and sterile or autoclaved materials and reagents. The human metastatic colon cancer cell line SW620 was cultured in vented 75cm² flask in 5% CO₂ in air, in 37°C incubator. Growth media consisted of RPMI containing L-glutamine, 10% v/v FCS, 100U/mL penicillin and 100µg/mL streptomycin. Cells were washed once in sterile PBS and covered in fresh total RPMI every 48-72 hours until it reached 70-90% confluency. Cells were then washed once in sterile PBS, and detached using trypsin. Detached cells were then washed once in total RPMI and counted for further experiments.

2.14. Preparation of SW620 conditioned media

SW620 cells were diluted to 1x10⁶cells/mL of total RPMI and 500 µL were loaded to each well of a 24-well plate. The plate was then incubated overnight in 5% CO₂ in air, in 37°C incubator to reach adherence. The media was then removed from the wells and one half of the plate was loaded with fresh total RPMI for the development of basal SW620 conditioned media. The other half of the plate was loaded with 10 µg/mL IFN-γ for the development of stimulated SW620 conditioned media. The plate was incubated in 5% CO₂ in air, in 37°C for 48 hours. Following incubation, supernatants from each well were collected and centrifuged at 350 rcf for 5 minutes. Supernatants were transferred to fresh eppendorfs and stored at -20°C until needed.

2.15. Chemotaxis assay

The chemotactic properties of liver conditioned media and the cell line, SW620, conditioned media were tested in a transwell chemotaxis assay following previously described protocol (Conroy et al., 2016). Inserts containing polycarbonate membranes with 5 μm pores were inserted in a sterile 24-well plate, creating an upper and lower chamber. The lower chamber was filled with 600 μL serum free RPMI supplemented with:

- A. 10% liver conditioned media, for our ex-vivo experiments.
- B. 40% SW620 conditioned media, for our in-vitro experiments.

PBMCs isolated from healthy donors were starved in serum free RPMI for two hours in 5% CO_2 in air, in 37°C incubator. PBMCs were then transferred to fresh serum free RPMI media and 1×10^5 cells were applied to the upper chamber of the wells and incubated for two hours in 5% CO_2 in air, in 37°C incubator. Inserts were then removed and media from the lower chamber was aspirated into flow tubes. Samples were centrifuged at 350 rcf for 5 minutes, and cell palettes were resuspended in PBA buffer (PBS containing 1.5% v/v BSA and 0.2% sodium azide (NaN_3)). Cells were stained with viability dye and markers of interest, and analysed by flow cytometry with the use of count beads (See section 2.8 for details). Experiment samples were run in duplicates for the liver conditioned media and triplicates for the cell line conditioned media.

2.16. Effect of blocking CXCL9 on immune cells' chemotaxis

To test the effect of blocking CXCL9 on PBMCs' chemotaxis, both liver conditioned media and IFN- γ -stimulated SW620 conditioned media were divided to two portions. One portion of each was incubated with 10 $\mu\text{g/mL}$ of anti-CXCL9 for one hour prior to loading the lower chamber of the transwell chemotaxis assay as mentioned above in section 2.13. the other untreated portion was used as a control. Experiment samples were run in duplicates for the liver conditioned media and triplicates for the cell line conditioned media.

2.17. Statistical analysis:

Statistical analysis was carried out using Prism GraphPad Version 8.0. Appropriate statistical tests were used to compare data in selected experiments. Due to small sample size, we could not assume the data followed normal distribution. Comparison of more than two groups was performed using Kruskal-Wallis test, with Dunn's multiple comparison test. For paired comparisons, Wilcoxon signed rank test was used. For comparison of two unmatched groups, Mann Whitney U test was used. A p-value < 0.05 was considered statistically significant. Within an experiment, * represent $p < 0.05$, and ** represent $p < 0.01$, and *** represent $p < 0.001$.

3. LOCALISATION AND QUANTIFICATION OF IMMUNE CELLS IN DONOR LIVER AND COLORECTAL LIVER METASTASIS

3.1. Introduction:

The liver's immunological roles are well described (Doherty et al., 1999, Crispe, 2009). It is designed to facilitate proficient innate immune recognition and effector function. It is also equipped to regulate early adaptive immune responses (Doherty et al., 1999). The immune system in the liver is also uniquely formed to induce tolerance to harmless stimuli including dietary antigens (Crispe, 2009). The liver's myeloid cells include resident macrophage populations, known as Kupffer cells, that accounts for up to 90% of total resident macrophages in the body (Bilzer et al., 2006). Kupffer cells are phenotypically and functionally distinct from circulating macrophages with an essential role in clearing blood of pathogens, and waste material (Naito et al., 2004). Myeloid populations in the liver also include myeloid and plasmacytoid DCs which drive both immunity and tolerance depending on cytokines in the microenvironment (Thomson and Knolle, 2010). The liver is also particularly rich in anti-tumour lymphoid populations including a high ratio of cytotoxic CD8+ to CD4+ T-cells, NK cells, $\gamma\delta$ T-cells, iNKT-cells, and MAIT cells (Robinson et al., 2016). MAIT cells, like iNKT-cells are not restricted by the classical MHC class I and II molecules. Instead, MAIT cells are restricted by the MHC-like molecule, MR1 which presents microbial-derived vitamin B metabolites (Böttcher et al., 2018), and may also have anti-tumour activity. These lymphoid populations can recognise wide range of stimuli. This includes abnormal antigens presented on MHC molecules, lack of normal MHC, stress signals, and tumour associated proteins. As discussed in detail in the chapter one; in response to tumour associated antigens and molecules, the liver's immune repertoire can initiate a potent cytotoxic response.

Despite the liver's substantially potent anti-tumour properties, a significant proportion of colorectal cancer patients develop liver metastases (Holch et al., 2017). In primary colorectal cancer, extensive work has been done to show that tumour infiltration of cytotoxic T-cells correlates to survival (Watt and House, 1978b, Galon et al., 2006, Galon et al., 2016). However, the immune environment of the gut is very distinct from the liver. Liver resident immune cells are phenotypically and functionally distinct (Norris et al.,

1998, Doherty et al., 1999). Different organs have specific immunological features, including proportions of different immune populations within the organ, and the structure of their cytokine microenvironment. Therefore, the prognostic value of immune cells in colorectal cancer may not translate to liver metastasis.

Very few studies have looked into the prognostic value of immune cell infiltration into liver metastasis. In 2009, tissue microarrays were produced from retrospectively retrieved FFPE blocks of tumours resected from patients with CRLM. These sections were then stained by immunohistochemistry for immune markers including CD3, CD8, CD45, and CD4. High CD8+ T-cells, and low CD4+ T-cells were found to be independent correlates of 10-year survival (Katz et al., 2009b). However, this group did not define different histological regions of the liver samples. They also depended on a highly automated protocol requiring sophisticated equipment, which enhances reproducibility and limits human errors but also limits availability to labs with access to this equipment. More recently, Galon's group in France quantified immune cells in more histologically defined areas of samples using fully automated IHC staining, and quantification analysis systems. Patient samples were identified as either high or low infiltrating TILs defined by "using the optimal cutoff at the cohort level calculated based on 3 hot spot (3HS) CD3 and CD8 densities from the lowest infiltrated metastasis per patient" (Mlecnik et al., 2016). A significant correlation was shown between the two groups and prognosis (Mlecnik et al., 2018). The analysis method Mlecnik *et al* used also relied on fully automated techniques with sophisticated analysis software which bears a high cost. This made their protocol highly sensitive and accurate, however it again limited the number of labs able to replicate their protocol. Both of these protocols limit the use of this analysis to well-resourced labs.

The value of assessing immune infiltration and how that links to prognosis is gathering momentum. Research has been focused on the type of immune cells accumulated in the tumours and their correlation to survival, prognosis, and response to therapy. However, researchers need to start investigating the reasons of immune exclusion in some tumours, and investigate how to enrich tumours with selected immune populations. In

order to study differences between tumours with high immune infiltration vs tumours with low immune infiltration, sophisticated protocols of immune infiltration assessment need to be simplified. Similarly, in order to use immune infiltration in clinical practice to guide patient care, we need a simplified protocol. Here we recognise the need for optimising a simplified and more manual based protocol to assess TILs in CRLM. Thus, making the assessment of immune infiltration more accessible to labs anywhere at the lowest cost possible. The quantification of TILs in CRLM may have a great prognostic value. Quantifying TILs may also present an opportunity to segregate CRLM patients into two groups independently from other clinical variables. These groups may then facilitate the investigation into the influence of the cytokine microenvironment on immune infiltration and prognosis.

Immune phenotypic analysis of the liver is mainly based on flow cytometry (Golden-Mason et al., 2000, Boisvert et al., 2003, Harmon et al., 2016). While flow cytometry is a technique that allows analysis of single cells with multiple cell markers at high throughput (Adan et al., 2017), histological techniques allows the visualisation of cellular arrangement and the localisation of cells of interest. This is important because the location of immune populations in different regions of tumour biopsies was shown to have a bearing on their prognostic value in colorectal cancer (Galon et al., 2016). Histopathological assessment is the gold standard for the diagnosis and staging of many tumours. Immunohistochemistry is a technique that utilises antibodies to bind and localise targets in a histological section; it allows visualisation of specific populations through targeting intracellular and membrane bound markers, and proteins with the use of specific antibodies (Dey, 2018). Many kits are available to link primary antibody to substrates. The Dako REAL EnVision Detection System is a powerful kit linking the primary antibody to the substrate and amplifying the signal through its unique horseradish peroxidase dextran backbone (See Fig. 3.1). Immunohistochemistry results can be visualised with immunofluorescence substrates or chromogenic substrates. On one hand, immunofluorescence allows the visualisation of several targets simultaneously through the use of different fluorochromes, however the use of chromogen substrates allows

the full visualisation of the target and surrounding cells, and may be preserved for review indefinitely (Karlsson and Karlsson, 2011). Limited immunohistochemistry studies localised immune cells in human liver tissue. One of the first studies localising leukocytes and T-cells in human liver was limited to healthy donor tissue; the study described some quantitative findings including the accumulation of T-cells around portal triads, but did not include much quantitative data (Smith et al., 2003).

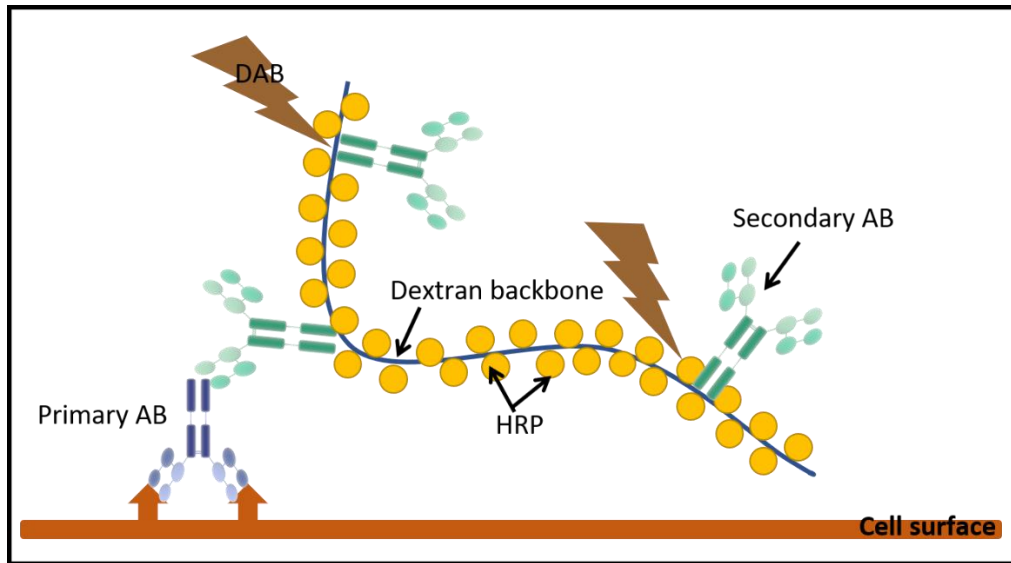


Figure 3.1: Dako™ EnVision™ Detection System.

The system consists of a dextran backbone coupled to a large number of horseradish peroxidase (HRP) and secondary antibodies. The results are visualised with diaminobenzidine (DAB) substrate giving a brown end product.

3.1. Specific hypothesis

We hypothesise that numbers of lymphocytes are altered in CRLM liver tissue compared to donor liver tissue, and that the density of T-cells in tumour tissue correlates to survival in CRLM patients.

3.2. Specific aims

- Phenotype classical T-cell populations by flow cytometry in donor liver tissue and different regions of CRLM tissue.
- Investigate quantitative alteration in MAIT cells between donor liver tissue and CRLM liver tissue.
- Define the histological features of different regions of CRLM liver tissue including tumour and morphologically normal adjacent and distal tissue.
- Localise leukocytes and T-cells in donor and CRLM liver tissue.
- Quantify leukocytes and T-cells in donor and CRLM liver tissue histologically by region.
- Analyse survival correlation of immune cells to survival months in CRLM patients at the different regions of the tumour bearing tissue.

3.3. Results

3.3.1. Study samples

A total of 110 human liver samples were collected for the work in this chapter through our collaboration with the liver unit at St Vincent's University Hospital in Dublin, the primary centre of hepatic resections and the only liver transplant centre in Ireland. The type of liver samples analysed are shown in Figure 3.2. Liver biopsies were obtained either from donor livers during transplantation, or from tumour bearing livers during CRLM resection. Donor liver biopsies were all obtained from transplantation approved organs of donors aged from 21 to 61 years. Fresh donor liver biopsies ranged in weight from 0.02-0.3g, and fresh CRLM biopsies ranged in weight from 0.05-1g. Tissue samples were digested to produce single cell suspensions, stored in formalin, and snap frozen in liquid nitrogen. Retrospectively retrieved FFPE blocks were obtained for both donor liver and CRLM tissue. CRLM liver samples, both fresh and retrospectively retrieved histology blocks, are included in the demographic table below (See table 3.1).

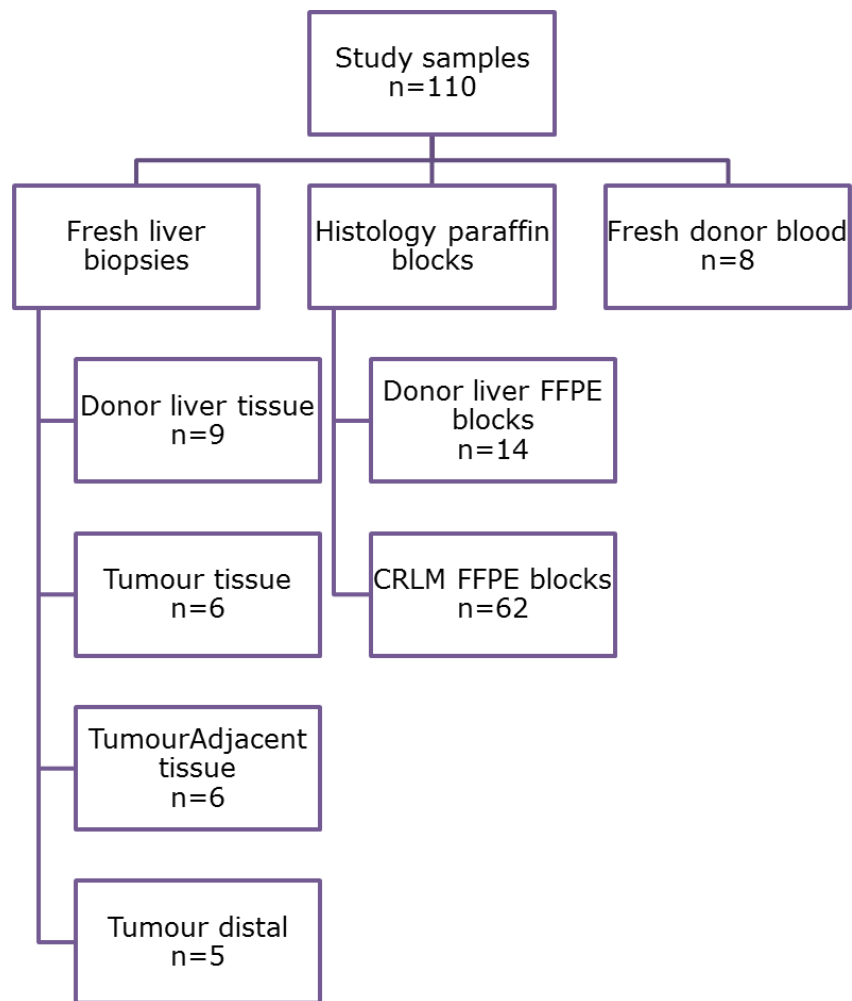


Figure 3.2: Summary of analysed patient samples in this study.

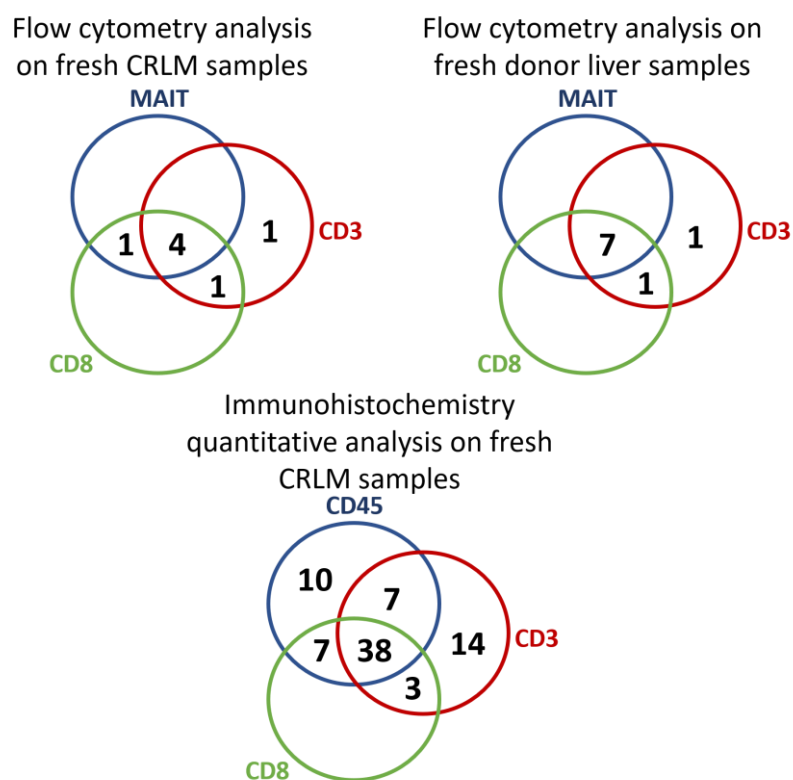


Figure 3.3: Performed flow cytometry and immunohistochemistry analysis on colorectal liver metastasis and donor liver samples.

Table 3.3.1: CRLM patients' demographics (n=63)

Characteristic		Overall survival p-value
Age		0.05
<55	25.5%	
55-65	49%	
>65	25.5%	
Number of tumours		<0.001
Median (range)	1 (1-14)	
> 1 tumour	43.6%	

Characteristic				Overall survival p-value
Tumour size (mm)				<0.001
Median (range)	32.5 (5-130)			
Neoadjuvant chemotherapy	YES (30%)	NO (24%)	UNKNOWN (46%)	0.09
Grade				0.03
Well	16%			
Moderate	76%			
Residual tumour	6%			
Steatosis				
No/yes	73.5% / 26.5%			
Overall survival				
<12 months	7.3%			
>12 months, < 5 years	38.1%			
> 5 years	54.6%			

3.3.2. Optimisation of hepatic mononuclear cell isolation

As the size of most biopsies was limited, we decided to examine two methods of dissociation and look at maximising immune cell yield. HMNC's were isolated as previously described using a collagenase-based method (Norris et al., 1998). As an alternative, we followed the same mechanical steps of the protocol but replaced the collagenase-based dissociation medium with the enzyme mix from the Human Tumour Dissociation Kit (Miltenyi Biotec) referred to as MACS, following the manufacturer's instructions only for the preparation of the enzyme mix. Analysis was carried out by flow cytometry with the use of count beads.

The same gating strategy in our flow cytometry analysis was used to assess the two dissociation protocols, as well as our liver phenotyping results (See Fig. 3.3). The FSC and SSC were used to identify lymphocytes based on their size and granularity. The gate of live cells was then drawn in the plot of SSC against viability dye. Doublets were then excluded in a plot of FSC-area vs FSC-height. A plot of SSC against the CD45 marker was then used to gate on Leukocytes; and leukocytes were then further subcategorised in a plot of CD3 against CD8 expression. Finally, MAIT cells were identified as CD45+CD161+Va7.2+ population.

We found the use of the enzyme mix from the MACS kit yielded more cells (See Fig. 3.4). Although this finding was not statistically significant, we decided to carry all forthcoming dissociations with the MACS enzyme mix.

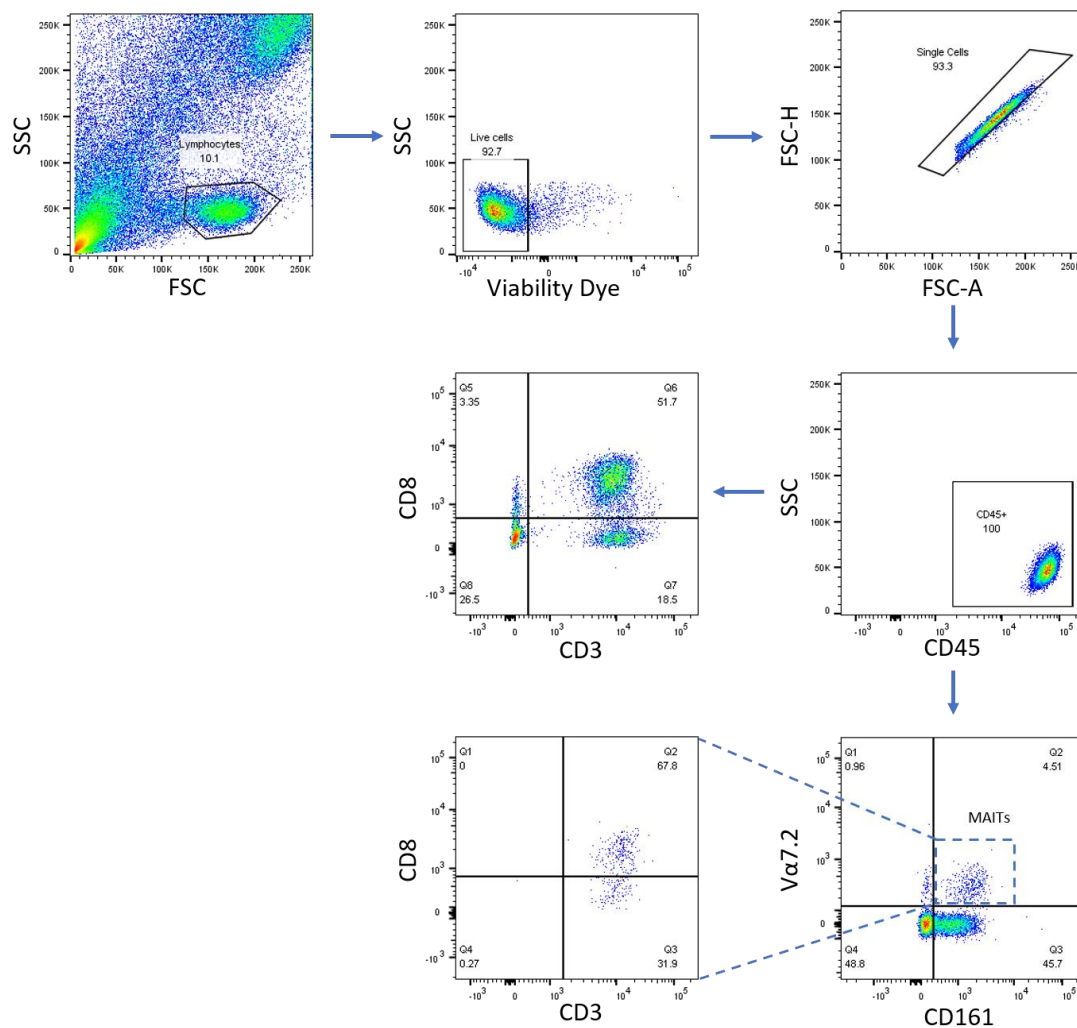


Figure 3.4: Flow cytometry gating strategy for immune phenotyping of liver tissue.

Hepatic mononuclear cells (HMNCs) were isolated by enzyme dissociation, followed by filtration through 70µm membrane eliminating debris, and low speed centrifugation to eliminate hepatocytes. HMNCs were stained with a viability dye followed by monoclonal antibodies for targets of interest. Following flow cytometry analysis, lymphocytes were identified by their size and granularity in a forward (FSC) vs side scatter (SSC) plot. Live cells were then gated through a plot of viability dye vs side scatter, and doublets were excluded in a plot of FSC-area vs FSC-height. Leukocytes were then identified as CD45+ cells vs SSC, and leukocytes were then subcategorized depending on their CD3 and CD8 expression. MAIT cells were identified as CD45+CD161+Va7.2+ population, then MAIT cells were gated on to show their CD3 and CD8 expression.

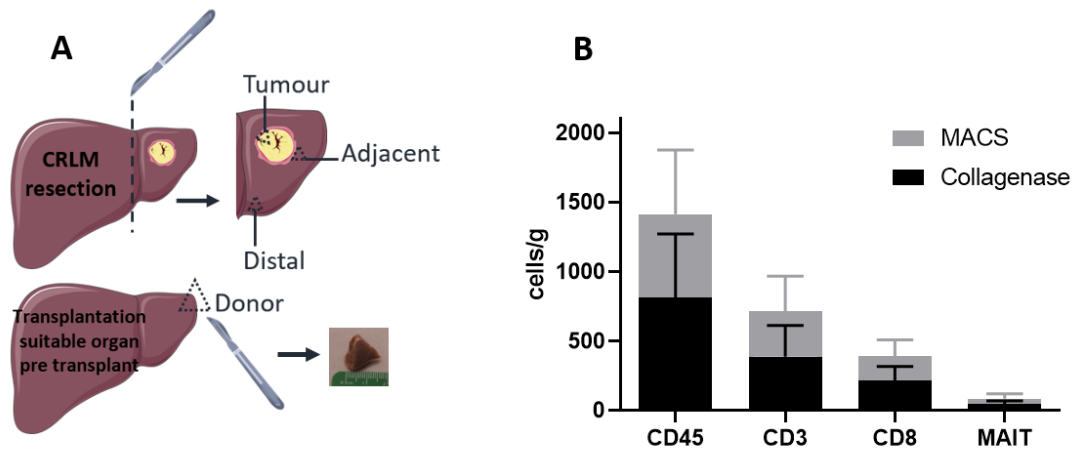


Figure 3.5: Higher numbers of leukocytes and lymphocytes were isolated using MACS enzymes.

