THE RECRUITMENT AND ROLE OF EFFECTOR AND REGULATORY T CELLS IN RENAL CELL CARCINOMA

by

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ABSTRACT

Immunotherapy for renal cell carcinoma (RCC) has yielded some clinical responses. However this approach frequently fails, possibly due to inefficient migration of T-cells to tumour tissue or immunosuppressive mechanisms within the tumour environment. To aid development of T-cell therapy for RCC I investigated how T-cells are recruited to this tumour, which T-cell subsets infiltrate, and how they function.

Analysis of the expression of all 19 chemokine receptors on matched TIL and PBMC demonstrated that CCR5, CXCR3 and CXCR6 were expressed at significantly higher levels on tumour-infiltrating T-cells than memory T-cells in PBMC, suggesting a role for these receptors in recruitment to RCC. Immunohistochemistry showed the corresponding ligands were present in RCC, and transwell assays confirmed the ligands induce migration of TIL. I demonstrated $Foxp3^{+}CD25^{hi}CD127^{low}$ Tregs were enriched within the tumour, and also expressed high levels of CCR5, CXCR3 and CXCR6, as well as CCR6. They lacked expression of IL-2 and IFN-γ post-stimulation, consistent with a regulatory phenotype. Functional characterisation of $Foxp3^{-}$ TIL demonstrated they can function *ex vivo*, however their high expression of the inhibitory molecule PD-1 may indicate exhaustion *in vivo.* Double positive $CD4^{+}CD8^{+}$ T-cells were also enriched in TIL and had a similar functional profile to CD8 T-cells.

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LIST OF ABBREVIATIONS

ACT	Adoptive cell therapy
ADCC	Antibody-dependent cellular cytotoxicity
AF700	Alexa-Fluor 700
AmCyan	Anemonia majano cyan
APC	Antigen presenting cell
APC (flow)	Allophycocyanin
APC-Cy7	Allophycocyanin-cyanine 7
BSA	Bovine serum albumin
CA-IX	Carbonic anhydrase IX
CAR	Chimeric antigen receptor
CCL	CC chemokine ligand
CCR	CC chemokine receptor
ccRCC	Clear cell renal cell carcinoma
CD	Cluster of differentiation
CLA	Cutaneous leukocyte antigen
CLIP	Class II associated invariant chain peptide
CML	Chronic myeloid leukaemia
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	Dendritic cell
DP	Double positive
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FACs	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GEF	Guanine exchange factor
GVHD	Graft-versus-host disease
HEV	High endothelial venule
HIFα	Hypoxia inducible factor α
HLA	Human leukocyte antigen
IFN	Interferon
IHC	Immunohistochemistry
Ii	Invariant chain
IL	Interleukin
iTreg	Inducible regulatory T cell
IQR	Inter-quartile range

MDSC	Myeloid derived suppressor cell
MFI	Median fluorescence intensity
MHC	Major histocompatability complex
MLN	Mesenteric lymph node
mRCC	Metastatic renal cell carcinoma
(m)RNA	(Messenger) ribonucleic acid
NICE	National institute for health and clinical excellence
NK	Natural killer cell
NKT	Natural killer T cell
nTreg	Natural regulatory T cell
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed death 1
PDGF	Platelet derived growth factor
PE	R-phycoerythrin
PE-Cy	R-phycoerythrin-cyanine
PerCPCy5.5	Peridinin chlorophyll protein complex-cyanine 5.5
PHA	Phytohaemagglutinin
PLC	Phospholipase C
PLN	Peripheral lymph node
PMA	Phorbol myristate acetate
PSGL-1	P-selectin glycoprotein ligand 1
RCC	Renal cell carcinoma
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SP	Single positive
TAA	Tumour associate antigen
TAP	Transporter associated with antigen processing
TBS	Tris-buffered saline
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
TGFβ	Transforming growth factor β
Th1/2	T helper cell 1/2
TIL	Tumour infiltrating lymphocyte
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tr1	T regulatory cell 1
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
VLA-4	Very late antigen 4

1. Introduction

The aims of this work are to investigate the phenotype and function of T cells infiltrating renal cell carcinoma (RCC), and to determine their mechanism of homing to the tumour site. The introduction will therefore begin by introducing RCC and discussing both current treatments and the need for better therapies for advanced stage disease. It will address the possibility of using the immune system, particularly T cell-based therapies, to treat RCC. A pre-requisite for effective treatment of tumours by T cells is their ability to home to the tumour site, therefore there will also be review of the mechanism by which T cells are recruited to different tissues, and the current understanding of T cell homing to cancers.

1.1. Renal cell carcinoma

In the UK kidney cancer is the 8th most common cancer in men and the 9th most common in women, and accounts for over 2% of deaths from cancer (Cancer Research UK 2008). 90% of kidney malignancies are renal cell carcinomas, which originate from the renal parenchyma (made up of the cortex and medulla) (**Figure 1**) (Chow et al. 2010). Due to the preponderance of renal cancers being RCCs, this study focuses on this cancer type.

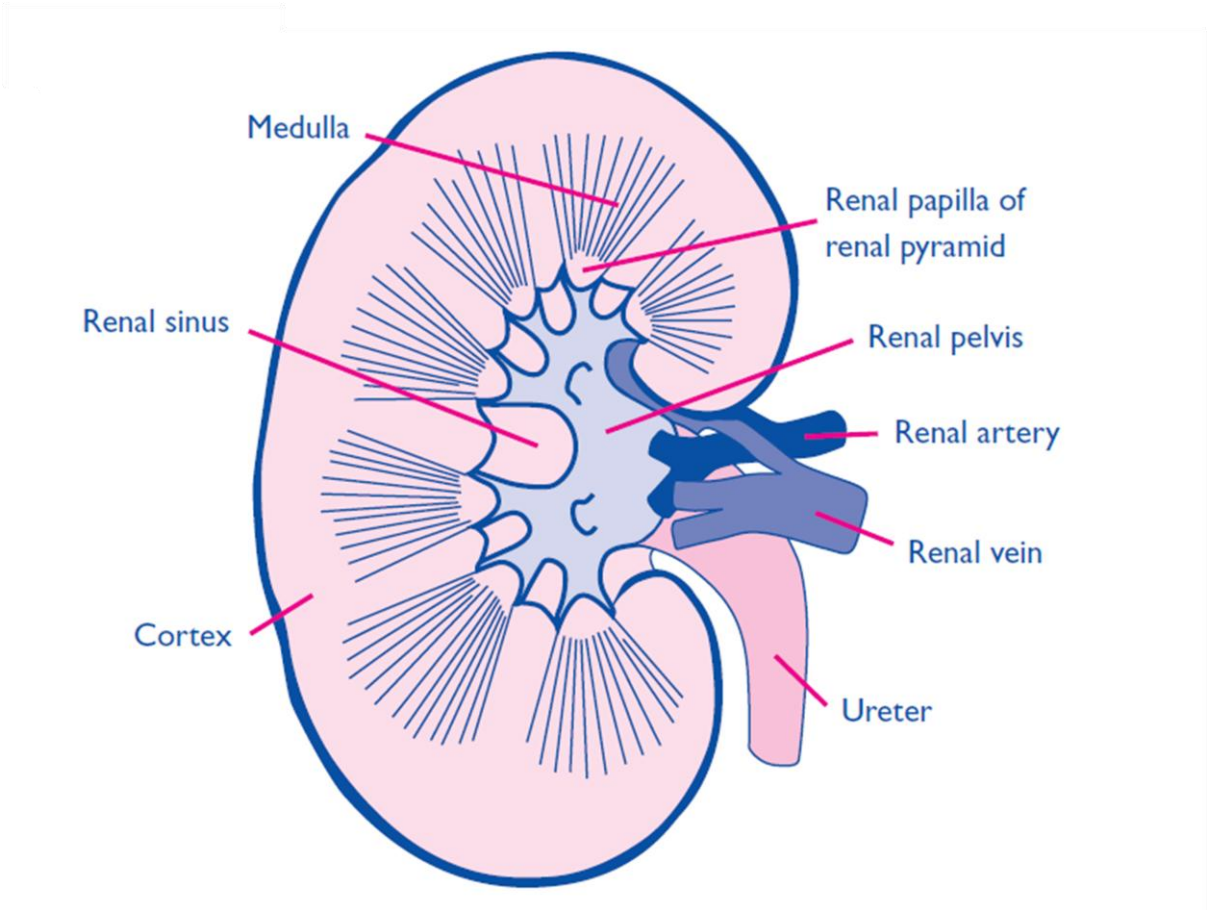


Figure 1: The kidney

RCC arises in the renal parenchyma, which is made up of the cortex and the medulla.
Picture taken from CancerStats Report – Kidney Cancer UK (Cancer Research UK 2008)

1.1.1. Pathology

Malignant RCC can be divided into subtypes based on histology. The subtypes differ in prognosis and disease progression, and diagnosis of the subtype can inform treatment choices. Clear cell RCC (ccRCC) is the most common form of the disease, accounting for 75% of RCC cases. It arises from the proximal tubules of the kidney and is characterised by cells with clear cytoplasm. The next most common subtype is papillary RCC, accounting for 10% of cases. Papillary RCC itself is sub-classified into Type I and Type II, the latter of which is associated with more aggressive disease and a worse patient outcome. 5% of RCCs are of the chromophobe type, which along with papillary type I have a better prognosis than ccRCC. Collecting duct RCC is rare and accounts for 1% of cases and carries a poor prognosis. The remaining cases consist of unclassified tumours and very rare subtypes: Xp11 translocation RCC, medullary carcinoma, RCC associated with neuroblastoma and mucinous, tubular and spindle cell carcinoma (Lopez-Beltran et al. 2006).

The staging of RCC tumours is classified using the TNM system, with T1-4 describing the size and spread of the primary tumour, N0-2 describing the number of regional lymph nodes involved and M0-1 detailing whether there are distant metastasis (Greene et al. 2011). Metastases are most common in the lung, followed by bone, liver and brain (Ritchie and Chisholm 1983). Fuhrman grading is also used as a description of the size and irregularity of the nucleus and the nucleolar prominence, and is a good predictor of prognosis (Fuhrman et al. 1982).

According to the British Association of Urological Surgeons (BAUS), in 2009 39.2% of patients were diagnosed at stage T1, 15.5% at T2, 27.3% at T3 and 17.5% at T4 (Fowler 2010). Survival rate is linked to the stage of the tumour, with patients diagnosed with stage one having a 75% 5-year survival rate, stage 2 a 63% survival rate, stage 3, 38% and stage 4, 11% (Guinan et al. 1995).

1.1.2. Epidemiology

In the UK kidney cancer accounts for 2-3% of all cancers diagnosed. Incidence varies worldwide, with Europeans and the North Americans more likely to have the disease, and lowest incidences of disease in Asians and South Americans. In all populations men are more likely to be diagnosed with RCC than women, with a male to female ratio of incidence of approximately 2:1 (Curado et al. 2011).

In the late 20th century incidence of RCC was rising, but in recent years rates have plateaued and may be declining (Curado et al. 2011;Levi et al. 2008). This could, in part, be due to increased early detection of RCC while it is at a low grade, however survival rates for all stages of the disease are improving (Chow and Devesa 2008;Kane et al. 2008).

1.1.3. Aetiology

There are a number of risk factors described for RCC. Smoking is one of the most studied, and male smokers in particular have an increased risk (50%) of developing RCC

(Hunt et al. 2005). Obesity is another clear risk factor, and is estimated to be a contributory factor in 30-40% of RCC cases in the USA and Europe (Calle and Kaaks 2004). The relationship is dose-dependent, with the risk of RCC increasing in proportion to body mass index (Adams et al. 2008).

A number of other risk factors have been suggested but have not been studied as thoroughly. Hypertension, renal disease, history of childbirth and occupational exposure to the chemical trichloroethylene have all been suggested to increase RCC incidence (Chow et al. 2010). The effect of diet has also been investigated with inconclusive results, however alcohol consumption has an inverse relationship with risk of developing the disease, with a 28% decreased risk in people consuming ≥ 15 grams of alcohol a day (Lee et al. 2007).

There are also a number of hereditary genetic conditions that can predispose people to developing RCC, and these account for approximately 3% of cases (Verine et al. 2010). The most well studied of these genetic diseases is von Hippel-Lindau (VHL) disease which is estimated to affect approximately 1 in 36000 live births in the UK. It is an autosomal dominant disease associated with a mutation in the VHL gene (Maher et al. 1991). As VHL is a tumour suppressor gene patients have an increased risk of developing various cancers, including ccRCC (Lonser et al. 2003). Up to 40% of patients develop RCC and it is thought the majority would go on to develop this tumour if they

did not first succumb to other complications (Levine et al. 1983;Maher et al. 1990;Malek et al. 1987).

74 % of sporadic ccRCC cases also have loss of function of both VHL alleles, highlighting the importance of this gene in the disease pathology (Banks et al. 2006). VHL is part of a ubiquitin ligase complex that binds to hydroxylated hypoxia inducible factor α (HIFα) and targets it for degradation (Pugh and Ratcliffe 2003). HIFα is normally only active under hypoxic conditions, where it is stabilised as it is no longer hydroxylated. It can then translocate to the nucleus and induce transcription of genes for angiogenesis and cell growth and survival, for the chemokine CXCL12 and its receptor, and for pH control. It also induces genes that alter glucose metabolism resulting in the Warburg effect, which ultimately supports the generation of new cells (Semenza 2003;Struckmann et al. 2008;Vander Heiden et al. 2009). If VHL is mutated HIFα is constitutively active resulting in pseudo-hypoxic conditions and inappropriate activation of angiogenic and cell proliferation pathways (Baldewijns et al. 2010;Semenza 2003), which promote tumour development.

HIFα is involved in a complex signalling pathway with many upstream factors. Other genetic disorders associated with RCC such as Birt-Hogge-Dubé syndrome, hereditary leiomyoma RCC and hereditary paranglioma syndrome also alter other components of the same signalling pathway and also predispose sufferers to RCC, highlighting the

importance of the pathway in control of cell proliferation and tumour suppression (Baldewijns et al. 2010;Verine et al. 2010).

1.1.4. Treatment

1.1.4.1. Current

Recommendations for treatment of RCC vary between countries. As the patients for this study were all treated in a UK NHS hospital, the following section focuses on guidelines given by the National Institute for Clinical Excellence (NICE) in the UK.

Surgery (full or partial nephrectomy) is considered the best option for RCC, and studies on stage 1 patients show it results in a greater than 90% cure rate (Lattouf et al. 2009). Even in stage 4, metastatic patients, cytoreductive surgery is considered as it eases disease burden and can improve quality of life (Wood 2003). Other methods of reducing tumour size such as radiofrequency ablation and cryotherapy are occasionally used for early stage tumours, particularly if patients have multifocal or bilateral tumours, as there is a better preservation of nephron function with these techniques (Kunkle and Uzzo 2008).

As RCC becomes more advanced, surgery is less likely to be curative and other treatment options are considered. Unfortunately chemotherapy has little efficacy in treating RCC (Yagoda et al. 1995) and so alternatives have been investigated. Immunotherapies using

interleukin-2 (IL-2) and interferon-α (IFN-α) have been used in the past, and although they have high toxicities and so were only indicated for use in fit patients, 10-20% of advanced RCC patients had a partial or complete response (Basso et al. 2009;Hutson 2011;Oudard et al. 2007;Reeves and Liu 2009). In fact, to date IL-2 treatment is the only curative therapy reliably documented for RCC with multiple metastasis. However due to the high toxicities associated with these therapies, the tyrosine kinase inhibitor (TKI) sunitinib is increasingly being used as a first-line therapy for metastatic RCC (mRCC) (NICE 2009).

Sunitinib targets vascular endothelial growth factor (VEGF) receptors and platelet derived growth factor (PDGF) receptors (Mendel et al. 2003), both of which are involved in pathways downstream of HIF activation (described earlier). Blockade of these receptors is thought to halt tumour growth by inhibiting the angiogenesis required for tumour cell survival. Sunitinib and another tyrosine kinase inhibitor (TKI), sorafenib, have also been shown to reduce the proportion of regulatory T cells (which correlate with a poor prognosis – see sections 1.3.6.3 and 1.4.3.2) in the peripheral blood and tumours of RCC patients (Adotevi et al. 2010;Desar et al. 2011), thereby potentially enhancing anti-tumour immunity. However eventually the tumour evolves to overcome this inhibition, as evidenced by the fact that patients on sunitinib eventually progress (Finke et al. 2011). Nevertheless it is one of the most successful treatments for mRCC, with a phase III trial showing improved response rate of 33% compared to 6% for IFN-α, and progression free survival of 11 months compared to 5 months (Faris and Michaelson 2010).

Recently a second TKI, pazoponib, has been recommended for use as a first-line therapy for RCC and its efficacy is currently being compared to sunitinib in a clinical trial (NICE 2011).

1.1.4.2. Future

Due to the lack of curative therapy for late stage RCC much work is still being carried out on potential treatments.

TKIs are a promising area of research and in addition to sunitinib, sorafenib (inhibitor of Raf, VEGF receptors, PDGF receptor β and the c-kit receptor) has been shown to have some efficacy in RCC (Escudier et al. 2009). Inhibitors to mTOR (which is upstream of VEGF) – temsirolimus and everolimus - have also shown some efficacy in RCC. A monoclonal antibody that affects the same pathway by neutralising VEGF, Bevacizumab, has also been investigated and appears to improve the length of progression free survival (Basso et al. 2009).

Further preliminary studies looking at using TKIs as adjuvants or neoadjuvants to reduce tumour burden and improve survival have taken place with some success (Bex et al. 2009;Thomas et al. 2009;Wood and Margulis 2009), and ongoing trials are investigating this further (Sciarra et al. 2011).Combining therapies such as bevacuzimab and IFN-α (Escudier et al. 2007;Rini et al. 2008) or using TKIs as radiosensitisers

alongside radiotherapy (Taussky and Soulieres 2009) have also had modest success and may warrant further investigation.

Other techniques being tested include high intensity frequency ultrasound (HIFU) for early stage patients (Caballero et al. 2010) but further study is required to determine its efficacy compared to existing treatments.

Finally immunotherapies are the subject of much research and various clinical trials have been performed using a range of approaches. These will be discussed in section 1.4.3, p41.

1.2. The immune system

The immune system has evolved from basic mechanisms of defence seen in plants and insects against pathogens, into a complex adaptive system in higher mammals capable not only of distinguishing between self and non-self, but also of recognising abnormal cells such as cancer. It is comprised of an array of specialised cells working in concert to protect against external and internal threats, and self-regulating to avoid inappropriate inflammation and tissue damage.

The immune system can be divided into innate and adaptive immunity. This project focuses on T cells, which are a branch of the adaptive immune system. Other aspects of

immunity will be discussed briefly, however this introduction will focus on T cell immunity.

1.2.1. Innate immune system

The innate immune system represents the most ancient type of immunity. Organisms from plants to mammals share the ability to produce antimicrobial peptides (defensins) that can kill bacteria (Ganz 2003), and carry receptors that stimulate a reaction in response to detection of pathogens (Hoffmann et al. 1999).

In humans the epithelium and mucus membranes offer a first line of defence against pathogens. However in case of a breach, macrophages patrol the body and destroy pathogens by phagocytosis (Aderem and Underhill 1999). Macrophages, along with other immune cells, are able to distinguish between self and non-self using pattern recognition receptors. One example of a class of pattern recognition receptor is the Toll-like receptors (TLRs), which can trigger cytokine and chemokine release upon activation, thereby promoting inflammation at the site of infection and recruiting more innate cells such as neutrophils and dendritic cells (Kawai and Akira 2011).

1.2.2. Adaptive

A significant function of the innate system in humans is to activate a second branch of the immune system: adaptive immunity. Adaptive immunity alone is not sufficient to protect against pathogens as the initial response can take days, however it does provide

key advantages. After the first challenge by a particular pathogen, immunological memory is generated allowing a much more rapid secondary response if the same pathogen invades again, and rapid clearance of the danger. In addition the adaptive system allows very specific recognition, with each adaptive immune cell carrying an antigen receptor recognising a particular peptide. There is a vast repertoire of receptors, and this diversity allows recognition of pathogens that have evolved to evade the innate immune system by avoiding or hiding expression of conserved molecular patterns recognised by innate receptors. The greater specificity of the adaptive system also allows greater selectivity, and may be a key mechanism in gut immunity where it is advantageous to permit survival and growth of beneficial bacteria while concurrently patrolling for pathogenic organisms (Lee and Mazmanian 2010).

The adaptive immune system consists of B and T lymphocytes that originate from the common lymphoid progenitor in the bone marrow. Each lymphocyte expresses one receptor specific for one antigen. When a naive lymphocyte recognises its antigen and receives appropriate stimulatory signals, it proliferates to create a clonal population. Once the danger has been resolved, a small proportion of the clonal cells, so-called 'memory' lymphocytes are maintained in case of re-infection.

1.2.2.1. B cells

B cells recognise antigen using a surface bound immunoglobulin protein, and function to kill extracellular pathogens. They do this by releasing soluble immunoglobulin (known

as antibodies) upon activation, of the same specificity to their surface receptor. Antibodies are then able to bind to the pathogens and either neutralise their function or opsonise them for ingestion by phagocytes. Bound antibodies can also act as a receptor for the first protein of the complement system with subsequent activation of the complement cascade that can lead to destruction of the pathogen.

1.2.2.2. NK and NKT cells

Natural killer (NK) cells recognise cells under physiological stress (for example infected cells or tumour cells) and kill them by releasing cytotoxic granules and producing cytokines upon activation. NK cells can be activated by stimulation via their activating receptors or by failing to receive signals through their inhibitory receptors (Hamerman et al. 2005). NKs are normally described as innate immune cells, however recent evidence has shown they have some features of adaptive immunity, including the ability to persist after stimulation and display enhanced function upon re-challenge (Vivier et al. 2011).

NKT cells are lymphocytes that express both NK receptors and an αβ T cell receptor (TCR). However, unlike T cells, NKTs have a very restricted TCR repertoire, all of which recognise lipids presented by the MHC-like molecule CD1d. They are able to activate DCs and rapidly release cytokines upon stimulation, which in turn influence and regulate the type of T cell response that occurs (Godfrey and Kronenberg 2004).

1.3. T cells

1.3.1. Introduction

In contrast to B cells, T cells migrate to the thymus where they mature and undergo selection processes to ensure they are capable of recognising self-major histocompatability complex (MHC) molecules and do not respond strongly to self-antigens (Klein et al. 2009). B cells, on the other hand, remain in the bone marrow, which is where pro-B cells develop into immature B cells by completing initial immunoglobulin gene (VDJ) rearrangement to form and express complete IgM molecules, before migrating to peripheral lymphoid organs where they mature into naive B cells that express both IgD and IgM. In common with B cells, T cells also have cell surface antigen receptors, known as T cell receptors (TCRs). The majority of T cells carry TCRs with α and β chains, and they recognise peptides bound to MHC molecules. All T cells also express the CD3 complex, which is associated with the TCR and is required for TCR expression and signalling (Reinherz et al. 1982). Most T cells also express either the CD4 or CD8 co-receptor, which associate on the T-cell surface with the TCR and bind to invariant sites on the MHC molecule that is presenting the antigen ligand, thereby improving the strength of the interaction and the T cell response (Ledbetter et al. 1981). T cells are known as either CD8 or CD4 T cells, accordingly.

T cell gene rearrangement occurs in the thymus. The β chain (in common with the immunoglobulin heavy chain) is encoded by three gene segments: the variable (V) segments, the joining (J) segments and the diversity (D) segments (Davis 1990). The α

chain (in common with the immunoglobulin light chain) is encoded by V and J segments. Initially the β chain rearranges (Saint-Ruf et al. 1994), firstly, by D to J gene segment rearrangement, followed by V to DJ rearrangement. It is then expressed alongside a surrogate α chain and CD3 (Mallick et al. 1993). At this stage CD4 and CD8 are both expressed (Petrie et al. 1990). β gene rearrangement then stops and the α gene is rearranged by V to J rearrangement. α gene rearrangement and expression continues until a successful αβ TCR is produced which survives positive selection in the thymus (Petrie et al. 1993). T cells then lose either CD4 or CD8 before undergoing negative selection, which ensures T cells don't recognise self-antigens strongly. T cells are then able to enter the circulation.

The repertoire of different αβ TCRs is vast as the variable (V) regions of the TCR are encoded in the genome in segments, and undergo gene rearrangement in order to form a complete coding sequence. There are multiple copies of the V gene segments for both the α and β chains, and variation can also arise at the junctions between gene segments and from different pairings between the α and β chains to make a complete TCR. This results in a potential diversity of up to 10^{15} different TCRs (Davis 1990).

1.3.2. The major histocompatability complex

The MHC, also known as human leukocyte antigen (HLA) in humans, plays a key role in antigen presentation. Prior to presentation antigen processing must occur, whereby

protein antigens are degraded into short peptide fragments that then bind to MHC molecules for subsequent presentation on the cell surface and interaction with specific TCRs.

In order to prevent pathogens evolving so that the MHC is unable to present peptides derived from their proteins, the MHC complex has itself evolved to be both polygenic and polymorphic. Everyone inherits multiple MHC class I genes (HLA-A, B and C) and MHC class II genes (HLA-DR, DP and DQ), which bind peptides differentially. In addition, within the human population there are hundreds of different alleles for each gene, resulting in huge variation in the exact MHC molecules, and therefore exact peptide binding specificities, within the population (Horton et al. 2004).

There are two classes of MHC molecules, MHC I and MHC II, which bind different peptides and are specialised for presenting peptides to different subsets of T cells.

1.3.2.1. MHC I antigen processing

MHC I molecules are expressed by every nucleated cell. They bind peptides of 8-10 amino acids in length and present them to a subset of T cells known as CD8 T cells. They bind peptides derived from proteins synthesised within the cell, which will include those from intracellular pathogens such as viruses, allowing killing of the infected cells upon recognition by T cells (Falk et al. 1990;Rotzschke et al. 1990). Proteins generated in the cytoplasm are degraded into peptides by the proteosome, before being transported into

the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP) proteins. MHC I proteins are formed in the ER, and only complete their folding upon binding of a peptide. They are then transported to the cell surface (Rock et al. 1994;Townsend et al. 1989;Townsend and Trowsdale 1993;Yewdell and Bennink 1989).

1.3.2.2. MHC II antigen processing

MHC II molecules bind longer peptides and are recognised by the CD4 subset of T cells. They are expressed primarily by cells of the immune system, but their expression can be induced in other cell types, including tumours (Steimle et al. 1994). They bind peptides generated from extracellular proteins, or proteins within intracellular vesicles of cells (Rudensky et al. 1991). They are therefore important in presentation of bacterial antigens. Extracellular proteins are taken up into the cell by endocytosis, into vesicles called endosomes. These endosomes then fuse with lysosomes. The proteins are gradually degraded into peptides by enzymes. MHC II is released from the ER and is transported to the endocytic vesicles, during which time the peptide binding groove is occupied by a protein called invariant chain (Ii). Once inside a new type of vesicle is formed, with both endosomal and lysosomal characteristics, known as the MHC class II compartment (MIIC) (Calafat et al. 1994). Within the MIIC Ii is cleaved to leave a small fragment — class II associated invariant chain peptide (CLIP) bound to the MHC molecule. HLA-DM, a protein closely related to the other MHC II molecules, then catalyses the release of CLIP and the binding of other peptides to the MHC II molecule (Denzin and Cresswell 1995;Roche and Cresswell 1991).

1.3.2.3. Cross-presentation

Proteins can be released from one cell and taken up by neighbouring cells through endocytosis, thereby entering the MHC II antigen processing pathway and being presented on MHC II molecules. Less obviously, such extracellular proteins enter the MHC I pathway. Dendritic cells (DCs) (see section 1.3.3.1, p19) are important in initiating a CD8 T cell response as they present co-stimulatory molecules required to correctly activate the T cells. Therefore if a virus does not infect DCs, or if CD8 T cell immune recognition is required of protein signatures from other threats (e.g. cancer), DCs need to have a mechanism to take up the proteins and present them on MHC I. Normally endocytosed proteins do not enter the MHC I pathway to avoid killing of bystander cells which may have taken up viral particles when an infected cell was killed, but certain DC subsets are able to perform this function by fusing phagosomes with the ER and hence bringing together TAP and MHC I molecules with exogenous peptides (Guermonprez et al. 2003).