Liver biopsies (n=3) were collected into total RPMI at the hospital and transferred to the lab for processing within two hours. Biopsies were divided into two, each was weighed, and then each piece was shaved and added onto either MACS enzyme mix according to manufacturer preparation instructions, or collagenase type IV (0.05% in hanks balanced salt solution (HBSS)). Samples were incubated at 37°C in a shaker rotating at 180rpm for 20mins, followed by filtration and centrifugation. (A) Representation of the different types of liver samples obtained during CRLM hepatectomy or orthotopic liver transplantation. (B) Absolute cell count of CD45+, CD3+, CD8+, and MAIT cells in samples using MACS enzyme cocktail vs collagenase. Data presented as mean $\pm$ SEM.

3.3.3. Phenotypic analysis of immune cells from normal and tumour bearing liver tissue by flow cytometry

We decided to start by phenotyping T-cells in donor liver tissue as well as the three different regions of CRLM tissue including the tumour, the adjacent, and the distal. Samples of CRLM tumour, adjacent, and distal tissue were identified macroscopically and sampled by a pathologist. Liver biopsies were divided into tumour tissue, adjacent tissue which is the

macroscopically normal tissue directly adjacent to the tumour, and distal which is the tissue obtained from the resection margin. HMNCs were isolated from the donor liver tissue, tumour, adjacent, and distal tissue. Cells were stained with monoclonal antibodies and analysed with flow cytometry following the gating strategy shown in figure 3.3, and immune populations were shown as a percentage of CD45+, the pan leukocyte marker.

The human donor liver has significantly more CD3+ ($70.8\% \pm 4.3\%$, $P=0.008$), CD8+ cells ($50.8\% \pm 5.3\%$, $P=0.003$), and MAIT cells ($15.4\% \pm 3\%$) compared to peripheral blood (CD3+: $34 \pm 4.9\%$, CD8+: $18.8 \pm 4.5\%$, MAITs: $1.4 \pm 0.5\%$; Fig. 3.5A,B,D). Overall, CD3+ and CD8+ populations are not significantly altered in CRLM, however looking closer into subpopulations reveals significant changes; CD3+CD8- subpopulation, which probably is mostly CD4+ T-cells, is significantly enriched in CRLM (tumour $46.7\% \pm 5\%$; donor $11.2\% \pm 5\%$; $P < 0.0001$; Fig. 3.5C), while CD3-CD8+ subpopulation, which probably is mostly NK-cells, is significantly depleted in CRLM (tumour $0.9\% \pm 4.4\%$; donor $43.6\% \pm 4.4\%$; $P < 0.0001$; Fig. 3.5C). MAIT cells were significantly depleted in CRLM tumour ($1.8 \pm 0.1\%$), CRLM adjacent ($3 \pm 0.4\%$), CRLM distal ($5.3 \pm 2\%$) compared to donor liver tissue (Fig. 3.5D). Further analysis of the CD45+CD161+Va7.2+ population the CD3+CD8+ MAIT cells were significantly decreased in CRLM ($74.4 \pm 7.2\%$) compared to donor liver tissue ($93.9 \pm 2.6\%$; $p=0.04$), while CD3+CD8- MAIT cells were increased in CRLM ($25.7 \pm 7.9\%$) compared to donor liver tissue ($6.1 \pm 2.6\%$; $p=0.06$; Fig. 3.5E).

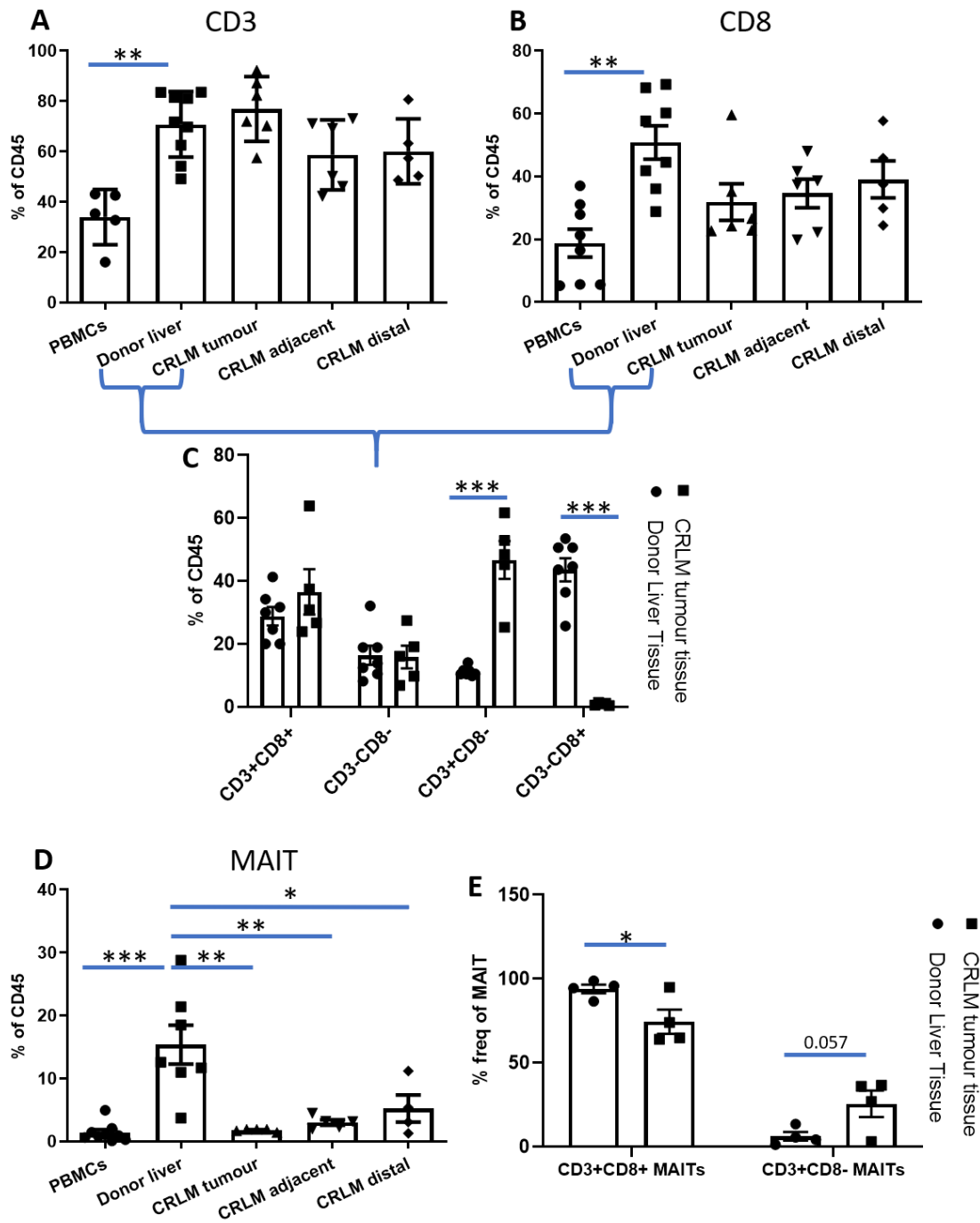


Figure 3.6: Human liver is enriched with immune populations, and these populations are affected in colorectal liver metastases (CRLM).

PBMCs ($n=8$) were isolated from fresh donor blood by density centrifugation using Ficoll-Paque™ PLUS, and hepatic mononuclear cells (HMNCs) were isolated by enzymatic dissociation from donor liver tissue ($n=8$), CRLM tumour tissue ($n=6$), CRLM adjacent ($n=6$), and CRLM distal tissue ($n=5$).

*Populations of interest were investigated through flow cytometry and analysed following the gating strategy shown in figure (3.). (A-C) Comparison of CD8+, CD3+, and MAIT cells in peripheral blood (PBMCs), donor liver HMNCs, and CRLM liver HMNCs. (D) Breakdown of CD3 and CD8 subpopulation expression in donor HMNCs vs CRLM HMNCs. (E) Breakdown of MAIT subpopulation expression in donor HMNCs vs CRLM HMNCs. Data presented as mean $\pm$ SEM. $P < 0.05$ indicated as *, $P < 0.01$ as **, $P < 0.001$ as ***.*

3.3.4. The histological features of donor and tumour bearing human liver tissue

To define and examine the histology of donor and CRLM liver biopsies, we stained sections from each with H&E. H&E is a basic histological stain known as the gold standard for diagnostics; haematoxylin stains nuclei blue and eosin stains extracellular matrix and cytoplasm pink (Chan, 2014). The liver's functional cells, the hepatocytes, are arranged in hexagonal lobules with a portal triad of a bile duct, a branch of portal vein, and a branch of hepatic artery at each corner, and a central vein in the centre. Blood from the gut, and the heart drain from circulation into the liver sinusoids through the portal triad then drain out of the liver through the central vein. Liver fatty change appears as inflated circular cells with the nucleus pushed to the side. A small degree of fatty change is still considered normal in the liver (Fig. 3.6A). changes in cellular arrangement, cell size, morphology, cytoplasm to nucleus ratio, and the uniformity of nucleus size are all indications of pathology. To investigate TILs in different regions of the tumour bearing liver, we defined each region histologically. Tumour tissue was defined as the active transformed area within the biopsy, invasive margin was defined as the junction between histologically normal and abnormal tissue, and the adjacent tissue was defined as the histologically normal area including hepatocytes, immune cells, and portal triads beyond the invasive margin (Fig. 3.6B)

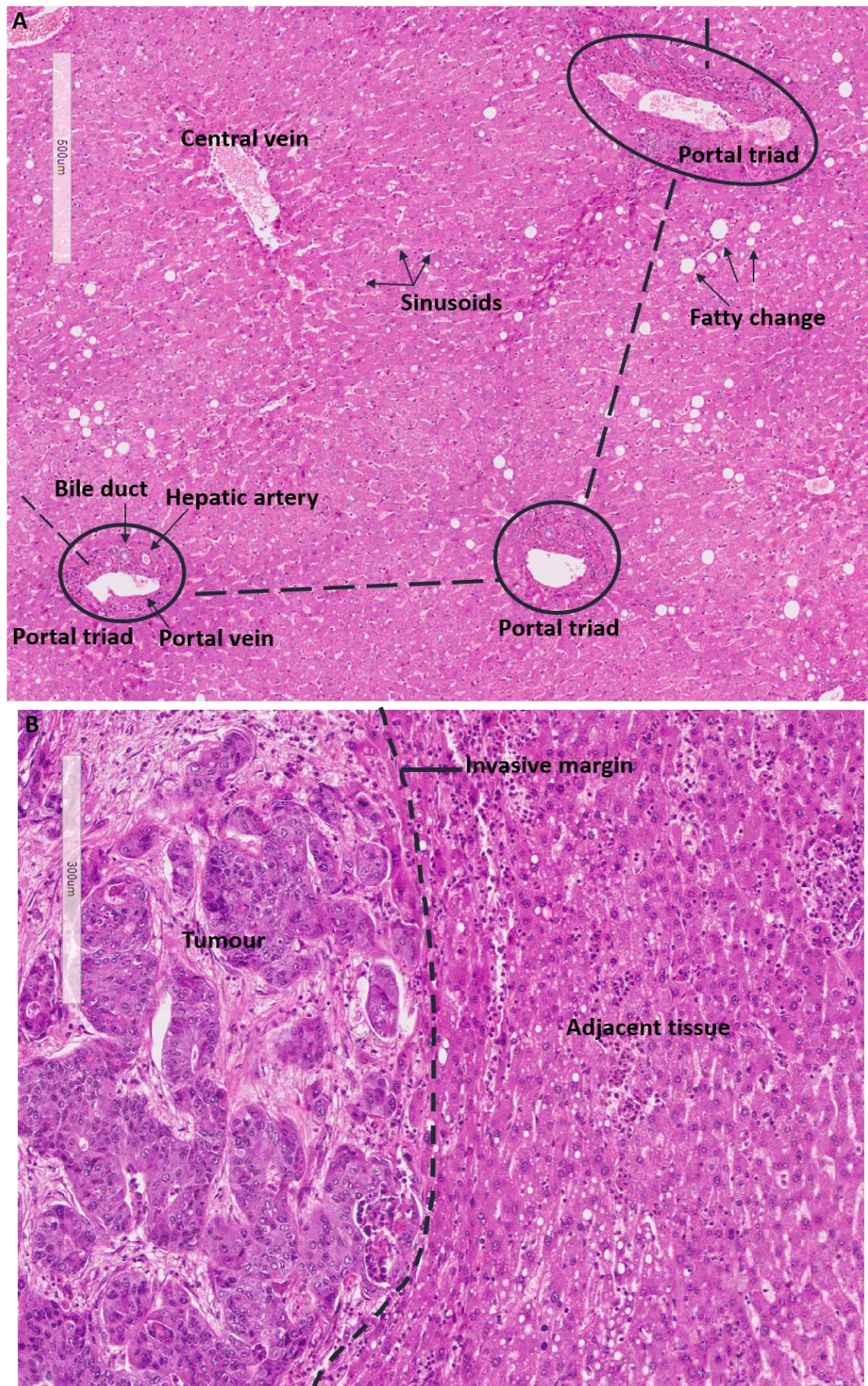


Figure 3.7: Representative histology image of normal liver and colorectal liver metastasis (CRLM) tumour tissue.

Formalin fixed paraffin embedded (FFPE) liver tissue were sectioned at 5µm and stained with haemotoxylin and eosin (H&E). Donor n=6, CRLM n=6. (A) Representative image of histologically normal liver tissue. (B) Representative image of histologically normal adjacent, invasive margin, and tumour tissue.

3.3.5. T-cell accumulate around portal triads in donor liver tissue.

CD45 is a leukocyte marker expressed by all myeloid and lymphoid populations, while CD3 is a specific marker for T-cells. Together, CD45 and CD3 allow the general and more specific localisation of the immune system in any tissue. Immune cells were targeted with CD45, and CD3 primary antibodies (Dako) in sections of donor liver, and tumour bearing liver tissue. CD45 and CD3 cells were found to cluster around portal triads compared to matched parenchyma (See Fig. 3.7). CD45 cells around portal triads (628.6 ± 126.9) were significantly higher than CD45 cells in the parenchyma (102.1 ± 16.3 ; $p=0.003$). Likewise, CD3 cells around portal triads (327.4 ± 43) were significantly higher than CD3 cells in the parenchyma (23.6 ± 4.4 ; $p=0.001$).

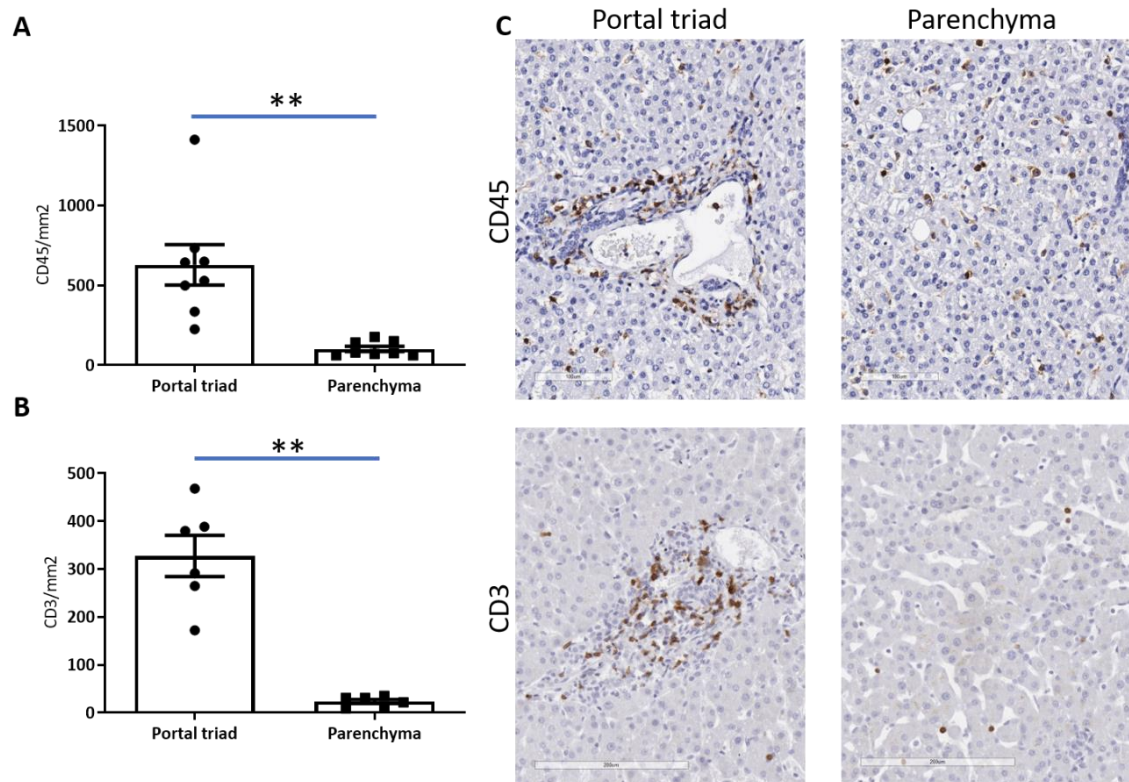


Figure 3.8: Leukocytes and T-cells accumulate around portal triads in donor liver tissue.

Formalin fixed paraffin embedded (FFPE) liver tissue were sectioned at 5 μ m and stained with monoclonal CD3 and CD45 antibodies. Absolute CD45 (n=8) and CD3 (n=6) cells were quantified in immunohistochemically stained sections of donor liver tissue and normalized to area. (A) CD45 cells (leukocytes) around portal triads vs liver parenchyma. (B) CD3 cells (T-cells) around portal triads vs liver parenchyma. (C) Representative image of positive cells around portal triads and within the liver parenchyma. Data presented as mean $\pm$ SEM. Using t-test; $P < 0.05$ indicated as *, $P < 0.01$ as **, $P < 0.001$ as ***.

3.3.6. Limitations of immunohistochemistry

Chromogen immunohistochemistry is a very challenging technique. Although the use of different chromogens as substrates to visualise more than one marker is possible, it is very difficult to optimise. Immunohistochemistry is also challenged by the area of the tissue stained,

limiting the analyses to only a small number of cells. Here we show examples of the limitations we faced and show results that prevented us from histologically quantifying NK-cells, as well as MAIT cells.

3.3.6.1. Human NK-cells in liver tissue

CD56 is a known and most widely used marker of NK-cells within the CD45+CD3+ population of cells (Montaldo et al., 2013). CD56 is also expressed by other cell types including $\gamma\delta$ T-cells, DCs, and activated CD8+ T-cells (Van Acker et al., 2017). CD56 positive cells in human hepatic tissue presented elongated morphology specifically around portal triads, and a mix of elongated and round morphologic features within hepatic sinusoids (See Fig. 3.8). based on morphology, using CD56 alone does not represent NK-cells and therefore is not a reliable marker to identify NK-cells. Positive cells shown in figure 3.8 may be other immune cells or even non-immune cells.

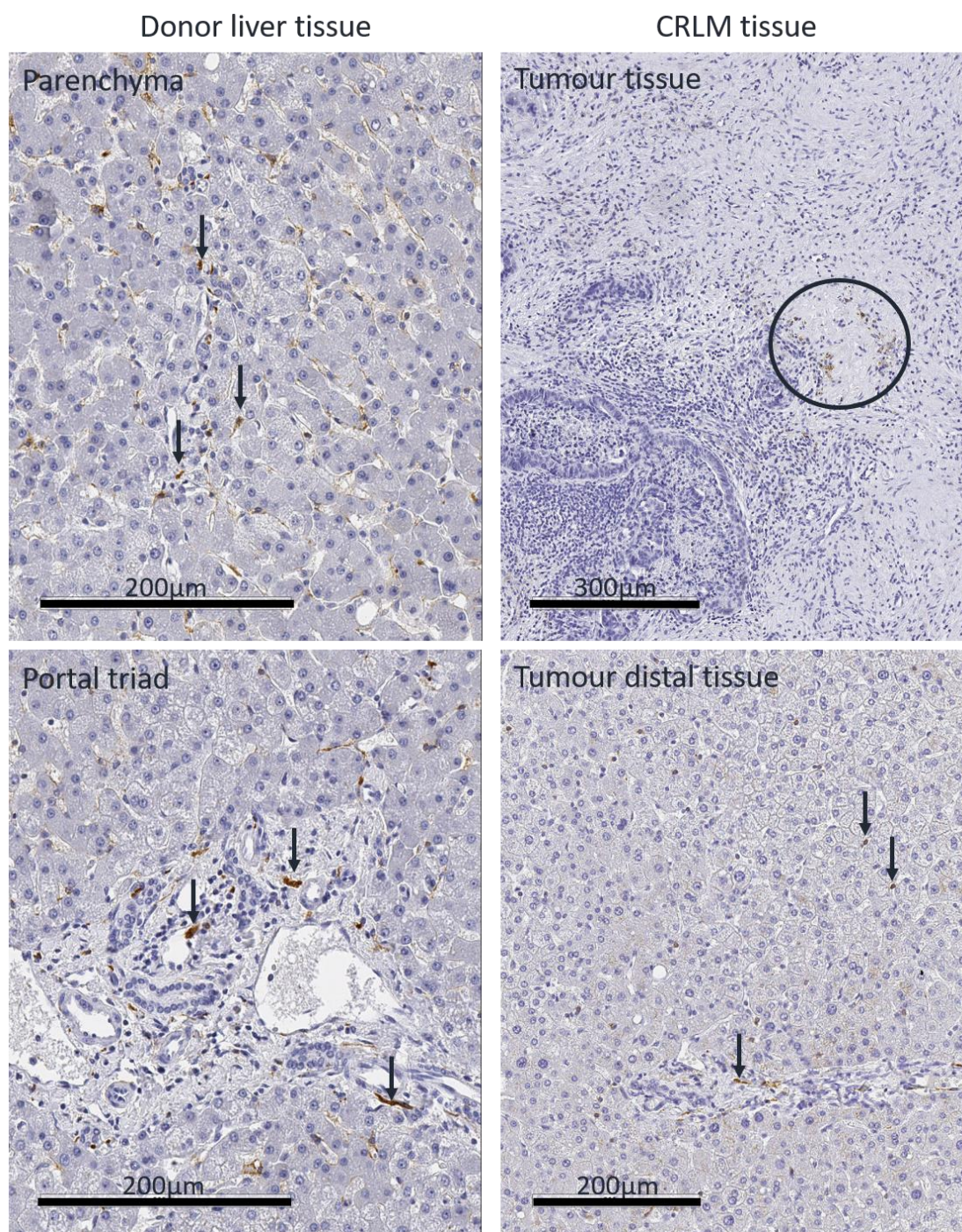


Figure 3.9: CD56+ cells are localized around portal triads and within the parenchyma in healthy and tumour bearing liver tissue.

Formalin fixed paraffin embedded (FFPE) liver tissue were sectioned at 5µm and stained with monoclonal CD56 antibody (n=3). Representative image of CD56 stained histological sections are shown here. Positive cells visualised with DAB. Few CD56+ cells are indicated by arrows in the parenchyma and around portal triads in donor liver tissue. In colorectal liver metastases

(CRLM) tissue, CD56+ cells are indicated by the circle at the edge of tumour tissue, and by arrows in histologically normal liver parenchyma distal to the tumour.

3.3.6.2. Immunohistochemistry restricts the quantification and analysis of rare cell populations

We found MAIT cells to be highly enriched in donor liver tissue and depleted in CRLM tissue (See Fig. 3.5D). However, it is still a very small population compared to T-cells in liver tissue. IHC is a technique that facilitates visualisation of target cells or proteins in biopsies that normally do not exceed 3cm³, cut into 5µm thick sections, which represents a single layer of cells. It is therefore very common to go through multiple sections without being able to localise any MAIT cells subsequently resulting in a false negative. The following figure is one example to illustrate this observation. The three pictures shown in figure 3.9 are from the same approximate location in a CRLM section stained with CD45 (A), CD3 (B), and Vα7.2 for MAIT cells (C). The pictures illustrate the infrequency of MAIT cells in comparison to other immune populations resulting in the difficulty of producing reliable quantitative data with IHC.

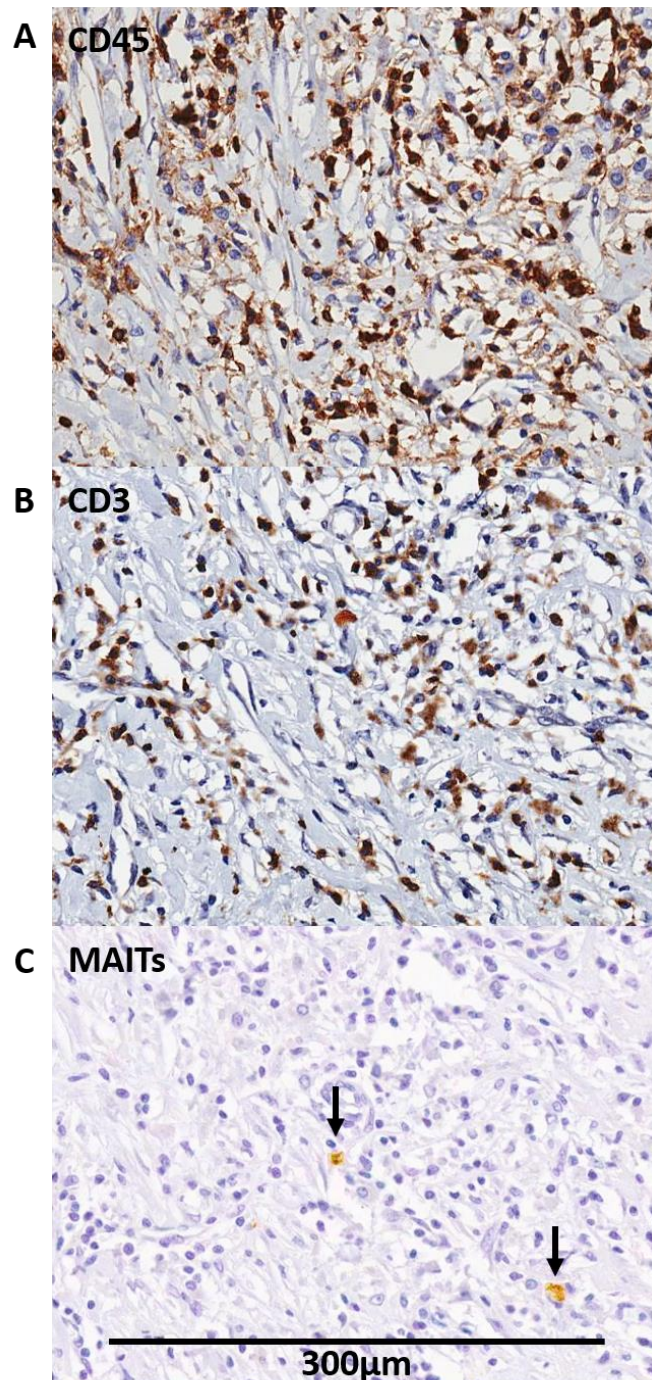


Figure 3.10: MAIT cells are identified in low numbers in CRLM FFPE sections.

Sequential colorectal liver metastasis sections were produced at 5µm thick from formalin fixed paraffin (FFPE) blocks (n=7), and stained for CD45 (A), CD3 (B), and Va7.2 (C) using the DAKO REAL™ EnVision™ Detection System kit (Agilent, USA). Pictures represent roughly the same location and illustrate the rarity of MAIT cells in comparison to CD45 and CD3 cells.

3.3.7. Leukocytes and T-cells accumulate at the tumour invasive margin

By localising leukocytes with the CD45 marker, and T-cells with the CD3 marker, we found that both populations accumulated at the invasive margin.

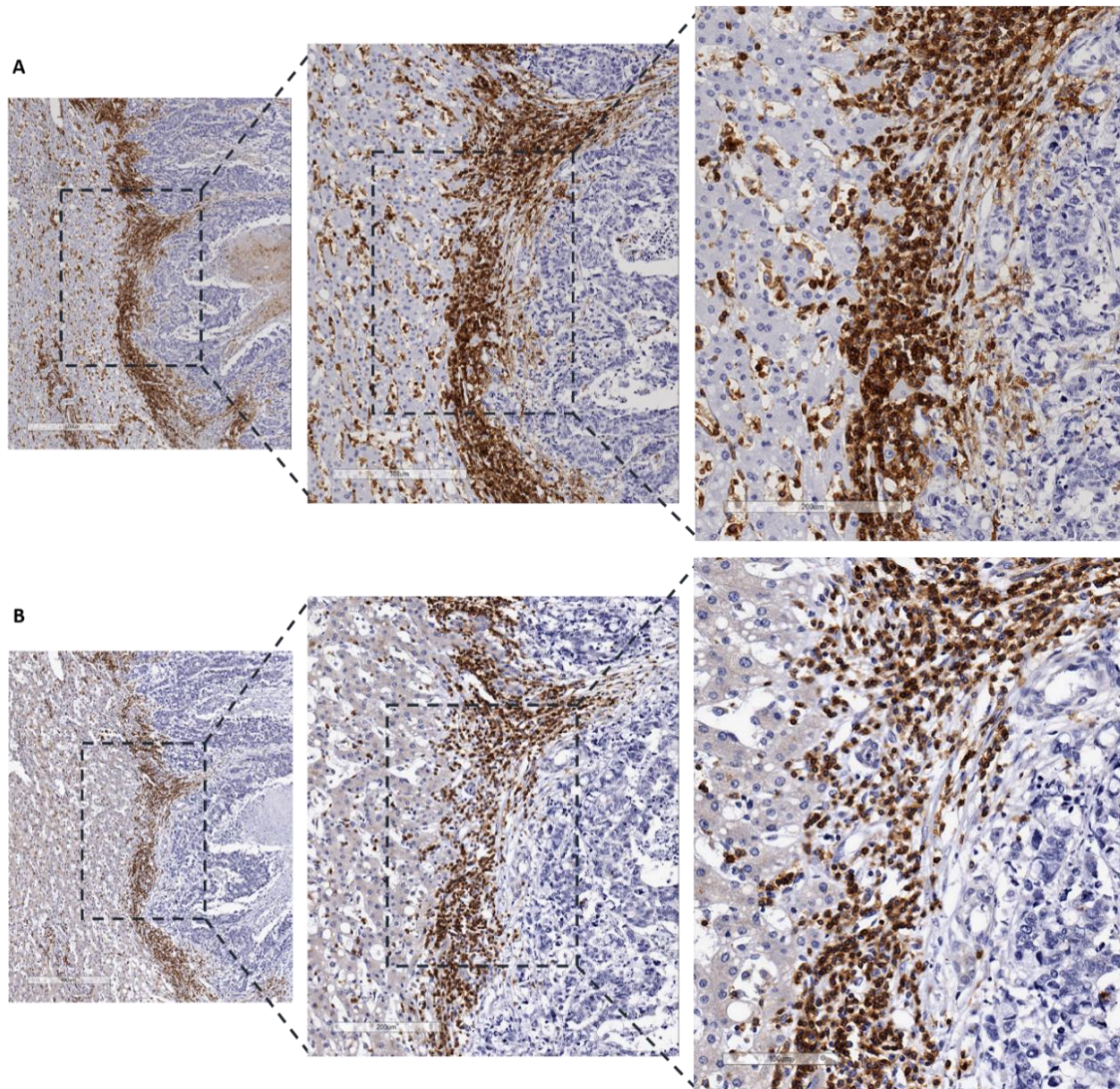


Figure 3.11: Leukocytes and T-cells accumulate at the tumour invasive margin in CRLM.

Formalin fixed paraffin embedded (FFPE) liver tissue were sectioned at 5 μ m and stained with monoclonal CD3 and CD45 antibodies (n=62). (A) Representative image of CD45 (leukocyte marker) stained CRLM section showing accumulation of CD45+ cells at the invasive margin through 3 increasing magnifications. (B) Representative image of CD3 (T-cell marker)

stained CRLM section showing accumulation of CD45+ cells at the invasive margin through 3 increasing magnifications (x5, x10, x20).

3.3.8. Infiltration of immune cells into CRLM tumours

The localisation of CD3, CD8, and CD45 cells in CRLM tissue showed most accumulation around the invasive margin separating the tumour from the morphologically normal tissue. The accumulation of immune cells at the invasive margin was interesting, and we started wondering whether their exclusion from the tumour centre is due to a physical barrier or whether it was influenced by other factors in the microenvironment. Upon closer examination, we also observed that immune cells appeared to break into the tumour bed through designated entry points. We labelled these areas as “penetration points”. This observation was evident through most of our cohort and was clear in all our high infiltration patients. Figure 3.11 is an example of the penetration points highlighted at low magnification on a CD45 stained section. Figure 3.11 also shows a penetration point at higher magnification, highlighting the same finding in both the corresponding CD3 and CD8 stained sections.

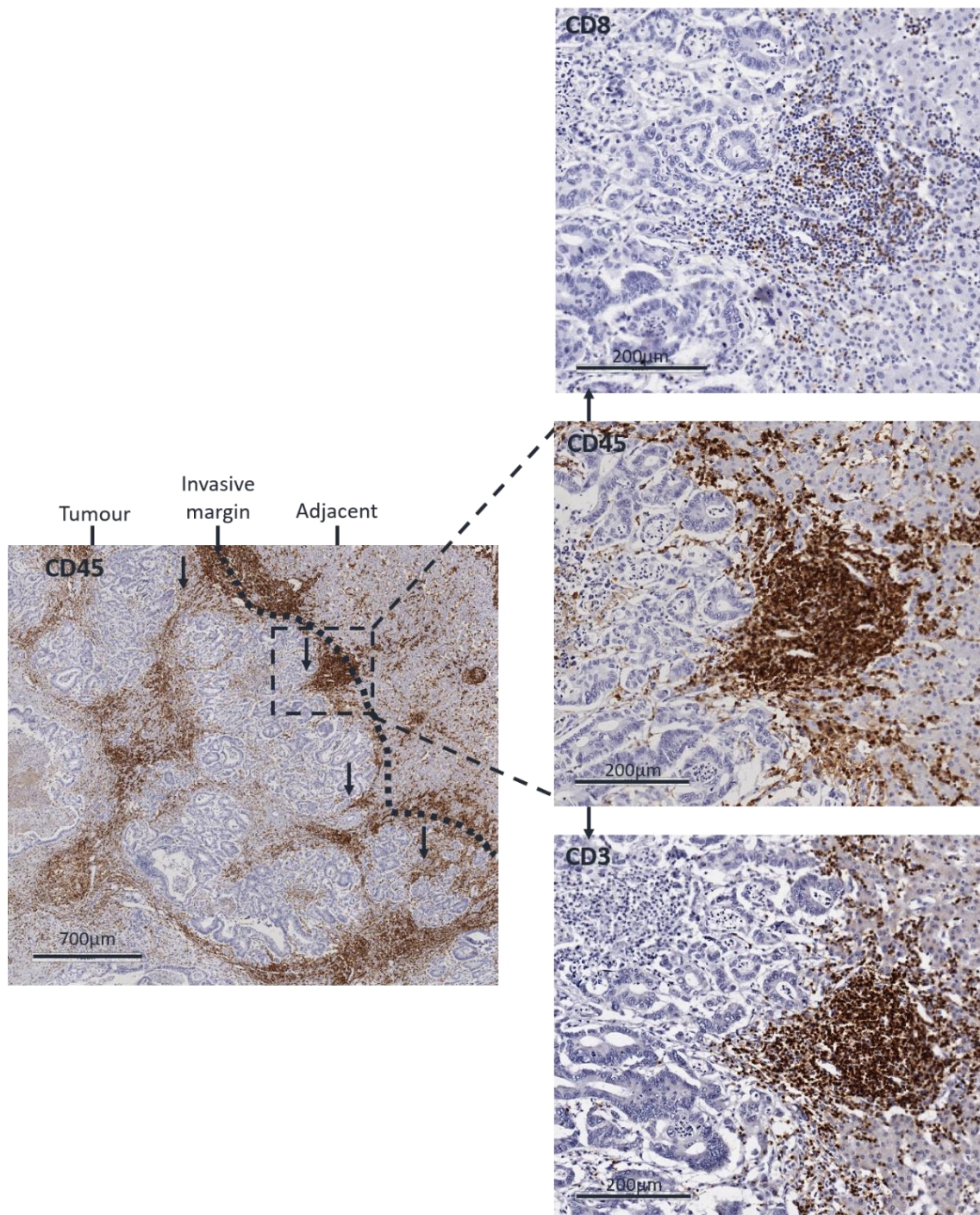


Figure 3.12: Immune cells infiltrate CRLM tumour through penetration points.

Formalin fixed paraffin embedded (FFPE) liver tissues were sectioned at 5µm and sequential sections were stained with monoclonal CD45, CD3 and CD8 antibodies (n=17). Representative image of immune accumulation of CD45, CD3, and CD8 cells at the invasive margin and their infiltration into tumour tissue through a few penetration points indicated by arrows at low magnification.

3.3.9. Quantification of tumour infiltrating lymphocytes in donor and CRLM liver tissue

We observed interesting trends in TILs in CRLM tissue with immune cells accumulating at the invasive margin and some patients having intensive immune infiltration while others exhibited no immune cells in their tumours. We decided to quantify cells in donor liver tissues as well as the different regions of CRLM tissues including the tumour, the adjacent, and the invasive margin regions. Our analysis was done on FFPE sections of donor liver, and resected CRLM tissue stained for the leukocyte marker, CD45, the T-cell marker, CD3, and the cytotoxic T-cell marker, CD8. Tumour tissue was identified as regions of abnormal cells that were morphologically similar to colon cells; tumour adjacent tissue is the morphologically normal liver tissue including hepatocytes, sinusoids, and portal triads surrounding the tumour tissue, and the invasive margin (IM) is the border at the edge of tumour and tumour adjacent tissue (Figure 3.12A). Stained sections were scanned using Aperio slide scanner (Leica). The scanned slides were then viewed on ImageScope software (Leica) and eight to ten random areas of the same size were selected at the lowest magnification possible, and extracted for further analysis from each of the tumour, adjacent, and IM regions. Extracted areas were then fed into TMARKER, a quantification software. Positive cells in the images were then quantified using the colour deconvolution plug in, and each count was verified manually.

Localisation and quantification of immune subsets highlighted a wide range of immune cell accumulation between patients. Means and standard error mean (SEM) are summarised in Table 2. The p-value in table 2 is calculated by one-way ANOVA (Kruskal-Wallis) test that to compare populations' numbers in the different regions of CRLM and donor liver. Overall, CD45, CD3, and CD8 were all significantly higher in adjacent morphologically normal tissue compared to donor tissue (see Table 2; CD45: $P<0.01$; CD3: $P<0.001$; CD8: $P<0.001$). All three cell populations were significantly higher in the IM compared to donor tissue (CD45: $P<0.0001$; CD3: $P<0.0001$; CD8: $P<0.001$). All three populations were depleted from tumour tissue when compared with the IM (see Table 2; CD45: $P<0.0001$; CD3: $P<0.001$;

CD8: $P < 0.001$). CD8+ and CD45+ populations were both significantly increased in the adjacent compared to tumour tissue (CD45: $P < 0.0001$; CD8: $P < 0.001$; See Fig. 3.12B). The cell numbers in all regions of the tumour bearing liver tissue reflected a wider range compared to cell populations in donor liver tissue (Fig. 3.12B).

Table 3.2: Mean and SEM summary for CD45, CD3, CD8 within tumour, adjacent, IM regions and donor liver tissue

	CD45 (cells/mm ²)			
Region	Tumour	Adjacent	IM	Donor
Mean	587.1	116.5	1778	474.1
SEM	72.6	81	190-7	79.7
<i>p</i> -value*	<0.0001			
	CD3 (cells/mm ²)			
Region	Tumour	Adjacent	IM	Donor
Mean	624.5	665.7	106.4	138.7
SEM	79.1	74.8	95.5	18.7
<i>p</i> -value*	<0.0001			
	CD8 (cells/mm ²)			
Region	Tumour	Adjacent	IM	Donor
Mean	215.9	410.4	512.9	79.5
SEM	43.1	52.7	85.5	14.9
<i>p</i> -value*	<0.0001			

*analysed by one-way ANOVA (Kruskal-Wallis test)

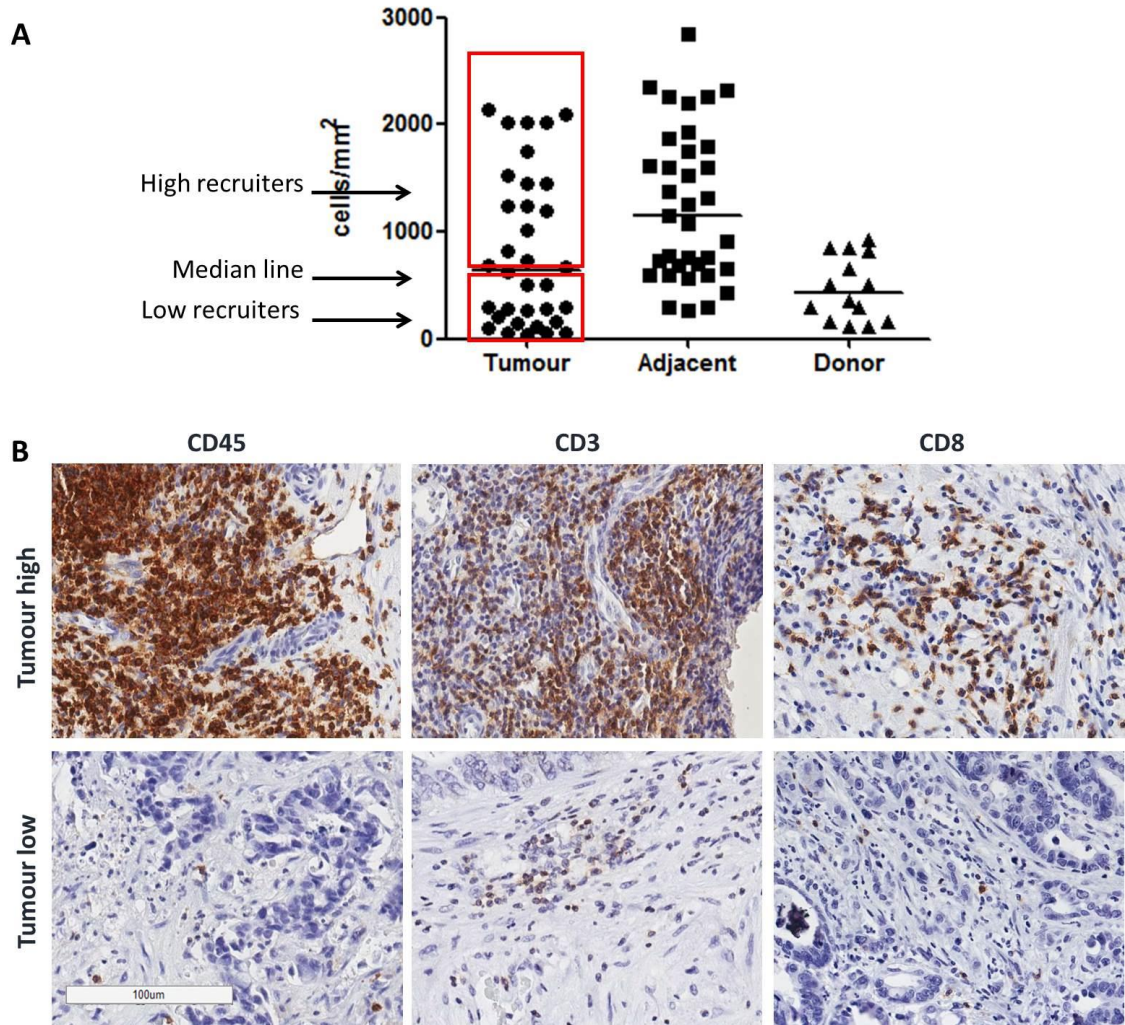


Figure 3.14: Immunohistochemistry staining of CD45, CD3, and CD8 identifies high and low immune recruiters.

(A) Hi and low recruiters in colorectal liver metastases were defined by the median. Patients who fell above the median line were identified as hi recruiters, while patients who fell below the median were identified as low recruiters. (B) Representative image of hi tumour infiltration vs low tumour infiltration for each CD45, CD3, and CD8.

3.3.10. Correlation of CD45, CD8 and CD3 populations within the tumour and at the invasive margin to overall survival in CRLM

To investigate the correlation of immune cell infiltration with survival, cells were quantified in each identified region; overall survival was calculated for all patients as the number of months of survival between resection and July 2018 or date of death. The correlation was then examined by linear regression, a test that shows whether the slope is significantly non-zero. The numbers of CD45 cells in CRLM adjacent region did not correlate to survival. Likewise, numbers of CD3 cells in CRLM adjacent region did not correlate to survival. Correlation plots of absolute cell counts to overall survival in months was significant for CD45 in tumour ($p=0.01$, $R^2=0.15$), CD8 in tumour ($p=0.002$, $R^2=0.25$), and also CD8 in adjacent ($p=0.048$, $R^2=0.13$; See Fig. 3.14).

The correlation between cell numbers and survival was then tested by log-rank (Mantel-Cox) test. Samples with cell counts above the median were considered “high recruiters” while samples that fell below the median were considered “low recruiters” (representative examples in figure 3.13)., and correlated with high and low recruitment using a Mantel-Cox test. CD8+ and CD3+ populations within the tumour were found to significantly correlate with survival (CD8: $P= 0.006$; CD3: $P= 0.039$; Fig. 3.15). CD45 at the invasive margin correlated significantly to survival ($P= 0.005$; Fig. 3.14). infiltration of CD8+, CD3+, and CD45+ in the adjacent region did not correlate to survival.

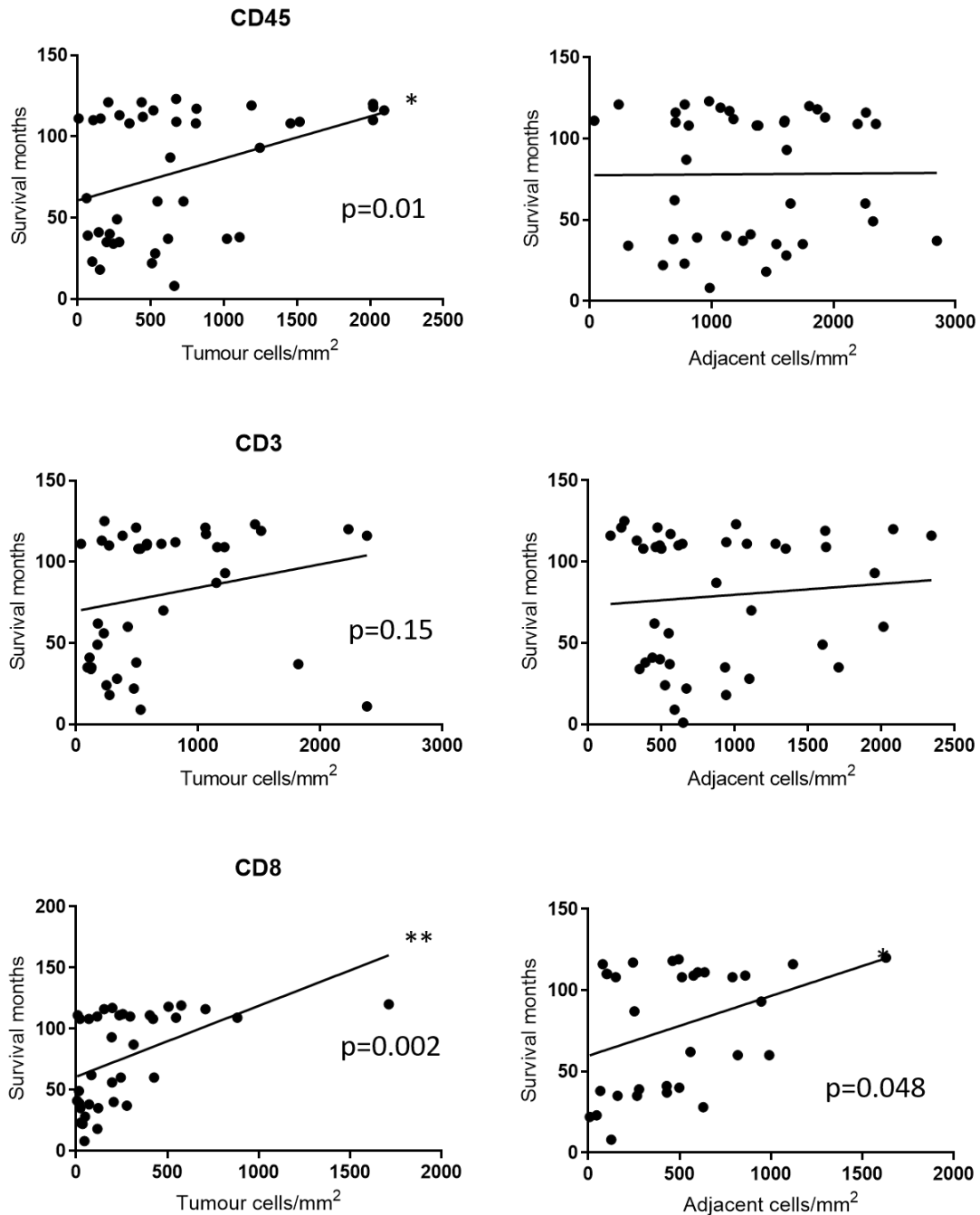


Figure 3.15: Leukocytes and CD8+ cell counts significantly and positively correlate with overall survival in patients with colorectal liver metastases (CRLM).

Linear regression graphs between overall survival in months and tumour absolute cell numbers as well as between overall survival in months and adjacent absolute cell numbers for CD45+ cells (n=41), CD3+ cells (n=41), and CD8+ cells (n=30).

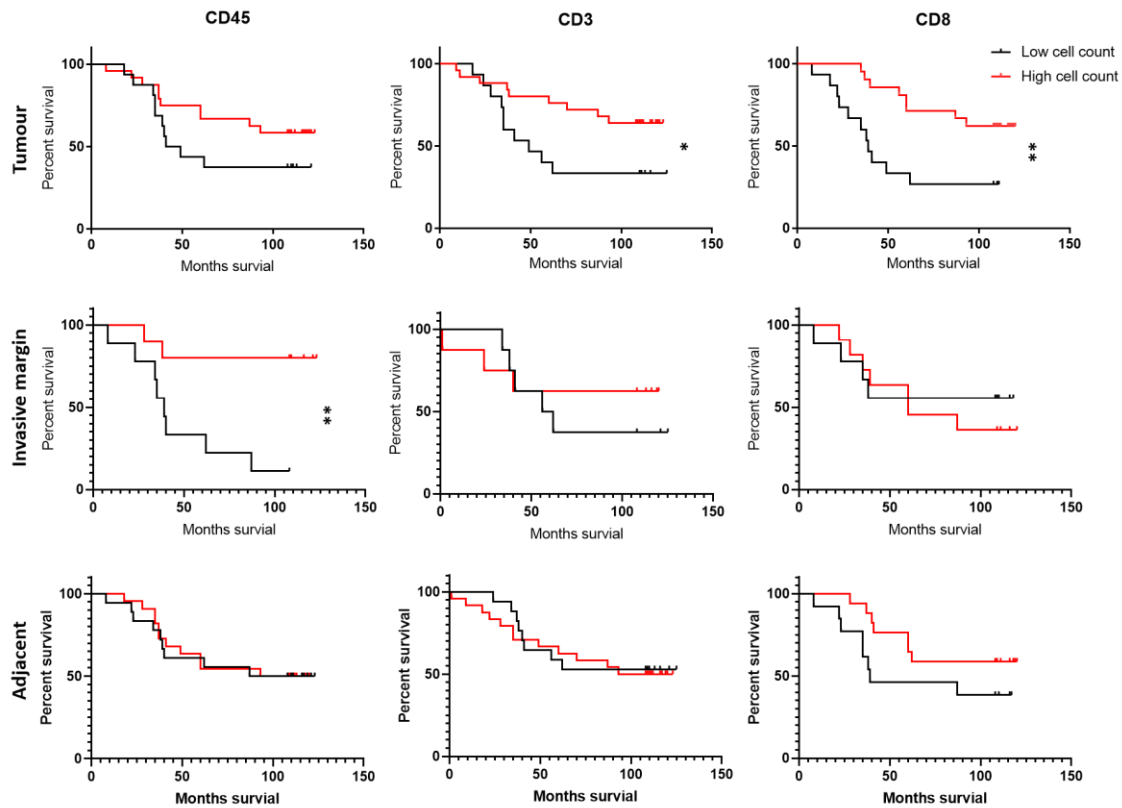


Figure 3.16: High tumour CD8 and CD3 cells, and invasive margin CD45 cells significantly reflect better overall survival.