1.3.3. Antigen presenting cells

While many cells can express MHC I and MHC II, in order to activate a naive T cell expression of co-stimulatory molecules that bind the CD28 receptor on T cells, namely CD80 and CD86, is required by the cell presenting antigen at the point of TCR:MHC interaction. Macrophages and B cells can both act as professional antigen presenting cells (APCs) and are able to up-regulate co-stimulatory molecules upon stimulation (Janeway, Jr. 1992). In fact the B cell immunoglobulin receptor enhances uptake of antigen and subsequent presentation on the B cell's MHC molecules, meaning that even

at low concentrations of antigen, if an antigen-specific B cell is present T cells can be activated (Lanzavecchia 1985).

However, mature dendritic cells are the most effective APC and constitutively express co-stimulatory molecules once they have matured (Heufler et al. 1988;Inaba et al. 1990).

1.3.3.1. Dendritic cells

Immature DCs migrate to peripheral tissues where they patrol the site by taking up pathogens by phagocytosis and extracellular fluid by macropinocytosis (Sallusto et al. 1995). If the immature DC encounters a danger signal, for example by ligation of a TLR, it matures, up-regulating chemokine receptors for migration to the lymph nodes (Dieu et al. 1998), increasing expression of MHC molecules (Cella et al. 1997) and up-regulating co-stimulatory B7 molecules (CD80 and CD86) (Caux et al. 1994;Rescigno et al. 1998). Once in the lymph node they produce the chemokine CCL18 to attract naive T cells and prime the antigen specific T cells to differentiate into the appropriate subset of effector T cell (Adema et al. 1997;Liu and MacPherson 1993). However, the receptors for this chemokine are not known (Chang et al. 2010;Schutyser et al. 2005). If an immature DC does not encounter a danger signal, or is exposed to IL-10, it will still home to the lymph node at the end of its lifespan, where it will present antigen in the absence of co-stimulatory molecules, thereby inducing anergy in CD8 T cells and a an anergic or

regulatory T cell (Treg) phenotype in CD4 T cells (Jonuleit et al. 2000;Steinbrink et al. 1999) (**Figure 2**).

1.3.4. T cell signalling

When a T cell recognises an MHC:ligand complex, CD4 or CD8 associate with the TCR resulting in a signalling cascade that, through the successive phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) and Zap-70, activates the phospholipase C (PLC) pathway (Denny et al. 2000;Visco et al. 2000). This results in an increase in intracellular calcium concentration, which in turn activates the transcription factor NFAT. It also causes the activation of protein kinase C, which activates the transcription factor NFκB (Jordan et al. 2003). Finally a MAP kinase pathway is initiated that activates Fos, a component of the transcription factor AP-1. However, in the absence of a co-stimulatory signal from a DC, AP-1 will not be fully formed, and IL-2 expression will not occur (Jain et al. 1992). Signalling through CD28, the receptor for the B7 co-stimulatory molecules, is required for the induction of a second MAP kinase pathway that activates Jun, the other component of AP-1 (Kempiak et al. 1999). The three transcription factors then translocate to the nucleus where they activate transcription of genes for cell proliferation and differentiation.

The cell surface molecule CD45 is also involved in the signalling pathway as it de-phosphorylates the inhibitory tyrosine residues on Lck and Fyn (the Src tyrosine kinases) that are constitutively phosphorylated in naive T cells (Cahir McFarland et al.

1993). This allows them to become activated and phosophorylate ITAMs, initiating the T cell signalling cascade.

CD45 itself has different isoforms as a result of alternative splicing, and these isoforms are differentially expressed depending on the stage of T cell differentiation (Trowbridge and Thomas 1994). Naive T cells express the CD45RA isoform, whereas effector or memory T cells lose CD45RA expression and gain CD45RO (Akbar et al. 1988). However some T cells regain CD45RA expression when they reach a late stage of differentiation, and are designated EMRA (effector memory RA) T cells (Di et al. 2011).

1.3.5. T cell differentiation

Once a naive T cell has encountered its antigen it differentiates into an effector T cell. After the initial immune response, a small proportion of these cells resist apoptosis and persist as either effector memory (T_{EM}), $CD8^+$ effector memory RA T cells (T_{EMRA}) or central memory (T_{CM}) T cells. These can be distinguished by their phenotype, as T_{CMs} express the lymph node homing receptor CCR7, which is absent on T_{EMs}. T_{EMs} patrol peripheral tissues and are able to function immediately upon antigen recognition, whereas T_{CMs} sample the lymphoid environment and differentiate into effect cells upon stimulation (Sallusto et al. 1999). T_{CMs} have the greatest proliferative potential, followed by T_{EMs} and finally T_{EMRAs} (Geginat et al. 2003).

It is unclear whether all effector T cells have the capacity to develop into memory T cells, or if just a subset are capable of doing so. Single naive T cells have been demonstrated to have the capacity to produce both effector and memory T cell progeny; therefore T cell fate is not decided prior to their first division (Stemberger et al. 2007). However it is unclear at what stage after their first division T cell fate is determined (Gerlach et al. 2011).

1.3.6. T cell subsets

1.3.6.1. CD8 T cells

CD8 T cells, often referred to as cytotoxic T lymphocytes (CTLs), recognise peptides bound to MHC I via their TCR. Their main function is to recognise virally infected cells, and as such upon binding of the TCR to the MHC complex, they initiate killing of the infected cell. This mechanism of direct killing means they may also be of use in fighting cancer.

CD8 T cells function by releasing cytotoxic granules upon stimulation. These granules contain perforin, which forms pores in the target cell membrane, and granzyme, which is a trypsin protease that enters the target cell through the perforin pores and induces apoptosis (Heusel et al. 1994;Kagi et al. 1994). In addition activated CD8 T cells express Fas ligand, which binds the Fas receptor expressed on the target cells, thereby inducing apoptosis (Rouvier et al. 1993). Upon activation CD8 T cells also produce the effector cytokines interferon-γ (IFN-γ), and tumour necrosis factors (TNF), which activate

macrophages, up-regulate MHC molecules and promote T cell survival and proliferation (Barber et al. 2006;Kasahara et al. 1983).

1.3.6.2. CD4 T cells

CD4 T cells are also known as T helper cells, as their principal function is to activate and promote the responses of other immune cells. Naive CD4 T cells differentiate into different functional subsets. The two major effector subsets are designated Th1 and Th2. Regulation of differentiation into either the Th1 or Th2 subset is dependent on both the antigen dose (Rogers and Croft 1999) and the cytokines present (**Figure 2**). Production of IL-12 or the type 1 interferons induces a Th1 phenotype by activating the transcription factor STAT4 (Cho et al. 1996;Manetti et al. 1993). This induces the expression of IFN-γ, which stabilises the phenotype by inducing expression of the transcription factor T-bet, which up-regulates the IL-12 receptor and IFN-γ production (Lighvani et al. 2001). Th2 differentiation, on the other hand, is regulated by IL-4, which induces expression of STAT6 (Zhu et al. 2001), which in turn up-regulates the transcription factor GATA-3. GATA-3 down-regulates IFN-γ and up-regulates IL-4, thereby inducing a Th2 phenotype (Ferber et al. 1999).

Th1 T cells activate macrophages through their expression of CD40 ligand or TNFα, to initiate fusing of their intracellular phagosomes with lysosomes. This is crucial in the case of infection of vesicles with bacteria, and results in the pathogens' destruction. Th1s also produce the effector cytokines IL-2 and IFN-γ, which augment the CD8 T cell

response by promoting their activation and up-regulating MHC I expression on APCs. In common with CD8 T cells, some CD4 T cells also express Fas ligand. Th1s can also activate some B cells to produce opsonising antibodies (Abbas et al. 1996;Mosmann and Coffman 1989).

Th2 cells produce IL-4 and in common with Th1 cells, function to activate B cells (Abbas et al. 1996;Mosmann & Coffman 1989). B cell activation by Th2 cells results in B cell production of neutralising antibodies and defence against extracellular pathogens.

Due to their ability to promote a cytotoxic T cell response, Th1 T cells are considered to me more beneficial than Th2s in tumour immunity.

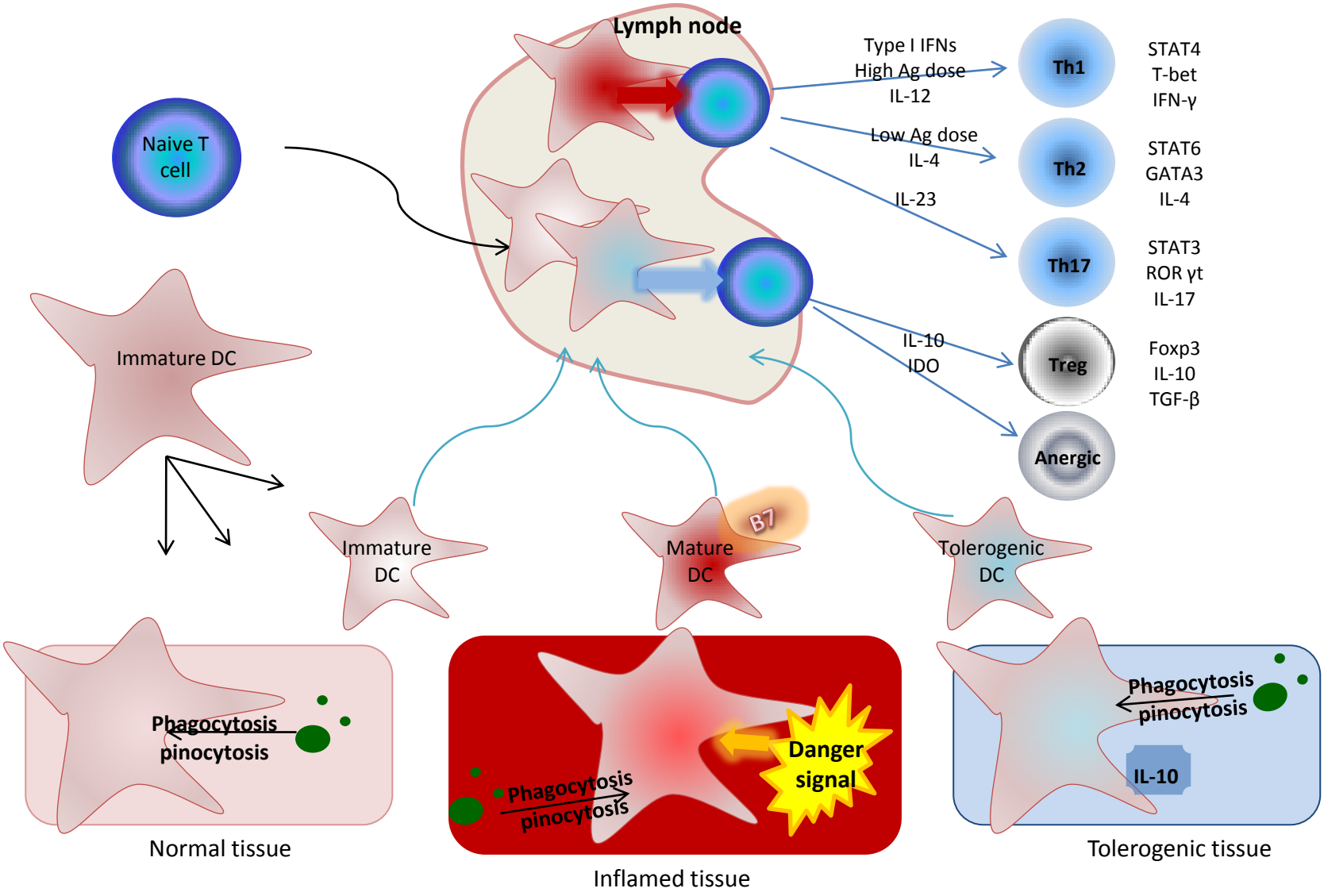

Lymph node
Naive T cell
Immature DC
Type I IFNs
High Ag dose
IL-12
Low Ag dose
IL-4
IL-23
IL-10
IDO
Th1
Th2
Th17
Treg
Anergic
STAT4
T-bet
IFN-γ
STAT6
GATA3
IL-4
STAT3
ROR γt
IL-17
Foxp3
IL-10
TGF-β
Immature DC
Mature DC
B7
Tolerogenic DC
Phagocytosis
pinocytosis
Danger signal
IL-10
Normal tissue
Inflamed tissue
Tolerogenic tissue

Figure 2: Naive CD4 T cells differentiate into different functional subsets

Immature DCs migrate to tissues where they sample the local environment, before homing to lymph nodes. If the DCs do not encounter a danger signal within the peripheral tissue, or if they are exposed to inhibitory cytokines such as IL-10, they do not up-regulate co-stimulatory molecules, and will induce a regulatory or anergic phenotype in the T cells they subsequently interact with in the lymph node.

If a DC does encounter a danger signal in the tissue it will up-regulate the co-stimulatory B7 molecules CD80 and CD86, which enable them to activate T cells to become effector cells. CD4 T cells will then differentiate into Th1, Th2 or Th17 T cells, depending on the cytokines and antigen dose present at the time of stimulation.

The transcription factors and cytokines expressed differ between these subsets and regulate their function.

1.3.6.3. Regulatory T cells

Regulatory T cells (Tregs) are a third subset of CD4 T cells. In humans Tregs can either arise in the thymus (natural, or nTregs), or be induced from peripheral naive CD4 T cells in response to transforming growth factor β (TGF-β) signalling (iTregs) (Chen et al. 2003;Qin et al. 1993;Sakaguchi et al. 2008). nTregs are selected for in the thymus by their recognition of self-antigen, but instead of being deleted are directed down a separate differentiation pathway so they become regulatory (Jordan et al. 2001).

Tregs can suppress CD8 and CD4 T cell proliferation and function, preventing inappropriate immune responses which would otherwise cause autoimmune disease such as rheumatoid arthritis (Behrens et al. 2007;Sakaguchi et al. 1995) and colitis (Sakaguchi et al. 1995;Takahashi et al. 2006). However, immunosuppressive activity may also affect anti-tumour T cell responses in cancer patients (Fujimoto et al. 1975).

Defining Tregs

The 'classic' Tregs, nTregs and iTregs, are now defined as $CD4^{+}CD25^{+}CD127^{low}FoxP3^{+}$ cells (Griffiths et al. 2007;Liu et al. 2006). Early studies on Tregs often used the $CD4^{+}CD25^{hi}$ phenotype as a marker of Tregs, as this does not require intracellular staining, and these cells have been shown to be functional in suppressing $CD4^{+}$ T cell proliferation in response to stimulation by antibodies to CD3 and CD28 (Cesana et al. 2006;Dannull et al. 2005). However, CD25 is also upregulated on effector T cells after activation, (Ortega et al. 1984) which means additional markers are required for more

accurate identification of Tregs. The Foxp3 transcription factor is critical for Treg development, and transduction of $CD4^{+}CD25^{-}$ T cells with Foxp3 induces Treg phenotype and function (Fontenot et al. 2003;Hori et al. 2003). In addition, mutations in *FOXP3* lead to autoimmune disease, as seen in immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) patients, indicating the vital role of Foxp3 expressing cells in immune regulation (van der Vliet and Nieuwenhuis 2007). Therefore it can be argued that Foxp3 is a better marker of Tregs than $CD4^{+}CD25^{hi}$. Nevertheless Foxp3 can also be transiently expressed on activated CD4 and CD8 cells so one cannot assume that every $Foxp3^{+}$ T cell is a Treg (Ahmadzadeh et al. 2007;Gavin et al. 2006). CD127 expression is consistently low on $Foxp3^{+}$ T cells and is useful as an additional marker, although it is transiently down-regulated upon T cell activation (Liu et al. 2006). Therefore the combination of the phenotypic markers $CD4^{+}CD25^{+}$ $CD127^{low}FoxP3^{+}$, combined with functional studies, present the most accurate method of defining a Treg population.

Other types of regulatory T cells have been identified and they may also have a role in cancer immunosuppression. T regulatory cells 1 (Tr1) cells have strong immunosuppressive properties, and can be induced in vitro through exposure to IL-10. However more recent studies suggest IL-27 is the main inducer of this T cell phenotype (Pot et al. 2009).Tr1s do not express Foxp3, and produce IL-10, TGF-β and IL-5, some IFN-γ and almost no IL-2 or IL-4 (Bacchetta et al. 1994;Groux et al. 1997;Roncarolo and Gregori 2008). They are important regulators of immunity and are thought to prevent autoimmune disease (Groux et al. 1997) and allergies (Akdis et al. 2004). Their

generation is promoted by squamous cell carcinoma of the head and neck tumours (Bergmann et al. 2008). In addition, some CD8 T cell subsets can have regulatory properties. CD8ααTCRαβ T cells can kill activated T cells in experimental autoimmune encephalitis and $CD8^{+}CD122^{+}$ regulate CD4 T cell mediated colitis through IL-10 expression (Endharti et al. 2011;Tang et al. 2006).

Function of classic Tregs

As with effector T cells, Tregs must be activated through their TCR in order for them to carry out their suppressive function, however subsequently they can suppress not only cells of the same antigen specificity, but also cells specific for different antigens, by a mechanism known as bystander suppression (Karim et al. 2005;Masuyama et al. 2002;Takahashi et al. 1998). The mechanism of Treg immune suppression is unclear, but is likely to be multifaceted.

Tregs express high levels of the IL-2 receptor (CD25) and it has therefore been suggested they act as an IL-2 sink, depriving effector T cells of the IL-2 they require for function (Pandiyan et al. 2007). Furthermore, mice lacking a functional IL-2 receptor develop autoimmune disease (Suzuki et al. 1995;Willerford et al. 1995) and the genes for IL-2 and CD25 are regulated by Foxp3 itself (Wu et al. 2006), suggesting IL-2 and its receptor are vital for effective Treg function. However CD25 is not required for suppressive function of Tregs *in vitro* (Fontenot et al. 2005), although it is required for Treg survival and maintenance (Fontenot et al. 2005;Setoguchi et al. 2005).

As with the IL-2 receptor, cytotoxic T-lymphocyte antigen 4 (CTLA-4) is also controlled by the Foxp3 transcription factor and is upregulated when Foxp3 binds its target (Wu et al. 2006). It binds to CD80 and CD86, thereby competing with CD28 and reducing co-stimulation. Its deficiency also causes autoimmune disease, improves anti-tumour immunity and decreases suppressive activity of Tregs (Wing et al. 2008). CTLA-4 expression does not simply produce a negative signal affecting the T cell expressing it. Murine studies have shown that whereas a CTLA-4 knockout has lethal lymphoproliferative disease and disregulated immunity, a mouse with both CTLA-4 $^{-/-}$ and CTLA-4$^{+/+}$ bone marrow will be completely normal (Bachmann et al. 1999). Therefore CTLA-4 regulates cells extrinsically, i.e. CTLA-4 expressed by one cell can affect its neighbours. It has recently been demonstrated that CTLA-4 can remove the co-stimulatory molecules CD80 and CD86 from APCs by trans-endocytosis, thereby limiting their capacity to stimulate a T cell response (Qureshi et al. 2011). In addition, binding of CTLA4 to CD80 and CD86 on DCs has been shown to trigger tryptophan catabolism in these cells, which suppresses T cell proliferation (Munn et al. 2004).

There is controversy over whether cell-cell contact is required for Treg mediated immune suppression. The majority of evidence suggests Tregs are incapable of suppressing CD4 T cell proliferation *in vitro* when separated from the proliferating cells by a semi-permeable transwell membrane, but need direct contact to exert their effect (Longhi et al. 2006;Sojka et al. 2008;Strauss et al. 2008;Zaiss et al. 2007). Mechanisms of action that require cell-cell contact include killing of target cells via granzyme and

perforin (Cao et al. 2007), and up-regulating cyclic AMP in target cells, which suppresses their proliferation and cytokine gene expression (Bopp et al. 2007).

However, other studies indicate that Tregs have a definite, although lessened effect through a transwell (Gad et al. 2004;Longhi et al. 2006;Strauss et al. 2007). IL-10 and TGF-β have been proposed as soluble mediators of immune suppression. Tregs can secrete TGF-β and IL-10 and these can inhibit T cell function in inflammatory bowel disease and endothelial cell inflammation (Asseman et al. 2000;He et al. 2010;Read et al. 2000). IL-10 does not appear to be required for immune suppression in every disease scenario (Suri-Payer and Cantor 2001), whereas knocking out TGF-β results in fatal autoimmune disease in mice (Marie et al. 2006). Studies in mice have identified a third cytokine, IL-35, that is highly expressed by Tregs and suppresses T cell proliferation *in vitro* and *in vivo* (Bardel et al. 2008;Collison et al. 2007). A recent study using human Tregs showed IL-35 was required in order for Tregs to reach their maximum level of suppression. Transwell assays also suggested the IL-35-mediated suppression was contact-independent (Chaturvedi et al. 2011).

1.3.6.4. Th17s

Th17s are another subset of effector CD4 cells, which are characterised by their production of IL-17 and expression of the transcription factors RORγt and STAT3 (Chen et al. 2007). TGFβ and IL-6 are both required for Th17 differentiation, with IL-1β, TNF-α and IL-23 having roles in amplification and stabilisation of the cells (Bettelli et al.

2006;Veldhoen et al. 2006). Analogous to the relationship between Foxp3 and Tregs, expression of the transcription factor RORγt induces CD4 differentiation into Th17s (Ivanov et al. 2006). In fact, Th17s appear to arise from the same cell lineage as Tregs, and the differentiation of a CD4 T cell into a Treg or Th17 depends on the relative expression of the transcription factors Foxp3 and RORγt, and on the cytokines present. If TGF-β is present at low concentrations, it may upregulate the IL-23 receptor which in turn helps stabilise a Th17 phenotype. At high concentrations, TGF-β upregulates Foxp3, which in turn inihibits RORγt expression. However if pro-inflammatory cytokines such as IL-6 and IL-21 are also present, upregulation of Foxp3 by TGF-β will be reduced and a Th17 differentiation will become more likely (Zhou et al. 2008). The cells are pro-inflammatory and provide defence against extracellular pathogens (Ye et al. 2001), although they also mediate autoimmune disease (Langrish et al. 2005).

The contribution of Th17s to anti-tumour immunity is controversial. Th17s negatively correlate with Tregs, known to adversely affect prognosis (Bettelli et al. 2006). A study on mice has shown that transgenic CD4 T cells with Th17 bias were better at causing tumour regression than Th1 cells, although they also induced autoimmune disease (Muranski et al. 2008). A study on human ovarian carcinoma patients found Th17 infiltration of the tumour to be negatively correlated with Treg infiltration and positively correlated with better prognosis, suggesting their function is anti- and not pro-tumour. The Th17 cells were associated with CXCL9 and CXCL10 expression and the study also suggested that the IL-17 and IFN-γ produced by Th17s were inducing production of these chemokines. CD8 T cells that infiltrate the tumour express CXCR3, the receptor for

these chemokines. Thus, production of CXCL9 and CXCL10 may be aiding recruitment of anti-tumour CD8 T cells (Kryczek et al. 2009a). Further study is required to determine whether Th17 infiltration of tumours always has positive consequences.

1.4. Immunotherapy for Cancer

Methods of harnessing the immune response in order to treat cancer were first used over a century ago, well before the interaction between the immune system and cancer was fully understood. Although the phenomenon of cancer regression coinciding with infection was first observed in the 1700s (Hoption Cann et al. 2002), it was William Coley who first refined a technique involving the injection of bacterial toxins as a therapy for tumours (Coley 1916).

In recent years, immunotherapy has once again attracted much interest as a cancer treatment. It now appears that the immune system surveys the body for cancerous cells and often eliminates them before they are clinically apparent. However natural selection results in some tumour cells evolving mechanisms to evade the immune response. This is highlighted from studies in mice, where tumours grown in immune deficient mice are more immunogenic upon transplant into immunocompetent animals, as the tumours have not developed mechanisms to avoid detection and destruction by immune cells (Shankaran et al. 2001).

Research now focuses on boosting the immune system to overcome the tumour immune evasion mechanisms. Various strategies are being explored and these will be discussed below. If successful, research into immunotherapies could result in a treatment that would be specific for tumours, work systemically and provide long lasting protection against recurrence.

1.4.1. Immunotherapy strategies

1.4.1.1. Innate immunity

As immune responses often commence with activation of the innate immune system, some therapies target TLRs to create a 'danger signal' to prompt a full immune response. Coley's toxins are likely to have worked in this manner, and in modern medicine Bacillus Calmette Guérin (BCG), made with extracts from bacteria, is used to treat bladder cancer (Morales et al. 1976). One of the bacterial wall peptidoglycans in the vaccine has been shown to activate TLR2 and TLR4, which initiates the production of inflammatory cytokines, thereby encouraging activation and recruitment of immune cells (Tsuji et al. 2000).

1.4.1.2. Antibodies

The innate immune system is also involved in antibody-based cancer therapies. Antibodies such as rituximab, which binds CD20 and is used to treat B cell non–Hodgkin's lymphoma and chronic lymphocytic leukaemia, and trastuzumab (Herceptin), which binds HER2/neu and is used in the treatment of breast cancer, are both thought to

work in part by opsonising cells for cytotoxic killing by NK cells, in a process called antibody-dependent cellular cytotoxicity (ADCC) (Barok et al. 2007;Beum et al. 2008;Eischen et al. 1996). Rituximab can also activate the complement cascade to aid tumour cell killing (Cragg et al. 2003).

Antibodies can also function by affecting cell signalling. Trastuzumab binding down-regulates the HER-2 receptor (a member of the epidermal growth factor receptor (EGFR) family) thereby inhibiting signalling and subsequent tumour cell proliferation (Nahta et al. 2004). Alternatively, as in the case of Bevacizumab which binds VEGF, the antibody can act in a neutralising role by blocking interactions between receptors and ligands, that would otherwise signal for cell proliferation (Wang et al. 2004).

1.4.2. T cell immunotherapy

Another promising area of cancer immunotherapy is the utilisation of T cells. T cells have an advantage over antibodies because they recognise antigens derived from all cellular compartments (see sections 1.3.2.1-1.3.2.3) and are therefore not restricted to those antigens expressed on the cell surface.

A variety of approaches have been tried to harness the power of T cells to fight cancer. One of the most successful treatments has been adoptive cell transfer (ACT). ACT using donor lymphocytes is successfully used in the clinic for chronic myeloid leukaemia (CML) patients who have relapsed following bone marrow transplant. However as

tumour-specific T cells are not isolated prior to transplant, graft-versus-host disease (GVHD) is a significant side effect (Collins, Jr. et al. 1997). ACT has also successfully been used to prevent and treat post-transplant lymphoproliferative disorder (PTLD). PTLD is caused when EBV, a virus which is normally asymptomatic and controlled by the immune system, is reactivated in bone marrow transplant patients, causing extreme lymphoproliferation. Infusion of EBV specific T cells derived from the donor and expanded in vitro, can effectively prevent and treat this disease (Rooney et al. 1998).

ACT has also been used in solid tumours, with particular success amongst melanoma patients. In this case, tumour-specific T cells taken from resected malignant tissue were expanded *in vitro* before re-introducing them into the patient. Initial attempts achieved an objective response rate of 34% (Rosenberg et al. 1994). Further study has revealed that tumour infiltrating lymphocytes (TIL) that have been cultured for a shorter time and therefore are less differentiated, with longer telomeres, will persist longer in the patient and be more effective (Robbins et al. 2004;Rosenberg et al. 1994;Zhou et al. 2005). In addition, conditioning patients with total body irradiation and/or chemotherapy to deplete their lymphocyte compartment increased response rates to up to 72%, by facilitating greater expansion of the infused cells *in vivo* and removing the regulatory T cells which otherwise hamper the response (Antony et al. 2005;Dudley et al. 2008). However there is significant toxicity associated with this combined therapy approach. Techniques currently being investigated to reduce the T cell differentiation status also promise to increase the response rate still further (Klebanoff et al. 2011).