Formalin fixed paraffin embedded (FFPE) tissue from resected colorectal liver metastasis (CRLM) that took place between 2005-2007 were recalled, sectioned at 5 μ m and stained by immunohistochemistry for CD45; n=40, CD3; n=40, and CD8; n=36. Absolute cells counts were normalised to area and Log-rank test was carried out for overall survival (months) in high vs low CD45, CD3, and CD8 counts. High and low cell counts defined as greater than the median, or lower than the median.

3.4. Chapter discussion

Here, we optimised the method of tissue dissociation to isolate hepatic mononuclear cells. In an effort to maximise the use of liver biopsies, we compared two dissociation methods and found that combining the enzyme mix by Miltenyi Biotec with the mechanical steps from our previous dissociation protocol yielded better cell numbers without increasing the total protocol time (Kelly et al., 2014). Yielding higher cell numbers meant we were able to carry out more experiments, and also allowed us to divide small biopsies to run both flow cytometry and protein analysis.

We phenotyped liver immune cells in donor liver tissue as well as CRLM tissue by flow cytometry. We demonstrated the enrichment of normal liver tissue with CD3⁺ T-cells, CD8⁺ T-cells, and MAIT cells as previously described (Doherty et al., 1999, Dusseaux et al., 2011) (Fig. 3.5A, B, D). We were also able to detect these populations in liver tissue from tumour, adjacent, and distal CRLM tissue but did not find any significant differences between the different regions of CRLM biopsies. We compared immune cells in CRLM tissue to donor liver tissue for the first time and found significant depletion of MAIT cells in CRLM. MAIT cells express MR1 antigen presenting molecule, which is highly conserved, highly responsive to bacteria and yeast, and is used for the specific detection of MAIT cells in humans and mice (Kurioka et al., 2016). Depletion of MAIT cells has been reported in autoimmune disease, however their role in autoimmune diseases is not clear yet (Kurioka et al., 2016). The role of MAIT cells in cancer is also not yet clearly described. In colorectal cancer, MAIT cells were significantly increased in tumours, and this increase correlated with unfavourable outcomes. Unlike primary colorectal cancer, we found MAIT cells to be depleted in CRLM. Although the role of MAIT cells is not clear in CRLM, their ability to produce gamma interferon, as well as their depletion in CRLM suggests they may have an important role in anti-cancer immunity. We found enrichment of CD3⁺CD8⁻ cells (which are mostly CD4 T-cells) in CRLM tumour tissue compared to donor liver tissue. CD4⁺ T-cells can be Th1 which activate cytotoxic CD8⁺ T-cells, Th2 which activate B-cells, and Tregs which suppress functions of effector T-cells (Khan et al., 2014). We did not

further investigate the CD3⁺CD8⁻ population because significant time was invested in developing the chromogen immunohistochemistry protocol, which only allowed investigating single targets. The different functions of these subpopulations make it hard to predict the overall influence on tumour progression. We also observed that CD3⁻CD8⁺ cells, which are mostly NK-cells and MAIT cells were also depleted in CRLM tumour tissue compared to donor liver tissue as previously described (Harmon et al., 2019a) (Fig. 3.5D). As CRLM biopsies have different densities than donor or healthy liver, and as CRLM biopsies often include necrotic portions, Flow cytometry is not a reliable technique for absolute cell count comparison. Normalising cell count to tissue weight is problematic when the compared tissues have different components with different densities. We therefore decided to determine absolute cell counts by immunohistochemistry.

With immunohistochemistry, we demonstrated overall increases in CD45⁺ leukocyte, CD3⁺ T-cells, and CD8⁺ cytotoxic T-cells absolute cell counts in the tumour bearing tissue when normalised to area. We also noted a high range of variation in T-cell infiltration within tumour tissue which correlated to survival (Fig. 12B, 15). This could help in the personalisation of patients' therapy plane, and may also lead to new therapeutic targets. Exclusion of TILs in some patients may be due to a physical barrier surrounding the tumour bed, and may also be due to a dysregulation in the tumour microenvironment which may lead to increased lymphocyte death, or alternatively may explain insufficient recruitment. We validated my simplified protocol for the quantification and assessment of TILs in CRLM. We also compared, for the first time, the absolute number of cells in CRLM to donor liver tissue. Interestingly, many patients who fell under the median line for the number of TILs still exhibited higher number of cells compared to donor liver tissue. We considered the source of tumour infiltrating leukocytes. One possible explanation would be local proliferation of resident cells. T-cell proliferation and an important and natural function. Loss of proliferation capability is possible and has been reported in aging (Franceschi, 1989). Proliferation can also be affected by components of the cytokine microenvironment including IL-10 and TGF- β (Taylor et al., 2006). Another explanation would be recruitment of cells into the liver and the

effect of chemokines in the tumour microenvironment (Balkwill, 2004). We believe that it is a combination of both and not one or the other.

Our CD45 IHC staining was carried out with a CD45 antibody rather than a CD45RO antibody. CD45 has a transmembrane structure, with an extracellular domain varying in length depending on combinations of exons used from the CD45 mRNA (Streuli et al., 1987). CD45RA contains exons 3, 4, and 7, and is known to be a naïve phenotype. CD45RO contains exons 3 and 7, and reflects a memory phenotype (Mahnke et al., 2013). Previous work done to assess the prognostic value of tumour infiltrating CD45 cells focused on CD45RO. Those previous studies reflected significant correlation to overall, and disease free survival in several solid tumours (Hu and Wang, 2017). This may explain why our assessment of CRLM infiltrating using our CD45 antibody did not reflect significant correlation to overall survival in both the tumour and adjacent regions. However, it is still very interesting that CD45 cells at the invasive margin significantly correlated to overall survival.

Having optimised a protocol for TIL assessment in CRLM tissue, and found that higher TIL recruiters have better overall survival, we decided to examine the cytokine microenvironment of CRLM aiming to identify key factors affecting immune recruitment in CRLM.

4. ALTERED INFLAMMATORY MICROENVIRONMENT IN COLORECTAL LIVER METASTASES

4.1. Introduction

In the previous chapter, we utilised immunohistochemistry to localise CD45+ leukocytes, CD3+ T-cells, and CD8+ cytotoxic T-cells. We developed a protocol to quantify positive cells and normalised cell counts to area. We then graphed the data and used the median to divide patients into one of two groups, high recruiters and low recruiters. Our analysis on the correlation between immune recruitment and survival showed that high recruiters of CD3+ and CD8+ cells in CRLM tumours had significantly higher survival. Simultaneously, we showed that patients who are low recruiters of CD3+ and CD8+ cells in CRLM tumours had significantly lower survival. Our data on the density and location of immune populations reflected a group of patients highly capable of recruiting significant numbers of T-cells to the site of the tumour, and another group of patients significantly less efficient in recruiting T-cells. This may be due to a defect in the production of trafficking signals in the form of chemokines. Alternatively, it may be that defects in the responses of immune populations to trafficking and activation signals leads to lower tumour infiltrating lymphocytes in some patients.

The liver's position and physiology makes it the first to encounter metastatic cells that originate in the gut (I Valderrama-Treviño et al., 2017). Blood from the gut including the spleen, stomach, pancreases, small intestine, and colon is filtered through the liver before it travels to the heart. Besides pathogenic and commensal derived molecules, blood from the gut also contains metastatic cells. The liver is therefore at high risk of metastasis. We mentioned earlier known facts about the enrichment of immune populations in the liver. We also validated these facts in the previous chapter. However, despite the liver's extensive anti-tumour immune repertoire, including both myeloid and lymphoid populations, it is highly susceptible to metastasis.

To support its functions, the human liver is in a state of constant physiological inflammation which is required for homeostasis and normal tissue activity in the presence of microbial and dietary stimulants coming from the gut (Knolle and Gerken, 2000). This haemostatic state is carefully

regulated and maintained by a network of cytokines (Robinson et al., 2016). As immune populations can be activated and inhibited by cytokines, the cytokine microenvironment is undoubtedly essential for tumour establishment and promotion (Balkwill et al., 2012). Studies highlighted roles for different cytokines in both tumour promotion, and tumour inhibition. One example is IFN- γ inhibition of tumour cell growth (Burke et al., 1999), while another example is the tumour promoting role of TNF- α which is mediated by increasing myeloid cell recruitment into the tumour microenvironment (Charles et al., 2009). Likewise, multiple cancer types have been associated with changes in their chemokine microenvironment. This dysregulation was linked to promoting tumour progression by facilitating immune evasion and formation of new blood vessels. on the other hand, chemokines can drive effector T-cell recruitment which is required to eliminate tumour cells (Balkwill, 2012). Changes in the tumour cytokine microenvironment are caused by mutations as well as changes in the immune repertoire.

Very little is known about the changes in the cytokine microenvironment of CRLM. A couple of earlier studies looked into levels of specific targets in extracted total protein in CRLM tissue and found that IL-10, IL-12, and IL-18 were elevated compared to donor liver total protein, while levels of IL-15 were lower in CRLM compared to donor liver (Kelly et al., 2004, Kelly et al., 2006a). More recent work investigated CRLM tissue conditioned media and described a shift in the cytokine milieu which is linked to numbers of neutrophils in CRLM tumours (Hand et al., 2018a). However, beyond this not much has been done on the effects of the cytokine microenvironment and specially on possible links to tumour lymphocyte infiltration in CRLM.

The cytokine microenvironment is an essential component of the tumour environment. We discussed in chapter one the different types of cytokines and their effect on shaping the immune environment including immune cell activation, proliferation, and expansion. Chemokines are a subset of cytokines known as principal drivers of cell migration and recruitment. Secreted chemokines and the expression of their corresponding receptors determine the density and type of recruited cells (Balkwill, 2004). In many cases, tumour cells are able to suppress chemokine expression by DNA

methylation to downregulate T-cell recruitment and facilitate immune evasion (Johnson et al., 2010). Studies of CRC previously linked the chemokine CXCL9 to T-cell enrichment and survival (Mlecnik et al., 2010, Ding et al., 2016, Wu et al., 2016). However, CXCL9 has not been studied in CRLM. CXCL9 is structurally related to CXCL10 and CXCL11. All three are IFN- γ inducible chemokines. CXCL9, CXCL10, and CXCL11 bind the receptor CXCR3 which is expressed on NK-cells, MAIT cells, and cytotoxic T-cells (Clark-Lewis et al., 2003, Tokunaga et al., 2018).

4.2. Specific hypothesis

To identify possible factors influencing immune cell recruitment into CRLM tumours, we decided to investigate and analyse the cytokine microenvironment of CRLM and compare it to the cytokine microenvironment of donor liver. We hypothesized that the liver cytokine microenvironment in CRLM is dysregulated.

4.3. Specific aims

- Examine changes in total protein expression in CRLM tissue compared to donor liver tissue.
- Compare total protein to secreted protein in liver tissue.
- Investigate general differences in the cytokine milieu between CRLM and donor liver tissue.
- Verify cytokines of interest with ELISA.
- Localise cytokines of interest in CRLM tissue.
- Test chemokine receptor expression in-vitro.

4.4. Results

4.4.1. Protein expression in donor liver and CRLM tissue

Besides specific changes in the cytokine milieu and its pathological effects, we wanted to investigate differences in the total amounts of protein in CRLM tumour and adjacent tissue compared to donor liver tissue. Six matched tumour and adjacent liver biopsies obtained from CRLM resections were weighed and homogenised for total protein. Five donor liver biopsies were weighed and homogenised for total protein. Total protein was measured with a BCA assay and protein expression was normalised to tissue weight (See Fig. 4.1). We found that protein expression in donor liver tissue ($224.5 \pm 39 \mu\text{g}/\text{mg}$) was significantly different from CRLM tumour tissue ($75.3 \pm 16.1 \mu\text{g}/\text{mg}$; $p=0.02$).

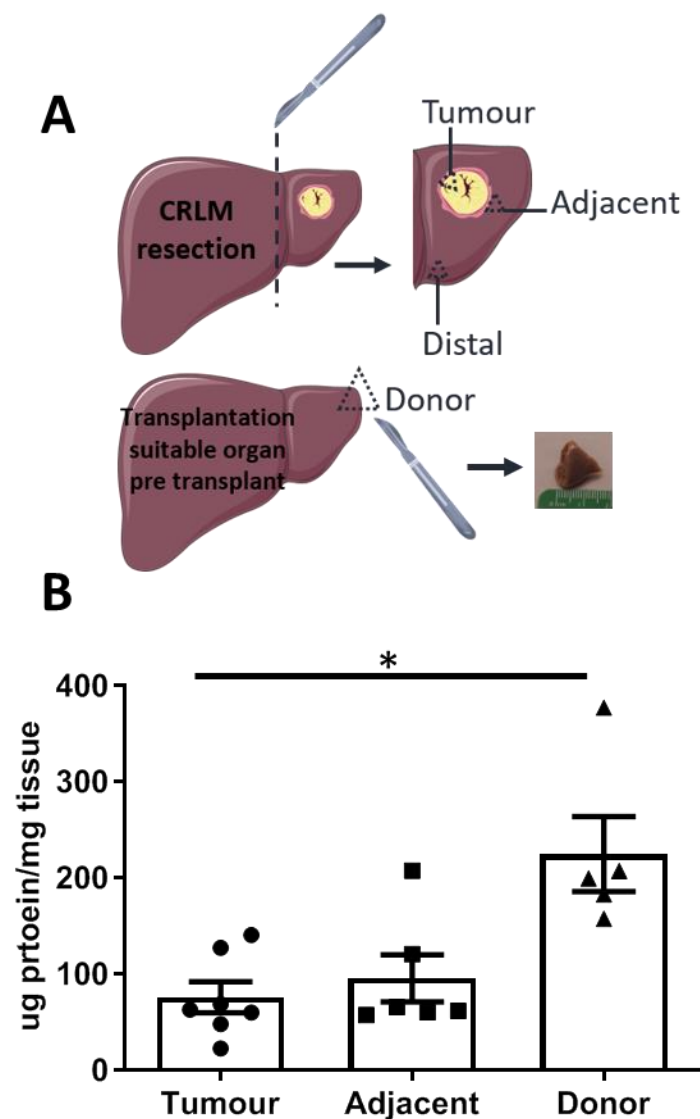


Figure 4.1: Total protein in donor liver tissue is significantly higher than CRLM tissue.

Matched tumour and adjacent tissue ($n=6$) and donor liver tissue ($n=5$) were weighed then homogenised for total protein, and the protein was measured through BCA and normalised to tissue weight. (A) Schematic of the different samples analysed. (B) Total protein of donor liver, CRLM tumour, CRLM adjacent tissue. Data presented as mean $\pm$ SEM. $P<0.05$ indicated as *, $P<0.01$ as **, $P<0.001$ as ***.

4.4.2. Protein expression varies in homogenates vs supernatants.

As previous investigation into CRLM microenvironment was carried out on tissue supernatants (Hand et al., 2018a), we wanted to investigate differences between tissue homogenates and tissue supernatants. Tissue homogenates samples are total protein extracted mechanically from tissue which was snap frozen in liquid nitrogen as fast as possible after collection. Tissue supernatants are collected 72 hours after tissue pieces are incubated in x-vivo media (Lonza) at 37°C. In theory, the cytokine milieu of homogenates total protein would include the cytokine signature of secreted and intracellular cytokines combined. On the other hand, the cytokine milieu reflected in our preparation of supernatants would represent the secreted cytokine signature of the liver microenvironment including actively produced cytokines during the 72 hours incubation period. To examine the difference of cytokine expression in supernatants and homogenates, the level of IL-6 and IL-10 were measured in both sample preparations of matched biopsies. IL-6 is essential in maintaining liver function and homeostasis, but is also linked to liver tumour development when persistently activated (Schmidt-Arras and Rose-John, 2016). IL-6 has also been linked to MDSC accumulation, and may be involved in progression of liver metastasis (Lin et al., 2019). IL-10 is involved in switching macrophages to their tumour-promoting M2 phenotype, and also promotes T-cell inhibition by inducing PD-L1 (Mikuła-Pietrasik et al., 2018).

IL-6 expression in matched tumour and adjacent tissue, as well as donor tissue seemed to be higher in supernatants compared to homogenates; IL-6 expression was higher in tumour supernatants (73.5 ± 19.7 pg/ μ g) compared to tumour homogenates (20.6 ± 6.2 pg/ μ g) and comparable in the adjacent supernatants (57.9 ± 34.5 pg/ μ g) and homogenates (46.9 ± 24.7 pg/ μ g). IL-6 expression was also higher in donor liver supernatants (69.5 ± 17.3 pg/ μ g). IL-10 expression was lower in tumour supernatants (32.6 ± 8.3 pg/ μ g) compared to tumour homogenates (77.9 ± 14.9 pg/ μ g) and lower in adjacent supernatants (56.3 ± 7.9 pg/ μ g) compared to homogenates (148 ± 46.5 pg/ μ g). Expression of IL-10 in matched tumour and adjacent tissue, as well

as donor tissue, were lower in supernatants compared to homogenates; IL-10 expression was also lower in donor liver supernatants (25.6 ± 5.3 pg/ μ g) compared to donor liver homogenates (84.1 ± 28.4 pg/ μ g). Within analysed supernatant, IL-10 was significantly higher in adjacent (56.3 ± 7.9 pg/ μ g) compared to donor (25.6 ± 5.3 pg/ μ g) supernatants ($p=0.02$). Overall it was clear that protein production and secretion may differ depending on the cytokine of question. Some cytokines may also be actively produced and/or affected by the x-vivo media and the incubation period in the process of supernatants development. The tumour microenvironment could essentially not only affect cytokine production, but also cytokine secretion from cells (See Fig. 4.2).

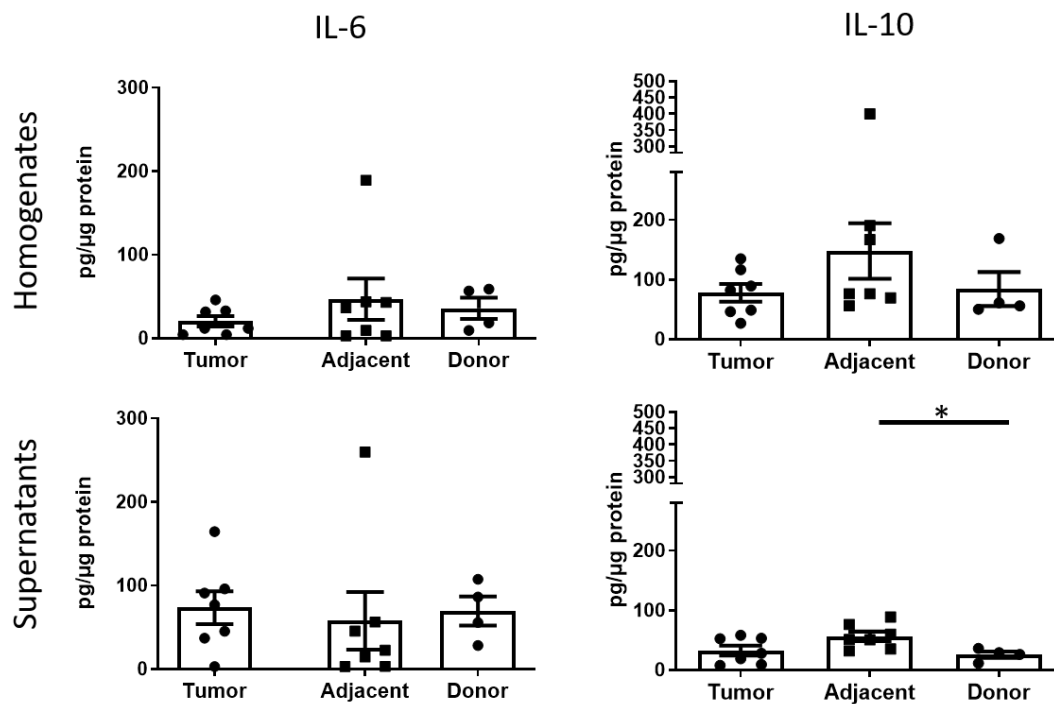


Figure 4.2: Total and secreted IL-6 and IL-10 levels vary.

Matched tumour and adjacent tissue (n=7) and donor liver tissue (n=4) were divided to two parts, one was homogenised for total protein, the other was incubated in x-vivo media (Lonza) for 72 hours and the supernatants were collected. IL-6 and IL-10 in both sample types were measured by ELISA (Peprotech) and normalised to the amount of protein in each sample.

*Data presented as mean $\pm$ SEM. $P < 0.05$ indicated as *, $P < 0.01$ as **, $P < 0.001$ as ***.*

4.4.3. Analysis of cytokine milieu in CRLM and donor liver homogenates highlights a distinct inflammatory signature

To further understand the liver microenvironment, we analysed the microenvironment of the homogenates of three CRLM tumour biopsies with their matched adjacent biopsies, and three donor liver biopsies with the human cytokine antibody array which includes 80 targets (Abcam, UK). We chose to use homogenates over supernatants based on two rationales. Our immune localisation and quantification analyses were done on biopsies that were fixed as soon as possible following sampling, so we wanted to analyse the cytokine environment in tissues snap frozen as soon as possible following sampling while avoiding any changes to the cytokine milieu which may result from incubation medium and time while preparing tissue supernatants. We also found that testing homogenates was logistically favourable. The protein array was a semi-quantitative technique based on a normalisation formula applied on intensity readings, and we added the same amount of total protein from each sample to each membrane. We found the liver microenvironment to be rich in cytokines. Interestingly, CRLM adjacent tissue had higher levels of most targets in the array compared to both CRLM tumour and donor liver tissues. On one hand, we were particularly interested in cytokines involved in T-cell proliferation including TGF, HGF, IL-2, IL-7, and IL-15. On the other hand, we were interested in trafficking mediators like CCL8 (MCP-2), CXCL10 (IP-10), and CXCL9 (MIG).

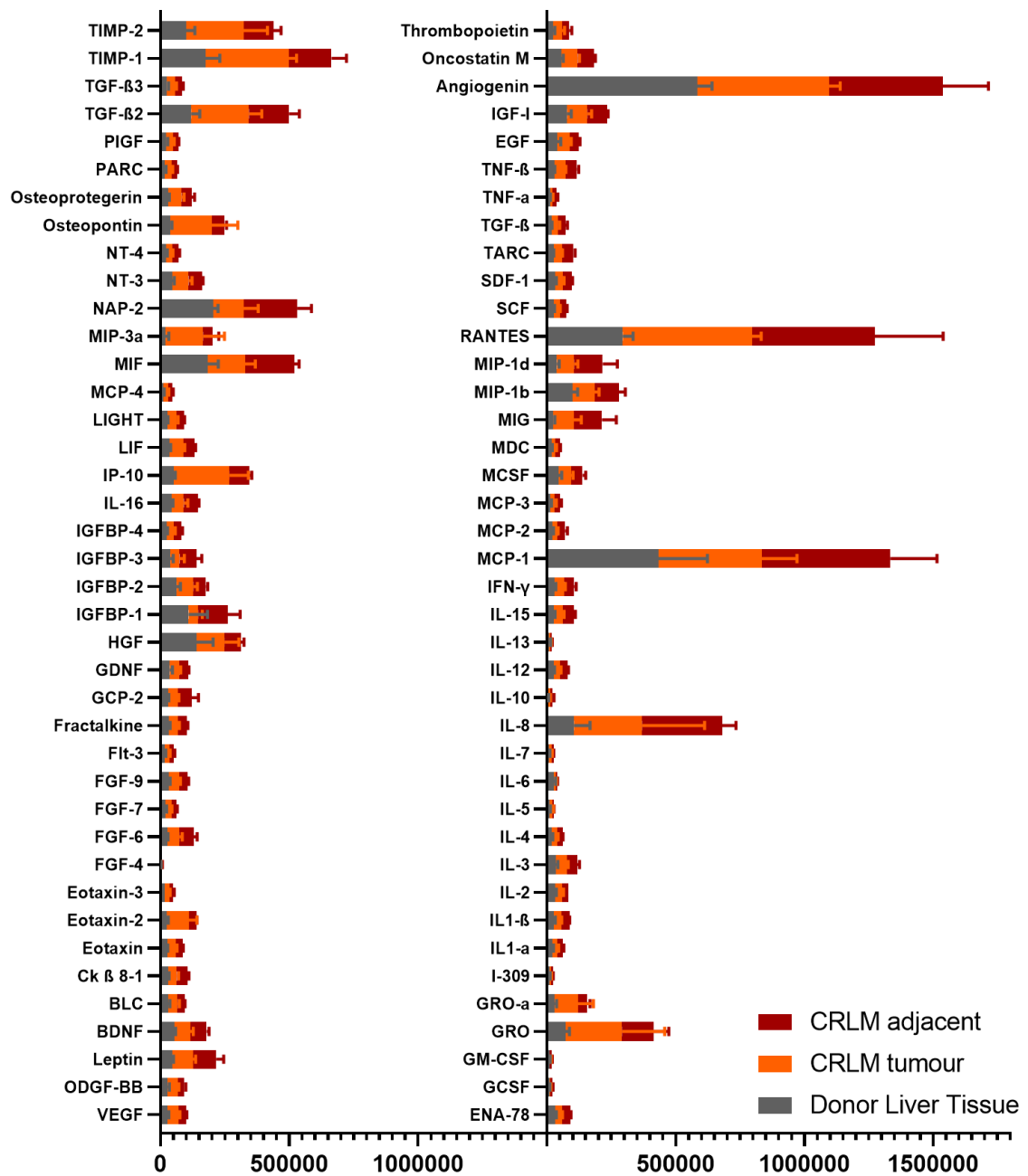


Figure 4.3: Colorectal liver metastases tumour and adjacent tissue have distinct protein profile compared to donor liver tissue.