In order to enhance T cell therapy further, genetic transfer of TCRs is being explored, where a high affinity TCR specific for a tumour antigen is cloned and transduced into patient T cells (Schmitt et al. 2009). Alternatively, where MHC restriction is a concern or MHC molecules have been down-regulated, patients T cells can be engineered to express a CAR (chimeric antigen receptor) which usually consist of a tumour-specific antibody fragment coupled to the CD3ζ chain (Thistlethwaite et al. 2005).

Another approach to activate a T cell response is to use vaccines. These can be with tumour cells with an adjuvant to activate DCs which in turn stimulate a T cell response (Thompson and Dessureault 2007). Alternatively DCs themselves can be cultured *ex vivo* and pulsed with peptide for the tumour associated antigen (TAA), so they present this to T cells once re-introduced into the patient (Gilboa 2007).

1.4.2.1. Tumour associated antigens

In order for a TAA to be useful it needs to have certain features to ensure T cells will respond efficiently and specifically. Ideally TAAs should be derived from proteins that are only expressed by the tumour, vital to tumour cell survival (so tumours don't evolve to evade an antigen specific T cell response), widely expressed in many patients' tumours and not targeted by immunological tolerance. TAAs fall into five broad categories. Mutated self-proteins, such as BCR-ABL which is formed from a fusion protein made when chromosomes translocate in CML (Bocchia et al. 1996), are good targets as there should be no immune tolerance to them and they are only expressed in

the tumour cells. Over-expressed self-proteins, for example Her2 in breast cancer (Peoples et al. 1995), can be good targets, however T cell tolerance or expression on normal tissues can limit their usefulness. T cell tolerance can also be a problem when targeting lineage specific antigens, such as melanoma antigen recognised by T cells 1 (MART-1), which is expressed in melanoma but also in melanocytes and the retina (Kawakami et al. 1994). Another class of TAA are aberrantly expressed self proteins, which are normally only expressed in immune-privileged sites such as the testes or placenta. RAGE and PRAME are cancer testes antigens which can also be expressed by tumours (Pellat-Deceunynck et al. 2000). 5T4 is an example of an oncofoetal antigen aberrantly expressed in some cancers (Griffiths et al. 2005).

1.4.2.2. Tumour Immune evasion

As they grow tumours evolve to evade and suppress the immune response. One way in which tumours can do this is by down-regulating MHC I expression, thereby hiding from T cell recognition. Loss of antigen processing machinery such as TAP also prevents antigen presentation by the tumour (Garcia-Lora et al. 2003). Tumours often express HLA-G in place of MHC I and II. HLA-G interacts with its ligand on NK cells, sending an inhibitory signal to prevent them killing the tumour cell (Lin et al. 2007;Tajima et al. 2003). Tumours also frequently have a loss of co-stimulatory molecules, thereby causing T cell anergy (Tirapu et al. 2006). T cells are also inhibited by tumour overexpression of gangliosides (which are glycosphingolipids) as they inhibit T cell proliferation and APC function, and can promote apoptosis (Jales et al. 2011;McKallip et al. 1999).

The programmed death 1 (PD-1) protein is expressed by T cells upon activation, however if it then binds either of its ligands, PD-L1 or PD-L2, T cell function becomes impaired and T cells become anergised (Barber et al. 2006;Freeman et al. 2000;Latchman et al. 2001). Various tumours express the PD-1 ligands (Wang et al. 2011b). Mouse studies have shown blocking PD-1 and PD-L1 can improve responses to tumour vaccines (Sierro et al. 2011), and clinical trials using the anti-PD-1 antibody CT-011 are currently underway.

Tumours also recruit other cell types which alter the tumour microenvironment into one which is immunosuppressive. One example of these are myeloid derived suppressor cells (MDSCs). MDSCs are thought to suppress T cells through their release of nitric oxide and arginase-1 (Nagaraj and Gabrilovich 2008). Another cell type enriched in many tumours is the Treg, which will be discussed below.

Tregs in cancer

Regulatory T cells pose a particular problem in cancer immunity, partly because tumour associated antigens normally derive from abnormally or over-expressed host proteins, and Tregs specific for those antigens can often be found within the T cell repertoire (Bonertz et al. 2009;Zou 2006). Tregs specific for other TAAs can also be induced if conditions are tolerogenic. It has been proposed that Tregs have a negative effect in cancer patients by suppressing anti-tumour T cell responses (Antony et al. 2005;Curiel et al. 2004;Turk et al. 2004). Indeed, depleting Tregs has been shown to increase tumour

rejection in mice (Onizuka et al. 1999;Shimizu et al. 1999). Elevated levels of Tregs have been detected in various cancers, including in the blood of pancreatic and breast cancer (Liyanage et al. 2002), lung cancer (Karagoz et al. 2010) and liver cancer (Feng et al. 2011) patients. Tregs have also been found in the TIL of breast, pancreatic (Liyanage et al. 2002), ovarian, colon, hepatic (Kryczek et al. 2009b) and gastric (Shen et al. 2010) cancer. Treg numbers are also higher in the TIL from patients with metastatic melanoma, compared to their peripheral blood (Jandus et al. 2008). Tregs from tumour draining lymph nodes in pancreatic cancer patients were able to suppress CD8 and $CD4^{+}CD25^{-}$ T cell proliferation, and IFN-γ production *in vitro* (Liyanage et al. 2002). Tregs from ovarian carcinoma tumours have also been shown to suppress tumour-specific T cell immunity in both *in vitro* experiments on human cells and an *in vivo* experiment using a mouse model (Curiel et al. 2004).

There is also evidence that in certain cancer settings, Tregs may actually be beneficial. The presence of Tregs within colorectal tumours correlates with an improved prognosis (Salama et al. 2009), as does increased proportions of Tregs in Hodgkin's lymphoma (Alvaro et al. 2005), follicular lymphoma (Carreras et al. 2006) and head and neck cancer (Badoual et al. 2006). It may be that in these cancers that the reduction of tumour-promoting inflammation by infiltrating Tregs may have a more significant effect than the suppression of tumour immunity by the same cells.

The precise mechanism of Treg enrichment in tumours is unclear. However it may be due to the chemokines produced by the tumour being complementary to chemokine receptors expressed on Tregs, thereby recruiting or retaining Tregs at the tumour site (Curiel et al. 2004;Ishida et al. 2006). In addition a Treg phenotype may be induced on effector CD4 cells due to exposure to TGF-β within the tumour tissue (Chen et al. 2003). Equally, Tregs may be better able to survive the tumour environment, for example it has been shown that Tregs have reduced sensitivity to oxidative stress, possibly due to greater expression of thiol groups (Mougiakakos et al. 2009). This resistance could explain the enrichment of Tregs at tumour sites where oxidative stress is prevalent.

1.4.3. Immunotherapy for renal cell carcinoma

In common with melanoma, RCC has high levels of TIL which suggests it is an immunogenic tumour (Bex et al. 2010;Capitanio et al. 2009;Cohen and McGovern 2005;Patard et al. 2003). In addition, 7% of patients with advanced disease can be cured by IL-2 therapy (Rosenberg 2007) and 10-20% respond to IL-2 or IFN-α immunotherapy treatments, which are thought to work by modulating the immune system in favour of an anti-tumour response (Oudard et al. 2007). Rare cases of spontaneous regression have been described (Oya 2009), and stem cell transplants have occasionally resulted in a graft vs. tumour immune response (Childs et al. 1999;Childs et al. 2000;Takahashi et al. 2008). This evidence, combined with other studies where the existence of functional anti-RCC T cells has been demonstrated in vitro, suggests T cell immunotherapy could be a potentially effective therapy for the disease (Alexander et al.

1990;Belldegrun et al. 1988;Finke et al. 1990;Hanada et al. 2011;Koo et al. 1991;Leisegang et al. 2010;Seliger et al. 2011).

However, so far the successes seen in melanoma have eluded RCC patients. Clinical trials using ACT of TIL, tumour cell vaccines and DC vaccines have reported varying degrees of success (summarised in (Shablak et al. 2009), (Draube et al. 2011) and (Itsumi and Tatsugami 2010) but no approach has produced significant complete responses.

1.4.3.1. Tumour antigens in RCC

One of the main challenges in developing effective immunotherapy for RCC is the lack of a defined target antigen. Work on the therapeutic effects of stem cell transplants has identified epitopes from the retrovirus HERV-E as potential targets, since HERV-E viral proteins are expressed in as many as 76% of clear cell RCCs, as a result of VHL gene inactivation (Cherkasova et al. 2011). To date the only T cell clone identified is restricted to the relatively rare HLA-A11 MHC I allele, but further studies hope to identify additional HERV-E specific TCRs (Takahashi et al. 2008). Carbonic anhydrase IX (CA-IX) expression is also regulated by VHL and is upregulated in 99% of RCCs but absent in normal kidney (Genega et al. 2010;Oosterwijk et al. 1986) and could therefore be an attractive target for immunotherapy. Approaches using an antibody for CA-IX to induce ADCC have had modest success (Bleumer et al. 2004;Bleumer et al. 2006), however ACT of T cells with a CAR resulted in severe toxicity due to expression of CA-IX in the bile duct (Lamers et al. 2006). A phase III trial using the antibody is currently taking place.

5T4 antigen is usually expressed in the placenta but is upregulated in various cancers, including RCC. Vaccines using modified vaccinia Ankara (MVA) engineered to deliver the 5T4 antigen elicit 5T4 specific antibody production and cellular responses (Hawkins et al. 2009), however a phase III trial (which unfortunately was terminated before all patients had the complete course of vaccines) found no difference in the overall survival of patients (with the possible exception of those with a good prognosis) (Amato et al. 2010). Recent studies have cloned TCRs specific for multiple RCC tumours without reactivity against normal tissue (Engels et al. 2005;Wang et al. 2011a). One of these TCRs is unconventional in that it does not recognise an MHC bound peptide but instead sees TRAIL bound to its DR4 receptor (Hanada et al. 2011). A clinical trial using patient T cells engineered to express this TCR is currently in progress

1.4.3.2. Immune evasion in RCC

Unlike other malignancies, in RCC there is often good expression of the MHC I (Romero et al. 2006;Saenz-Lopez et al. 2009) and MHC II (Brasanac et al. 1999;Tomita et al. 1990) molecules needed for T cell recognition, and therefore it may be possible to promote an anti-tumour response if immunosuppressive mechanisms could be overcome.

In RCC, as with other cancers, the tumour microenvironment appears to encourage the enrichment of Tregs, which can predict a worse prognosis and potentially reduce anti-tumour responses (Griffiths et al. 2007). The tumour may also influence systemic immunity, as the proportion of $CD4^{+}CD25^{hi}$ cells in the peripheral blood was found to be

higher than in the blood of healthy donors (7.5% vs. 2.24% of lymphocytes and 2.47% vs. 1.50% of T cells) (Cesana et al. 2006;Griffiths et al. 2007). When Tregs were identified by staining for Foxp3 as well as CD4 and CD25, they were found to represent 2.02% of the peripheral blood of RCC patients compared 0.73% in healthy donors. A correlation between higher levels of circulating $CD4^{+}CD25^{high}$ cells and a worse prognosis has also been demonstrated (Griffiths et al. 2007).

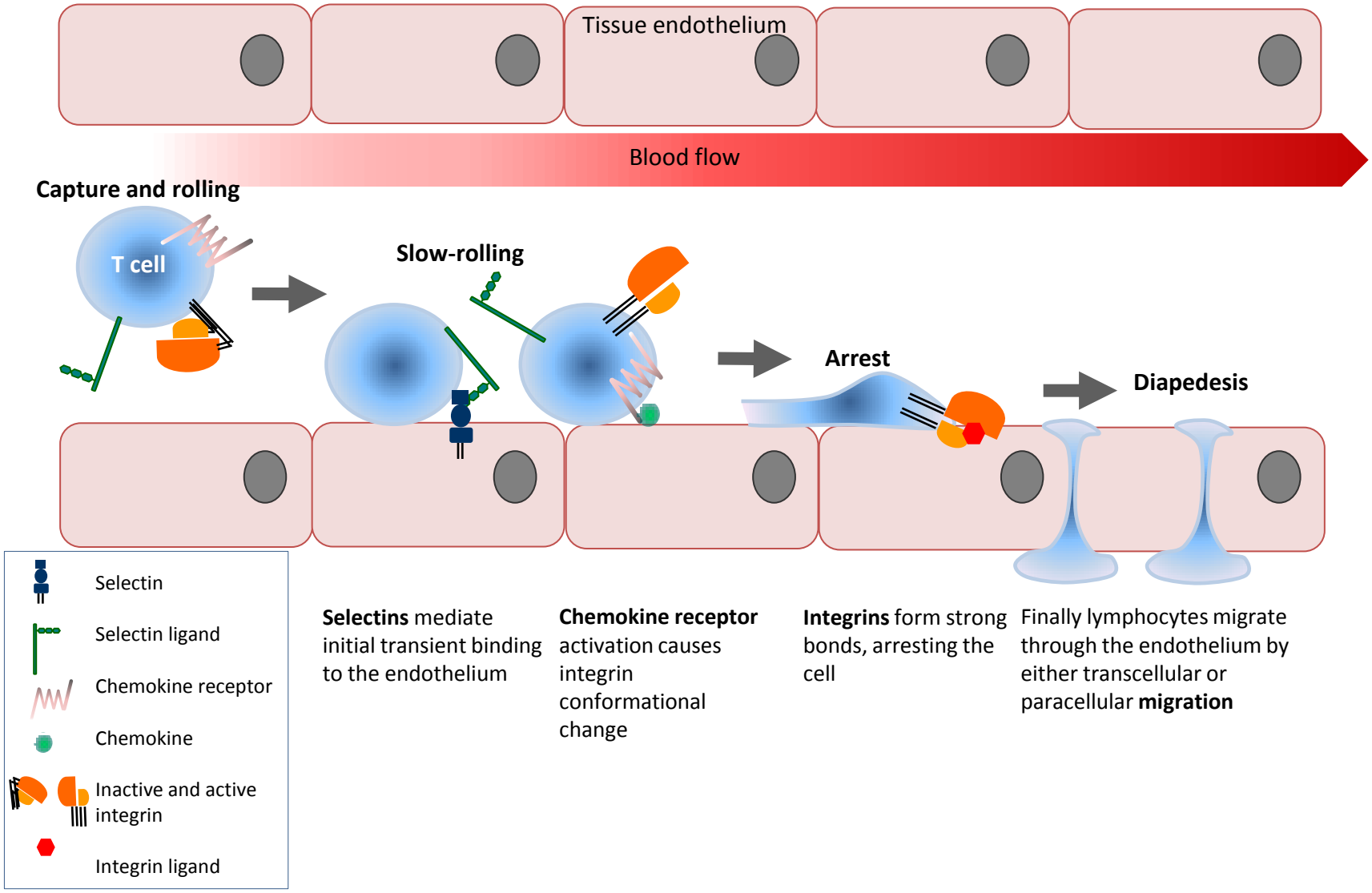
Evidence from RCC patients show the ganglioside GM2 can be found on TIL, despite the fact the cells do not make GM2 synthase, indicating the GM2 is tumour-derived. Cells from blood of healthy donors also pick up GM2 after incubation with supernatant from RCC cell lines. GM2 positive cells apoptose more frequently than GM2 negative cells suggesting tumour GM2 release is an immunosuppressive mechanism (Biswas et al. 2009). There is also substantial evidence that tumour cells from RCC express the inhibitory molecule PD-L1 (Thompson et al. 2004;Thompson et al. 2006;Thompson et al. 2007).

I have investigated markers of the functional status of RCC TIL, and examined the potential contribution of PD-1:PD-L1 and Tregs to immune suppression in RCC. I will discuss immune evasion in RCC in more detail in later chapters in the context of my own results.

1.5. T Cell migration

A pre-requisite for effective T cell therapy is that tumour specific T cells must be able to home to the tumour site (Breart et al. 2008;Mukai et al. 1999;Quezada et al. 2008). High levels of tumour-specific T cells in the circulation will not function unless they can migrate to, and access, the tumour cells (Rosenberg et al. 2005). Until now the homing phenotype required for a T cell to migrate to RCC was not well defined. This work aims to identify the mechanisms by which T cells migrate into RCC to aid the development of more effective immunotherapies for RCC.

In order for naive T cells to mount an effective immune response they must first localise to the lymphoid tissue where they can encounter their cognate antigen and become activated, after which they must migrate to the tissue where they are required. This T cell homing is orchestrated by a variety of ligand and receptor molecules expressed both on the T cell surface and the tissue itself. These molecules have to facilitate initial adhesion of the T cell to the tissue endothelium, arrest them further in order to retain them and finally aid transendothelial migration. This stepwise model is known as the 'adhesion cascade' (**Figure 3**).



Selectins mediate initial transient binding to the endothelium

Chemokine receptor activation causes integrin conformational change

Integrins form strong bonds, arresting the cell

Finally lymphocytes migrate through the endothelium by either transcellular or paracellular **migration**

Figure 3: The adhesion cascade

T cells within blood vessels can form transient bonds with the vessel endothelium via selectins and their ligands. If a chemokine receptor expressed by the T cell binds its receptor integrins on the T cell will become activated. If they encounter their ligand the T cell will form a strong bond and be arrested on the endothelium. It can then migrate through the endothelium into the tissue where it is required.

1.5.1. The adhesion cascade

1.5.1.1. Selectins

The initial transient binding of the T cell to the vessel endothelium is mediated by a family of C-type lectins known as 'selectins', which bind sugar moieties on specific glycoproteins (Cummings and Smith 1992). Most naive and central memory T cells express high levels of L-selectin (CD62L) (Sallusto et al. 1999), which recognises PNAd, a molecule consisting of a protein backbone and a sulphated oligosaccharide. PNAd is present on the high endothelial venules (HEVs) of peripheral lymph nodes (PLNs), and therefore the PNAd:CD62L interaction is involved in capturing circulating lymphocytes and directing them to the PLNs where they can encounter APCs (Geoffroy and Rosen 1989;Michie et al. 1993). MAdCAM-1 is a similar molecule, also a ligand of CD62L, and is present on the HEVs of Peyer's patches and mesenteric lymph nodes (MLNs) thereby directing lymphocytes to those tissues (Kraal et al. 1995). Therefore the expression of CD62L directs cells to lymphoid organs (Gallatin et al. 1983).

Two other types of selectin, P and E-selectin, are both found constitutively in the skin and are upregulated during inflammation (de Vries et al. 1998). It is therefore unsurprising that skin-homing T cells express the receptor for these selectins: cutaneous lymphocyte antigen (CLA) (Fuhlbrigge et al. 1997). Therefore the selectins and ligands a T cell expresses can start to direct its migration to a specific tissue site.

1.5.1.2. Integrins

Ligand:selectin bonds are transient and due to the nature of the bonds require shear stress force provided by blood flowing over the endothelium to occur (Alon et al. 1995). However, for T cell migration through an HEV into a tissue, a strong bond between the cell and the endothelium is needed. Integrins are heterodimeric receptor proteins with α and β glycoprotein chains (Law et al. 1987;Sanchez-Madrid et al. 1983). Their first role in the adhesion cascade is to slow down rolling by forming stronger bonds between the lymphocytes and endothelium. Patterns of integrin expression are also tissue specific. T lymphocytes migrating to the MLNs must possess $\alpha_4\beta_7$ integrins that interact with MAdCAM-1 present on the MLN HEVs (Berlin et al. 1993;Hamann et al. 1994). They also express the $\alpha_1\beta_2$ integrin LFA-1, which interacts with ICAM-1 on the MLN (Bargatze et al. 1995;Marlin and Springer 1987). Migration to PLNs also requires an LFA-1:ICAM-1 interaction (Stein et al. 2000). Additionally an interaction occurs between the $\alpha_4\beta_1$ (VLA-4) integrin on lymphocytes and the adhesion molecule VCAM-1 on skin HEVs (Berlin-Rufenach et al. 1999;Elices et al. 1990;Santamaria Babi et al. 1995).

Integrins need to be in an active conformation to bind their ligands and form strong bonds. This conformational switch is mediated by signalling downstream of chemokine receptor:ligand interactions.

1.5.2. Chemokines and chemokine receptors

1.5.2.1. Introduction

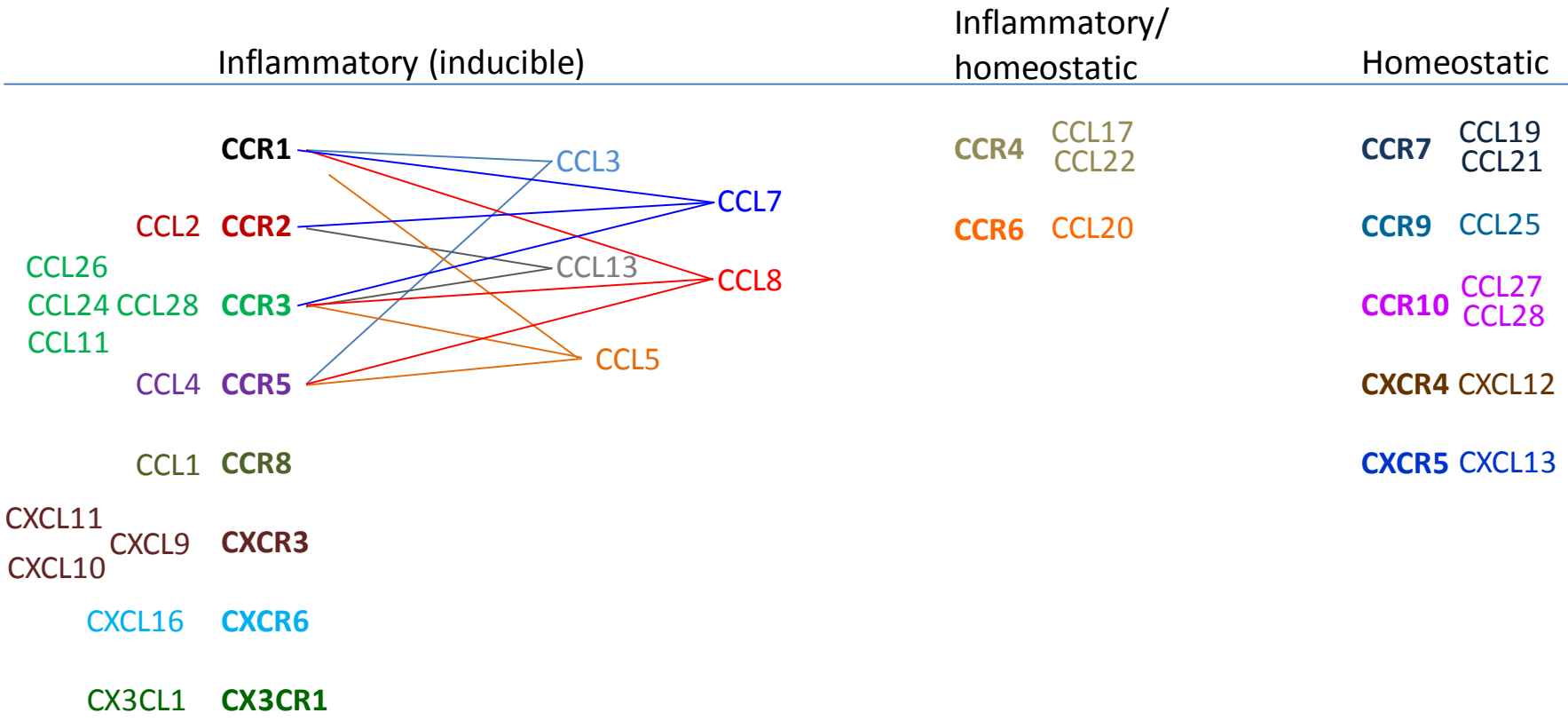
In order to obtain complete lymphocyte arrest from rolling, chemokines and their receptors are necessary. Chemokines are a family of small chemoattractant proteins that bind to G protein-coupled receptors (GPCRs) (Kelvin et al. 1993). They are grouped by their amino acid composition, specifically on the position of the first two cysteine residues of the chemokine conserved tetra-cysteine motif, with the CC and CXC chemokines forming the two largest groups (Murphy et al. 2000). The molecules CX3CL1, XCL1 and XCL2 are also regarded as chemokines. Chemokines can be grouped according to whether they are "inflammatory" or "homeostatic", that is whether they are produced by inflamed tissue and so recruit innate immune cells and effector T cells, or are constitutively produced by tissues and so maintain normal immune cell trafficking (Bono et al. 2007;Cyster 1999;Oppenheim et al. 1991;Schall and Bacon 1994;Yoshie et al. 1997a).

Homeostatic chemokines and receptors function to maintain health and immune function. CCR7 and CXCR5 are required for secondary lymphoid homeostasis (Forster et al. 1999;Voigt et al. 2000), and CCR9 and CCR10 for gut and skin immunosurveillance (Homey et al. 2000;Zabel et al. 1999). CXCR4 has many roles including intestinal homeostasis (Werner et al. 2011). CCR4 and CCR6 also play some roles in immune surveillance and lymphoid homeostasis (Campbell et al. 1999;Cook et al. 2000), however they can also play a role in inflammatory disease (Katou et al. 2001;Welsh-Bacic et al. 2011).

Inflammatory chemokines are produced in inflammatory settings and their receptors are induced on lymphocytes upon activation. Some chemokine receptors are more commonly induced on different subsets of T cells: type 2 T cells more commonly express CCR3, CCR4 and CCR8 (D'Ambrosio et al. 1998;Sallusto et al. 1998), whereas CCR5, CXCR3, CXCR6 and CX3CR1 are more often found on type 1 T cells (Fraticelli et al. 2001;Kim et al. 2001a;Loetscher et al. 1998b;Sallusto et al. 1998;Unutmaz et al. 2000). Other receptors, CCR1, CCR2, and CCR6, are also upregulated in response to inflammation, but their expression patterns are less distinct (Bonecchi et al. 1998;Ebert and McColl 2002).

As is evident from table 1, the chemokine and chemokine receptor system is complex with many ligands binding multiple receptors and vice versa. Therefore there is redundancy in the system. This is most evident for the inducible or inflammatory receptors and chemokines, and mouse knockouts of these receptors produce mild or undetectable phenotypes (Hancock et al. 2000;Unutmaz et al. 2000). In fact, humans with mutated CCR5 not only have no obvious phenotypic defects, but actually have a survival advantage as wild type CCR5 is one of the receptors used by HIV to infect cells (Huang et al. 1996;Samson et al. 1996). In contrast, knockouts of the homeostatic chemokine receptors have more severe effects, with $CXCR4^{-/-}$ being embryonic lethal (Zou et al. 1998), and CCR7 and CXCR5 knockouts displaying disrupted secondary lymphoid tissue architecture and impaired splenic primary follicles, respectively (Forster et al. 1999;Voigt et al. 2000).

Table 1:Chemokine receptors expressed by T cells, and their ligands



1.5.2.2. Function

The binding of chemokines to their receptors not only activates integrins to enhance their binding to adhesion molecules, but also mediates cell migration via a concentration gradient. When a chemokine binds its receptor an intracellular signalling cascade is set up, beginning with dissociation of G protein subunits which activate PLC. Activated PLC then cleaves phosphotidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to generate inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ then triggers an influx of Ca^{2+}, which together with DAG activates guanine-nucleotide-exchange factors (GEFs) (Li et al. 2000;Wu et al. 2000). GEFs then activate small GTPases(del Pozo et al. 1999;Vicente-Manzanares et al. 2005). This then leads to induction of transitional integrin conformational changes through either the direct or indirect (via intermediate effectors) association of a GEF with actin-binding proteins such as talin-1. Talin-1 then stabilises the high-affinity integrin conformation by coming between the α and β integrin chains, thereby opening the ligand-binding pocket. This results in stronger integrin-ligand bonds that can arrest the lymphocyte from rolling (Ley et al. 2007;Sampath et al. 1998;Wegener et al. 2007). The lymphocytes then transmigrate into the tissue where they are required.