Donor liver tissue (n=3) and matched CRLM tumour and adjacent tissue (n=3) were mechanically homogenised in RIPA buffer for total protein. 1000µg/mL from each sample was analysed with an 80-target protein array (Abcam) following manufacturer's instructions. Quantitative results were calculated and normalised following manufacturer's instruction. Data presented as mean ± SEM.

4.4.4. Validation of protein expression of selected cytokines in CRLM and donor liver tissue by ELISA

Sequential to our protein array analysis, we decided to verify the expression of CXCL9, CXCL10, and IFN- γ with ELISA. CXCL9 and CXCL10 are structurally related chemokines and are both induced by IFN- γ (See Fig. 4.4.D). Although CXCL10 did not show any significant difference between donor liver tissue and CRLM tissue (See Fig. 4.4B), we observed significant differences in CXCL9. We found CXCL9 to be significantly enriched in CRLM tumour (282.3 ± 22.5 pg/mg) compared to adjacent (103.7 ± 20.2 pg/mg), distal (117.2 ± 11.9 pg/mg), and donor tissue (172.9 ± 21 pg/mg; $p < 0.0001$; See Fig. 4.4A). We found IFN- γ to be slightly higher in the donor (178.1 ± 27.9 pg/mg) compared to CRLM (tumour: 107.4 ± 21 pg/mg; adjacent: 79.5 ± 15 pg/mg; distal: 106.2 ± 14.6 pg/mg) but that was not significant.

We also decided to verify levels of CCL8, also known as MCP2. CCL8 is involved in the recruitment of tumour associated macrophages, and Treg-cells (Cassetta et al., 2019)(See Fig.4.5A). CCL8 is induced by IL-1 and IFN- γ , secreted mainly by tumour cells and macrophages, and its effects are mediated through four different receptors including CCR1, CCR2, CCR3, and CCR5 (Ge et al., 2017). We found CCL8 to be significantly higher in CRLM tumour (155.4 ± 31.4 pg/mg) compared to donor liver (39.6 ± 9.3 pg/mg; $p = 0.008$), and also significantly higher than the adjacent (78.7 ± 26.1 pg/mg; $p = 0.046$; See Fig. 4.5B).

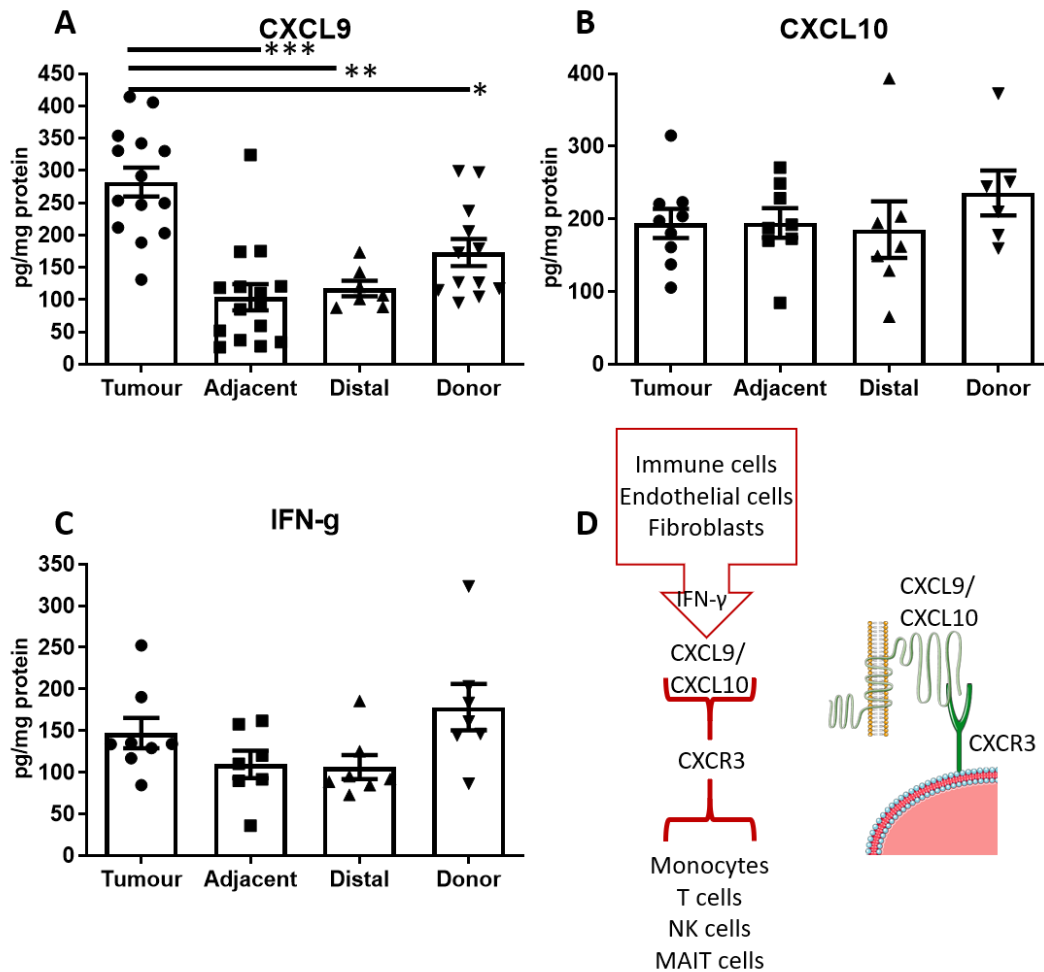


Figure 4.4: CXCL9 levels are significantly altered in colorectal liver metastases but not its structurally close chemokine, CXCL10 or their inducer IFN-γ.

*Liver biopsies from donor liver (n= 12), and CRLM tissue (n=14) were mechanically homogenised in RIPA buffer for total protein extraction. Proteins of interest were measured by ELISA (Peprotech). (A-C) levels of CXCL9, CXCL10, and IFN-γ. Data presented as mean ± SEM. P<0.05 indicated as *, P<0.01 as **, P<0.001 as ***. (D) Schematic of CXCL9 and CXCL10 expressers, inducer, and their receptor, CXCR3, expression.*

4.4.5. Expression of CXCR3

As CXCL9 was increased in CRLM tumour, we wondered whether the receptor for it, CXCR3, was affected in the tumour microenvironment. To investigate, CRLM and donor liver biopsies were dissociated into single cell suspensions, stained with a viability dye, and with an antibody mix including CD45, CD3, CD8, CD161, Vα7.2, and CXCR3 for flow cytometry analysis. Gating for CXCR3 expression was based on FMO's (See Fig 4.6). Within the CD45+ population (Fig. 4.7A), CXCR3 expression was lower overall in CRLM compared to donor liver. Within the three regions of CRLM, tumour adjacent and distal, CXCR3 expression was at its lowest in the distal. Similar trends were seen within CD3+ population (Fig. 4.7B), CD8+ population (Fig. 4.7C), and the MAIT population (Fig. 4.7D). Differences within the 4 populations were not statistically significant.

We then decided to localise both CXCL9 and CXCR3 in the tumour and adjacent tissue of CRLM. We stained for CXCL9 and CXCR3 by IHC, and identified both lymphocytes and tumour cells, as well as other structures within the histology sections based on morphology. In the adjacent tissue we found both CXCL9 and CXCR3 to be expressed by what morphologically appeared to be lymphocytes, and these cells were seen primarily around portal triads (See Fig 4.8A, D). Within CRLM tumour, we observed CXCL9 to be expressed on lymphocytes as well as cuboidal cells which resembled bile ducts (See Fig. 4.8B,C). Simultaneously, CXCR3 appeared to be expressed by both lymphocytes and tumour cells (See Fig. 4.8E,F).

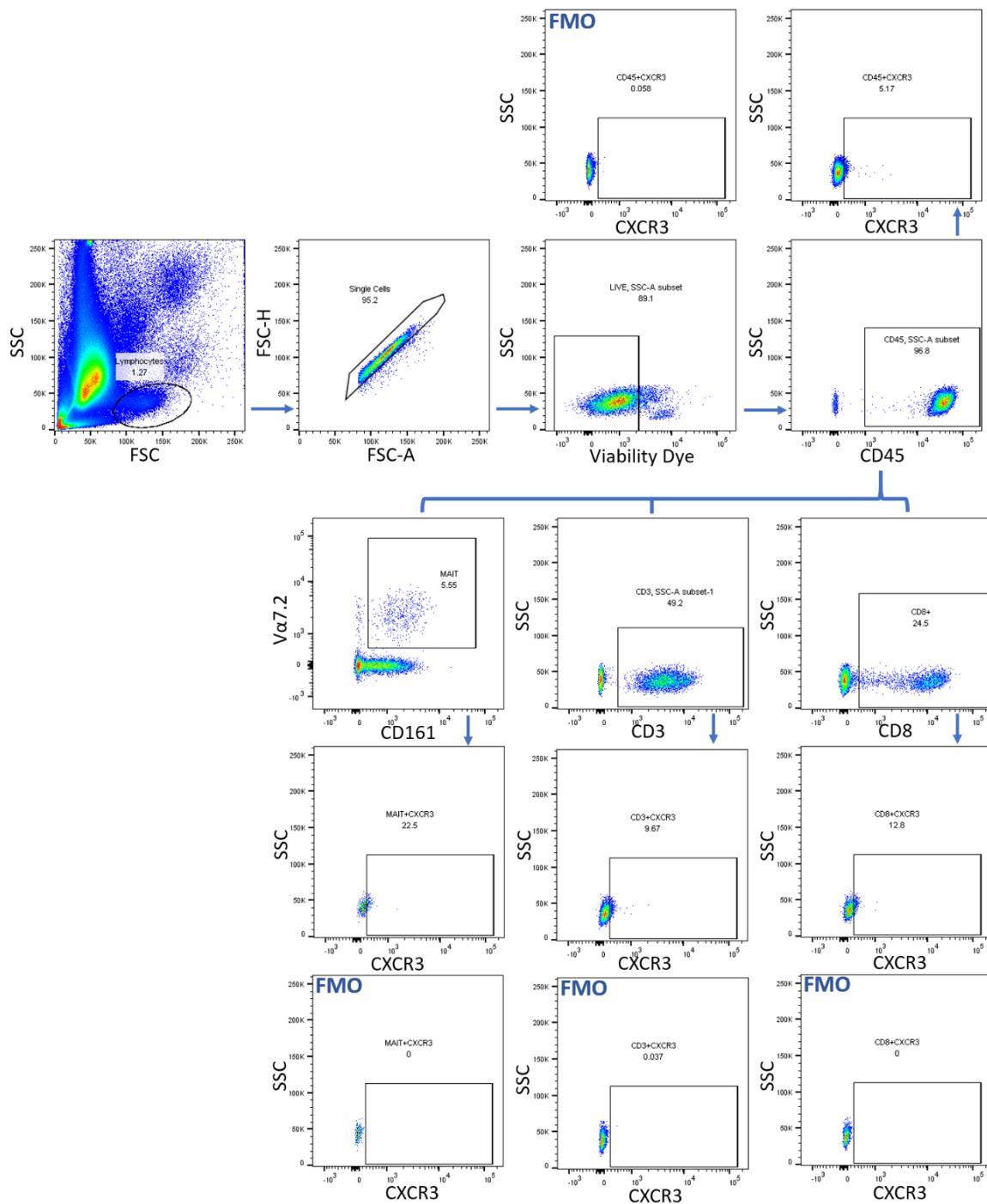


Figure 4.6: Flow cytometry gating strategy for CXCR3 expression.

Lymphocytes were identified by their size and granularity in a forward (FSC) vs side scatter (SSC) plot, followed by identifying single live cells through an FSC-area (FSC-A) vs FSC-height (FSC-H) plot and a viability dye plot. CD45+ cells were then gated on followed by gating on CD3+ and then CD8+ cells. CXCR3 positive gates were then drawn based on an FMO for each population.

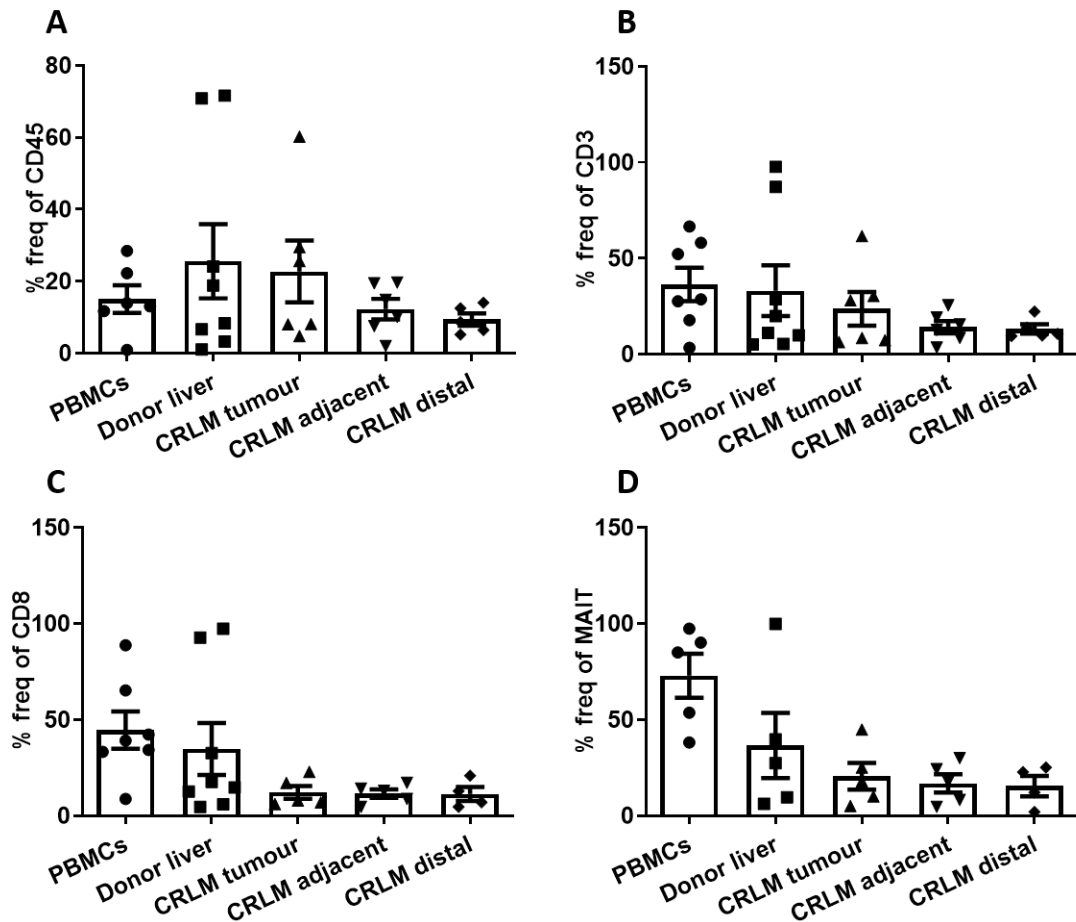


Figure 4.7: CXCR3 expression is not significantly altered in colorectal liver metastases (CRLM) compared to donor liver.

Hepatic mononuclear cells (HMNCs) were isolated by enzyme dissociation, followed by filtration through 70µm membrane eliminating debris, and low speed centrifugation to eliminate hepatocytes. HMNCs from Liver donor (n=8) and CRLM biopsies (n=6) were stained with a viability dye followed by monoclonal antibodies for CD45, CD3, CD8, CD161, Va7.2 for flow cytometry analysis. CXCR3 positive gates were then drawn based on an FMO for each population. Data presented as mean ± SEM.

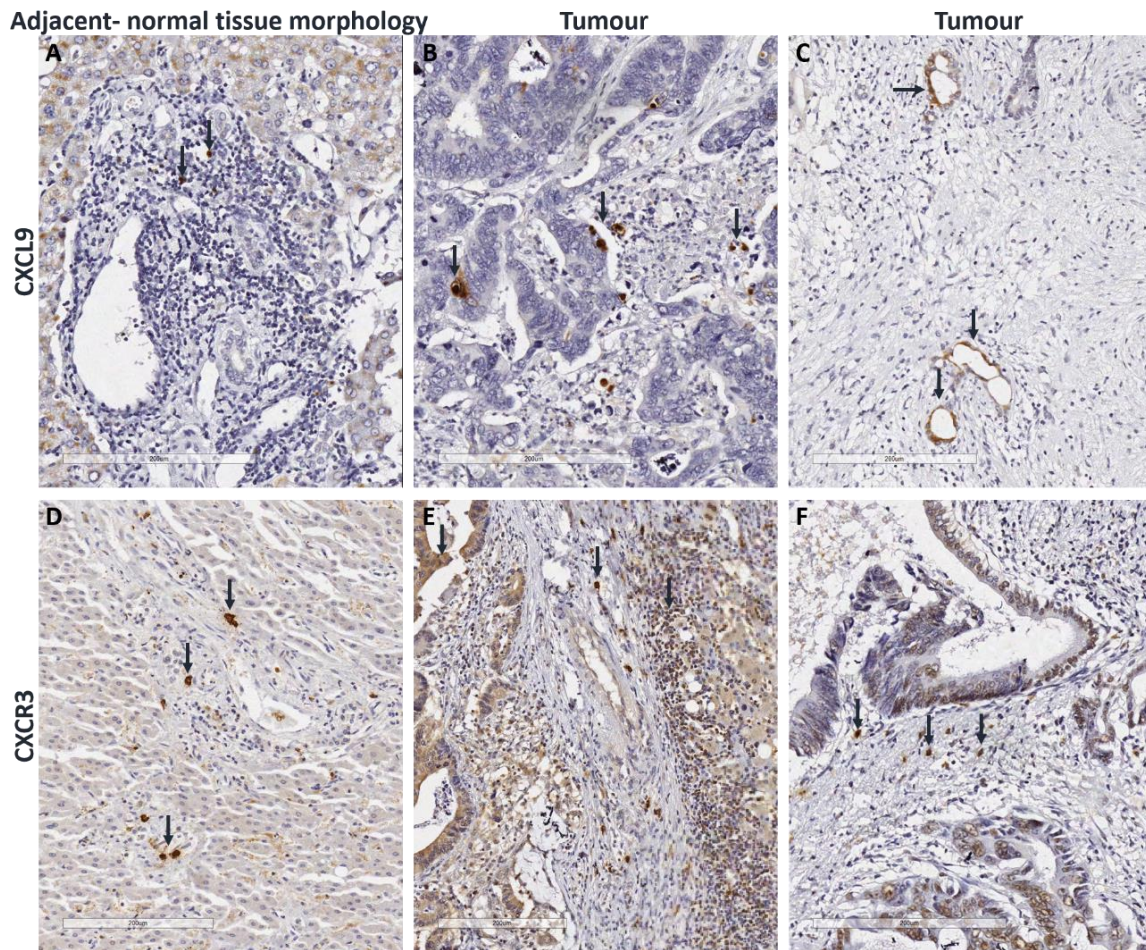


Figure 4.8: Localisation of CXCL9 and CXCR3 in CRLM tumour and adjacent tissue.

Representative images of CXCL9 (n=8) and CXCR3 (n=8) stained sections by IHC. Positive cells visualised with DAB. Examples of positive cells indicated are indicated here by arrows.

4.4.6. Expression of CXCR3 in-vitro

We found that our flow cytometry analysis of CXCR3 expression was inconclusive, did not show any significant differences in expression between donor liver and CRLM HMNCs (Fig. 4.7). Therefore, we decided to test CXCR3 expression on CD8+ cells in-vitro with and without T-cell activation. PBMCs were isolated from donor liver blood and incubated for three days with and without activation by anti-CD3 and anti-CD28. Cells were incubated for 72 hours at 37°C then they were collected, and stained with a viability dye as well as CD45, CD8, and CXCR3 for flow cytometry analysis. We found that CXCR3 expression was enhanced upon activation in-vitro (See Fig.4.9).

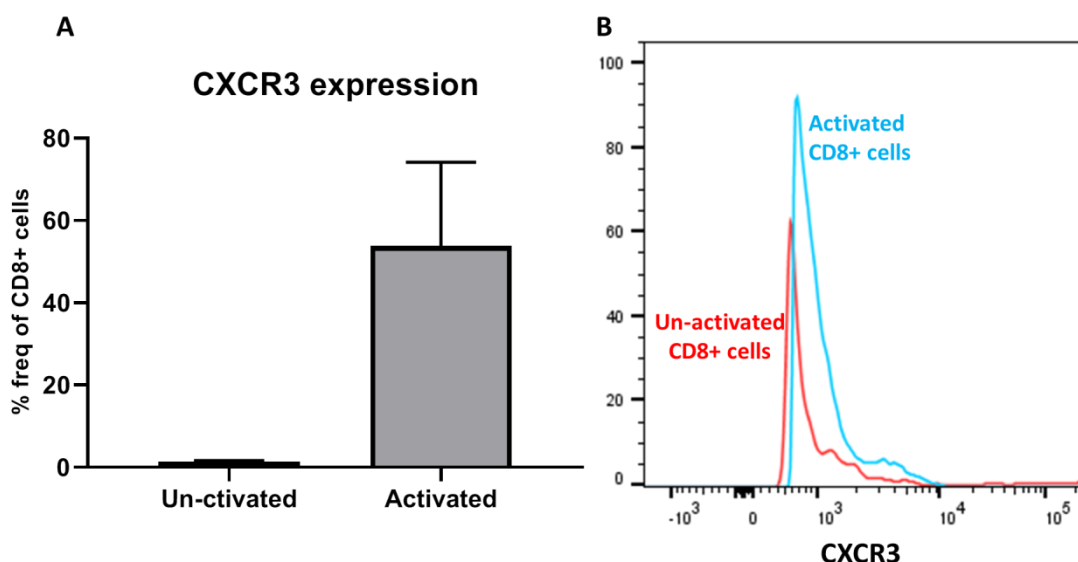


Figure 4.9: Effect of activation on CXCR3 expression on CD8+ cells in-vitro.

PBMCs were isolated from fresh donor blood ($n=2$). 1×10^6 cells/mL were incubated for 72 hours at 37°C with or without activation with soluble anti-CD3 ($1 \mu\text{g/mL}$) and anti-CD28 ($3 \mu\text{g/mL}$). After incubation cells were collected and stained with a viability dye as well as CD45, CD8, and CXCR3 for flow cytometry analysis. (A) Percentage of CXCR3 positive cells within CD8+ population. (B) Modal histogram analysis of CXCR3 expression on activated and un-activated CD8+ cells.

4.5. Chapter discussion

In the previous chapter, we localised and quantified immune populations in CRLM. We divided patients into two groups of high and low recruiters based on the number of immune cells in CRLM tumours. We examined the correlation between overall survival and immune cell recruitment and found that the group of patients with high numbers of infiltrating immune cells had significantly better overall survival. Based on our findings, we decided to investigate changes in the cytokine microenvironment of tissues from CRLM bearing livers.

In this chapter, we examined two methods of protein preparation. The first was the mechanical homogenisation of tissues in lysis buffer. The second was the incubation of tissues in X-VIVO media for 72 hours at 37°C, and collecting supernatants which represent tissue conditioned media. We also examined levels of cytokines using a protein array, which is a general semi-quantitative technique. Finally, we validated selected cytokines by ELISA as a specific quantitative technique.

We measured total protein in donor liver tissue, CRLM tumour, and CRLM adjacent tissue with a BCA assay following mechanical homogenisation. Donor biopsies had significantly more total protein compared to CRLM tumour when normalised to weight (Fig. 4.1). This may suggest the decrease in total protein is a reflection of pathology. However, as protein levels were normalised to weight, it is worth considering that the tumour may include necrotic portions which contribute to weight but not protein production. Alternatively, it is also worth considering that the tumour is denser than donor liver tissue, which also contributes to weight but not necessarily to protein expression.

To examine the differences between the two protein preparations, we measured levels of IL-10 and IL-6 in both protein preps of matched tissues. We found some interesting differences including significantly increased levels of IL-10 in donor liver supernatants compared to CRLM adjacent supernatants (Fig.4.2). This finding was not reflected in tissue homogenates. Several factors could contribute to differences in cytokine

levels between the two protein preparations including stability of cytokines during different processing conditions.

There are several advantages to using tissue supernatants and one of them is the exciting prospect of only examining secreted cytokines. In theory, supernatants may be argued as a better representative of what is happening in the tumour microenvironment. However, there are also several disadvantages. The composition of the X-VIVO media by Lonza is patented which prevents us from accounting for possible undesired effects on the samples during the 72 hours incubation period including its effects on cytokine stability. Furthermore, the preparation of tissue supernatants requires fresh biopsies which contribute to intersample variation. This is because samples were obtained singularly with varied time gaps in between depending on dates of transplants and resections. Likewise, total protein homogenisation also has its advantages and disadvantages. Homogenisation can be done on frozen tissue making it possible to process all samples at once which is a big advantage. However, it represents the sum of extracellular and intracellular protein which could be argued not to be the best representation of the cytokine microenvironment. After looking at IL-6 and IL-10 in both protein preparations, and considering advantages and disadvantages of both, we decided to use homogenates for the protein array analysis of cytokine microenvironment in donor liver vs CRLM. We also decided to use supernatants for our functional studies shown in chapter five.

The protein array we performed on tissue homogenates included 80 targets, most of which were altered in CRLM compared to donor liver (Fig. 4.3). The liver is rich in immune populations, and we believed that the liver's immune repertoire probably leads to a rich inflammatory microenvironment. We observed that the liver is rich in inflammatory cytokines including chemokines, and growth factors. The highest five cytokines in donor livers were angiogenin, RANTES (CCL5), MCP-1 (CCL2), NAP-2 (CXCL7), and MIF. Angiogenin is mainly an inducer of new blood vessel formation (Lyons et al., 2016). RANTES is important in migration of effector and memory T-cells (Crawford et al., 2011). MCP-1 is important in migration of monocytes and macrophages (Deshmane et al., 2009). NAP-2 is important for neutrophil

recruitment (Brown et al., 2017). Macrophage migration inhibitory factor (MIF) is involved in phagocytosis, and anti-tumour activity (Nishihira, 2000). Within CRLM, the majority of the cytokines examined by the array were elevated in homogenates from the tumour as well as homogenates from the adjacent CRLM tissues. The five cytokines with the highest levels in CRLM are angiogenin, RANTES (CCL5), MCP-1 (CCL2), TIMP-1, and IL-8. As we closely examined differences in expression of each cytokine in donor liver relative to CRLM adjacent and tumour, we found CXCL10 (IP-10), CXCL9 (MIG), and CCL8 (MCP-2) to be particularly interesting because of their fold change as well as their function in recruiting immune populations.

We decided to verify CXCL9, CXCL10, and CCL8 by ELISA, and we also verified IFN- γ as an inducer of all three chemokines (Fig. 4.4, 4.5). We found no significant differences in CXCL10 between donor liver and CRLM tissue. We also found CXCL9 to be significantly increased in the tumour. Interestingly, CXCL9 levels were low in tissues adjacent to CRLM. IFN- γ was highest in donor liver and at its lowest in CRLM distal tissue. CXCL9, CXCL10, and IFN- γ all exhibited large range of variation between samples similar to the variation in immune cell counts which we showed in chapter three. CCL8 was also significantly higher in CRLM tumour compared to both donor liver, and CRLM adjacent samples with a significantly wide range of interpatient variation.

CXCL9 and CXCL10 bind the CXCR3 receptor expressed by monocytes, T-cells, NK-cells, and MAITs. We wondered whether receptor expression was also altered in the tumour microenvironment. To investigate, we dissociated donor liver and CRLM tumour, adjacent, and distal tissue into single cell suspensions. We then stained cells for viability and markers of interest with monoclonal antibodies for flow cytometry analysis. Expression of CXCR3 by flow cytometry did not show any significant differences between sample types (Fig. 4.7) when comparing percentage of positive cells. We were not able to compare expression based on histogram analysis of the flow cytometry data because we lacked adequate controls in our experiment design. Then, we localised both CXCL9, and its receptor, CXCR3 by IHC (Fig. 4.8). We observed CXCL9 on lymphocytes within both tumour and adjacent (morphologically normal) tissues. We also observed CXCL9

expression on cuboidal cells that looked like bile ducts within tumour tissue. Similarly, CXCR3 expression was observed on lymphocytes within tumour and adjacent tissues, and it was also expressed by tumour cells.

In the previous chapter, we demonstrated that CD8⁺ cell infiltration into the tumour microenvironment of CRLM is linked to improved survival, so we decided to test expression of CXCR3 on CD8⁺ cells in vitro in response to the presence and absence of activation signals. To do so, we isolated fresh donor PBMCs and incubated them for 72 hours with or without the activation molecules, anti-CD3 and anti-CD28. We then collected the cells, and stained them for flow cytometry analysis of CXCR3 expression. We found that CXCR3 expression is markedly higher in activated CD8⁺ cells.

In this chapter, we showed the rich inflammatory microenvironment of donor liver tissue. We also observed differences in CRLM tissues compared to donor liver tissues. We believed that the dysregulation in cytokine microenvironment is linked to the dysregulation of immune recruitment in patients with low T-cell tumour infiltration. The significant interpatient variation in CD8⁺ cell numbers we showed in chapter three, combined with the significant interpatient variation in CXCL9 that we showed in this chapter excited us. In the next chapter, we investigate functional aspects of CXCL9 in CRLM microenvironment and its effect on immune cell migration.

5. CXCL9 AND MIGRATION IN COLORECTAL LIVER METASTASIS

5.1. Introduction

In chapter three, we stained histology sections from CRLM FFPE blocks for leukocyte and T-cell markers. We quantified these cells and showed that CRLM patients could be divided into either a group of high recruiters of T-cells or a group of low recruiters of T-cells. When we correlated the number of T-cells to overall survival in CRLM we found that the group of patients with high T-cell infiltration had significantly better survival. We then looked into the cytokine microenvironment of CRLM in chapter four. We analysed total protein extracted from CRLM tumour tissue, CRLM adjacent tissue, and donor liver tissue through a protein array that included 80 cytokine targets. The protein array reflected the rich cytokine microenvironment of donor liver. The array also reflected that the cytokine microenvironment of CRLM is altered. This was expected as the immune repertoire of CRLM is distinctly different from donor liver. We were particularly interested in a group of chemokines that exhibited higher levels in the tumour microenvironment including CXCL9. Further validation of CXCL9 showed significant overall increase of protein in CRLM tumour compared to donor liver total protein. We also observed a wide range of variation in CXCL9 similar to the variation seen in T-cell absolute counts. CXCL9 is a T-cell chemoattractant induced by IFN- γ . CXCL9 was a part of a described prognostic gene expression signature in colorectal cancer (Kistner et al., 2017, Li et al., 2019). However, CXCL9 has not been studied in CRLM. CXCL9 mediates its function through its CXCR3 receptor. We tested CXCR3 expression on CD8+ T-cells isolated from blood and found it to be significantly upregulated on activated cells. We believe that the immune environment of CRLM has great bearings on patient prognosis and response to therapy. We observed that both CD8+ T-cells, and CXCL9 were high in CRLM tumour compared to donor liver. We also observed that both CD8+ T-cells and CXCL9 expressed wide range of interpatient variation. These two observations combined made us wonder about the functional significance of CXCL9 in the microenvironment of CRLM.

Chemokines drive leukocyte migration which is essential in both homeostasis and disease (Chen et al., 2018). In cancer, chemokines have a

role in leukocyte recruitment, angiogenesis, tumour proliferation, and metastasis (Mollica Poeta et al., 2019). Trafficking of immune cells involves a multistep adhesion cascade (See Fig. 5.1). The first step of tethering and rolling is driven by adhesion molecules that belong to one of several families including the family of selectins, and the family of integrins (Luster et al., 2005). The second step drives activation of integrins to increase binding avidity to their specific endothelial ligands including chemokines like CXCL9; this process is initiated by G protein-coupled receptors like the CXCL9 receptor, CXCR3 (Campbell and Humphries, 2011). Integrins come into play again at the next step of arrest which then leads to leukocyte polarisation and diapedesis (Luster et al., 2005).

The efficacy of many immunotherapies depends on the number of TILs in the tumour microenvironment (Tong et al., 2018). Immune checkpoint inhibitors are passive immunotherapies designed to reverse T-cell inhibition, thus enhancing tumour cell elimination by immune cells (Pennock and Chow, 2015). We believe we need to promote inflammation and enrichment of effector immune cells within the CRLM microenvironment in order to improve efficacy of therapies that target immune cells.

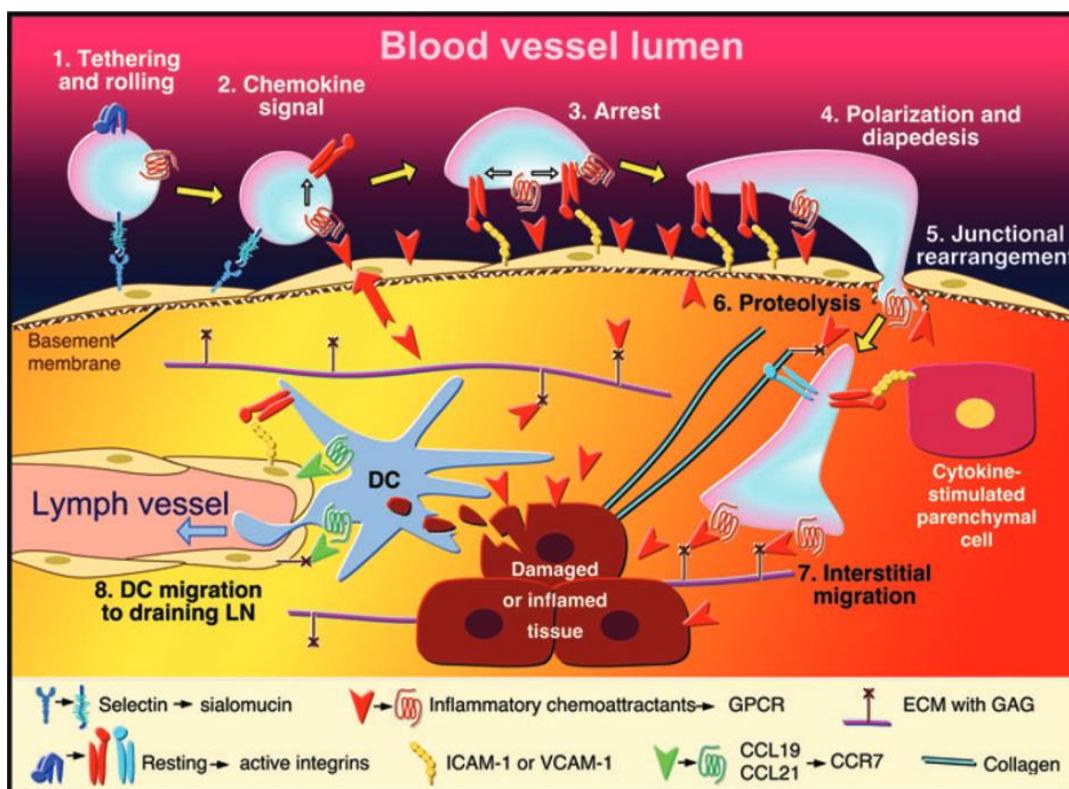


Figure 5.1: Migration steps of immune cells (Luster et al., 2005).

We decided to run functional experiments to examine the influence of CRLM microenvironment on immune cell migration. We know now that CXCL9 is expressed in both CRLM tissue and donor liver tissue as shown with our protein array, ELISA, and IHC. However, we did not know the role that CXCL9 plays in the microenvironment of the liver. Nor do we or others know how its expression is regulated in metastatic liver, and whether other components in the tumour microenvironment affect CXCL9 function.

The transwell migration and invasion assay is a widely used system to test directional response of single cells towards a migration stimulus. It may also help define migratory potential of different cells which is affected by their receptor expression. Transwell chemotaxis assays are carried out in sterile conditions using 96 or 24-well plates loaded with a transwell with a porous membrane (See Fig. 5.2). The transwell pore size depends on the size of cells. The top well is loaded with single cell suspension, while the lower well is loaded with media containing stimulatory signals of interest. Once both components are loaded, the wells are incubated at 37°C. The incubation period depends on the type of cell and stimulus (Justus et al., 2014). Test result is assessed by either fixing and staining cells in the lower well at the end of the assay, or alternatively by collecting media from the lower well and staining cell with monoclonal antibodies for flow cytometry analysis and quantification.

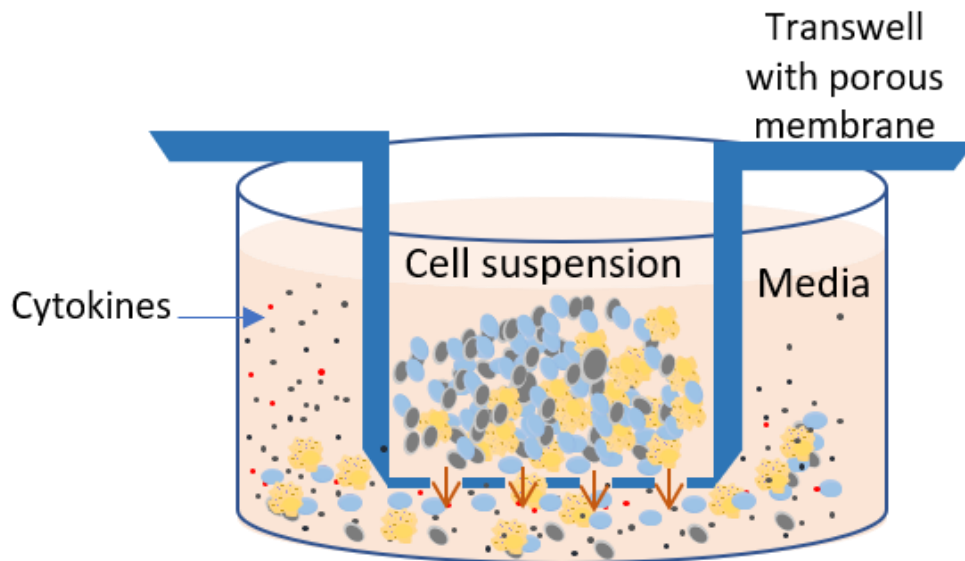


Figure 5.2: Experimental setup of chemotaxis assay.

5.2. Specific hypothesis

We hypothesized that dysregulation of the chemokine microenvironment is linked to insufficient immune cell recruitment and survival in CRLM. We specifically hypothesise that CXCL9 is significant for immune infiltration and therefore survival in CRLM patients.

5.3. Specific aims

- To test the effect of donor and CRLM liver conditioned media on chemotaxis of immune cells.
- To generate an alternative model of liver conditioned media for optimisation of subsequent experiments.
- To test the effect of blocking CXCL9 on chemotaxis.
- To test the correlation between CXCL9 levels in CRLM tumour and absolute immune cell numbers.

5.4. Results

To investigate the role of CXCL9 in lymphocyte chemotaxis and its significance, we carried out in-vitro and ex-vivo experiments.

5.4.1. The effect of liver conditioned media on chemotaxis of immune cells

Based on the significant interpatient variation observed in CD45+ leukocytes numbers, CD3+ T-cell numbers, CD8+ T-cell numbers, as well as CXCL9 levels in total protein, we decided to investigate the effects of CRLM tumour liver conditioned media (LCM), CRLM adjacent LCM, CRLM distal LCM, and compare it to donor LCM in respect to immune cell chemotaxis. Each tissue type was incubated in 500µL X-VIVO media for 72 hours at 37°C. After the incubation period, supernatants were collected and spun down to remove debris. These tissue supernatants made up our LCM and were stored at -20°C until needed. For the chemotaxis assay, total protein in our LCM samples was measured by BCA and samples were normalised by their total protein content. Fresh blood donors were age matched to our cohort of CRLM. Age matched donor PBMCs were isolated, starved, then placed in the top compartment of a transwell with 5µm pores with LCM in the bottom well to test cell migration. The contents of the lower wells were collected and stained for flow cytometry analysis and numeration. Lymphocytes, CD45+ leukocytes, and CD8+ T-cells were gated on as shown in figure 3.3 shown in the chapter three. Cell numbers were calculated with the use of CountBright™ absolute counting beads.

We found lymphocytes to have slightly higher migration in response to donor LCM compared to tumour LCM (See Fig. 5.3A). Interestingly, we also observed that both CRLM adjacent LCM and CRLM distal LCM induced higher migration of lymphocytes compared to both donor LCM and CRLM tumour LCM. Chemotaxis of CD45+ cells was higher in response to tumour LCM compared to donor LCM (See Fig. 5.3B). Chemotaxis of CD8+ cells was

lower in response to tumour LCM compared to donor LCM (See Fig. 5.3C). None of the differences in chemotaxis were statistically significant.

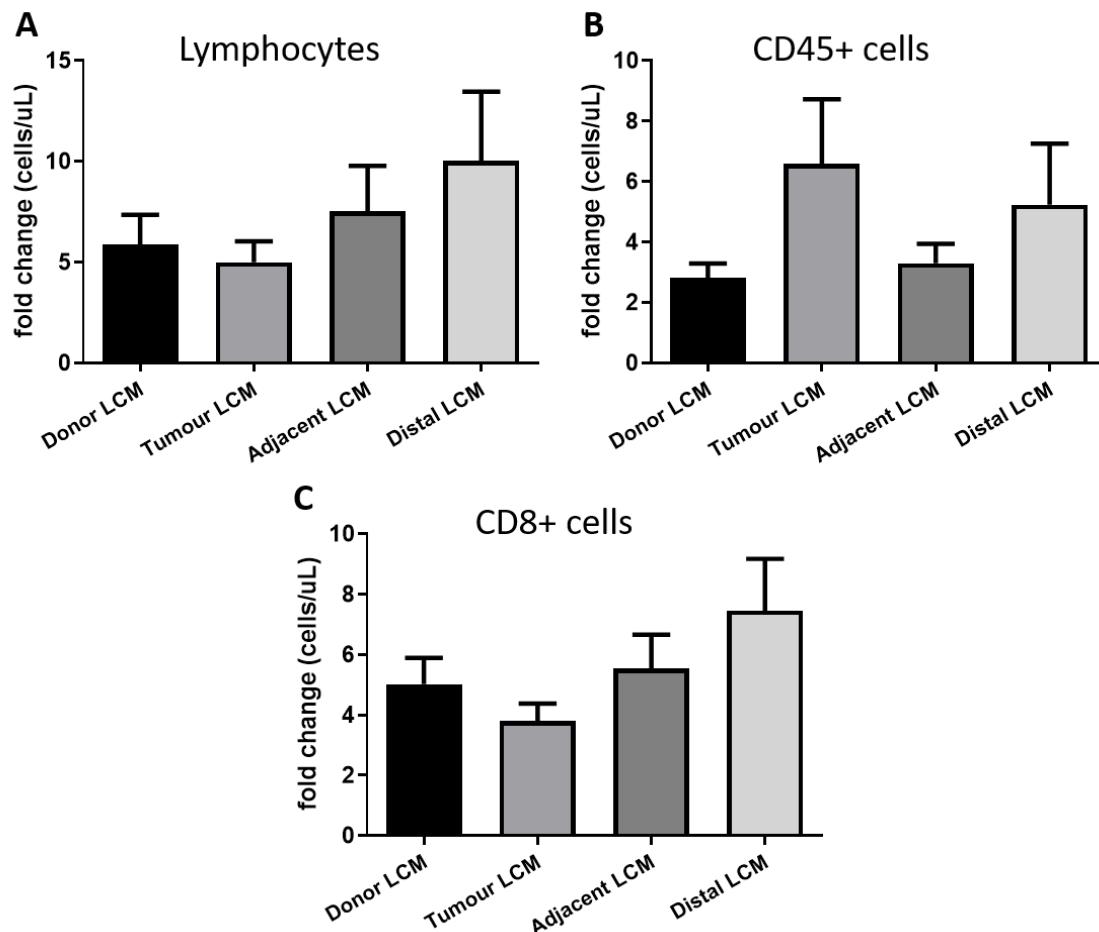


Figure 5.3: Chemotaxis of PBMCs towards liver conditioned media.

PBMCs were isolated from two age matched donors. PBMCs from each donor were tested against LCM from Donor liver tissue ($n=7$), LCM from CRLM tumour tissue ($n=9$), LCM from CRLM adjacent tissue ($n=7$), and CRLM distal tissue ($n=3$). LCM from CRLM tumour, adjacent, and distal tissue were not matched. Cell migration was tested in response to liver conditioned media with transwell inserts containing 1×10^5 starved PBMCs in the upper chamber and 10% conditioned media in the lower chamber and incubated for two hours. Cells were collected from the lower chamber after incubation, stained with monoclonal antibodies for viability, CD45, and CD8 and analysed with flow cytometry. This was done in duplicate for each

conditioned media. Data shown as fold change over x-vivo media control. Data shown as mean $\pm$ SEM. (A) Chemotaxis of lymphocytes (B) Chemotaxis of CD45+ cells (C) Chemotaxis of CD8+ cells.

5.4.2. Chemotaxis of immune cells in response to pooled liver conditioned media from hi and low immune recruiters

Our first experiment showed that CD8+ cells' migration was lower in response to CRLM tumour LCM compared to donor LCM, but unfortunately the difference was not statistically significant. To further investigate our hypothesis on the possible correlation between the chemokine microenvironment of CRLM and the number of TILs in CRLM, we decided to alter our chemotaxis experiment. To decrease the effect of interpatient variation we decided to pool LCMs. As we divided CRLM patients into a high TIL group and low TIL group, we decided to divide the six tumour supernatants we had available into two groups as well. We counted CD8+ cells in FFPE sections corresponding to the six available tumour supernatants we had. We then pooled the three supernatants with the highest counts of CD8+ cells (referred to as Hi LCM pool). Similarly, we pooled the supernatants with the three lowest CD8+ cell counts. We also pooled four donor LCMs (referred to as Low LCM pool). Chemotaxis of four isolated donor PBMCs was tested against the three pools of LCM.

Results shown with each connecting line reflecting the migration of cells from the same donor in response to the different LCM pools (See Fig. 5.4). Chemotaxis of CD45+ cells was higher in response to the high LCM pool compared to donor LCM pool (See Fig. 5.4A). Similarly, chemotaxis of CD45+ cells was higher in response to the low LCM pool compared to donor LCM pool. Chemotaxis of CD45+ cells was not different in response to the two tumour LCM pools. Chemotaxis of CD8+ cells was higher in response to the high LCM pool compared to donor LCM pool (See Fig. 5.4B). Similarly, chemotaxis of CD8+ cells was higher in response to the low LCM pool compared to donor LCM pool. Chemotaxis of CD8+ cells was not different in

response to the two tumour LCM pools. We expected the two tumour LCM pools to have different chemotaxis potential but that was not evident in our experiment.

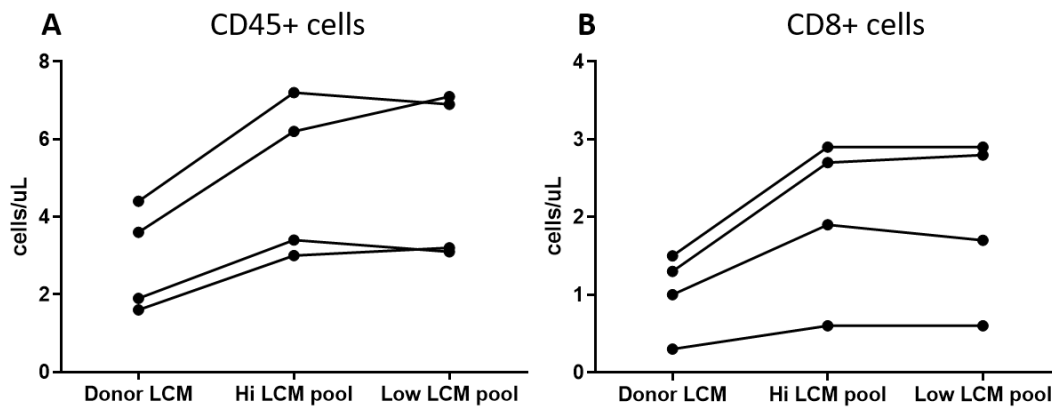


Figure 5.4: Chemotaxis of PBMCs towards pooled liver conditioned media.

Cell migration was tested in response to liver conditioned media with transwell inserts containing 1×10^5 starved PBMCs in the upper chamber and 10% conditioned media in the lower chamber and incubated for two hours (PBMCs $n=4$). Cells were collected from the lower chamber after incubation, stained with monoclonal antibodies for viability, CD45, and CD8 and analysed with flow cytometry. Chemotaxis was tested in response to donor liver LCM, pool of three LCM from patients with high tumour immune infiltration, and a pool of three LCM from patients with low tumour immune infiltration. Experiments were carried out in triplicate. Data shown as mean for each PBMC donor.

5.4.3. In-vitro model of LCM

Our supply of tissue supernatants was limited. Tissue samples were used to flow cytometry, protein extraction and supernatants development depending on the size of the biopsy. Because liver biopsies were precious

limited samples, we decided to develop an alternative model of LCM in-vitro. We grew the metastatic CRC cell line, SW620 in vitro. CXCL9 is induced by IFN- γ so we stimulated SW620 cells with different concentrations of IFN- γ , then we measured CXCL9 by ELISA (Peprotech). We found that SW620 produced CXCL9 in response to 10 ng/mL IFN- γ (226.2 ± 31 pg/mL) and 100 ng/mL IFN- γ (316.5 ± 6.5 pg/mL) in a dose dependent manner. CXCL9 was significantly higher in the stimulated SW620 by 100 ng/mL IFN- γ compared to basal SW620 ($p=0.04$). We also found that levels of CXCL9 in donor liver LCM (292.1 ± 85.6 pg/mL) were similar to that in our cell line media (See Fig. 5.5).

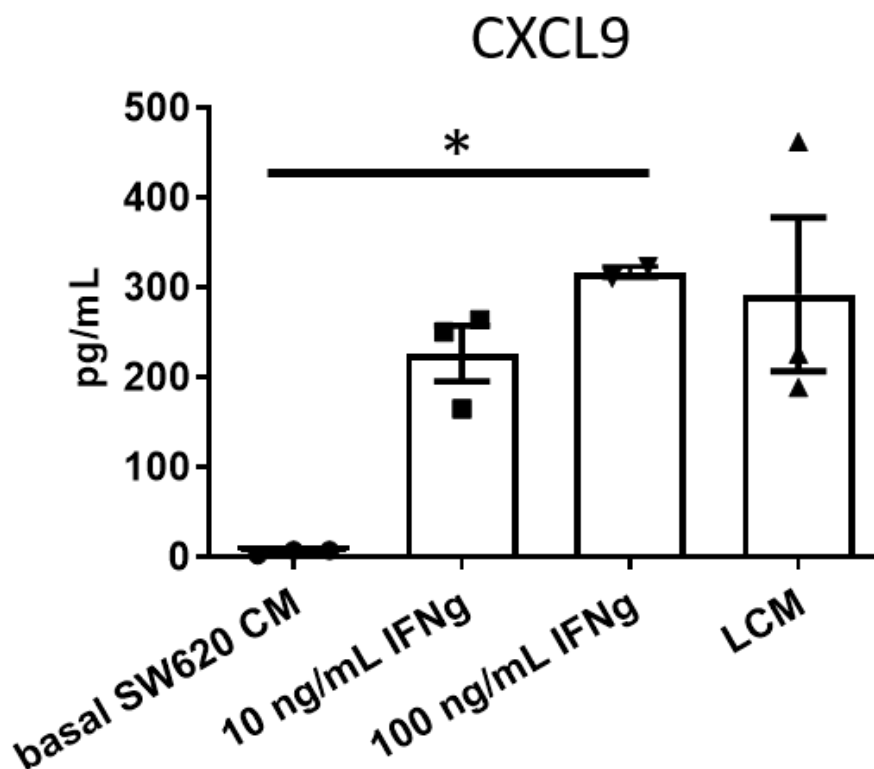


Figure 5.5: Colorectal metastasis cell line, SW620, produces CXCL9 in response to IFN- γ .