Chemokine receptor ligation can also lead to cell proliferation and survival. This can be a direct effect, activating the phosphoinositide 3-kinase (PI3K) signalling pathway, as in the case of CCL5 binding (Choi et al. 2007). Other chemokines that can act in this fashion include CCL2, CCL3, CCL21 and to a lesser extent CCL4 (Flanagan et al. 2004;Taub et al. 1996). Chemokines can also improve T cell stimulation by APCs, thereby indirectly

increasing T cell proliferation. CCL2, CCL3, CCL4 and CCL5 binding to DCs increased expression of the co-stimulatory molecule CD80 (Taub et al. 1996).

Chemokines can also affect whether an APC primes a T cell to be type 1 or type 2. Binding of CCL2, CCL7, CCL8 or CCL13 to a receptor on APCs decreases IL-12 production, thereby biasing towards a type 2 response (Braun et al. 2000;Chensue et al. 1996). All these ligands bind CCR2, indeed CCL2 has no other known receptor, indicating that this effect is mediated through CCR2. In contrast, ligation of CCR5 on APCs increases production of IL-12 and favours a type 1 response (Aliberti et al. 2000).

Chemokines can also directly influence polarisation if present at the time of TCR stimulation. CCL2 induces cells that produce IL-4 and not IFN-γ, i.e. type 2 T cells. The presence of CCL3 (ligand for CCR1 and CCR5) on the other hand, results in Th1 T cells that produce IFN-γ (Karpus et al. 1997). It is likely that more ligands and receptors influence T cell polarisation, but they have not yet been investigated. The reason different chemokines result in different polarisation is unclear but it has been speculated that they may result in different G_i proteins being activated in the downstream signalling pathway (Luther and Cyster 2001).

1.5.3. Chemokine receptor expression

Chemokine receptor expression is influenced by TCR stimulation. Naive T cells down-regulate CCR7 and CXCR4 on initial stimulation, to direct them away from lymph node homing and allow them to migrate to peripheral tissue (Bleul et al. 1997;Gunn et al. 1998a).

The chemokine receptor expression profile of an activated T cell also depends on the cytokine and chemokine signals it receives (**Figure 4**). Exposure to IFN-α increases CCR1 expression while decreasing CCR3 and CCR4 (Sallusto et al. 1997;Sallusto et al. 1998), and IL-2 increases expression of the Th1 receptors, CCR5, CXCR3 and CXCR6 (Loetscher et al. 1998a;Unutmaz et al. 2000). It also increases CCR6 expression (Unutmaz et al. 2000). IL-15 has a similar effect, up-regulating CCR5, CCR6 and CXCR6 (Norii et al. 2006;Unutmaz et al. 2000).

The tissue a DC has migrated from can determine the chemokine receptor profile it will imprint on T cells. DCs from the gut can induce CCR9 expression and those isolated from skin induce the expression of E-selectin ligands. Signals from the secondary lymphoid tissue in which a T cell is primed also have a similar effect (Dudda et al. 2005).

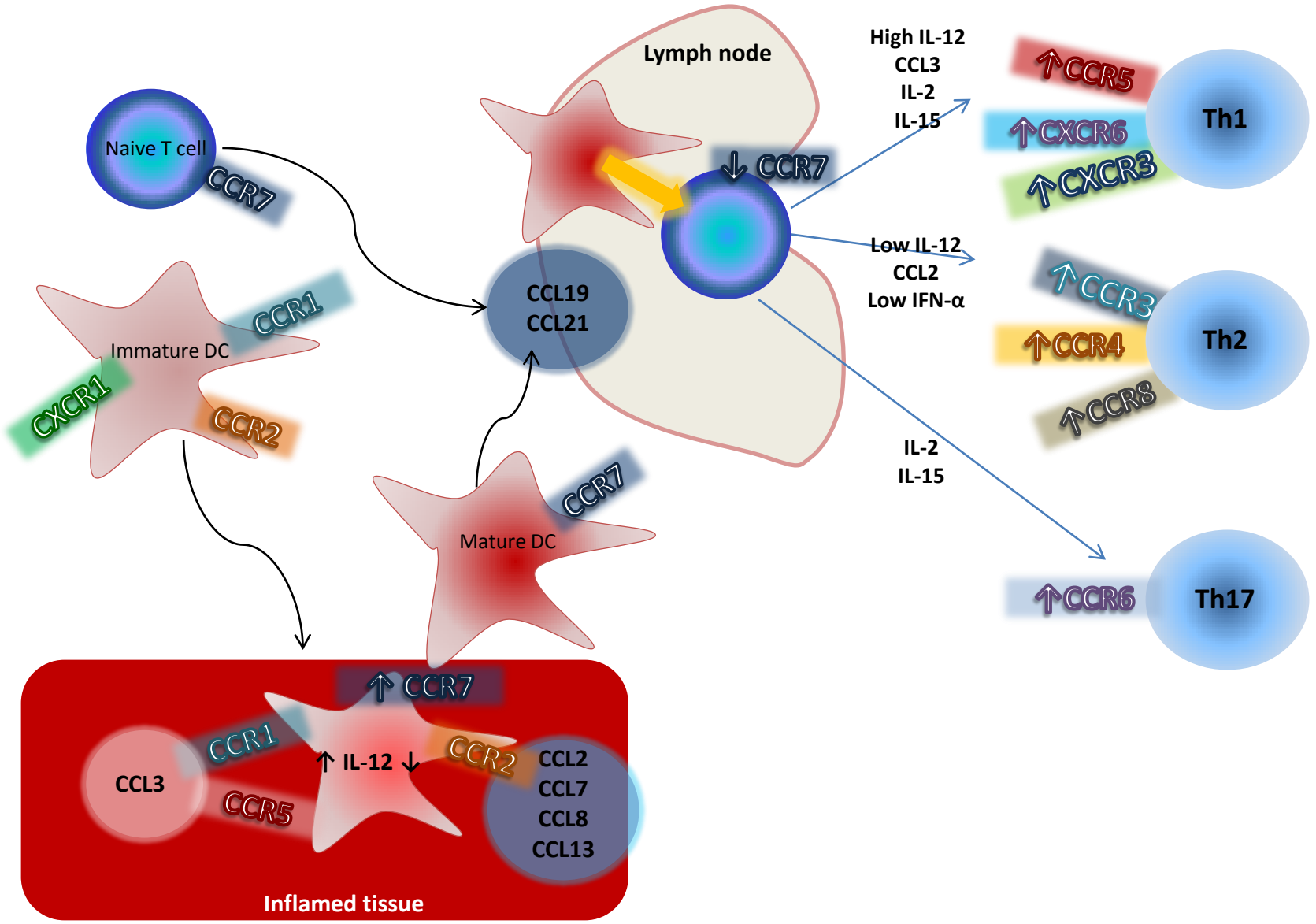

Lymph node
Naive T cell
CCR7
↓ CCR7
CCL19
CCL21
Immature DC
CCR1
CXCR1
CCR2
Mature DC
CCR7
↑ CCR7
CCR1
CCR5
↑ IL-12 ↓
CCR2
CCL3
CCL2
CCL7
CCL8
CCL13
Inflamed tissue
High IL-12
CCL3
IL-2
IL-15
↑CCR5
↑CXCR6
↑CXCR3
Th1
Low IL-12
CCL2
Low IFN-α
↑CCR3
↑CCR4
↑CCR8
Th2
IL-2
IL-15
↑CCR6
Th17

Figure 4: Regulation and expression of chemokine receptors on T cell subsets

Naive T cells express CCR7, which directs them towards the ligands CCL19 and CCL21 that are expressed in the lymph nodes. Immature DCs express tissue homing chemokine receptors, which interact with their ligands within the tissue. These interactions influence IL-12 production by DCs and both down-regulate the tissue homing receptors and up-regulate CCR7. Therefore the DCs also migrate to the lymph nodes, where they can prime naive T cells. The chemokine and cytokine signals the T cells receive determine their chemokine receptor expression and subsequent homing capacity.

Regulatory T cells are also affected by their chemokine receptor repertoire. It appears imperative that Tregs are primed in the lymph node, and therefore naive Tregs must express lymph node homing molecules CCR7 and CD62L, in order for them to be effectively activated and subsequently suppress the immune response (Schneider et al. 2007;Szanya et al. 2002;Taylor et al. 2004;Tosello et al. 2008). Murine studies also found CCR5 expression on Tregs is required to prevent GVHD and Tregs expressing CCR4 are required for tolerance to allografts (Lee et al. 2005;Taylor et al. 2004;Wysocki et al. 2005). Some experiments have show the majority of peripheral blood Tregs from humans express CCR4, CCR6, CCR7, CCR8, CXCR4, CD62L and CLA (Hirahara et al. 2006;Iellem et al. 2001). Activated Tregs express CXCR4, which is involved in Treg trafficking to bone marrow (Zou et al. 2004), where T cells can also be primed for an immune response (Feuerer et al. 2003). CCR4 and its ligand, CCL22, have been implicated in Treg trafficking both ovarian carcinoma and Hodgkin's lymphoma (Curiel et al. 2004;Ishida et al. 2006).

1.5.4. Tissue specific homing

T cells need to home to the lymph node in order to encounter APCs and interact with B cells. T cells homing to the lymph node express the chemokine receptor CCR7, and its ligands, CCL19 and CCL21, have been shown to be involved in T cell recruitment to secondary lymph nodes (Campbell et al. 1998b;Gunn et al. 1998b;Yoshida et al. 1997).

The chemokine receptor homing phenotypes required for migration to cutaneous and mucosal/intestinal tissue have also been defined. In addition to specific selectins and integrins being involved in T cell recruitment to the skin, particular chemokine receptors and chemokines are involved. CCR4 is expressed on most CLA^+ CD4 circulating T cells and its ligand CCL17 is constitutively expressed by skin tissue and is upregulated during inflammation (Campbell et al. 1999). CCR10 is also expressed by a subset of T cells and its ligand CCL27 is secreted by skin keratinocytes (Homey et al. 2000). The chemokine receptors required for intestinal homing have been similarly defined. The chemokine CCR9 is expressed on a group of $\alpha_4\beta_7^+$ T cells and its ligand CCL25 is constitutively secreted by the small intestine (Svensson et al. 2002). However T cells from CCR9 deficient mice still show a small but significant migration to the small intestine (Stenstad et al. 2006), highlighting the redundancy in the system and indicating that it is the co-expression of several molecules rather than their individual expression that controls T cell homing in these instances.

The chemokine receptors required for homing to other tissues, particularly in the context of inflammatory diseases or cancer, have also been investigated. Inflammation up-regulates the inducible chemokines, and their receptors play varying roles in T cell migration to the sites of disease. CXCR3 ligands have been shown to be up-regulated in chronic hepatitis and infiltrating T cells show high CXCR3 expression and enhanced migration to their ligands (Curbishley et al. 2005).

T cells trafficking to bronchoalveolar (BAL) fluid in allergic asthma patients were found to show higher expression of CCR5, CCR6, CXCR3, and CXCR4 compared with peripheral blood T cells (Thomas et al. 2007). Another study on patients with various lung diseases found CXCR6 to show the greatest up-regulation when comparing infiltrating and circulating T cells, and also showed its ligand CXCL16 was highly expressed in the lung (Morgan et al. 2005). CXCR6 has also been demonstrated to be involved in lymphocyte homing to the aortic wall (Galkina et al. 2007). Although CCR4 is usually involved in T cell recruitment to cutaneous tissue, it also has a role in regulatory T cell trafficking to MLNs in inflammatory bowel disease (Yuan et al. 2007).

The chemokine receptors required for homing to cancer tissue is of great interest in the context of improving T cell therapies. Machado et al (Machado et al. 2009) carried out flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) and TILs from Hodgkin's Lymphoma (HL) patients and found that $CXCR3^+$ and $CXCR4^+$ T cells were enriched in TIL. They also showed CXCR5 and CCR7 were expressed on at least 33% of TILs. 10-20% TILs showed expression of CXCR6, CCR4 and CCR5, whereas CCR1, CCR2, CCR3 and CCR6 were virtually absent. The adhesion molecules CD62L LFA-1, PSGL-1 and VLA-4 were detected on the majority of TILs. By contrast, TILs from the colorectal cancer cases studied had higher expression of CXCR6, CCR5 and CCR6 and lower expression of CCR7 and CD62L. Hepatocellular carcinoma infiltrating lymphocytes also showed high expression levels of CXCR3 and CCR5 and the corresponding chemokine ligands were expressed in the tumour, suggesting that lymphocytes are recruited there by these chemokine interactions (Yoong et al. 1999).

1.5.4.1. T cell migration into renal cell carcinoma

Integrins and their ligands are vital for T cell recruitment to RCC. An immunohistochemical study of 22 RCC samples and normal tissue found low levels of ICAM-1 and VCAM-1 expressed, and that the levels of ICAM-1 were significantly lower than on normal tissue (Hemmerlein et al. 2000). In contrast another study found ICAM-1 expression to be higher on ccRCC compared to normal tissue. VCAM-1 expression was comparable on normal and tumour tissue, as was β1 integrin expression which could be an indicator of VLA-4 expression. Two studies comparing VLA-4 expression on metastatic and non-metastatic RCC found expression to be very low on non-metastatic tumours but upregulated on both the primary and metastatic tumours in patients with mRCC (Gilcrease et al. 1996;Tomita et al. 1995). Therefore, at least to some degree, RCC can express the integrin ligands required for T cell homing.

In order to attract T cells via chemokine receptors, the tumour must produce chemokines. CXCL16 has been shown to be expressed in RCC tumours, as has CCL20, CXCL9, CXCL10, CCL5 and CCL4 (Gutwein et al. 2009;Kondo et al. 2004;Kondo et al. 2006;Middel et al. 2010;Suyama et al. 2005a).

It is currently thought that T cells migrate into RCC using the receptors CCR5 and CXCR3 (Attig et al. 2009;Cozar et al. 2005;Kondo et al. 2006;Suyama et al. 2005a). However, previous studies have only examined the expression of a limited number of chemokine receptors, and did not discriminate between naive and memory T cells when comparing

TIL with peripheral blood mononuclear cells (PBMC), which could potentially bias the results as PBMC contain a much greater proportion of naive cells. In addition no study has investigated the homing phenotype of the distinct T cell subsets infiltrating RCC. This study therefore aimed to screen TIL and PBMC with a comprehensive panel of chemokine receptor antibodies to determine the homing mechanisms of various T cell subsets to RCC, selecting only memory T cells for more accurate comparison between TIL and PBMC.

1.6. Hypotheses

The first chapter of my thesis deals with the hypothesis that T cells are recruited into RCC using particular homing receptors. The second chapter concerns the hypothesis that the T cells in TIL have been anergised or rendered dysfunctional by the tumour, and that the tumour has recruited regulatory T cells in order to evade the immune system. The third chapter investigates the hypothesis that other T cell subsets, Th17s and Tr1s, are recruited into RCC tumours and play a role in tumour immunity.

1.7. Aims

The aims of this work were:

- To investigate the mechanisms of T cell recruitment into RCC, determining which receptors are required and whether the same receptors are relevant for effector and regulatory T cell homing
- To investigate the functional capacity of T cells that home to RCC

- To investigate the presence and function of regulatory T cells in the tumour, and to investigate how the tumour may be evading the immune response
- To determine if other T cell subsets are present at the tumour site and how they may function

2. Materials and Methods

2.1. Materials

2.1.1. Clinical material

Samples of human RCC tissue, adjacent normal kidney tissue and 120ml heparinised whole blood were obtained from patients undergoing surgical resection (nephrectomy) at the Queen Elizabeth Hospital, Birmingham, UK. Paraffin-embedded tissue blocks were obtained from the department of pathology, Queen Elizabeth Hospital.

Written informed patient consent and local ethical committee approval (South Birmingham research ethics committee LREC reference 06/Q2706/82) were obtained prior to sample collection. Inclusion criteria stated patients must be aged 18 years or over, competent to give full informed consent and have a haemoglobin level of >10g/dL. Patients were excluded if they were taking corticosteroids or other medication that suppresses the immune system. Patients were also excluded if they were known to be infected with HIV, HBV or HCV.

2.1.2. Patient characteristics

Heparinised blood samples and tissue biopsies used in this study were collected from 78 RCC patients undergoing nephrectomy, of which the vast majority (80%) were clear cell, 14% papillary, 3.4% translocation, 1.7% collecting duct and 1.7% chromophobe. 71% were male, and the mean age of the patients at the time of nephrectomy was 61 years.

According to the TNM system 5.6% of tumours were classed as stage 4, 41% were stage 3b, 15% stage 3a, 1.9% stage 2, 20% stage 1b and 17% stage 1a. 23% of the tumours were Fuhrman grade 4, 28% grade 3, 46% were grade 2 and only 3.5% of tumours were grade 1.

2.1.3. Buffers, media and solutions

Complete medium: RPMI 1640 (Gibco) containing 10% v/v heat-inactivated foetal calf serum (FCS) (Invitrogen) and 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco).

Cryopreservation media: RPMI 1640 containing 40% v/v FCS and 10% v/v dimethyl sulphoxide (DMSO) (Fisher scientific).

FACs media: PBS containing 0.1% w/v bovine serum albumin (BSA) (Miltenyi biotech) and 0.1% v/v NaN_3 (Fisher scientific).

EDTA antigen retrieval buffer (pH8): 1mM ethylenediaminetetraacetic acid (EDTA) (Abcam) in distilled H_20, with 0.1% Tween20 (Promega), adjusted to pH8.

Citrate antigen retrieval buffer (pH6): 10mM citrate buffer (Abcam) in distilled H_20, with 0.1% Tween20, adjusted to pH6.

Migration assay medium: RPMI 1640 containing 0.1% w/v BSA and 100 UI/ml penicillin and 100 μg/ml streptomycin.

2.1.4. Antibodies

2.1.4.1. Flow cytometry

Table 2: Primary and conjugated antibodies (flow cytometry)

Antibody	Source	Catalogue number	Final concentration
Anti-human CCR1	R&D	MAB145	1 in 100 dilution of stock
Anti-human CCR2	R&D	MAB150	1 in 250 dilution of stock
Anti-human CCR3	R&D	MAB155	1 in 100 dilution of stock
Anti-human CCR4	BD	551121	1 in 100 dilution of stock
Anti-human CCR5	BD	555990	1 in 500 dilution of stock
Anti-human CCR6	R&D	MAB195	1 in 100 dilution of stock
Anti-human CCR7	BD	552175	1 in 250 dilution of stock
Anti-human CCR8	Abcam	ab1666	1 in 5 dilution of stock
Anti-human CCR9	R&D	MAB179	1 in 100 dilution of stock
Anti-human CCR10	Abcam	ab3904	1 in 5 dilution of stock
Anti-human CXCR1	R&D	MAB330	1 in 250 dilution of stock
Anti-human CXCR2	R&D	MAB331	1 in 80 dilution of stock
Anti-human CXCR3	BD	557184	1 in 500 dilution of stock
Anti-human CXCR4	R&D	MAB172	1 in 500 dilution of stock
Anti-human CXCR5	R&D	MAB190	1 in 250 dilution of stock
Anti-human CXCR6	R&D	MAB699	1 in 250 dilution of stock

Anti-human CXCR7	R&D	MAB4227	1 in 20 dilution of stock
Anti-human XCR1	R&D	AF857	1 in 20 dilution of stock
Anti-human CX3CR1	MBL	D070-3	1 in 40 dilution of stock
PE-Cy5 conjugated anti-human CD3	Beckman Coulter	A07749	1 in 20 dilution of stock
ECD cojugated anti-human CD8	Beckman Coulter	737659	1 in 20 dilution of stock
FITC conjugated anti-human CD4	BD	555346	1 in 20 dilution of stock
Pacific blue conjugated anti-human CD3	Beckman Coulter	558117	1 in 50 dilution of stock
Am-Cyan conjugated anti-human CD8	BD	339188	1 in 50 dilution of stock
PerCP-Cy5.5 conjugated anti-human CD4	BD	332772	1 in 50 dilution of stock
PE-Cy7 conjugated anti-human CD4	BD	557852	2 in 50 dilution of stock
PE conjugated anti-human CD3	BD	555333	1 in 50 dilution of stock
FITC conjugated anti-human CD3	BD	555332	1 in 50 dilution of stock
APC-Cy7 conjugated anti-human CD3	BD	557832	1 in 50 dilution of stock
AF700 conjugated anti-human CD45RA	Biolegend	304120	1 in 50 dilution of stock
FITC conjugated anti-human CD107a	BD	555800	1 in 10 dilution of stock
PE conjugated anti-human CTLA-4	BD	555853	1 in 2.5 dilution of stock
AF700 conjugated anti-human HLA-DR	BD	560743	1 in 2.5 dilution of stock
APC conjugated anti-human IFN-γ	BD	341117	1 in 100 dilution of stock
APC conjugated anti-human IL-2	BD	341116	1 in 10 dilution of stock
APC conjugated anti-human TNF-α	BD	340534	1 in 10 dilution of stock
FITC conjugated anti-human CD27	BD	555440	1 in 2.5 dilution of stock
PE-Cy7 conjugated anti-human CD127	Ebioscience	25-1278	1 in 5 dilution of stock

PE-Cy5 conjugated anti-human CD25	BD	555433	1 in 5 dilution of stock
PE conjugated anti-human CD25	Miltenyi	120-001-311	1 in 5 dilution of stock
APC conjugated anti-human PD-1	Ebioscience	17-9969	1 in 5 dilution of stock
PE conjugated anti-human IL-17	Ebioscience	Dec-78	1 in 10 dilution of stock
FITC conjugated anti-human Foxp3	Ebioscience	11-4776	1 in 5 dilution of stock
PerCP-Cy5.5 conjugated anti-human Foxp3	Ebioscience	45-4776	1 in 10 dilution of stock
PE conjugated anti-human IL-10	Invitrogen	RHCIL-1004	1 in 10 dilution of stock
Pacific blue conjugated anti-human CD45RA	Biolegend	304123	1 in 50 dilution of stock
AF700 conjugated anti-human Ki67	BD	561277	1 in 10 dilution of stock
PE-Cy7 conjugated anti-human IFN-γ	BD	557643	1 in 25 dilution of stock
AF700 conjugated anti-human granzyme B	BD	561016	1 in 17 dilution of stock
PE conjugated anti-human perforin	BD	556437	1 in 10 dilution of stock
PE conjugated anti-human IL2	BD	559334	1 in 17 dilution of stock

Table 3: Isotype controls (flow cytometry)

Antibody	Source	Catalogue number	Final concentration
mouse IgG1 negative control	R&D	MAB002	as appropriate
mouse IgG2a negative control	R&D	MAB003	as appropriate
mouse IgG2b negative control	R&D	MAB004	as appropriate
mouse IgG3 negative control	DAKO	M060501	as appropriate
rat IgG2a negative control	R&D	MAB006	as appropriate

FITC conjugated rat IgMk negative control	BD	555951	as appropriate
goat IgG negative control	R&D	AB-108-C	as appropriate
AF700 conjugated mouse IgG1 negative control	BD	557882	as appropriate
AF700 conjugated mouse IgG2a negative control	BD	557880	as appropriate
PE conjugated mouse IgG1 negative control	BD	555749	as appropriate
FITC conjugated mouse IgG1 negative control	BD	555748	as appropriate
APC conjugated mouse IgG1 negative control	BD	554681	as appropriate
PE conjugated rat IgG1 negative control	AbD Serotec	MCA1123PE	as appropriate
PE conjugated mouse IgG2a negative control	BD	551438	as appropriate

Table 4: Secondary antibodies and reagents (flow cytometry)

Antibody	Source	Catalogue number	Final concentration
PE-Cy5.5 conjugated anti-mouse IgG1	Invitrogen	M32018	1 in 100 dilution of stock
PE conjugated anti-mouse IgG2b	Invitrogen	M32404	1 in 100 dilution of stock
Biotinylated goat anti-mouse IgG2a	Invitrogen	M32315	1 in 100 dilution of stock
Biotinylated goat anti-rat IgG	Southern biotec	305208	1 in 100 dilution of stock
Biotinylated goat anti-mouse IgG	Southern biotec	102008	1 in 100 dilution of stock
Biotinylated rabbit anti-goat IgG	Abcam	ab6740	1 in 100 dilution of stock
PE conjugated streptavidin	Invitrogen	SNN1007	1 in 100 dilution of stock
APC conjugated Streptavidin	Invitrogen	SA1005	1 in 100 dilution of stock

2.1.4.2. Immunohistochemistry

Table 5: Primary antibodies (IHC)

Antibody	Source	Catalogue number	Final concentration
Anti-human CCL4	R&D	AF-271	0.5µg/ml
Anti-human CCL5	R&D	AF-278	5µg/ml
Anti-human CCL20	R&D	AF360	5µg/ml
Anti-human CXCL9	R&D	AF-392	5µg/ml
Anti-human CXCL10	R&D	AF-266	5µg/ml
Anti-human CXCL11	R&D	AF-260	5µg/ml
Anti-human CXCL16	R&D	AF-976	5µg/ml
Anti-human PD-1	Abcam	ab52587	10µg/ml
Anti-human CD3	Dako	M7254	1/200
Anti-human Foxp3	ebioscience	14-4777	2.5µg/ml
Anti-human CCR5	Abcam	ab1673	3.3µg/ml
Anti-human CCR6	R&D	MAB195	0.3µg/ml
Anti-human CXCR3	BD	557184	2.5µg/ml
Anti-human CXCR6	R&D	MAB699	2.5µg/ml

Table 6: Isotype controls (IHC)

Antibody	Source	Catalogue number	Final concentration
Rabbit IgG negative control	R&D	AB-105-C	as appropriate
mouse IgG1 negative control	Dako	X0931	as appropriate
mouse IgG2a negative control	Dako	X0943	as appropriate
mouse IgG2b negative control	Dako	X0944	as appropriate
goat IgG negative control	R&D	AB-108-C	as appropriate

Table 7: Secondary antibodies (IHC)

Antibody	Source	Catalogue number	Final concentration
ImmPRESS Universal antibody anti-mouse Ig/anti-rabbit Ig peroxidase	Vector	MP-7500	undiluted
ImmPRESS anti-goat Ig peroxidase	Vector	MP-7405	undiluted

2.2. Methods

2.2.1. Collection and preparation of blood and tumour tissue

2.2.1.1. Isolation of lymphocytes from normal and tumour tissue

Tissue was manually disaggregated using scalpels in complete medium. Necrotic or haemorrhagic tissue was excluded from the study. The supernatant was collected, passed through a 70μm filter to remove tissue aggregates, and analysed for the presence of lymphocytes using light microscopy. A minimum of $0.4x10^6$ tumour-infiltrating lymphocytes were required in order to perform the smallest of the subsequent experiments, therefore samples with fewer TIL were unusable.

2.2.1.2. Isolation of lymphocytes from peripheral blood

Whole blood was mixed at a ratio of 1:1 with RPMI 1640 and layered over Lymphoprep™ density gradient solution (Axis-Shield) at a ratio of 2:1, prior to centrifugation at 800 x g for 20 minutes (brake off). PBMCs were removed from the density gradient interface and washed by first re-suspending in RPMI 1640 and centrifuging at 600 x g for 10 minutes, then re-suspending in RPMI 1640 and centrifuging at 400 x g for 5 minutes.

2.2.1.3. Cryopreservation of tissue infiltrating lymphocytes and PBMC

Cells were cryopreserved at -180°C for long term storage. Cell suspensions were pelleted by centrifugation at 400 x g for 5 minutes, and resuspended in cryopreservation medium. Cells were frozen overnight in a Mr Frosty freezing container (Nalgene

labware) at -80^{o}C, before being stored over liquid nitrogen. When required cells were thawed in a 37^{0}C waterbath and immediately washed twice by re-suspending in complete medium and centrifuging at 400 x g for 5 minutes.