The metastatic CRC cell line SW620 was grown to confluency in tissue culture flask then 5×10^5 cells were plated into each well of a 24-well plate, and stimulated after confluency with RPMI, RPMI with 10%FBS,

RPMI+10%FBS+10ng/mL IFN- γ , and RPMI+10%FBS+100ng/mL IFN- γ for 48hrs (n=3). Liver biopsies from transplant donor organ and CRLM resections were cut into 5mm³ pieces and incubated for 72hrs in x-vivo media and supernatants were collected. CXCL9 was measured by ELISA (Peprotech). Data shown as mean $\pm$ SEM.

5.4.4. The effect of blocking CXCL9 in SW620 CM on PBMC chemotaxis

We wanted to investigate the effect of blocking CXCL9 on chemotaxis of CD45+ leukocytes and CD8+ T-cells. To do so, we grew the metastatic CRC cell line SW620 then equal cell number was seeded into each well of a 24-well plate and left to adhere overnight. The media was then replaced with fresh total RPMI, or fresh total RPMI containing 10ng/mL IFN- γ and incubated for 48 hours. The supernatants were then collected, spun down to remove any cells, and used for our chemotaxis as we explained earlier. Chemotaxis of three isolated donor PBMCs was tested in response to total RPMI as a control, basal SW620 conditioned media, IFN- γ stimulated SW620 conditioned media, and IFN- γ stimulated SW620 conditioned media which was pre-treated for 1 hour with anti-CXCL9. Experiment was carried out in duplicate.

No cells migrated was evident in response to the RPMI control. We found that IFN- γ stimulated SW620 CM induced significantly higher chemotaxis than basal SW620 CM (46 ± 1.5 cells/ μ L) for CD45+ cells (79.7 ± 12.8 cells/ μ L; $p=0.04$). Similarly, significantly higher number of CD8+ cells (73 ± 5 cells/ μ L; $p=0.04$) migrated in response to IFN- γ stimulated SW620 CM. Neutralising CXCL9 in the stimulated SW620 media led to a decrease in CD45+ cells (45 ± 4.4 cells/ μ L; $p=0.055$) compared to IFN- γ stimulated SW620 CM. and also, significantly less CD8+ cell (44.3 ± 4.6 cells/ μ L; $p=0.04$) migrated in response to the CXCL9 neutralised IFN- γ stimulated SW620 CM in our chemotaxis assay (See Fig. 5.6).

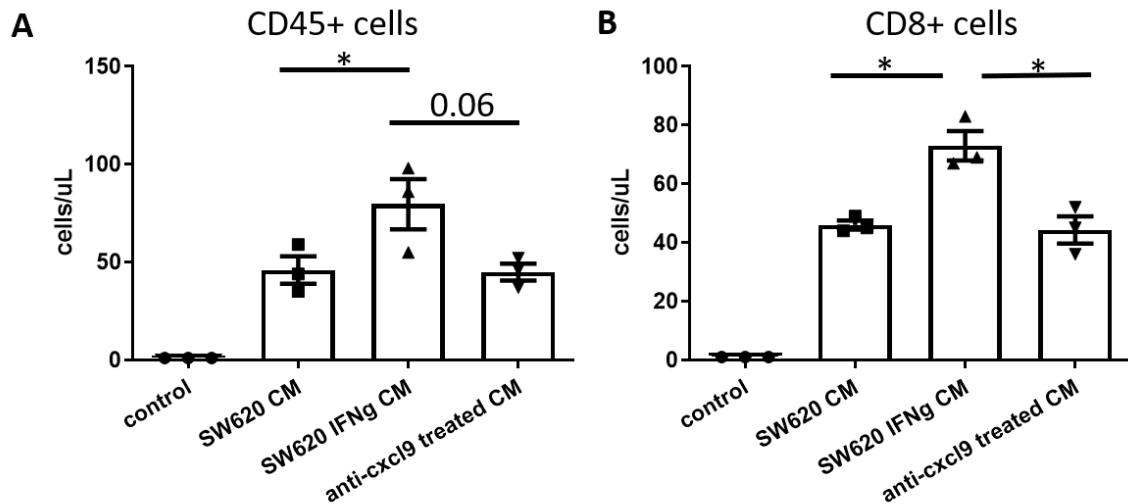


Figure 5.6: Blocking CXCL9 in SW620 CM significantly reduces cell migration.

Cell migration was tested in response to liver conditioned media with transwell inserts containing 1×10^5 starved PBMCs in the upper chamber and conditioned media (CM) in the lower chamber and incubated for two hours. Matched media was treated with anti-CXCL9 for one hour prior to the start of the chemotaxis assay ($n=3$). Cells were collected from the lower chamber after incubation, stained with monoclonal antibodies for viability, CD45, and CD8 and analysed with flow cytometry. Data shown as mean $\pm$ SEM. $P < 0.05$ indicated as *. Chemotaxis stimulation media was made up of total RPMI and 40% cell line conditioned media. Migration was tested in response to RPMI alone (control), basal SW620 CM (SW620 CM), IFN- γ stimulated SW620 CM (SW620 IFN- γ CM), and matched anti-CXCL9 treated IFN- γ stimulated SW620 CM (anti-CXCL9 treated CM). Cell migration was significantly higher in the IFN- γ stimulated SW620 CM and significantly decreased in response to CXCL9 blockade.

5.4.5. The effect of blocking CXCL9 in CRLM tumour LCM on PBMC chemotaxis

Based on our findings with the SW620 CM (Fig. 5.6), we decided to repeat the experiment using pooled CRLM tumour LCM. We used pooled LCM to minimise interpatient variation, and also to be able to test the effects against multiple donor PBMCs. Similar to our previous experiment, CXCL9 in a portion of our pooled LCM was neutralised with anti-CXCL9 for an hour. Chemotaxis of PBMCs was tested in response to matched tumour LCM and neutralised tumour LCM. We found that neutralising CXCL9 in tumour LCM significantly reduced chemotaxis of donor CD45+ cells ($p=0.04$). We also found that neutralising CXCL9 in tumour LCM significantly reduced chemotaxis of donor CD8+ cells ($p=0.04$) compared to matched un-neutralised tumour LCM (See Fig. 5.7).

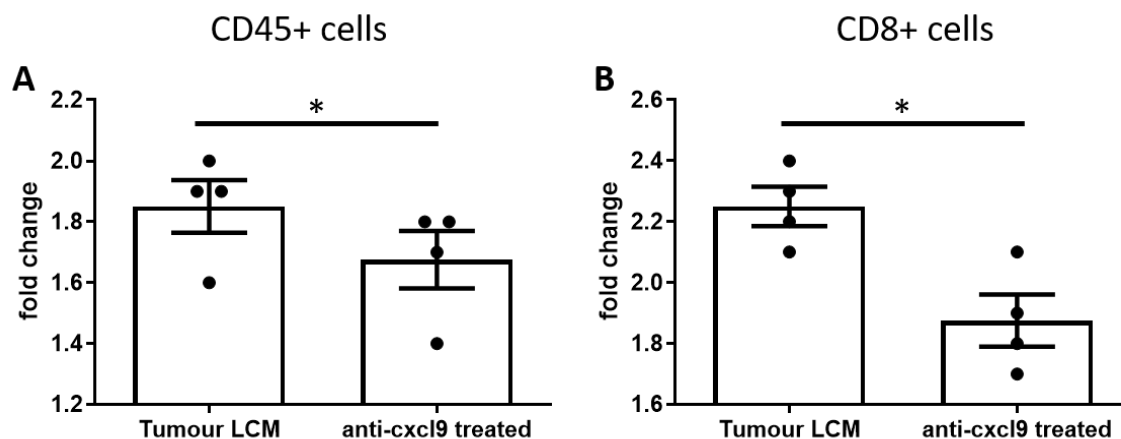


Figure 5.7: Blocking CXCL9 in CRLM tumour LCM significantly reduces cell migration.

Cell migration was tested in response to liver conditioned media with transwell inserts containing 1×10^5 starved PBMCs in the upper chamber and conditioned media (CM) in the lower chamber and incubated for two hours. Matched media was treated with anti-CXCL9 for one hour prior to the start of the chemotaxis assay ($n=4$). Cells were collected from the lower chamber after incubation, stained with monoclonal antibodies for viability, CD45, and

*CD8 and analysed with flow cytometry. Data shown as mean $\pm$ SEM. $P < 0.05$ indicated as *. LCM was used at 10% to stimulate migration. Migration was tested in response to matched tumour LCM and anti-CXCL9 treated tumour. Cell migration was significantly lower in response to neutralised media for both CD45+ cells, and CD8+ cells.*

5.4.6. Correlation between CXCL9 levels and absolute immune cell counts

Since blocking CXCL9 alone significantly reduced the migration of immune cells (Fig. 5.7), we decided to measure CXCL9 in as many CRLM biopsies as possible by ELISA and correlate it back to IHC absolute cell counts. We compared tumour cell counts to both tumour and adjacent CXCL9 levels (See Fig. 5.8). CD45+ cell numbers did not correlate to CXCL9 levels in neither the tumour nor the adjacent CRLM tissue. CD3+ cell numbers correlated significantly with CXCL9 levels in both the tumour ($R^2=0.42$, $p=0.02$), and adjacent tissue ($R^2=0.45$, $p=0.02$). CD8+ cells did not show any significant correlations with either tumour or adjacent CXCL9 levels.

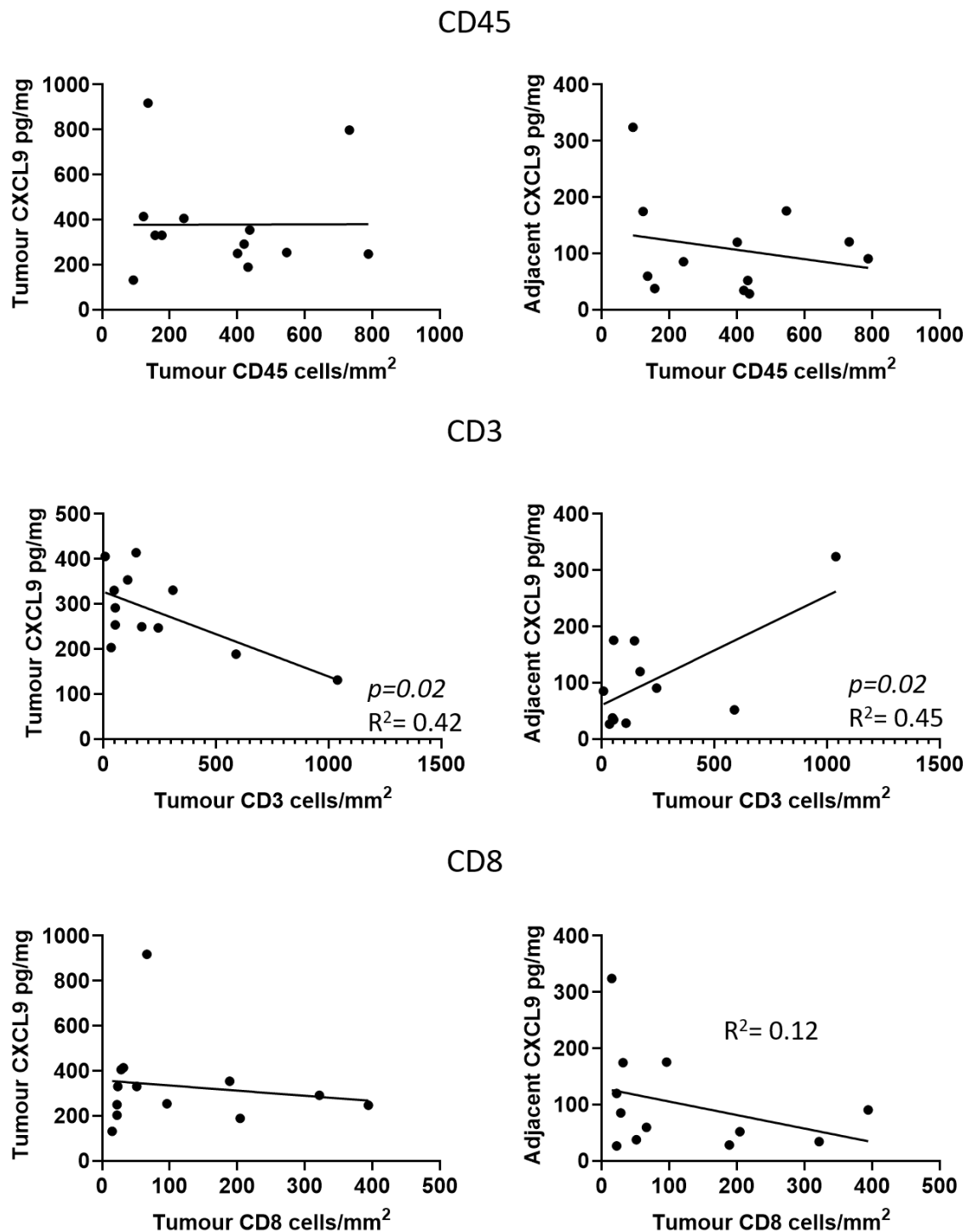


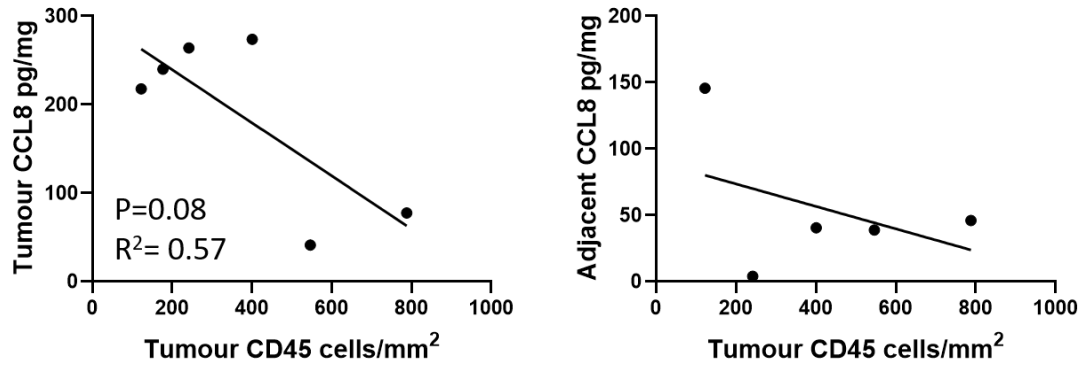
Figure 5.8: Correlation between CXCL9 and immune cell count in CRLM tumour and adjacent tissue.

Linear regression graphs between absolute number of cells in CRLM tumour and CXCL9 levels in protein of CRLM tumour, and CRLM adjacent tissue. CD45+ cells (n=13), CD3+ cells (n=12), and CD8+ cells (n=13).

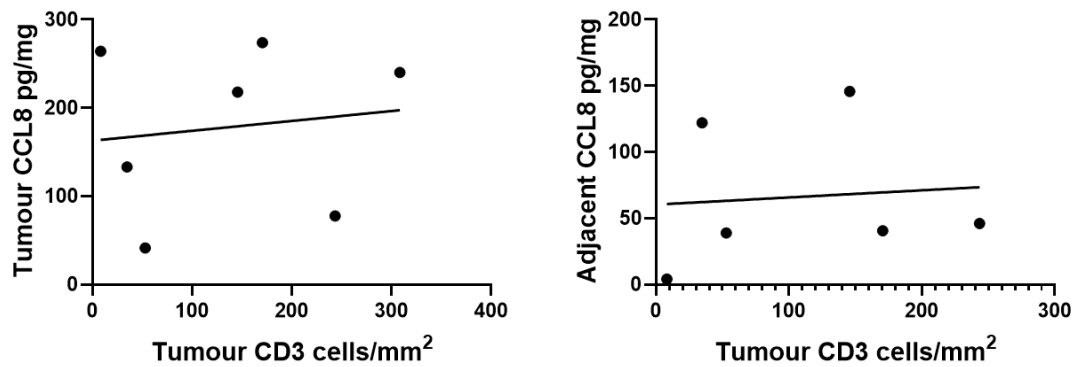
5.4.1. Correlation between CCL8 levels and absolute immune cell counts

The effects of blocking CCL8 on monocyte migration was not examined. However, we decided to investigate any correlation between CCL8 levels of absolute cells counts in CRLM tissue. We compared tumour cell counts to both tumour and adjacent CCL8 levels (See Fig. 5.9). As mentioned earlier, our biopsy samples were very limited. The testing of CCL8 was only performed on spare samples after finishing our higher priority protein analysis. Higher sample n number is required to increase reliability of linear progression analysis. Some trends of correlation were evident, with the most interesting being an inverse correlation between CCL8 levels in total protein from the tumour tissue and CD45+ cell numbers in CRLM tumour (See Fig. 5.9).

CD45



CD3



CD8

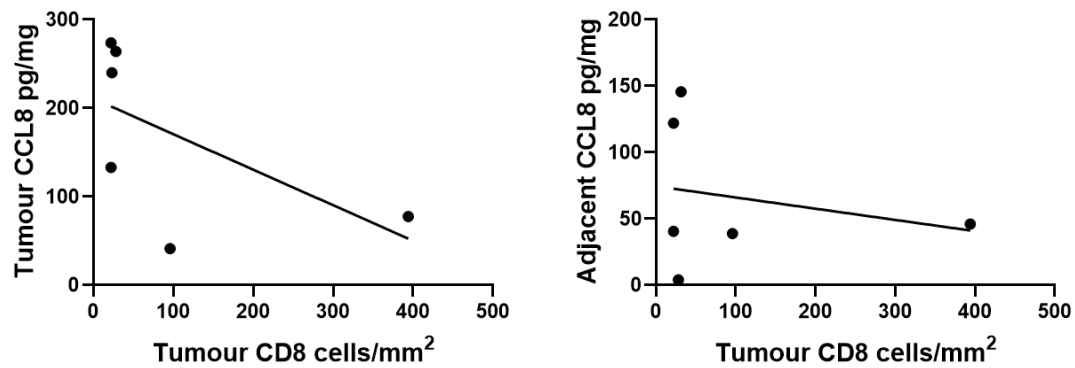


Figure 5.9: Correlation between CCL8 and immune cell count in CRLM tumour and adjacent tissue.

Linear regression graphs between absolute number of cells in CRLM tumour and CXCL9 levels in protein of CRLM tumour, and CRLM adjacent tissue. CD45+ cells ($n=13$), CD3+ cells ($n=12$), and CD8+ cells ($n=13$).

5.5. Chapter discussion

In the previous chapter, we chose to test the cytokine microenvironment in total protein obtained from tissue homogenates rather than tissue supernatants for several reasons. We did not know the components of the X-VIVO media (Lonza) used in the process of developing tissue supernatants. We wanted to limit the risk of denaturation of any cytokine over the incubation period of supernatant development. We also decided that extracting total protein from frozen tissue was logistically favourable. In contrast, in this chapter we carried out our experiments with tissue supernatants (conditioned media). We were able to use the X-VIVO media as a negative control in these experiments to account for any chemotactic factors that may be a part of its' ingredients. We also had a significant target in mind, CXCL9, which we were able to measure in our preparations of conditioned media and believed is adequately stable. In this chapter, we favoured the use of conditioned media as a representative to secreted cytokines in CRLM and donor liver microenvironment.

For our first experiment, we chose two donors whose age fell within the range of our CRLM cohort. We isolated PBMCs from these two donors then tested their chemotaxis in repose to seven individual donor LCMs, nine individual CRLM tumour LCMs, seven individual CRLM adjacent LCMs, and three individual CRLM distal LCMs (Fig. 5.3). We observed that lymphocytes and CD8+ cells chemotaxis was lower in CRLM tumour compared to donor Liver. The difference here in chemotaxis was not significant. However, any decrease in cell migration is not favourable to patient prognosis and response to therapy. The chemotaxis of CD45+ cells on the other hand was markedly higher in response to CRLM tumour LCM compared to donor LCM. This may suggest that the tumour is secreting chemokines that target monocytes rather than lymphocytes. This may contribute to the tumour's recruitment of M2 macrophages. M2 macrophages are tumour associated macrophages that promote tumour progression by suppressing the immune response, promoting angiogenesis, and driving metastasis (Yang and Zhang, 2017).

We then decided to repeat our chemotaxis experiment with pooled LCMs. We counted CD8+ cells in corresponding histological sections for each of the six CRLM tumour LCMs we had available. We then pooled together the three LCMs that corresponded to the highest three CD8+ cell counts (Hi LCM pool), and pooled the three LCMs that corresponded to the lowest three CD8+ cell counts (Low LCM pool). We chose to base our pooling on CD8+ cells because it had the most statistically significant correlation to survival (Fig 3.15). We tested the pooled LCMs against four fresh donor PBMCs and hypothesised that we would observe reduced chemotaxis in LCM pooled from low CD8 recruiters compared to the LCM pooled from high CD8 recruiters. However, there was no difference in chemotaxis between the two pools (Fig 5.4). This may be because we were limited by the six tumours' LCMs we had available, and the differences in CD8+ cells in the tissue corresponding to each LCM were similar, and not enough to show a difference. We believe that repeating this experiment with pooled LCMs with higher distinction in CD8+ cell counts might reveal interesting findings. In this experiment, we decided not to prime peripheral blood isolated T-cells before testing their chemotaxis in response to LCM pools. The priming of cells prior to testing chemotaxis would affect receptor expression. Our experiment was designed to investigate whether chemotactic factors in the liver had significant effects on immune cell chemotaxis in isolation from other variables including the extent of T-cell priming in the tumour microenvironment.

We then wanted to create an alternative model to LCM. One that is less complex and more abundant. To do so, we resorted to a cell line and in-vitro stimulation with IFN- γ as CXCL9 is known to be induced by it. We grew the CRC metastatic cell line SW620. We then counted these cells and reseeded them in a 24-well plate at a consistent concentration. After allowing them to adhere overnight, we stimulated some well with IFN- γ , and left some well unstimulated as a basal control. We then measure levels of CXCL9 by ELISA. Basal SW620 had very low levels of CXCL9. IFN- γ stimulated SW620 supernatant had similar CXCL9 levels to donor liver conditioned media. The protocol was repeated three times for validation. We decided to use 10ng/mL IFN- γ stimulation for 48 hours to produce our

alternative LCM model. Media from unstimulated SW620 media was also developed each time to use as a control.

Now that we had an abundant substitute for LCM, we decided to block CXCL9 and test the difference on chemotaxis of immune populations. We expected to see a marked reduction in chemotaxis because our model in theory, only had tumour cells-IFN- γ induced chemotactic factors. We divided our SW620 IFN- γ stimulated CM into two portions. One was left as is, and the other was treated with anti-CXCL9 to neutralise CXCL9. Chemotaxis of donor PBMCs was then tested against basal (unstimulated) SW620 CM, IFN- γ stimulated SW620 CM, and neutralised CM (Fig. 5.6). we observed significant reduction in chemotaxis in response to CXCL9 neutralised CM compared to the un-neutralised CM.

Once we were able to show the significant role of CXCL9 in CD45+ and CD8+ cell chemotaxis, we wondered about the significance of it in a more complex microenvironment that include many other chemokines. We repeated the experiments with CRLM tumour LCMs. One portion from each LCM was treated with anti-CXCL9 to neutralise CXCL9. Chemotaxis was then tested against a control of the X-VIVO media, CRLM tumour LCM, and the CXCL9-neutralised CRLM tumour LCM (Fig. 5.7). results were shown as fold change compared to the X-VIVO control. We were able to show a statistically significant reduction of chemotaxis in response to CXCL9-neutralised CRLM tumour LCM. This result suggests that CXCL9 plays a significant role in CD8+ cell recruitment, and that its role is not redundant in the presence of other chemotactic factors in the tumour microenvironment.

We then correlated the levels of CXCL9 to immune cell absolute numbers (Fig 5.8). CD45 cell numbers did not correlate to CXCL9 in the tumour or the adjacent tissue of CRLM. CD3+ cells in CRLM tumour significantly inversely correlated to CXCL9 in the CRLM tumour. The abundance of CXCL9 and lack of CD3+ cells may suggest that chemokine production is adequate but CXCR3 expression may have been inadequate. On the other hand, CXCL9 levels in CRLM adjacent tissue directly correlated with CD3+ cell numbers in CRLM tumour. There was a slight trend of correlation between

CD8+ cell numbers in CRLM tumour and CXCL9 levels in CRLM adjacent tissue. We also showed significant interpatient variation in CCL8 levels in the previous chapter (Fig. 4.5), so we decided to correlate cell numbers to CCL8 levels (Fig.5.9). We observed interesting trends between CD45+ cells in CRLM tumour and CCL8 levels in CRLM tumour. We also observed an interesting trend between CD8+ cells in CRLM tumour and CCL8 levels in CRLM tumour. Both correlation analyses were based on a limited number of samples. Reliable correlation analysis would require many more samples. These results may suggest interesting leads to be followed but do not provide definitive answers.

6. OVERALL DISCUSSION AND FUTURE DIRECTIONS

6.1. Overall discussion

The immune system is our strongest anti-tumour asset. Immune populations are highly capable of identifying transformed cells. Classical T-cells can recognise tumour associated antigens presented on MHC molecules expressed on tumour cells or by antigen presenting cells (Hadrup et al., 2013). NK-cells can be activated by the absence of normal self-antigens and without prior sensitisation (Murphy and Weaver, 2016). $\gamma\delta$ T-cells can recognise tumour associated molecules and stress signals (Harly et al., 2012). iNKT-cells can recognize tumour associated glycolipids presented through CD-1d, which is also recognised by MAIT-cells (Sundstrom et al., 2015, Krovi and Gapin, 2018). Neutrophils, macrophages, and dendritic cells are phagocytes that can encounter tumour associate antigens while ingesting extracellular fluid and can be activated against tumour cells (Murphy and Weaver, 2016). In response to activation, immune cells can directly kill tumour cells by secreting FasL, TRAIL, IFN- γ , and TNF- α . Alternatively, subsets of lymphocytes and monocytes can further activate other subsets to kill immune cells.

The liver is a unique immunological organ with the largest tissue resident populations of macrophages and NK-cells, and with more CD8 than CD4 T-cells reflecting potent cytotoxic capacity and substantial anti-tumour mechanisms (Robinson et al., 2016). The liver also has a significantly expanded population of MAIT cells and an expanded population of iNKT-cells (Kurioka et al., 2016). Despite the liver's extensive immune environment, over half of primary colorectal cancer patients develop liver metastasis (Coimbra et al., 2015). This presents a tremendous problem and clinical burden, especially with primary colorectal cancer being the third most prevalent cancer worldwide.