2.2.1.4. Preparation of tissue slides from paraffin blocks

Formalin fixed, paraffin embedded sections of 5μm were cut from a tissue block using a microtome, and mounted onto X-tra® Adhesive Snowcoat slides (Surgipath). Slides were incubated at 60^{O}C for 1 hour, before storage.

2.2.2. Flow cytometric analysis

Details of antibodies used are in section 2.1.4.1, p66.

Flow cytometry was performed using a Coulter XL (for preliminary examination of chemokine receptor expression on TIL) or BD LSRII flow cytometer (all other flow assays). Antibodies were diluted in 50μl FACs media and added for 20 minutes on ice then washed by dilution in FACs media followed by centrifugation at 400 x g for 5 minutes.

Concentration matched isotype controls were used to confirm specific staining.

Data were analysed using FlowJo software (Treestar Inc.).

2.2.2.1. Chemokine receptor expression on T cell subsets

To analyse cell surface phenotype, TIL from 35 patients and matched PBMC from 12 RCC patients were stained. Up to $1x10^5$ Cells were stained using individual or combinations of antibodies to: CCR1-10, CXCR1-7, XCR1 or CX3CR1, CD11b, CLA, CD62L, PSGL-1, VLA-4 and BLT-1. Where appropriate, biotinylated secondary antibodies were then used: goat anti-mouse, goat anti-rat or rabbit anti-goat, followed by Streptavidin-PE or Streptavidin-APC; or goat anti-mouse IgG1-PECy5.5, goat anti-mouse IgG2b-PE and goat anti-mouse IgG2a-biotin.

Cells were then blocked with 5% mouse serum (Invitrogen) in FACs buffer for 20 minutes then stained with CD3-PECy5, CD8-ECD and CD4-FITC (Coulter XL analyses), or CD3-Pacific Blue, CD4-PerCPCy5.5/CD4-PECy7, CD8-AmCyan, CD45RA-AF700 and LIVE/DEAD® Fixable Red Dead Cell Stain Kit (Invitrogen) (LSRII analyses) and gated accordingly.

To analyse Treg homing phenotype cells were also stained for CD25-PECy5 or CD25-PE and CD127-PECy7. They were then fixed and permeabilised using Foxp3 Fixation/Permeabilisation Concentrate and Diluent kit (Ebioscience) then stained with an anti-Foxp3-FITC antibody.

2.2.2.2. Expression of markers of immune function and intracellular cytokine staining of T cells subsets

$1x10^5$ TIL or PBMC were re-suspended in 100µl of either a stimulation mix of complete media containing 0.02µg/ml PMA, 2µg/ml Ionomycin, and 4µg/ml DNase IV (to prevent clumping due to released DNA) (all Sigma); or a no-stimulation mix of complete media containing 4µg/ml DNase IV. CD107a-FITC or the relevant concentration matched isotype control were added and the cells were incubated in the dark for 1 hour at 37°C, 5% CO_2. 100µl of complete media containing 40µg/ml Brefeldin-A, 4µl/ml GolgiStop (both BD) and 4µg/ml DNase IV were then added to each well and cells were incubated for a further 4 hours. Alternatively T cells were incubated at a 1:1 ratio with Dynabeads® Human T-Activator CD3/CD28 beads (Invitrogen), with Brefeldin-A, GolgiStop and DNase IV, for 5 hours at 37°C.

Cells were then all stained for CD3-Pacific Blue/CD3-PE/CD3-FITC/CD3 APC-Cy7, CD4-PECy7/CD4-FITC, CD8-AmCyan, CD45RA-AF700/CD45RA-Pacific Blue and LIVE/DEAD viability stain and gated accordingly. Cells were also stained for combinations of CD27-FITC, CTLA-4-PE, HLA DR-AF700, CD25-PE, PD-1-APC and CD127-PECy7.

Cells were then fixed and permeabilised, as before. Combinations of antibodies to IFN-γ-APC, IL-2-APC, TNFα-APC, Foxp3-PerCPCy5.5, IL-10-PE, granzyme B-AF700, perforin-PE, IL-17-PE and Ki67-AF700 were then used.

2.2.3. Immunohistochemistry

Details of antibodies used are in section 2.1.4.2 p70.

Sections were deparaffinised by placing in either Xylene or Histoclear (Fisher Scientific) for 10 minutes, then rehydrated in industrial methylated spirit (IMS) (Fisher Scientific) for 10 minutes. Sections were rinsed in tap water then placed in 0.3% hydrogen peroxide solution (30% H_20_2 (Sigma) dissolved in H_20) to block endogenous peroxidase activity. Where antigen retrieval was required, sections were either placed in EDTA buffer or citrate buffer that had been pre-heated in a microwave for 10 minutes at 800 Watts. The sections were then heated by microwave (800 Watts) for a further 20 minutes. After the slides had cooled, they were washed with Tris-buffered saline (TBS). A blocking step was performed using 2X casein solution (10X casein solution (Vector) diluted in TBS) or normal horse serum (Vector).

Sections were then stained with antibodies to CD3, Foxp3, PD-1, CCL4, CCL5, CCL20, CXCL9, CXCL10, CXCL11, CXCL16, CCR5, CCR6, CXCR3 or CXCR6, or isotype matched controls. EDTA antigen retrieval was used before staining for CD3, Foxp3, CCL4, CCR6, CXCR3 and CXCR6 for one hour at room temperature. Citrate buffer antigen retrieval was used before staining for PD-1 overnight at room temperature, CXCL9 and CXCL11 overnight at 4^{o}C, or CCR5 for 1 hour at room temperature. For CCL20 staining, slides were pre-treated overnight in EDTA buffer on a hot-plate stirrer at 65^{o}C before staining for 1 hour at room temperature. CCL5, CXCL10 and CXCL16 antibodies were used overnight at 4^{o}C.

Slides were then washed twice for 10 minutes on a stirring platform with TBS containing 0.1% Tween, and bound antibody was detected using an immunoperoxidase method (Vector IMPRESS anti-mouse or anti-goat kits) and visualised using DAB (Vector), used according to the manufacturer's instructions. Sections were mounted using DPX and a coverslip and air-dried. Slides were recorded digitally using a Nikon Eclipse E400 microscope.

The identification and tissue distribution of positively staining cells was determined with the help of a pathologist, Neeta Deshmukh (Department of Pathology, Queen Elizabeth Hospital, Birmingham, UK).

2.2.4. Migration assay

T cells (0.25-1 x 10^5) in 75μl migration assay medium were added to the top chamber of a 3μm pore HTS-Transwell-96 (Corning) and the same medium containing recombinant CCL4, CCL5, CXCL16 or the CXCR3 ligands (CXCL9, CXCL10 and CXCL11) (R&D Systems), or combinations of ligands for multiple chemokine receptors, was added to the lower chamber. Where cell numbers permitted, T cells (0.25-1 x 10^5) in 75μl migration assay medium were also added to wells of a 96-well plate in triplicate, and incubated under the same conditions, in order to have a record of the number of cells inputted into the assay. Transwells were incubated at 37^{o}C for 4 hours before cells from the lower chamber were harvested and stained using anti-CD3-PE. Migrated cells were counted using a BD

LSRII flow cytometer and Flow-Count™ fluorospheres (Beckman Coulter). The number of cell inputted were also measured in the same way. Background migration was assessed using migration media alone as a stimulus. The migration index was calculated as the ratio of cells migrated towards ligand compared to cells migrated towards media alone.

The assay was performed in triplicate.

2.3. Statistical analysis

Statistics were calculated using GraphPad Prism (GraphPad Software Inc.). The majority of data did not reach a normal distribution, therefore data were compared using a Wilcoxon signed rank test or a Wilcoxon matched pairs test. Data were not corrected for multiple comparisons as, due to the nature of the assays, the resulting estimates of significance would be quite conservative and could mask interesting trends. For example, if I used the Bonferroni correction for the comparison of chemokine receptor expression in TIL and PBMC examined in chapter 3, where 26 comparisons were made, only a p value of 0.0019 or less would be considered significant. In most cases, further investigations were undertaken to determine whether observations that had reached significance were important.

Correlations were calculated using Spearman correlation tests. Details of all the correlation calculations performed are in Appendix A.

In all cases p values of less than 0.05 were considered significant.

3. Molecular mechanisms of T cell recruitment into renal cell carcinoma

3.1. Introduction

This chapter aims to investigate the mechanisms by which T cells migrate into RCC, and which T cell subsets are recruited. The specific combination of receptors and ligands required for homing to many tissues has been well characterised, however the molecules required for homing to RCC are currently unknown. This information is important in the context of developing T cell immunotherapies for the cancer, as in order for an anti-tumour T cell to be effective it must be able to migrate to the tumour site (Mukai et al. 1999). It also allows us to block the homing of undesirable cell subsets, such as Tregs, to improve tumour immunity.

If a particular homing receptor is involved in recruiting T cells into RCC the TIL may show an enrichment of that receptor compared to the corresponding population of cells in PBMC. Therefore to determine which receptors might contribute to T cell recruitment to the tumour I compared the homing phenotype of matched TIL and PBMC from RCC patients. I also compared the chemokine receptor expression on CD4 and CD8 effector T cell subsets, and on Tregs, to determine whether they were recruited by similar or distinct mechanisms.

Unless otherwise stated, throughout this chapter 'TIL' and 'PBMC' refer to the T cell compartment within the respective populations. All significances were calculated using Wilcoxon matched pairs tests.

3.2. T cells infiltrate RCC

RCC has been reported to contain a significant T cell infiltrate. I performed immunohistochemical analysis (IHC) of $CD3^+$ cells in 10 RCC patient samples and compared the staining to isotype matched controls. I saw a clear T cell infiltrate in RCC tissue (**Figure 5**A). The majority of T cells were clustered near vascular areas, with only a few T cells able to penetrate the tumour nests. A similar observation was made by Wittnebel et al. (Wittnebel et al. 2007)

I performed multi-colour flow cytometry on T cells from tumour-infiltrating lymphocytes (TIL) and PBMC from matched patient samples to determine their chemokine receptor and adhesion molecule expression profile. For the 22 TIL samples initially studied I was only able to use 4-colour flow cytometry, so the analysis was first gated on lymphocytes based on the forward:side scatter profile, and then on CD3 and either CD4 or CD8. Later the BD LSRII FACs machine became available, allowing a larger panel of antibodies to be used. In addition to allowing gating using a LIVE/DEAD stain, the larger antibody panel available using the LSRII meant I could gate on the $CD45RA^-$ (memory) fraction, which is beneficial as this constitutes the majority of T cells within TIL and ensured I was comparing the equivalent population in peripheral blood.

Excluding CD45RA$^+$ T cells means I would not have included CD8 T_{EMRA} cells in my analysis, however the low proportions of CD45RA$^+$ T cells in TIL suggest this population is rare in RCC TIL.

Previous studies have shown that >65% of TIL are T cells, and they are chiefly CD8$^+$ (Kopecky et al. 2007;Kowalczyk et al. 1997;Van den Hove et al. 1997a). Other studies have demonstrated that even when CD8 T cells are not the predominant T cell population, the CD8:CD4 ratio is still higher in T cells from TIL than from PBMC (Balch et al. 1990;Cozar et al. 2005). In concordance with those studies the mean ratio of CD8:CD4 T cells in my samples was significantly higher in TIL (1.13 inter-quartile range[IQR]: 0.81-1.60) than matched blood (0.30 IQR: 0.21-0.40) (**Figure 5**B). Statistically CD8s represented a greater proportion of TIL than CD4s (p=0.0187), but the difference was small (median 62% IQR: 53%-70% vs. 55% IQR: 45%-70%) (**Figure 5**C).

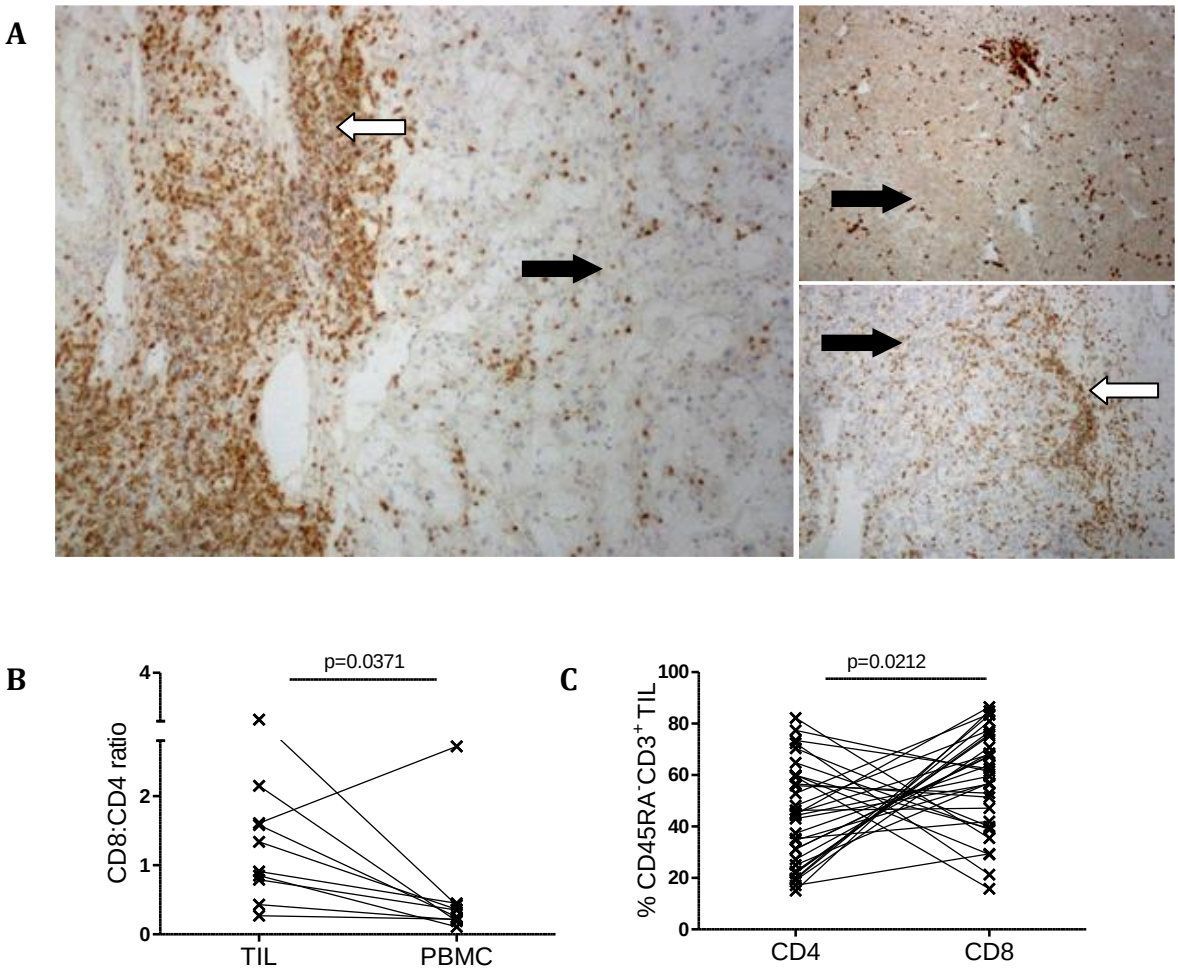


Figure 5: T cells infiltrate RCC

5µm paraffin sections from RCC tumours were stained with an antibody to CD3. Photos are representative of 10 RCC cases. Black arrows indicate tumour nests, white arrows indicate fibrous/stromal areas (A). Paired TIL and PBMC were stained with antibodies to CD4 and CD8, and analysed by flow cytometry to determine the ratio of CD8 T cells: CD4 T cells. (B) and the relative proportions of CD4 and CD8 T cells (C). Significance was calculated using a Wilcoxon matched pairs test.

3.3. Homing phenotype of TIL

I used flow cytometry to analyse chemokine receptor and adhesion molecule expression on up to 22 samples of RCC TIL using the 4-colour Coulter XL (

Figure 6, **Figure 7**), and subsequently 10 samples of RCC TIL and 10 matched PBMC using the BD LSRII (**Figure 8** and **Figure 9**). I stained for all 19 chemokine receptors (CCR1-10, CXCR1-7, XCR1 and CX3CR1) and the adhesion molecules CD11b, CLA, CD62L, LFA-1, PSGL-1, VLA-4 and BLT-1. Both methods of analysis gave similar results however the LSRII appeared more sensitive and produced results with higher percentages of homing marker-positive cells and higher levels of homing molecule expression, denoted by larger median fluorescence intensities (MFI). In addition I believe the LSRII data are a more accurate representation as I was able to exclude dead cells and naive T cells. Therefore the figures quoted in the following text are from the LSRII analysis. A table describing chemokine receptor expression by TIL according to the Fuhrman grade and RCC subtype of the sample can be found in Appendix B.

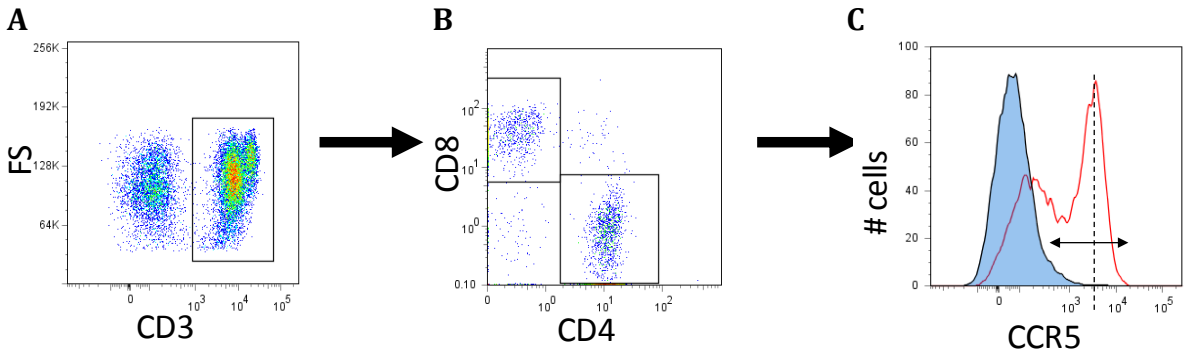


Figure 6: Gating strategy for defining chemokine receptor positive cells

The figure illustrates the gating strategy used to firstly define $CD3^+$ cells (A), then to define the $CD4^+$ and $CD8^+$ cell subsets (B), and finally to define the percentage of cells positive for a receptor (horizontal arrow) or the median fluorescence intensity of the positively-staining cells (dotted vertical line), using an isotype control to determine the level of non-specific staining (blue shaded area) (C).

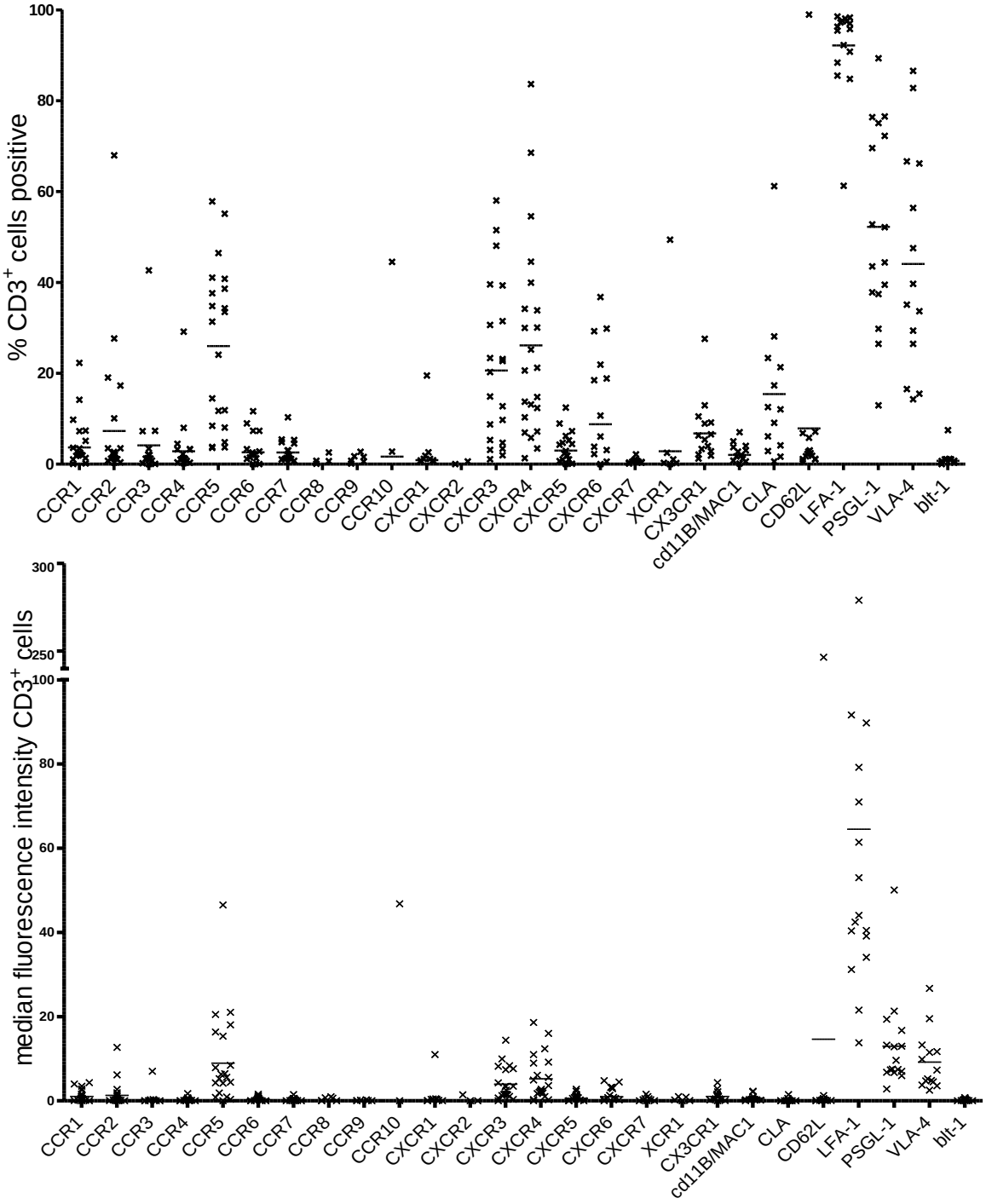


Figure 7: Initial analysis of homing phenotype of RCC TIL using the Coulter XL cytometer

RCC TIL samples were stained with antibodies specific for 19 chemokine receptors and 7 adhesion molecules. Graphs display the percentage of T cells positive for each marker and the median fluorescence intensity of the staining, both corrected for non-specific staining using concentration matched isotype controls. The number of RCC cases stained was as follows: CCR2 and CXCR4 n = 22; CCR1, 5 and CXCR3 n = 21; CXCR1, 5, 6 n = 20; CCR4 and CCR6 n = 19; CCR7 and CXCR7 n = 18, CCR9 and CD62L n = 17; CCR3 CXCR2, LFA-1 and PSGL-1 n = 16; CCR8, 10 and CX3CR1 n = 15; XCR1, CD11B, VLA-4 and BLT-1 n = 14; CLA n = 13.

3.3.1. Expression of adhesion molecules on RCC TIL

Integrins and selectins play key roles in T cell recruitment into tissues. Adhesion molecule expression on $CD45RA^{-}CD3^{+}$ CD4 and CD8 T cells was determined by flow cytometry. I found LFA-1, PSGL-1 and VLA-4 were expressed on ≥50% of TIL, with LFA-1 being expressed on a mean of 84 ± 13 % of TIL, PSGL-1 on 50 ± 22 % and VLA-4 on 53 ± 24 %. CLA and CD62L were also expressed, on 23 ± 23 % and 16 ± 20% of TIL respectively (**Figure 8**). All adhesion molecules except for CLA were expressed at high levels (MFI) compared to the chemokine receptors studied (see section 3.3.2, p88). Adhesion molecule expression was similar on CD4 and CD8 T cells (**Figure 8**)

Adhesion molecule expression did not correlate with RCC subtype or grade.

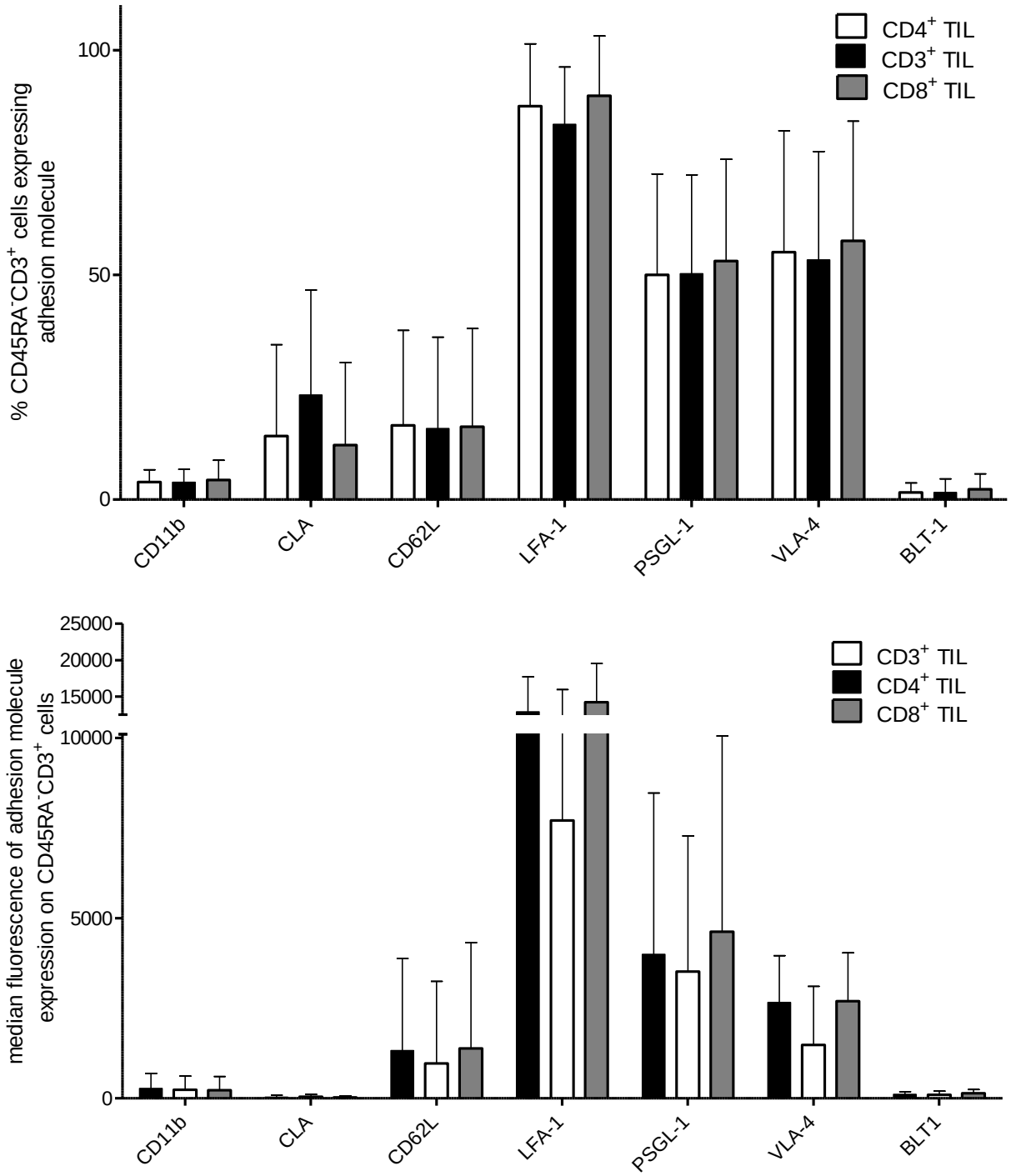


Figure 8: Adhesion molecule expression on RCC TIL: LSRII analysis

RCC TIL were stained with antibodies to 7 adhesion molecules. Graphs display the mean percentage of T cells positive for each marker and the mean median fluorescence intensity of the staining (+ SD). Results are corrected for non-specific staining using concentration matched isotype controls. The number of RCC cases stained was as follows: CD11b n = 7; CD62L and PSGL-1 n = 6; LFA-1, CLA, VLA-4 and BLT-1 n = 5.