Available treatments for patients with CRLM include thermal ablation, regional hepatic intraarterial chemotherapy, and radiation therapy (Rachdi et al., 2019, Adam and Kitano, 2019). However, the main curative option for patients with CRLM is liver resection, which aims to remove portions of the liver that contain the tumour or tumours. This is unfortunately not an option for everyone. CRLM liver resection is only performed if the clinical

team believes it will improve over-all survival significantly. Patients with prohibitive operative risks including comorbidities that may complicate the surgery or the aftercare are excluded. Patients with chronic liver disease are also excluded. The number of lesions or tumours developing within the liver vary between patients and therefore eligibility for surgical resection will also depend on the number of lesions within each lobe of the patient's liver (Weiss and D'Angelica, 2012). Even for those fortunate enough to be eligible for hepatic resection, the recurrence rate following liver resection is over 70% (Nierop et al., 2019, Xu et al., 2018). This highlights the need for better treatment strategies, and provides a rationale for focusing on colorectal cancer metastasis into hepatic tissue.

For many years, the majority of clinicians ignored the influence and the role of the immune system in tumour development and progression. However, the link between immune cell numbers and survival has been shown (House and Watt, 1979b). Different groups looked into the number of immune cells both in the blood and within tumours in different types of cancer following different protocols and showed a correlation to survival. As early as 1970, a group identified a link between peripheral blood lymphocytes and neutrophil levels and curability in different cancer types (Riesco, 1970b). In 1978, another group quantified lymphocyte in H&E stained histological sections of colorectal cancer based on morphology, and observed significant differences between patients (Watt and House, 1978b). The same group went on to show a link between the number of tumour infiltrated lymphocytes and survival (House and Watt, 1979b). Surprisingly these findings did not attract much attention at the time, and it wasn't until a couple of decades later that more groups became interested in the tumour's immune environment and its links to survival. In 2006, Gerome Galon showed that the type, density, and location of immune cells correlated to survival in colorectal cancer (Galon et al., 2006). He was able to challenge the narrative of the negative association of immune populations in tumours, and highlighted the positive correlation of tumour infiltrated lymphocytes with survival. In the following decade, a team led by Galon was able to develop a sophisticated protocol that produces what he called an "immunoscore" to assess the type, location, and density of immune cells in primary colorectal

cancer and validated its prognostic value in a worldwide study of 1336 patients (Galon et al., 2016).

Meanwhile, the interest of scientists had also exploded in the field of immunotherapy. As scientists understood the potency of the immune system's anti-tumour response, they started wondering about the possibility of manipulating it. We have known that tumour cells manage to escape immune surveillance by suppressing its effector functions. In 2018, the Nobel Prize in Physiology or Medicine was awarded to Tasuku Honjo and James P. Allison for their discovery of immunotherapy to checkpoint inhibitors. Immunotherapies are now approved for several types of cancer (Guo, 2018). They target immune checkpoints in the cancer microenvironment that inhibit the function of immune populations. Monoclonal antibodies have been designed to target CTLA-4, PD-1, and LAG-3 (Wei et al., 2018). These antibodies target the same ligands the tumour cells exploit to inhibit immune cells by blocking their activation and proliferation. Unfortunately, the efficacy of these treatment is still low. A significant contributor to the efficacy of immunotherapies is the number of immune cells in the tumour environment (Bonaventura et al., 2019). The lack of the primary target of the therapy used would logically reduce the chance of it performing at its maximum capacity.

The overall hypothesis for this project revolved around deficient immune accumulation in CRLM and its link to the cytokine microenvironment. We were first interested in the immune environment of CRLM. This was drawn from our consideration of the high incidence of CRLM and the liver's high vulnerability to colorectal metastasis, the need for better therapeutic strategies in CRLM, the definitive value of the immune system's anti-tumour capacity, and the currently low efficacy of immunotherapies. The liver's rich and diverse immune repertoire suggests it would be highly responsive to immunotherapies. However, the efficacy of immunotherapies in patients with liver metastasis is low (Tumeh et al., 2017, Ciardiello et al., 2019). We hypothesized that the numbers of lymphocytes in CRLM liver tissue is affected, and that the density of T-cells in tumour tissue of CRLM patients correlates to survival. To investigate, we decided to start by examining

immune cell infiltration in biopsies obtained from resections of colorectal liver metastasis.

Despite the intensive amount of work carried out to quantify immune populations in primary colorectal cancer, and the work carried out to correlate T-cells to prognosis and response to therapy, very little was done to confirm these findings in liver metastasis. The liver is a unique lymphoid organ (Doherty et al., 1999, Crispe, 2009), and so the findings on immune infiltration in CRC may not be reflected in the liver. When we started this investigation, few studies had investigated immune infiltration in liver metastasis. Indeed earlier studies from our lab localised CD45+ leukocytes, CD3+ T-cells, and CD20+ B-cells in donor liver histology sections but did not compare cell numbers to CRLM sections (Smith et al., 2003). Our lab also phenotyped some immune populations by flow cytometry and reported on their frequency as a percentage of CD45+ cells in both donor liver and CRLM biopsies. CD3+Va24+V β 11+ cells, which were T-cells with positive NK markers were found depleted in CRLM (Kenna et al., 2003b). $\gamma\delta$ T-cells, a subset of $\gamma\delta$ T-cells was found depleted in CRLM (Kenna et al., 2004a). NK-cells were found depleted in CRLM (Harmon et al., 2016). However, previous work done by our lab did not quantify absolute numbers of leukocytes and T-cells in both donor liver and CRLM tissues, and patients with CRLM were not stratified into groups based on their immune infiltration. We wanted to adapt Galon's main principles into a simpler protocol requiring less automation and fewer resources. We aimed to investigate whether his findings in colorectal cancer would translate into CRLM. We define four histological regions for our CRLM biopsies. The first being the tumour region, which was the abnormal cells and structures within the biopsy. The second was the adjacent region, which was the normal cells and structures directly adjacent to the tumour. The third was the invasive margin, which referred to the junction where the adjacent and tumour tissues meet. Finally, the distal region and that was the normal tissue obtained from the resection margin; it represented the histologically normal tissue as far away from the tumour as possible. We also had unique access to biopsies from donor liver organs obtained during liver transplantations. We believed the donor liver is a better representation of

healthy control compared to distal tissue, which is used as “healthy liver” in most studies.

We first phenotyped hepatic mononuclear cells isolated from each CRLM region and donor liver tissue (Fig 3.5). We found donor liver to be enriched in lymphoid populations as previously described (Doherty et al., 1999, Doherty and O'Farrelly, 2000b). We also found CD8+CD3- cells, which are probably mostly NK-cells, were depleted in CRLM as previously described (Harmon et al., 2019a). An interesting and novel finding was the depletion of MAIT cells in CRLM compared to donor liver. The role of MAIT cells in cancer is not clear however their ability to produce IFN- γ as well as their depletion in CRLM suggests they have an important role. We did not further investigate the role of MAIT cells in CRLM because it was beyond the scope of this research project. The use of flow cytometry did not allow the assessment of absolute cell numbers. Most studies assessing absolute cell numbers through flow cytometry normalise data based on tissue weight. Tumours are made of abnormal cells that have different morphology, and most often different densities which contributes to total weight. Tumours also have necrotic centers in many cases which contribute to weight but are void of viable cells. While running our experiments, we found that CRLM tumour tissue weighed more than donor liver tissue of similar size. Therefore, to reliably assess absolute cell numbers and compare immune repertoire in CRLM compared to donor liver tissue we resorted to immunohistochemistry. The technique of immunohistochemistry allowed us to exclude necrotic centers, and allowed us to normalise our data by tissue area rather than weight.

We then manually produced histology sections from retrospectively recalled CRLM FFPE blocks and stained them by IHC with monoclonal antibodies for the visualisation and quantification of CD45+ cells (leukocytes), CD3+ cells (T-cells), and CD8+ cells (cytotoxic T-cells). We similarly, manually produced histology sections from FFPE blocks of donor liver tissue and stained them by IHC with monoclonal antibodies for the visualisation and quantification of leukocytes, T-cells, cytotoxic T-cells (Fig. 3.12). To date, only a few studies quantified absolute immune cell numbers in CRLM, and none quantified immune cell numbers in donor liver for comparison. Cell

counts were assessed using a free and publicly available software, and verified manually following the detailed protocol explained in the materials and methods chapter. When compared to donor liver, total counts of CD45+ cells, CD3+ cells, and CD8+ cells were all higher in CRLM. The increase of CD3+ T-cells and cytotoxic CD8+ T-cells in most CRLM patients suggests that the liver almost always reacted against the tumour, and also suggests the liver isn't oblivious to the threat. We found significant interpatient variation for all three markers, with the highest mean of cell counts found at the invasive margin. This validated an observation that we described while visually inspecting our stained sections (Fig 3.10). We now believe that one of the factors contributing to the accumulation of immune cells at the invasive margin could be a physical barrier that prevented these cells from infiltrating the tumour. The formation of a wall of connective tissue surrounding solid tumours has been described to form around benign tumours, and was linked to favorable patient outcome as it isolates and constricts the tumour (Perumpanani et al., 1999). Further work will be required to investigate the frequency of CRLM tumours with a surrounding band of connective tissue and whether it links to prognosis.

We observed that immune cells penetrated the tumour bed through what seemed to be designated entry points. Cells accumulated roughly evenly alongside the invasive margin, but seemed to penetrate the tumour through certain points scattered along the margin. We called these "penetration points" (Fig. 3.11). Ablation therapy is a minimally invasive technique involving the use of thermal energy to either heat or cool tissue to cytotoxic levels (Knavel and Brace, 2013). The use of ablation has been used in the clinic to replace or support surgical resection of CRLM and was shown to have favorable outcome (Li et al., 2016, Tsitskari et al., 2019). We now believe that one mechanism for the success of ablation therapy is boosting immune penetration points into tumours.

We divided patients into two groups; high recruiters and low recruiters based on the median for each marker's cell counts. Patients that fell below the median were classified as low recruiters, while patients that fell above the median were classified as high recruiters (Fig. 3.13). We investigated the link between high and low recruiters and overall survival and found that

high recruiters of CD8 and CD3 cells within the tumour region had significantly longer survival. Although our protocol was less sensitive, the approach was simpler and our findings were similar to those described in a recent paper based on a far more complex and automated approach (Mlecnik et al., 2018). We also found that high recruiters of CD45 cells at the invasive margin had significantly higher survival. We wondered whether CD45+ monocytes contributed to immune infiltrating mechanisms and whether they were needed to help lymphocytes penetrate through the invasive margin into the tumour. This has not been investigated and our speculation is only based on the drastic improvement in survival in patients with high CD45+ cells' specifically at the invasive margin (Fig 3.15). Investigating penetration points and the mechanisms linked to them was beyond the scope of this research project.

Having established that the immune repertoire in CRLM was altered, and that deficient accumulation of lymphocytes affected survival, we started considering possible reasons contributing towards immune enrichment or depletion in the tumour environment. One possible reason would be increased immune cell death due to accumulation of apoptosis inducers in the tumour microenvironment (Harmon et al., 2019a). Another would be compromised cell migration and recruitment due to altered cytokine microenvironment in CRLM (Balkwill, 2012, Hand et al., 2018a). Based on previous findings of microenvironment dysregulation in CRLM, we decided to look into the cytokine microenvironment (Hand et al., 2018a).

The liver is under constant pressure to induce tolerance towards harmless dietary and commensal antigens while eliminating pathogenic threats. This is done by maintaining a state of inflammation under careful control. This control is attributed to a complex cytokine microenvironment. Metastasis is the seeding of abnormal and foreign cells in a secondary location. These new cells as well as the altered immune repertoire would naturally alter the cytokine microenvironment (Balkwill et al., 2012). As the cellular and structural composition of the liver is altered in CRLM, we believed that the cytokine microenvironment is also altered. This would in return affect the liver's anti-tumour capacity. We also believed that the dysregulation of the cytokine microenvironment is linked to lymphocyte accumulation in CRLM.

In the past, individual cytokines were measured in total protein extracted from homogenised CRLM tissue. Previously, the group showed that IL-10, IL-12, and IL-18 were increased, while IL-15 was decreased in CRLM (Kelly et al., 2004, Kelly et al., 2006a). More recently, a broader investigation was carried out on CRLM tissue conditioned media using a protein array and highlighted the 12 most altered cytokines, most of which were chemokines including CXCL5, CXCL1, CCL3, CCL4, CCL5 and CXCL10 (Hand et al., 2018a). We considered the advantages and disadvantages of different protein preparations and decided to investigate the cytokine microenvironment in total protein extracted from tissue homogenates. We chose tissue homogenates over tissue supernatants (or conditioned media) because we believed it was a more controlled preparation technique and logistically better as we were able to freeze all tissues and process them together. We knew that human samples naturally have a big range of interpatient variation, and therefore sought to reduce handling steps in sample preparation that may increase background noise and mask potential differences between groups.

We analysed total protein from homogenates of three liver donor tissues, three CRLM tumour tissues, and three CRLM adjacent tissues (Fig. 4.3). Protein microarrays are a semi-quantitative technique that enables investigation of small samples for many targets simultaneously. The protein array we ran highlighted the healthy liver's rich and complex cytokine microenvironment. The array also pointed to the extensive dysregulation of the cytokine microenvironment in CRLM tissue, and even between the different regions of CRLM tissue. It was particularly interesting that the biggest distinction was not between the donor liver tissue and the tumour tissue, but rather between the donor liver tissue and CRLM adjacent tissue, which morphologically looks normal. Our attention was particularly drawn to CXCL9 (or MIG), CXCL10 (or IP-10), and CCL8 (or MCP-1).

Immune cell enrichment in the tumour microenvironment has been linked to better response to checkpoint inhibitors (Darvin et al., 2018). As chemokines are principal drivers of cell migration, we decided to look into chemokines in CRLM tumour microenvironment. CXCL10 and CXCL9 are structurally related IFN- γ induced chemokines that bind the receptor,

CXCR3. CXCR3 is expressed on NK-cells, NKT-cells, and cytotoxic T-cells (Clark-Lewis et al., 2003, Tokunaga et al., 2018). Although some studies highlighted the role of CXCL9 in metastasis, others highlighted the role of CXCL9 in anti-tumour responses. Mouse models showed increased TILs and decreased tumour angiogenesis in response to CXCL9 induction therapies (Tokunaga et al., 2018). CXCL9 knockout mice with fibrosarcoma tumours showed significantly less tumour T-cell infiltration compared to control mice (Gorbachev et al., 2007). CXCL9 induction by macrophages in mice models of glioblastoma and ovarian cancer led to increased tumour infiltration of CD8 T-cells (Pascual-García et al., 2019). CXCR3 knockout mice with tumours of colon cancer origin showed significant reduction in survival (Chow et al., 2019). In human studies, melanoma patients with good checkpoint inhibitor response had elevated CXCL9 levels in their plasma (Chow et al., 2019). Several studies have linked CXCL9 in CRC to T-cell enrichment and survival (Mlecnik et al., 2010, Ding et al., 2016, Wu et al., 2016). However, not much is known about CXCL9 in CRLM. Even less is known about CCL8 in CRLM. CCL8 was shown to be expressed by tumour associated macrophages, and was linked to tumour progression (Cassetta et al., 2019). It is a macrophage and Treg-cell chemoattractant, induced by IL-1 and IFN- γ , and can bind several receptors including CCR1, CCR2, CCR3, and CCR5.

We validated levels of CXCL9, CXCL10, CXCL8, and IFN- γ by ELISA (Fig. 4.4, 4.5). Levels of CXCL8 were significantly higher in the tumour compared to donor liver, and also significantly higher in the tumour compared to adjacent tissue (Fig 4.5). Levels of CCL8 in CRLM tumour showed significant interpatient variation not seen in donor liver tissue highlighting the heterogeneity of impacts exerted by tumours on patients. We also correlated CCL8 levels in the tumour to the number of CD45+ cells in the tumour and found they might be linked (Fig 5.9). We decided not to pursue this any further because our samples were very limited, and because our primary interest was the recruitment of cytotoxic T-cells. We found CXCL9 levels to be significantly higher in tumour tissue compared to adjacent, distal, and donor tissues (Fig. 4.4). CXCL9 also showed significant interpatient variation in both the tumour and adjacent tissue. When

correlated to the absolute number of immune cells in CRLM, we found significant correlation between CXCL9 levels and CD3+ cells (Fig 5.8).

We wondered whether we could link CXCL9 to CD8+ cell migration, and whether CXCL9 had a primary or redundant role in CD8+ cell migration in CRLM. To investigate this, we measured CXCR3 expression in CRLM and donor liver tissue, tested CXCR3 expression in activated and un-activated CD8+ cells, and ran several chemotaxis experiments.

We isolated hepatic mononuclear cells from CRLM and donor tissue. We then stained HMNCs for immune cell markers and for CXCR3 for flow cytometry analysis. We unfortunately did not include proper controls to be able to compare histograms across samples, and we were only able to compare percentage of positive cells for CXCR3 within each tissue type. Our flow cytometry analysis of CXCR3 expression did not show any significant differences in CXCR3 expression by CD45+ cells, CD3+ cells, CD8+ cells, or even MAIT cells (Fig.4.7). We then tested the expression of CXCR3 in-vitro in response to T-cell activation with anti-CD3 and anti-CD28. We found that the expression of CXCR3 on cytotoxic T-cells increases significantly in response to activation. This may suggest that tumour cells can reduce immune recruitment into the tumour by reducing or suppressing T-cell activation.

Chemotaxis assay is an in-vitro assay that allows the measurement of cell migration in response to a stimulant. The assay we performed was a transwell version which incorporates 24-well plate with inserts that hang into the well and include a porous membrane. Normally, the membrane pore size would be based on the size of tested cells. The cells are loaded into the top-hanging well, and the stimulus would be included into the media placed into the main and lower well. We chose to use samples of conditioned media as the stimulus in our assay, and we analysed the results by flow cytometry. We first tested the migration of two donor PBMCs towards nine tumour conditioned medias, and seven donor liver conditioned medias. We found the migration of CD8+ cells to be lower than the migration in response to donor liver conditioned media (Fig. 5.3), though the assay showed significant interpatient variation and was not statistically

significant. We then decided to pool conditioned medias and test multiple donor PBMCs. We defined the number of CD8+ cells in the histological sections corresponding to each available tumour conditioned media, then we pooled the highest three together, and the lowest three together. We hypothesised that migration in response to the media from the three patients with the highest CD8+ cells in their tissues would be higher than that of the low pool. However, cell migration in response to the two pools of tumour conditioned media was not different (Fig.5.4). The chemotaxis assay done with the pooled conditioned media also showed that migration of CD8+ cells is higher in response to pooled tumour conditioned media compared to pooled donor liver conditioned medias. This complements the observation we mentioned before about the absolute number of cells in CRLM compared to donor liver; the majority of patients with CRLM had higher immune cell numbers compared to donor liver, including some of the patients that fell below the median line of cell numbers.

To investigate the significance of CXCL9 in immune cell chemotaxis. We ran a chemotaxis assay in response to tumour conditioned media and our in-vitro model of tumour conditioned media with and without CXCL9 neutralisation. When we tested donor PBMCs migration in response to our in-vitro model of tumour conditioned media, we found that chemotaxis of CD45+ and CD8+ was significantly reduced in response to CXCL9 neutralisation (Fig 5.6). We repeated the experiment with pooled CRLM tumour conditioned media and validated our in-vitro result ex-vivo; we found that migration of CD45+ and CD8+ cells was significantly reduced in response to CXCL9 neutralisation (Fig. 5.7). CRLM tumour conditioned media is a complex mix of cytokines including growth factors and chemokines. We found significant reduction in chemotaxis of cytotoxic T-cells in response to the blocking of a single chemokine. This suggests that the role of CXCL9 in the migration of cytotoxic T-cells is not redundant, and that it can't be compensated for by other chemokines in the cytokine microenvironment of CRLM.

We finally examined the correlation of CXCL9 to immune cell numbers (Fig 5.8). We were limited by a small sample size of 12-13 and therefor were not too discouraged by the lack of significant correlation between CXCL9

levels and CD8 cell numbers. We found a significant negative correlation between CRLM tumour CD3 cell numbers and tumour CXCL9 levels contradicting our hypothesis. One possible explanation is that levels of the chemokine, CXCL9, are not reduced in the tumour microenvironment of low recruiters, but rather levels of the receptor, CXCR3, were low. CXCR3 expression is upregulated in response to T-cell activation, which in turn may be inhibited by the tumour microenvironment. Another reason may be our technical limitation of separating the invasive margin from our tumour and adjacent samples, since that is a microscopic region that can only be defined histologically. Nevertheless, it is essential to point out that this observation is based on a sample size of 12 with 10 of the samples aggregating on one side of the graph. Levels of adjacent tissue CXCL9 also significantly and positively correlated to CD3 cell numbers in the tumour. These findings highlight the value of defining different histological regions and their bearing on our investigations. They also highlight the need to increase our sample size in our correlation analysis before we can draw more definitive conclusions.

In this research project, we compared absolute counts of leukocytes and lymphocytes in patients with CRLM to donor liver for the first time. We observed significant interpatient variation within our counts of leukocytes (CD45+), T-cell (CD3+), and cytotoxic T-cells (CD8+). This variation allowed the classification of CRLM patients into "high recruiters" and "low recruiters". We also showed that lymphocyte infiltration correlates to survival in CRLM. We then showed that the cytokine microenvironment was dysregulated in CRLM compared to donor liver with a protein array of 80 targets. We further validated levels of CXCL9 in CRLM and donor liver and showed that CXCL9 levels were significantly higher in CRLM tumour. We showed that cytotoxic T-cells upregulated the expression of CXCR3 in response to activation. Finally, we showed that neutralising CXCL9 significantly reduces migration of cytotoxic T-cell. We believe that identifying targets to improve lymphocyte infiltration into the tumour will improve responsiveness to immunotherapies. Our work here indicates that CXCL9 may be one such target, and that enhancing CXCR3 expression

followed by enhanced CXCL9 levels in the tumour microenvironment could boost favourable tumour-immune infiltration.

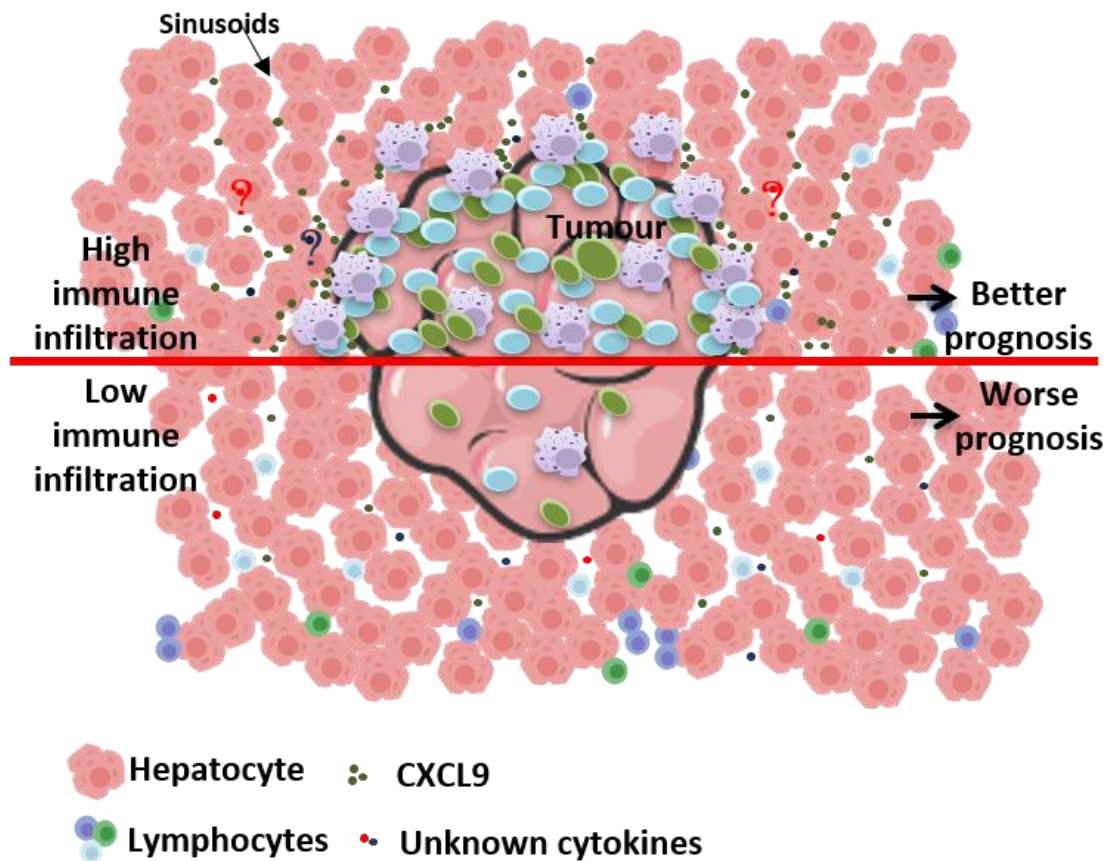


Figure 6.1: Model of the link between tumour-immune infiltration and the cytokine microenvironment.

Tumours with high infiltration of lymphocytes have been shown to have better prognosis. Lymphocyte infiltration is influenced by the local cytokine microenvironment, which in return is also influenced and altered in the tumour microenvironment. We believe that manipulating the chemokine microenvironment may boost lymphocyte infiltration, and that one significant chemokine in particular is CXCL9.

6.2. Future directions:

In the course of investigating our hypothesis, we identified several findings we could not pursue or further study because they opened up separate questions beyond the scope of this PhD thesis. In particular the finding of:

- A. Capsule like band surrounding some CRLM tumours.
- B. Immune cell penetration points.
- C. Depletion of MAIT cells in CRLM.
- D. The correlation of CD45+ cells at the invasive margin with favorable survival.
- E. Significantly high levels of CCL8 in CRLM

We believe that these findings warrant several new investigations which include:

- Quantification of “capsule” positive CRLM tumours and correlation to survival.
- Investigate lymphocyte exclusion at the invasive margin and further examine “penetration points”.
- Investigating the source of lymphocytes in CRLM tumours in order to determine whether they are primarily recruited from circulation, or proliferate from tissue resident cells.
- Investigate the role of MAIT cells in CRLM, and determine the cause of their depletion.
- Investigate the significance on CCL8 in CRLM.

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