3.3.2. Expression of chemokine receptors on RCC TIL

The chemokine receptor profile of TIL gives an indication as to what mechanisms they used to migrate into and within the tissue, and as to whether the T cells are Th1 or Th2 biased. Chemokine receptor expression on $CD45RA^{-}CD3^{+}$ CD4 and CD8 T cells was determined by flow cytometry, as before (**Figure 9**).

Not surprisingly, the number of T cells expressing homeostatic chemokine receptors was low, with only CXCR4 being expressed on over 10% of T cells (37 ± 17 %), and at high levels (MFI 639 ± 504). CCR7, CCR9, CCR10 and CXCR5 were expressed only by very small populations of T cells (**Figure 9**).

CCR4 and CCR6 can perform homeostatic functions but have also been shown to be involved in inflammatory scenarios (Katou et al. 2001;Welsh-Bacic et al. 2011;Yoshie et al. 1997b). CCR4 was expressed on very few T cells (3.1 ± 3.5 %), however a slightly larger T cell subset expressed CCR6 (12 ± 7.4 %), although the levels of expression were not high (MFI 175 ± 158).

The Th1 associated inflammatory chemokine receptors were expressed on a larger proportion of TIL: CCR5 was on 49 ± 17 %, CXCR3 on 25 ± 18 % and CXCR6 on 21 ± 14 %. In addition they were expressed at high levels (MFI 1778 ± 519, 639 ± 504 and 519 ± 786) respectively. The Th2 associated receptors CCR3 and CCR8 were expressed by only

a minority of TIL. Very few cells expressed the remaining 'inflammatory' receptors, CCR1, CCR2, CXCR1, CXCR2, XCR1 and CX3CR1.

The literature is controversial on the role and expression of CXCR7 on leukocytes (Berahovich et al. 2010;Hartmann et al. 2008), and I detected very few T cells in the TIL population expressing this receptor.

Homing receptor expression was comparable on CD4 and CD8 T cells (**Figure 9**). However CCR6 was expressed more frequently ($p=0.0020$) and at higher levels ($p=0.0078$) on CD4 than CD8 T cells in TIL. CCR4 and CCR7 were on significantly more CD4 than CD8 T cells ($p = 0.0488$ and $p = 0.0117$), however they were only expressed on a small minority of CD4s (3.1 ± 2.2 % and 5.2 ± 4.3 %) (**Figure 10**). CCR7 was also expressed at higher levels on the CD4 subset, however the MFI was still very small (52 ± 61).

Chemokine receptor expression did not correlate with RCC subtype or grade.

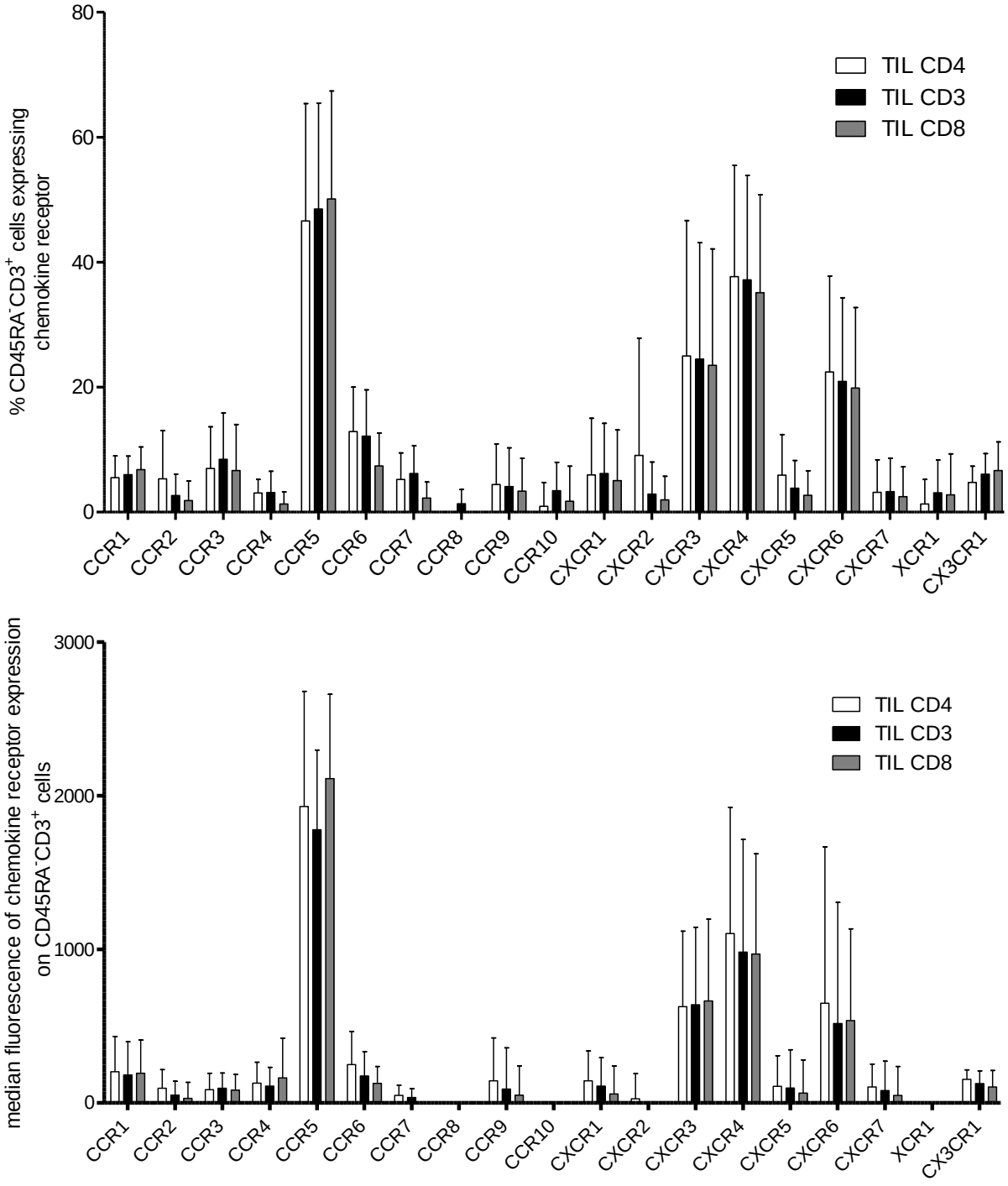


Figure 9: Chemokine receptor expression on RCC TIL: LSRII analysis

RCC TIL were stained with antibodies to all 19 chemokine receptors. Graphs display the mean percentage of T cells positive for each marker and the mean median fluorescence intensity of the staining (+ SD). Results are corrected for non-specific staining using concentration matched isotype controls. The number of RCC cases stained was as follows: CCR3, 4 5 6 7, CXCR3, 4 and 6 n = 10; CCR8 and CX3CR1 n = 8; CCR1, 2, 9, 10, CXCR2, 5, 7 and XCR1 n = 7; CXCR1 n = 6.

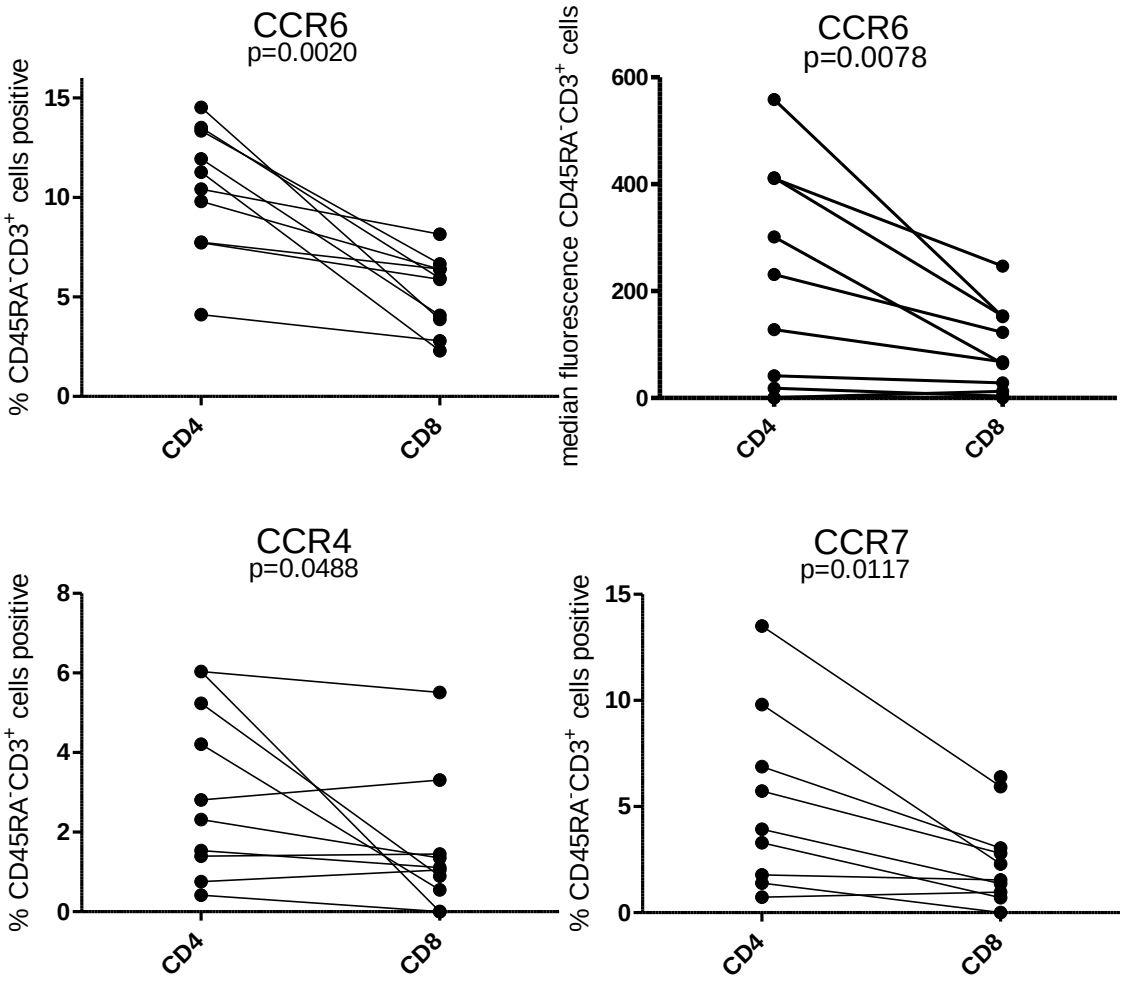


Figure 10: CCR4, CCR6 and CCR7 had different patterns of expression on CD4 and CD8 T cell subsets from TIL

RCC TIL were stained with antibodies to all 19 chemokine receptors. Graphs display the percentage of T cells positive for each marker and the median fluorescence intensity of the staining. Results are corrected for non-specific staining using concentration matched isotype controls. Chemokine receptor staining on CD4 and CD8 T cells was compared and results are shown for those receptors for which the expression was significantly different between the subsets. Significance was calculated using a Wilcoxon matched pairs test.

3.3.3. Chemokine receptor expression on TIL compared to matched peripheral blood

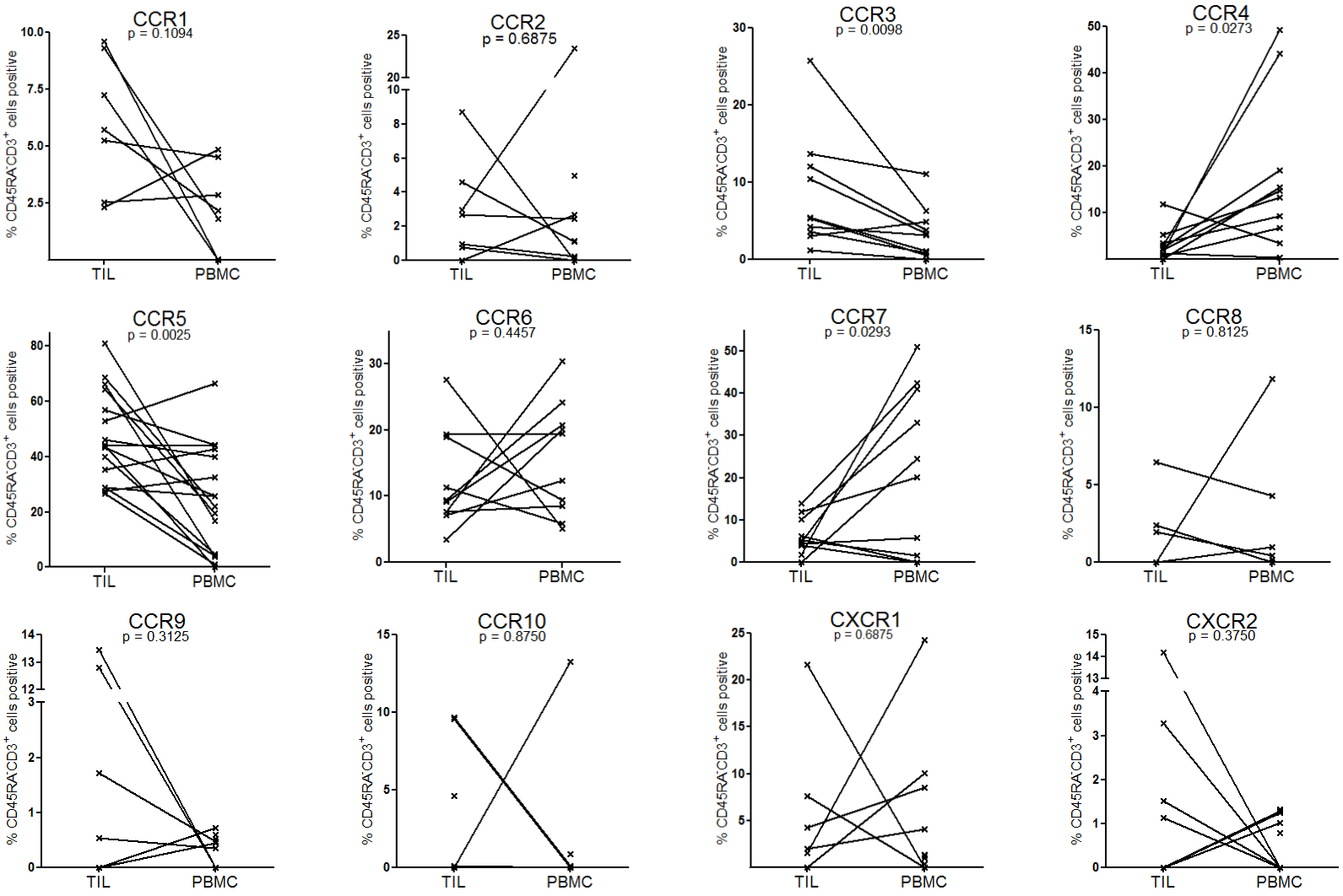
If a particular homing receptor is involved in recruiting T cells into RCC, the TIL may show an enrichment of that receptor compared to the corresponding population of cells in PBMC. I compared the expression of the aforementioned adhesion molecules and chemokine receptors on up to 16 samples of matched TIL and PBMC. There were no significant differences in adhesion molecule expression between TIL and PBMC (**Figure 11** and **Figure 12**)

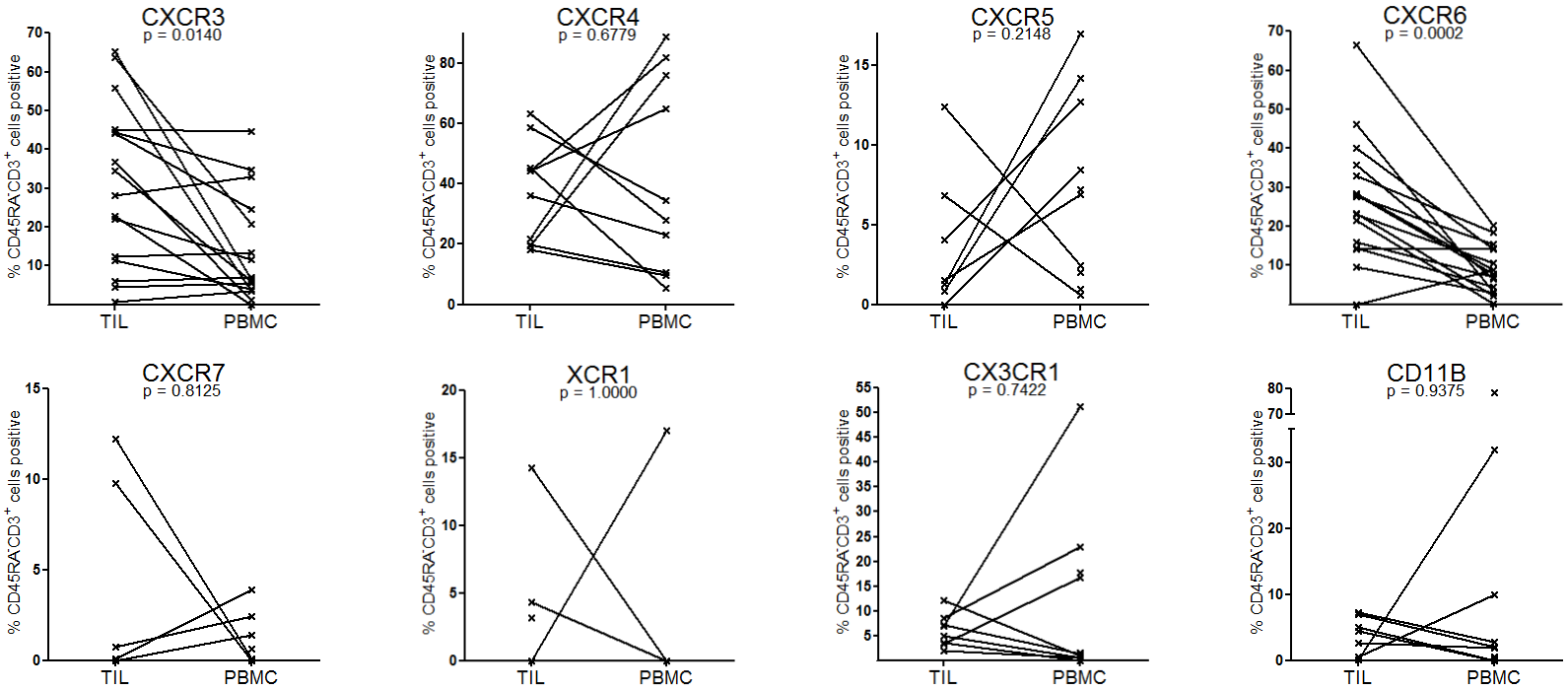
Compared to matched peripheral blood, $CD3^+$ TIL had significantly higher expression levels of the receptors CCR1 and CCR3. CCR3 was also expressed on significantly more T cells. CCR5, CXCR3 and CXCR6 were also expressed more frequently in TIL than PBMC. These three receptors were also expressed at higher levels on TIL compared to matched blood (**Figure 11** and **Figure 12**).

When I compared chemokine receptor expression on CD4 and CD8 T cells in PBMC, I found CXCR6 was expressed on significantly more CD8 T cells than CD4 T cells in PBMC, and CCR5 was expressed at significantly higher levels on CD8s than CD4s. CXCR6 was also expressed at higher levels and CCR5 at greater frequencies on CD8s from blood than CD4s, but the difference was not significant (**Figure 13**). If CCR5 and CXCR6 do function in T cell migration to RCC, this may explain the enrichment of CD8 T cells within the tumour.

These data suggest CCR5, CXCR3 and CXCR6 receptors play a role in selective recruitment or retention of T cells in RCC tumours. Whilst they may make a significant contribution to the T cells that express them, CCR1 and CCR3 were expressed on such a small proportion of TIL that I decided not to investigate them further. Instead further research focussed on the receptors enriched on a larger proportion of TIL— CCR5, CXCR3 and CXCR6 —as these are more likely to be involved in recruiting the majority of TIL into the tumour.

In addition, some receptors were enriched on PBMC compared to TIL. CCR4 was expressed on a greater percentage of T cells in PBMC than TIL ($p = 0.0273$), as was CCR7 ($p = 0.0293$). Neither of these receptors were expressed at significantly higher levels on PBMC T cells than TIL, however the difference in MFI for CCR7 was approaching significance (**Figure 11** and **Figure 12**).


CCR1
p = 0.1094
CCR2
p = 0.6875
CCR3
p = 0.0098
CCR4
p = 0.0273
CCR5
p = 0.0025
CCR6
p = 0.4457
CCR7
p = 0.0293
CCR8
p = 0.8125
CCR9
p = 0.3125
CCR10
p = 0.8750
CXCR1
p = 0.6875
CXCR2
p = 0.3750
% CD45RA-CD3+ cells positive
TIL
PBMC


CXCR3
p = 0.0140
CXCR4
p = 0.6779
CXCR5
p = 0.2148
CXCR6
p = 0.0002
CXCR7
p = 0.8125
XCR1
p = 1.0000
CX3CR1
p = 0.7422
CD11B
p = 0.9375
% CD45RA-CD3+ cells positive
TIL
PBMC

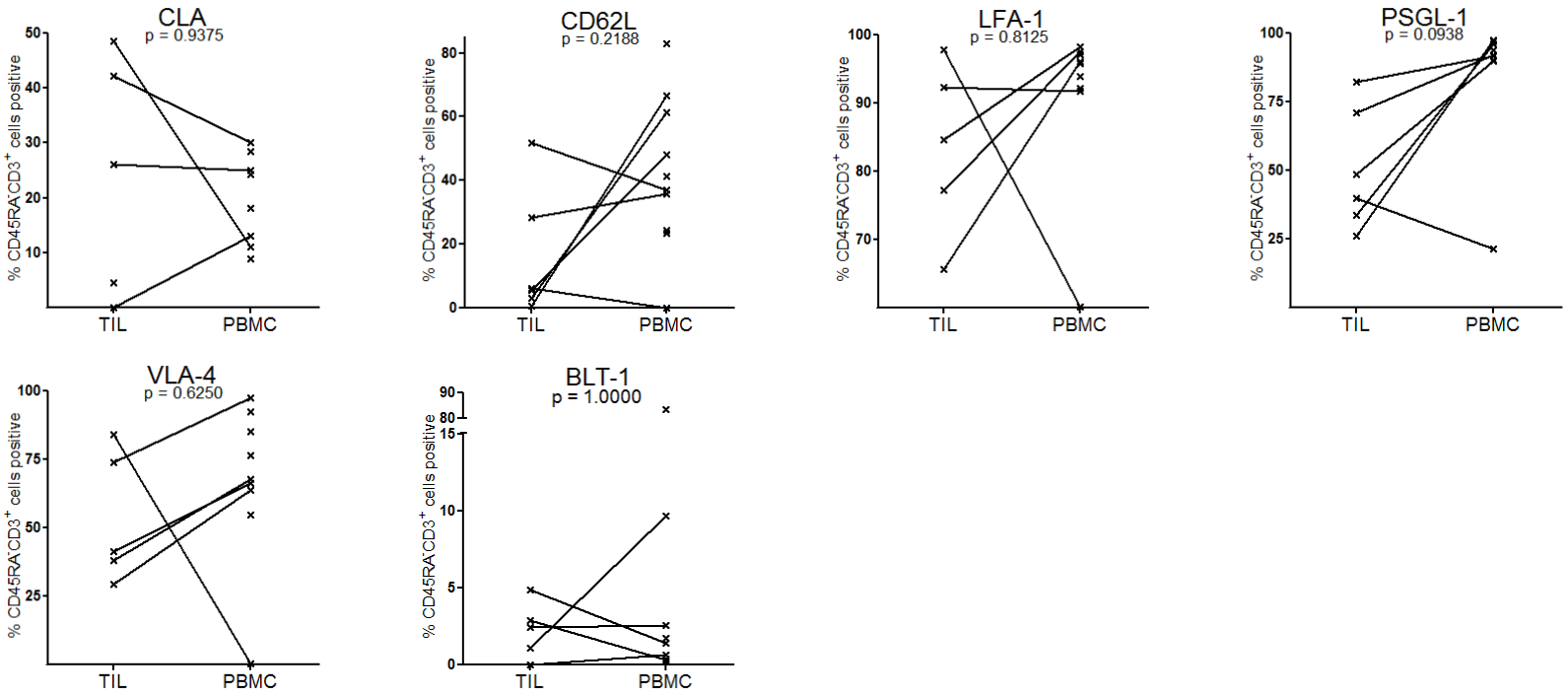
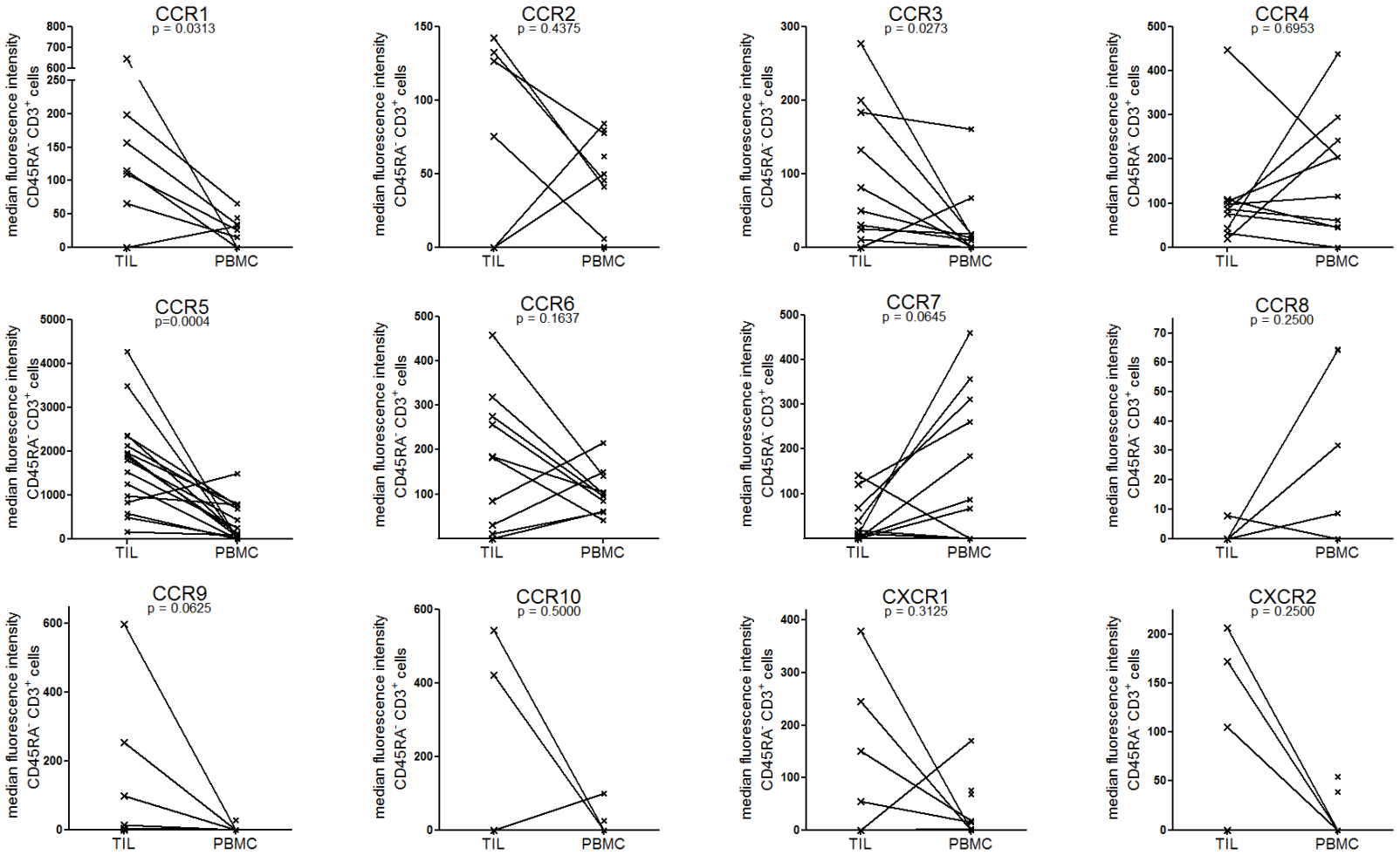
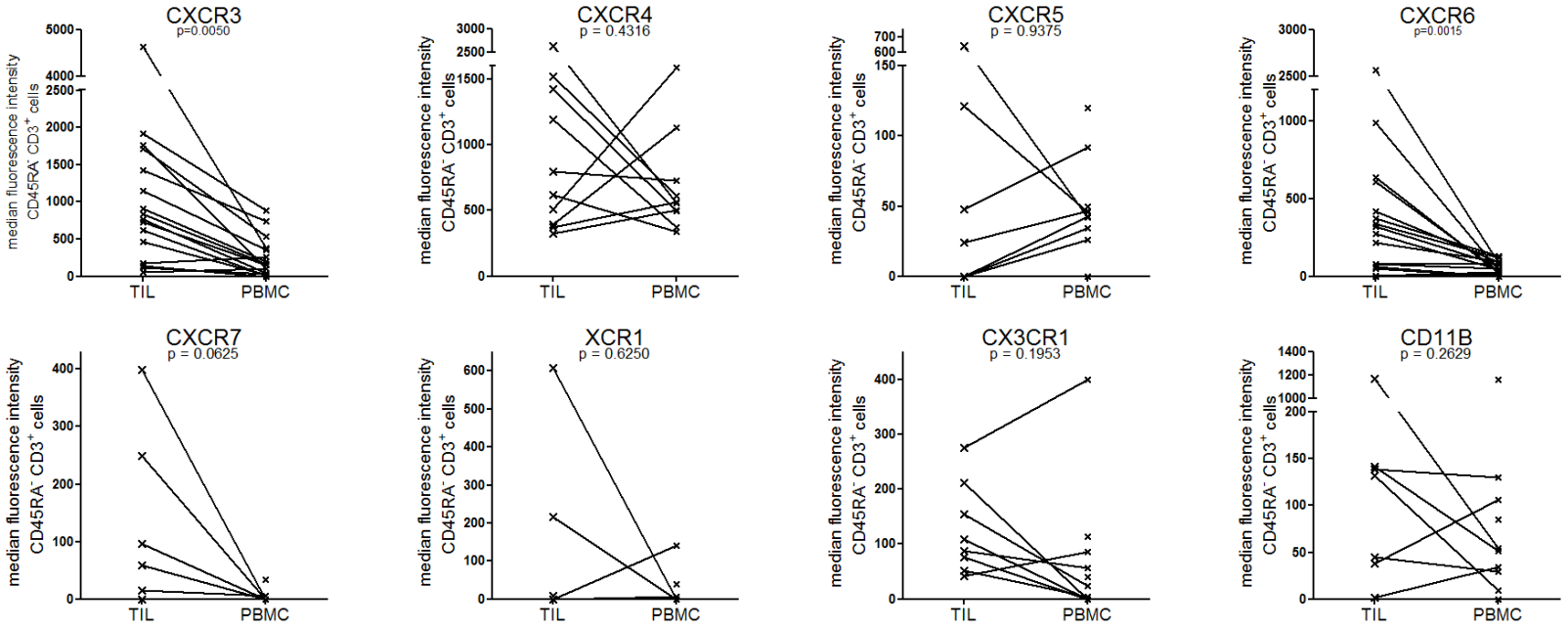


Figure 11: Paired analysis of the percentage of T cells from RCC TIL and matched PBMC expressing chemokine receptors and adhesion molecules

RCC TIL and PBMC were stained with antibodies to all 19 chemokine receptors and 7 adhesion molecules. Graphs display the percentage of viable $CD3^{+}CD45RA^{-}$ cells positive for each marker. Results are corrected for non-specific staining using concentration matched isotype controls. Significance calculated using a Wilcoxon matched pairs test.


CCR1
p = 0.0313
CCR2
p = 0.4375
CCR3
p = 0.0273
CCR4
p = 0.6953
CCR5
p=0.0004
CCR6
p = 0.1637
CCR7
p = 0.0645
CCR8
p = 0.2500
CCR9
p = 0.0625
CCR10
p = 0.5000
CXCR1
p = 0.3125
CXCR2
p = 0.2500
median fluorescence intensity CD45RA- CD3+ cells
TIL
PBMC


CXCR3
p=0.0050
CXCR4
p = 0.4316
CXCR5
p = 0.9375
CXCR6
p=0.0015
CXCR7
p = 0.0625
XCR1
p = 0.6250
CX3CR1
p = 0.1953
CD11B
p = 0.2629
median fluorescence intensity CD45RA- CD3+ cells
TIL
PBMC

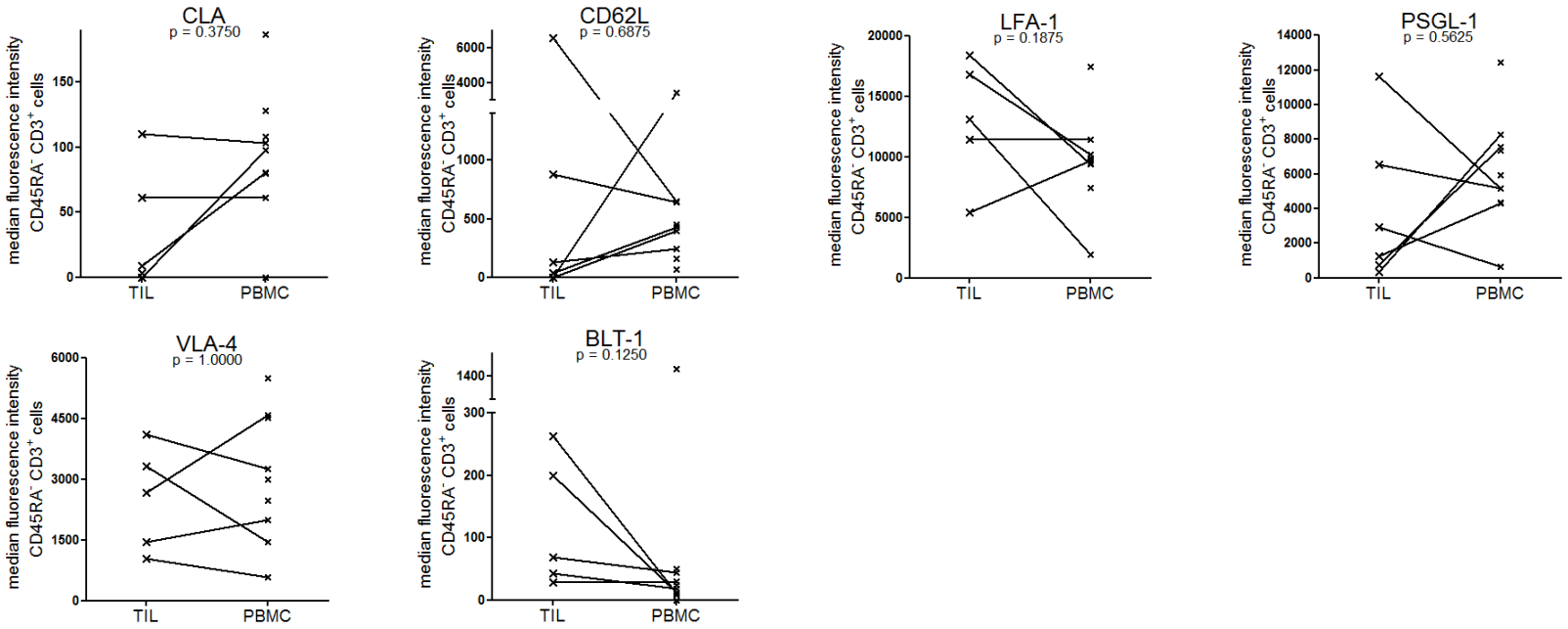


Figure 12: Paired analysis of the median fluorescence intensity of the staining for chemokine receptors and adhesion molecules on TIL and matched PBMC

RCC TIL and PBMC were stained with antibodies to all 19 chemokine receptors and 7 adhesion molecules. Graphs display the MFI of staining for each marker on viable $CD3^+CD45RA^-$ cells. Results are corrected for non-specific staining using concentration matched isotype controls. Significance calculated using a Wilcoxon matched pairs test.

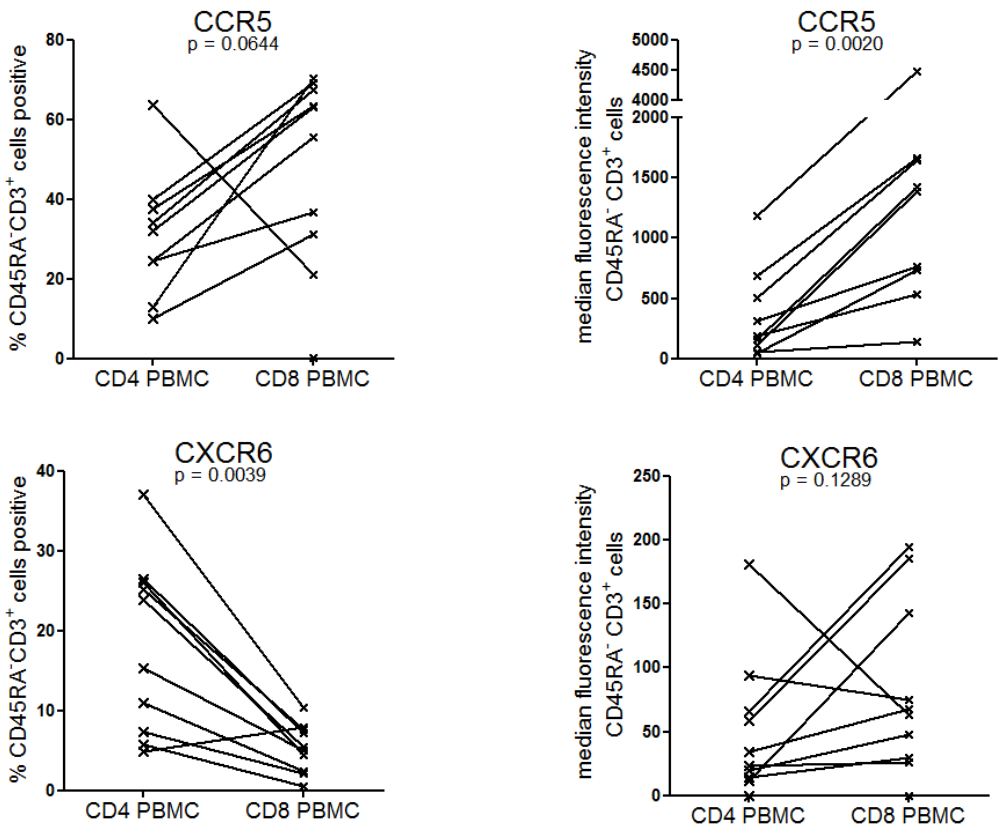


Figure 13: CCR5 and CXCR6 had different patterns of expression on CD4 and CD8 T cells from PBMC

RCC patients' PBMC were stained with antibodies to all 19 chemokine receptors. Graphs display the percentage of T cells positive for each marker and the median fluorescence intensity of the staining. Results are corrected for non-specific staining using concentration matched isotype controls. Chemokine receptor staining on CD4 and CD8 T cells was compared and results are shown for those receptors for which the expression was significantly different between the subsets. Significance was calculated using a Wilcoxon matched pairs test.

3.3.4. Multiple chemokine receptor expression

The previous experiments did not allow us to determine if particular receptors were co-expressed on the same T cell. The combinations of receptors expressed give an insight into whether just one type of receptor is required for homing to RCC, or whether several steps involving multiple receptors are involved. There may also be redundancy in the system, and T cells expressing just one of the three receptors of interest may have the capability to enter RCC tumours.

Therefore I co-stained for CCR5, CXCR3 and CXCR6 on $CD45RA^{-}CD3^{+}$ lymphocytes on 6 matched TIL and PBMC samples. This revealed 63 ± 31 % of TIL to express more than one of these receptors, compared to 14 ± 5.5 % in PBMC (p=0.0313) (**Figure 14**). In TIL $CCR5^{+}$ cells made up the majority of single receptor positive T cells, accounting for 10 ± 18 % $CD3^{+}$ TIL. CXCR3 and CXCR6 single positive T cells were almost non-existent in TIL. In contrast the majority of PBMC were negative for all three receptors (71 ± 15 %), and there were significantly more single positive $CXCR3^{+}$(8.8 ± 10 %, p = 0.0313) and $CXCR6^{+}$ (2.0 ± 0.8 %, p = 0.0313) T cells.

The proportion of $CCR5^{+}CXCR3^{+}$ and $CCR5^{+}CXCR6^{+}$ $CD3^{+}$ T cells was significantly greater in TIL than PBMC (p = 0.0313 for both comparisons). There was also a greater frequency of $CXCR3^{+}CXCR6^{+}$ T cells and $CCR5^{+}CXCR3^{+}CXCR6^{+}$ T cells in TIL than PBMC (**Figure 14**), however this did not reach significance (p values of 0.0625 and 0.0938 respectively).

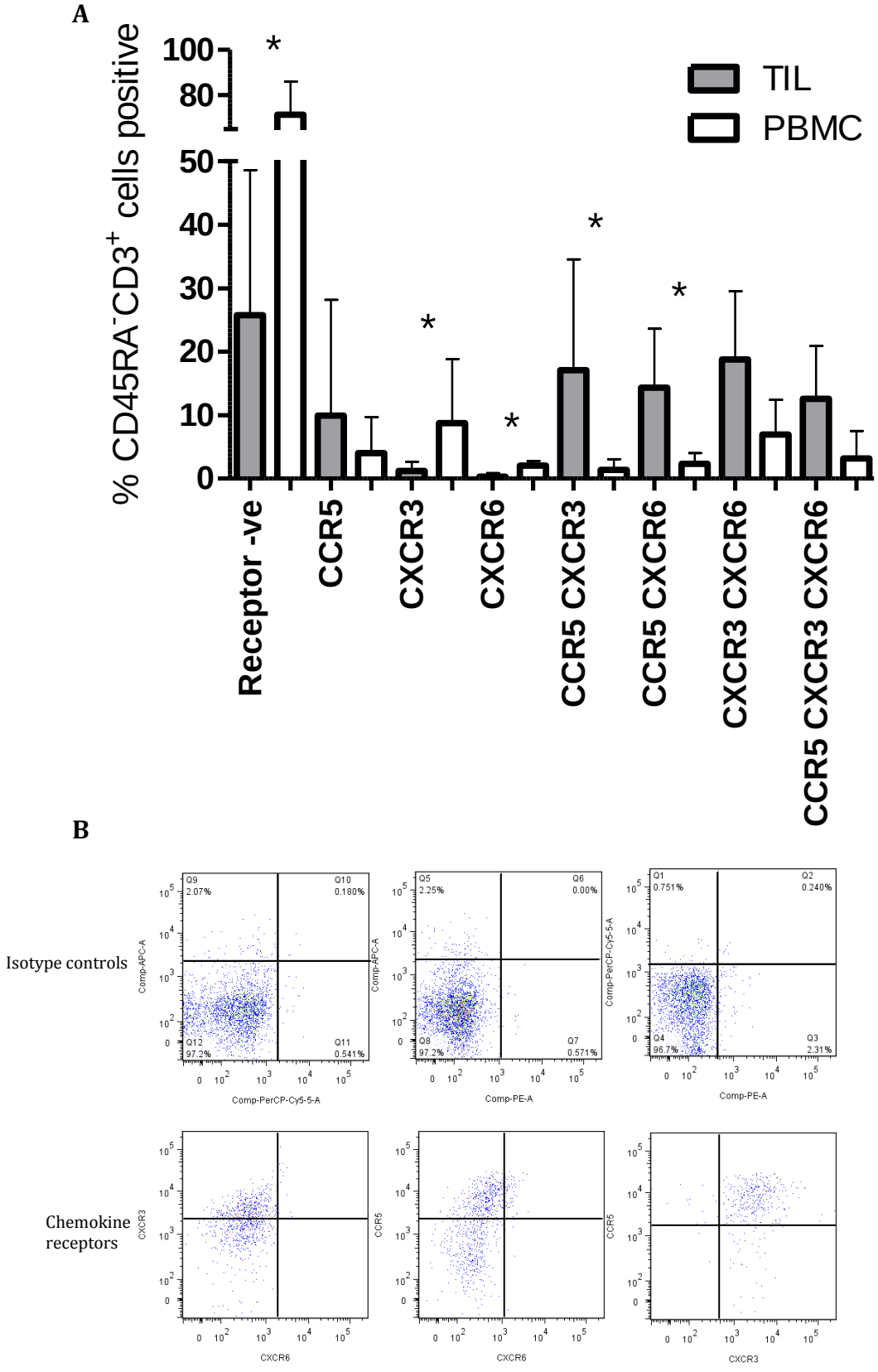

A
% CD45RA-CD3+ cells positive
100
80
50
40
30
20
10
0
TIL
PBMC
*
*
*
*
Receptor -ve
CCR5
CXCR3
CXCR6
CCR5 CXCR3
CCR5 CXCR6
CXCR3 CXCR6
CCR5 CXCR3 CXCR6
B
Isotype controls
Chemokine receptors
Comp-APC-A
Comp-PerCP-Cy5-5-A
Comp-PE-A
CXCR3
CCR5
CXCR6

Figure 14: Co-expression of CCR5, CXCR3 and CXCR6 on T cells from TIL and matched PBMC.

The relative proportion of cells expressing none, one, two or all three of the chemokine receptors was determined using flow cytometry. Results show the mean of data from 6 cases (+ SD). Significance was calculated using a Wilcoxon matched pairs test (A). Representative plots for RCC TIL are also shown (B)

3.4. Classic Tregs are recruited into RCC

There is evidence to suggest that regulatory T cells (Tregs) can suppress anti-tumour T cell responses at the tumour site. Elevated levels of Tregs have been found in the blood and TIL of patients with various cancers including pancreatic, breast, hepatocellular and ovarian cancer, and Tregs from ovarian cancer ascites, hepatocellular carcinoma and RCC have been demonstrated to be capable of suppressing effector T cell proliferation *in vitro* (Curiel et al. 2004;Fu et al. 2007;Liotta et al. 2010a;Liyanage et al. 2002). As Tregs may be suppressing anti-tumour immune responses in RCC, I sought to determine whether they may be selectively recruited to this tumour.

Using intracellular flow cytometry I found a mean of 11 ± 6.0 % of CD4 TIL expressed the Treg marker Foxp3 , which is significantly greater than the 6.6 ± 4.3 % of $Foxp3^+$ CD4 T cells in PBMC (p=0.0013) (**Figure 15**). I also used immunohistochemistry for Foxp3 to investigate the distribution of the putative Tregs within 10 RCC tumours. Compared to CD3 staining, it appeared $Foxp3^+$ cells are more evenly distributed throughout the tumour and do not aggregate in fibrous areas to the same extent (**Figure 16**). However the small sample size did not allow a quantitative evaluation of the differences in cell distribution.

When I compared the percentage of $Foxp3^+$ cells in TIL and the Fuhrman grade of the tumours, I found a significant positive correlation ($r = 0.7096$, $p = 0.0097$) (**Figure 17**).

Flow cytometry of lymphocytes from 6 samples of adjacent normal renal tissue showed that in 5 out of 6 samples, fewer than 0.5% of $CD4^+$ $CD45RA^-$ T cells express Foxp3. In one sample 1.37% of $CD4^+$ T cells were $Foxp3^+$, however all were $CD127^{hi}$, suggesting they are not true Tregs (**Figure 18**).

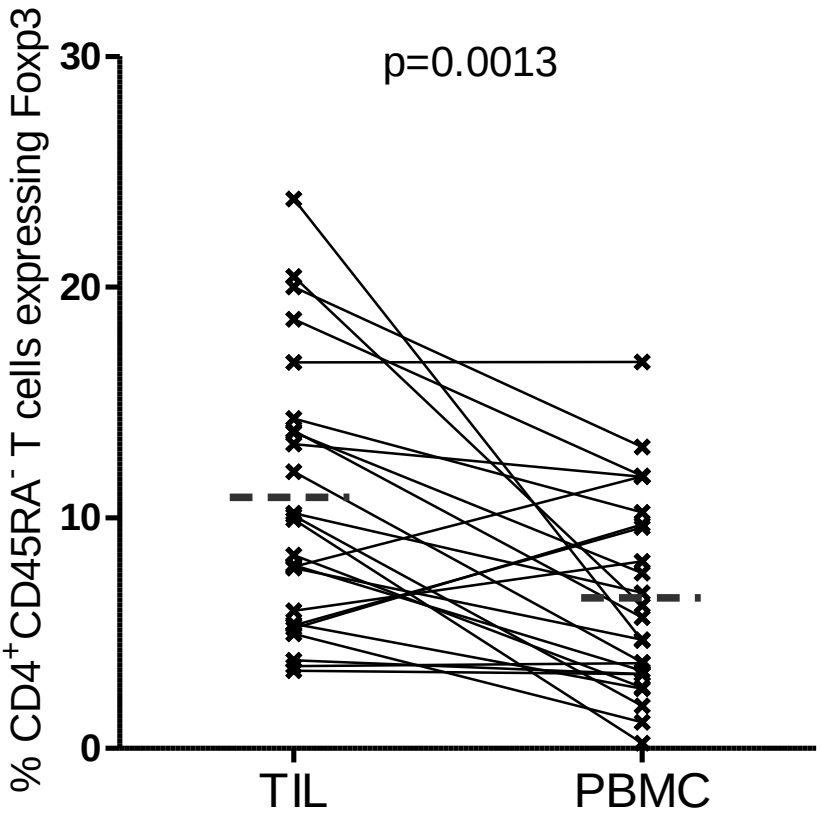


Figure 15: Foxp3⁺ T cells are enriched in RCC tumours

CD4⁺ T cells from TIL and PBMC were stained intracellularly for Foxp3. Significance calculated using a Wilcoxon matched pairs test.

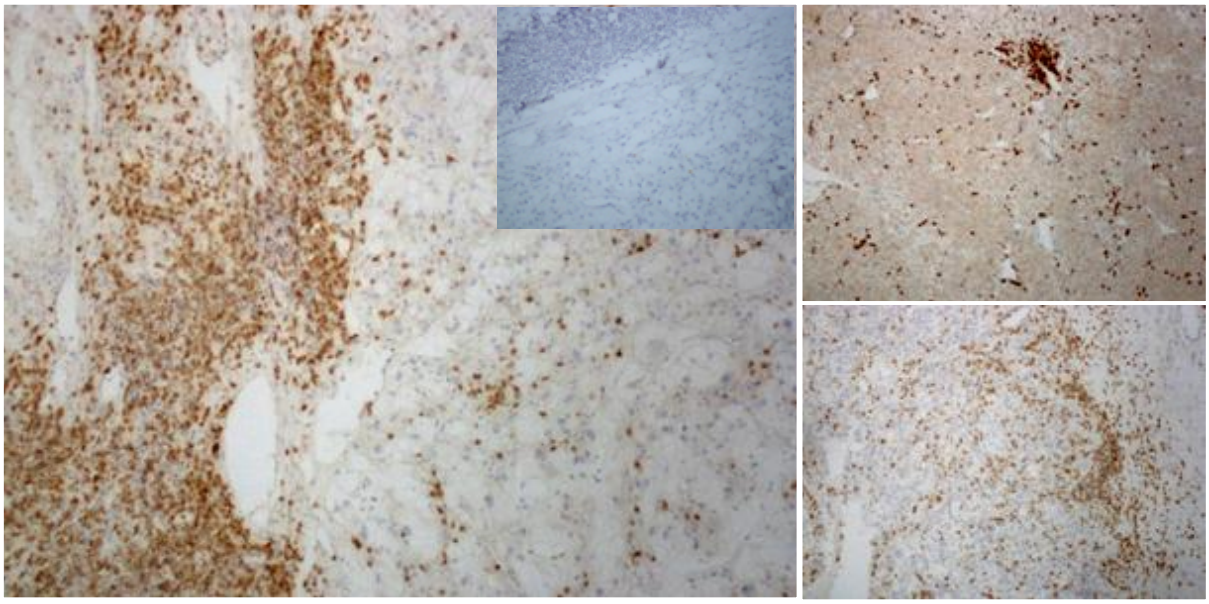


Figure 16: Immunohistochemistry for Foxp3

5µm paraffin sections from RCC tumours were stained with an antibody to Foxp3. Photos are representative of 10 RCC cases. Results were compared to concentration matched isotype controls (inset large photo).

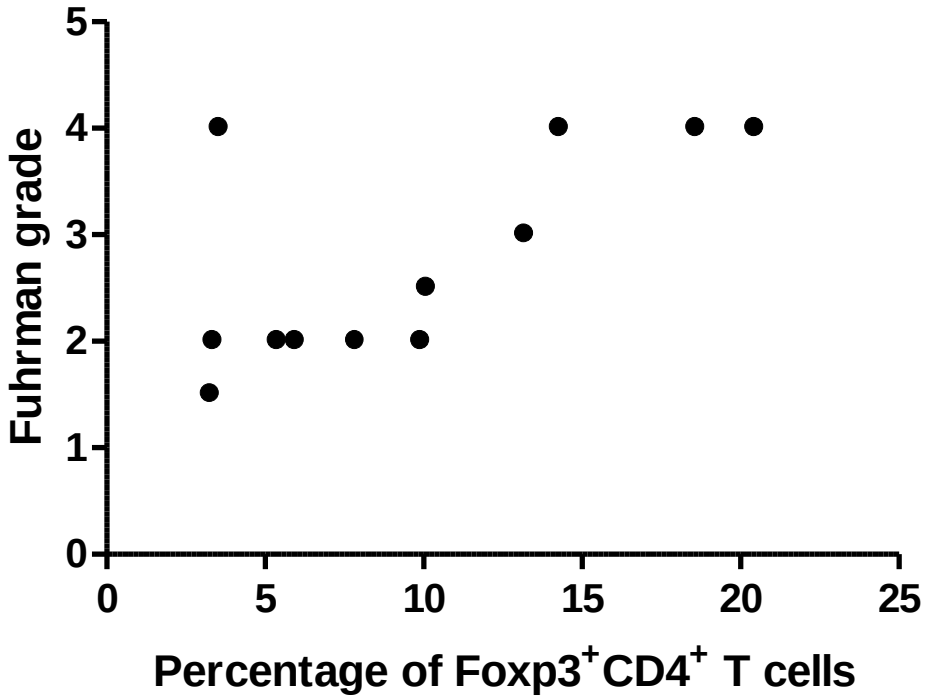


Figure 17: The percentage of Foxp3⁺CD4⁺ T cells in TIL correlates with the grade of the tumour

A Spearman correlation coefficient was calculated to determine the correlation between the percentage of Foxp3⁺CD4⁺ T cells and the Fuhrman grade of the tumour.

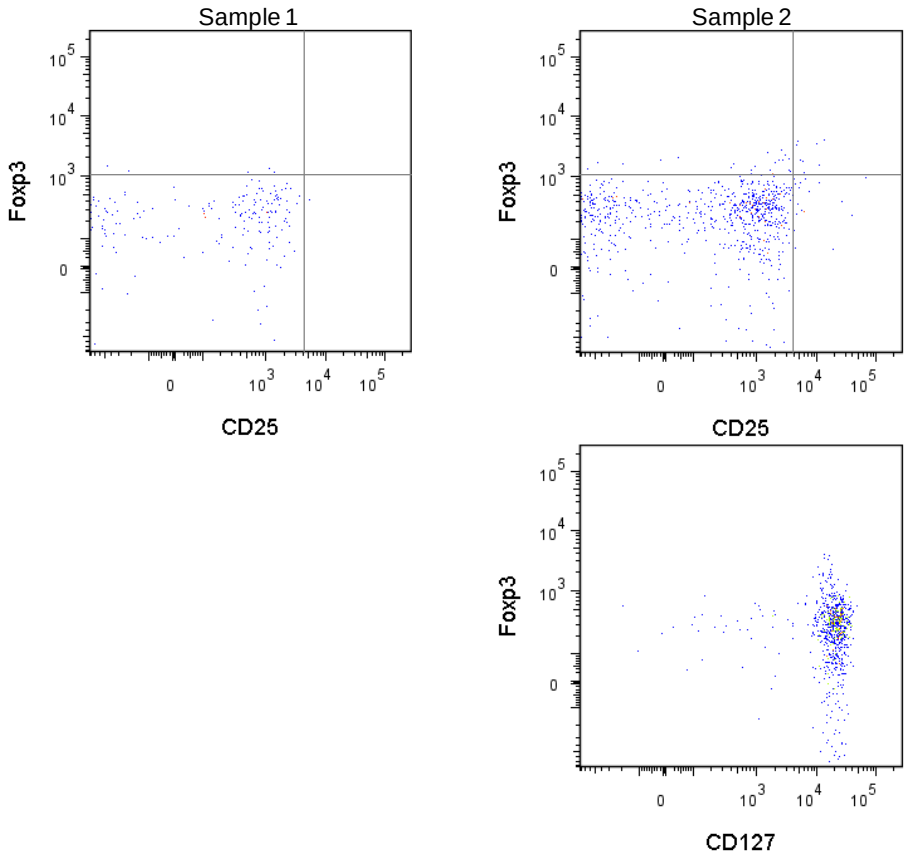


Figure 18. Foxp3 expression in T cells from normal kidney tissue

T cells isolated from normal kidney adjacent to tumour were isolated and stained for T cell markers and Foxp3, and analysed by flow cytometry. Cells are gated on $CD3^+$ LIVE $CD45RA^-$ $CD4^+$. Sample 1 is representative of the 5 out of the 6 samples stained in which $Foxp3^+$ cells made up fewer than 0.5% of CD4 T cells. Sample 2 contained some $Foxp3^+CD4^+$ T cells, however the $Foxp3^+$ cells were $CD127^+$ and therefore not true T cells.

However, Foxp3 can be upregulated transiently when T cells are activated (Ahmadzadeh et al. 2007;Gavin et al. 2006), so in order to further ascertain whether these cells were regulatory or not, I measure the surface levels of CD25 and CD127. Studies have shown classical Tregs have high levels of CD25 and low levels of CD127, and my data matched this pattern. (**Figure 19**). I also carried out additional phenotypic and functional studies on the Tregs to further demonstrate their regulatory phenotype, which are described in section 4.3.

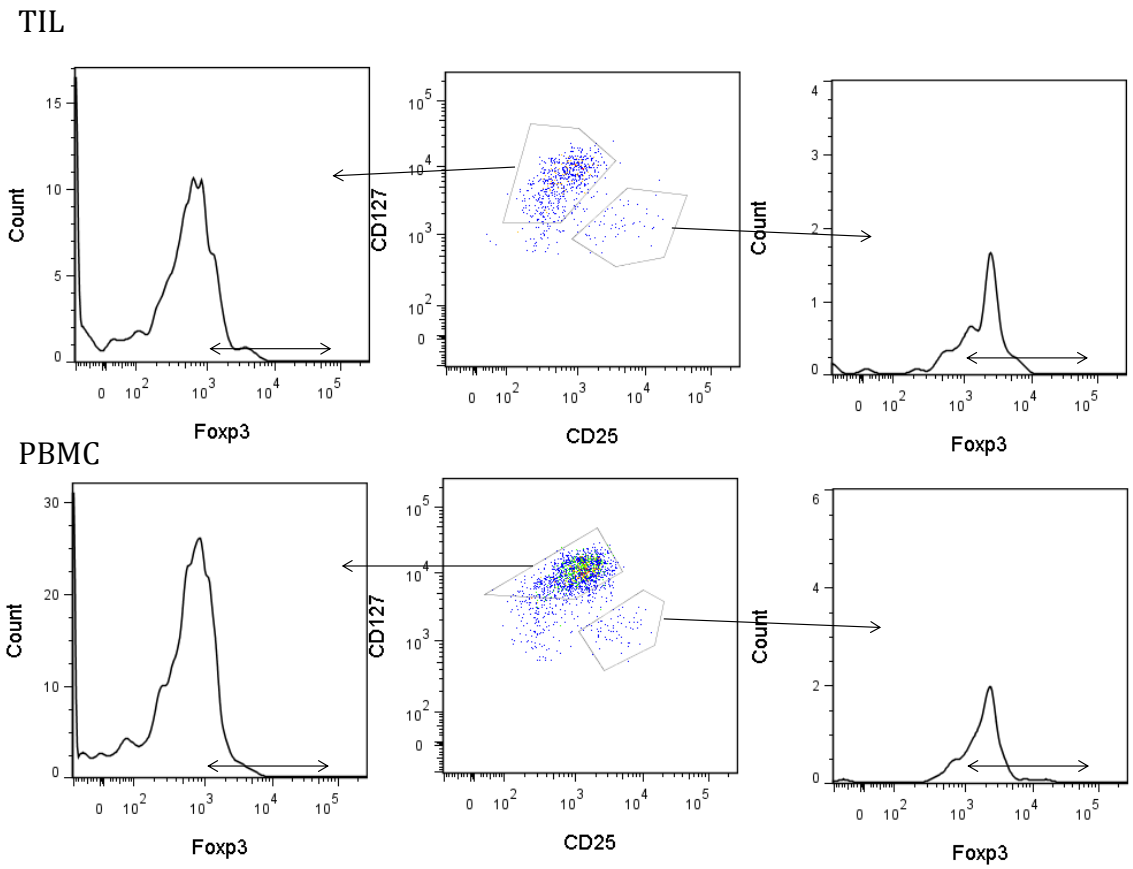


Figure 19: Foxp3⁺ T cells in RCC TIL and PBMC have a classic Treg phenotype

Levels of putative Tregs in TIL and PBL were examined by flow cytometry for co-expression of CD127 and CD25. $CD25^{hi}CD127^{lo}$ T cells from TIL and PBMC were $Foxp3^{+}$ (indicated by horizontal arrow). Data shown are representative of 25 RCC cases

3.4.1. Homing phenotype of Tregs

I studied the homing phenotype of Foxp3$^+$ cells in parallel with the phenotyping of the whole T cell population within TIL. Overall chemokine receptor and adhesion molecule expression on Foxp3$^+$ cells followed a similar pattern to that on all CD3$^+$ TIL (**Figure 20**, **Figure 21**). However, the frequency of cells expressing CCR6 was significantly higher within the Foxp3$^+$ population than the whole CD3$^+$ population ($p = 0.0488$) and the percentage of CXCR3$^+$ cells among the Foxp3$^+$ subset was significantly lower ($p = 0.0488$) (**Figure 22**).

The MFI for CCR4 expression, a marker commonly found on Tregs, was significantly higher on Foxp3$^+$ cells than the total CD3$^+$ population ($p = 0.0020$). This was also true for the receptors CCR5 ($p = 0.0098$), CCR6 ($p = 0.0039$) and CXCR6 ($p = 0.0020$), indicating Foxp3$^+$ cells may be recruited more effectively via these receptors than other T cells (**Figure 22**).

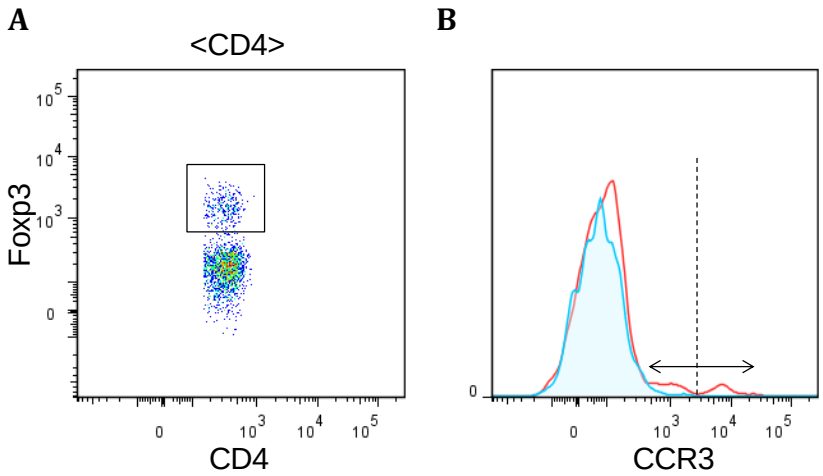
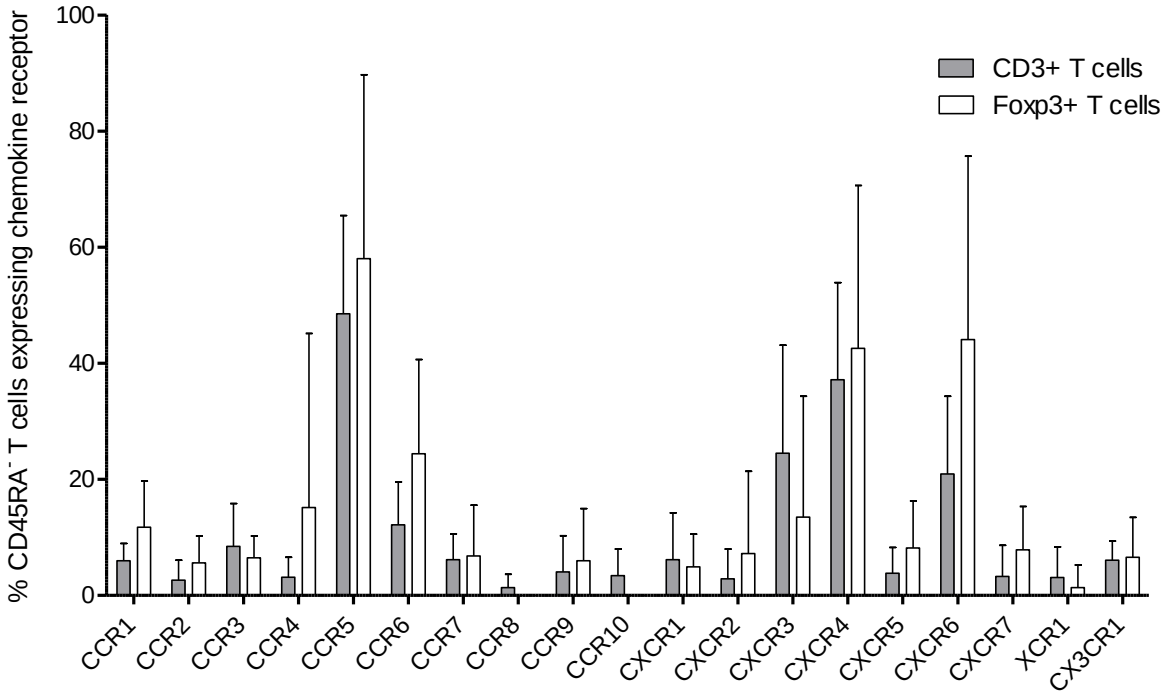
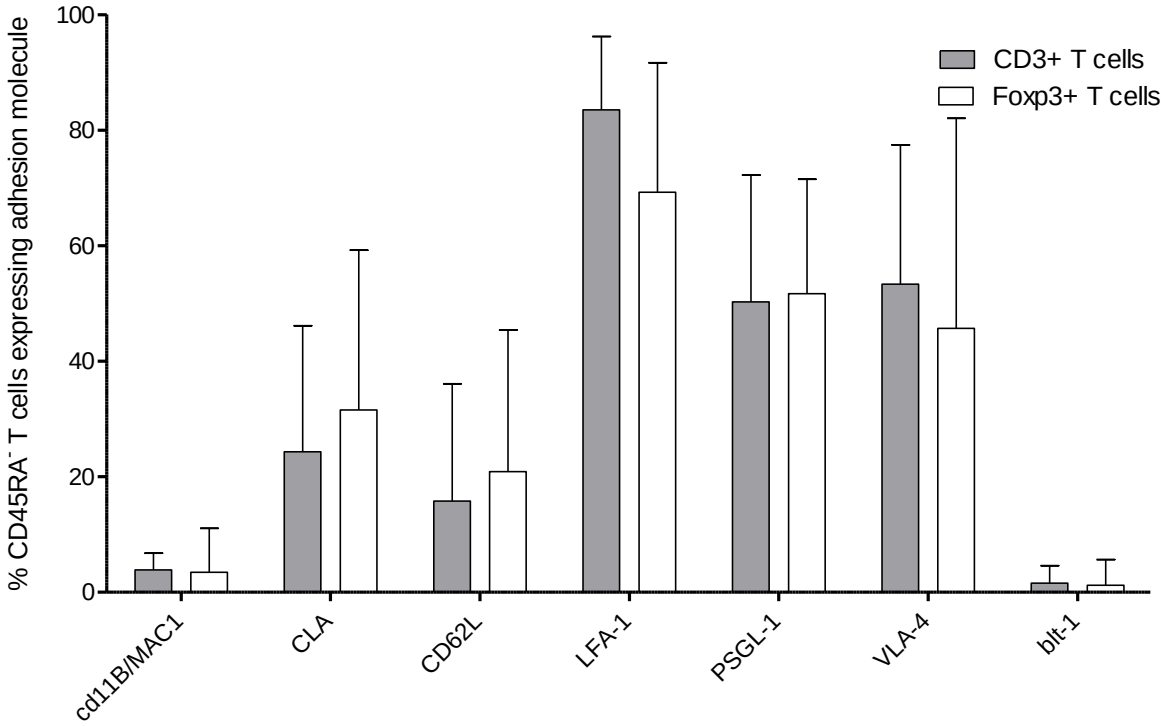


Figure 20. Gating strategy for defining chemokine receptor positive Tregs

After gating for $CD4^+$ T cells, Foxp3 was used to define Tregs (A), the percentage of cells positive for a receptor (horizontal arrow) and the median fluorescence intensity of the positive cells was then determined, using an isotype control to determine the level of non-specific staining (blue shaded area) (B).


% CD45RA- T cells expressing chemokine receptor
100
80
60
40
20
0
CD3+ T cells
Foxp3+ T cells
CCR1
CCR2
CCR3
CCR4
CCR5
CCR6
CCR7
CCR8
CCR9
CCR10
CXCR1
CXCR2
CXCR3
CXCR4
CXCR5
CXCR6
CXCR7
XCR1
CX3CR1


% CD45RA- T cells expressing adhesion molecule
100
80
60
40
20
0
CD3+ T cells
Foxp3+ T cells
cd11B/MAC1
CLA
CD62L
LFA-1
PSGL-1
VLA-4
blt-1

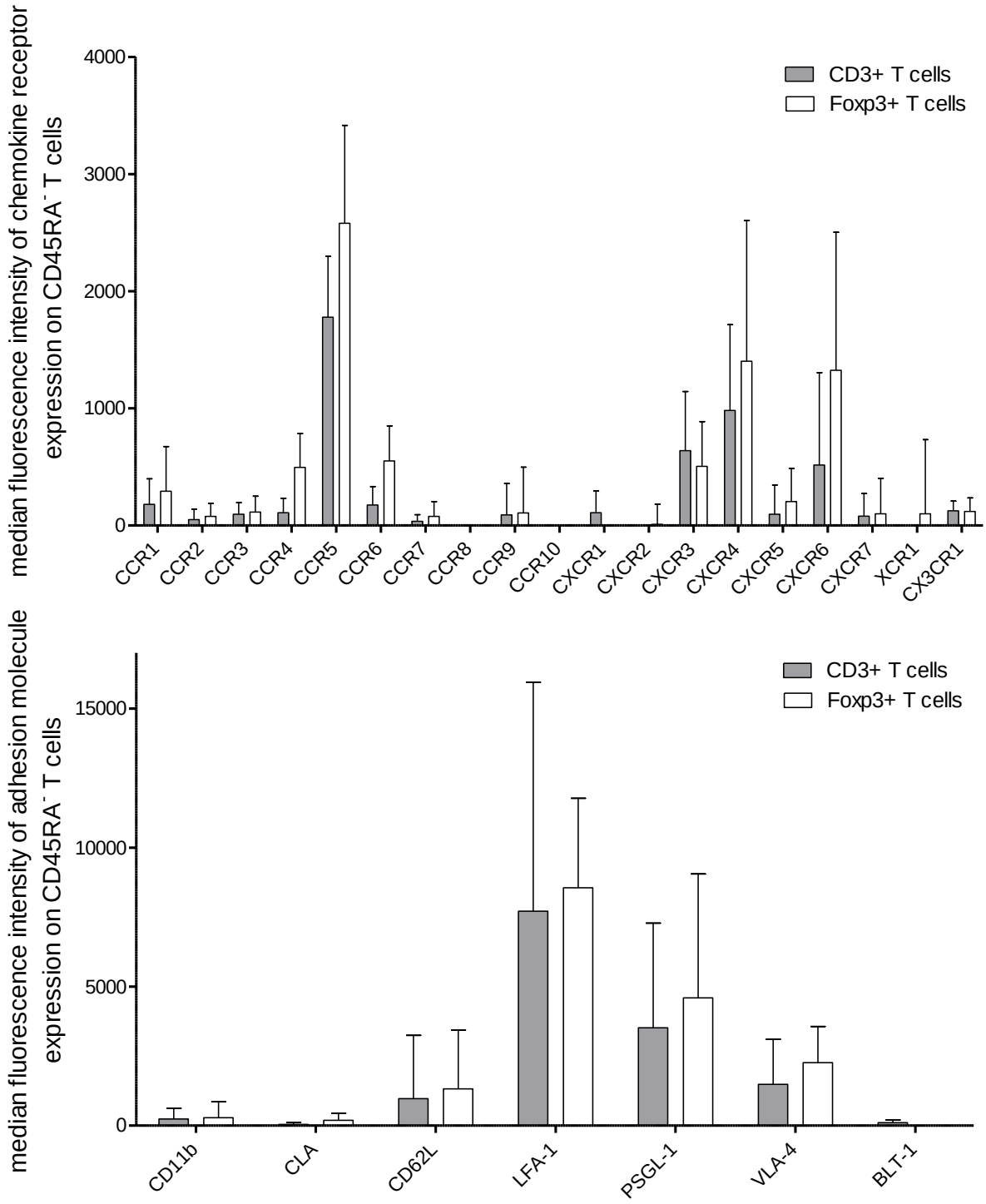


Figure 21: Homing phenotype of Foxp3⁺ TIL

RCC TIL samples were stained with antibodies specific for 19 chemokine receptors and 7 adhesion molecules. Graphs display the mean percentage of T cells and the CD4⁺ Foxp3⁺ subset positive for each marker and the mean median fluorescence intensity of the staining (+ SD), both corrected for non-specific staining using concentration matched isotype controls. The number of RCC cases stained was as follows: CCR3, 4 5 6 7, CXCR3, 4 and 6 n = 10; CCR8 and CX3CR1 n = 8; CCR1, 2, 9, 10, CXCR2, 5, 7, XCR1 and CD11b n = 7; CXCR1, CD62L and PSGL-1 n = 6; LFA-1, CLA, VLA-4 and BLT-1 n = 5.

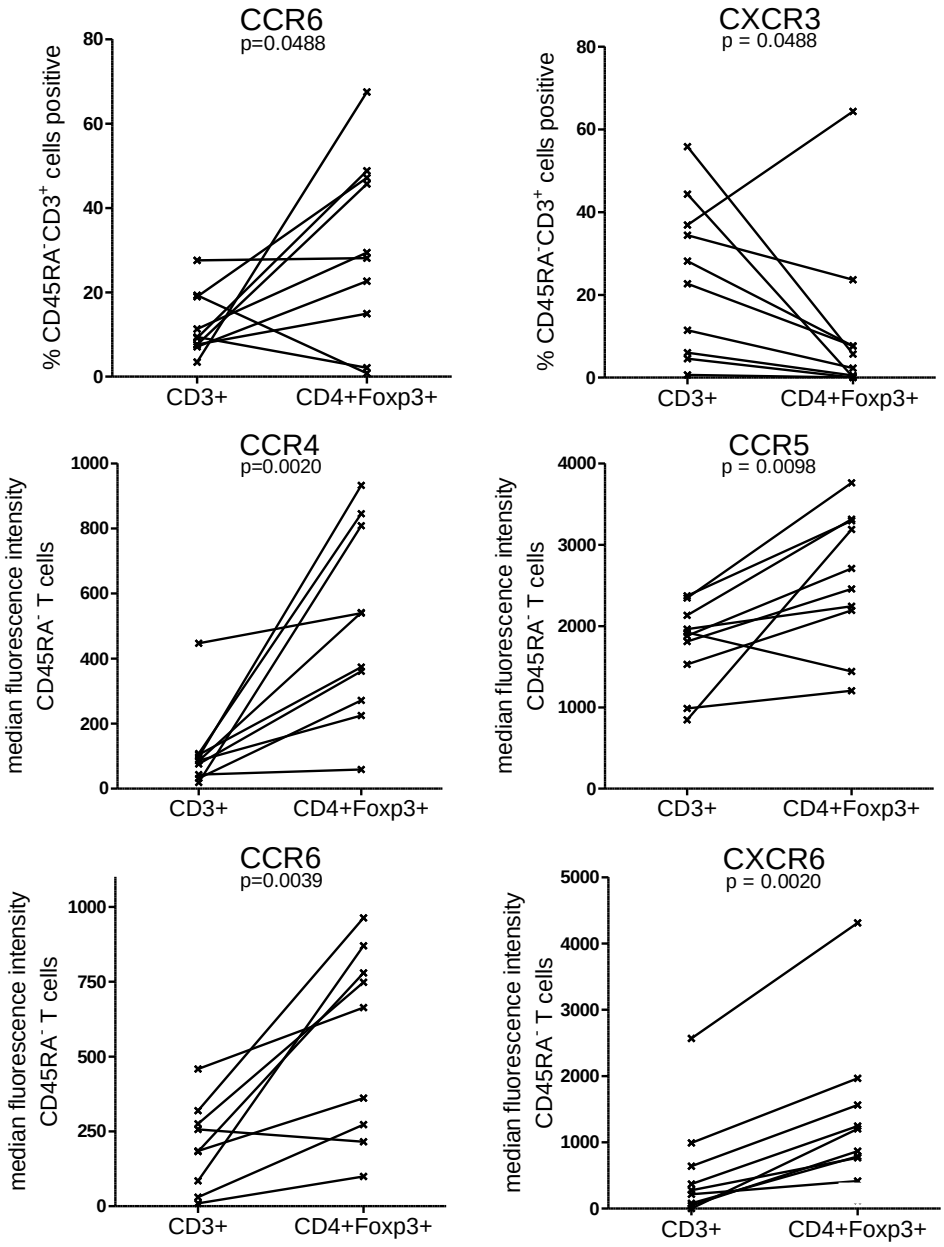


Figure 22: Different patterns of chemokine receptor expression on CD4⁺Foxp3⁺ T cells and the whole T cell population

RCC patients' PBMC were stained with antibodies to all 19 chemokine receptors. Graphs display the percentage of T cells positive for each marker and the median fluorescence intensity of the staining. Results were corrected for non-specific staining using concentration matched isotype controls. Chemokine receptor staining on CD4⁺Foxp3⁺ and CD3⁺ T cells was compared and results are shown for those receptors for which the expression was significantly different between the subsets. Significance was calculated using a Wilcoxon matched pairs test.

3.4.2. Comparison of chemokine receptor expression on Foxp3$^+$ T cells from TIL and matched peripheral blood

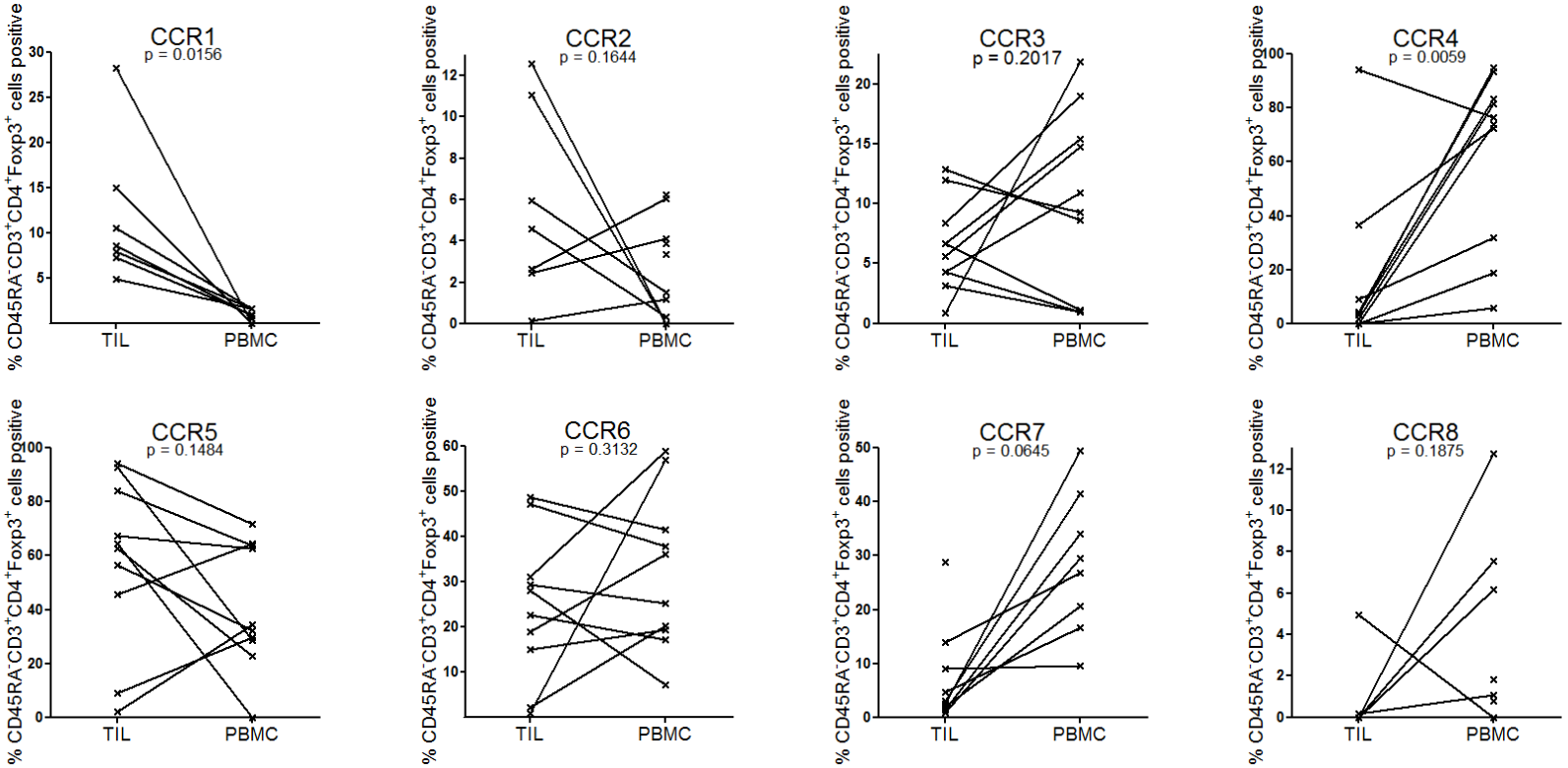
Foxp3$^+$ cells in PBMC show the conventional Treg chemokine receptor profile, with CCR4 and CCR7 being expressed on a greater percentage of Foxp3$^+$ cells in PBMC than TIL ($p = 0.0059$ and $p = 0.0645$) (**Figure 23**). The Foxp3$^+$ T cells in blood also expressed higher levels of CCR4 compared to matched TIL ($p = 0.0316$) (**Figure 24**).

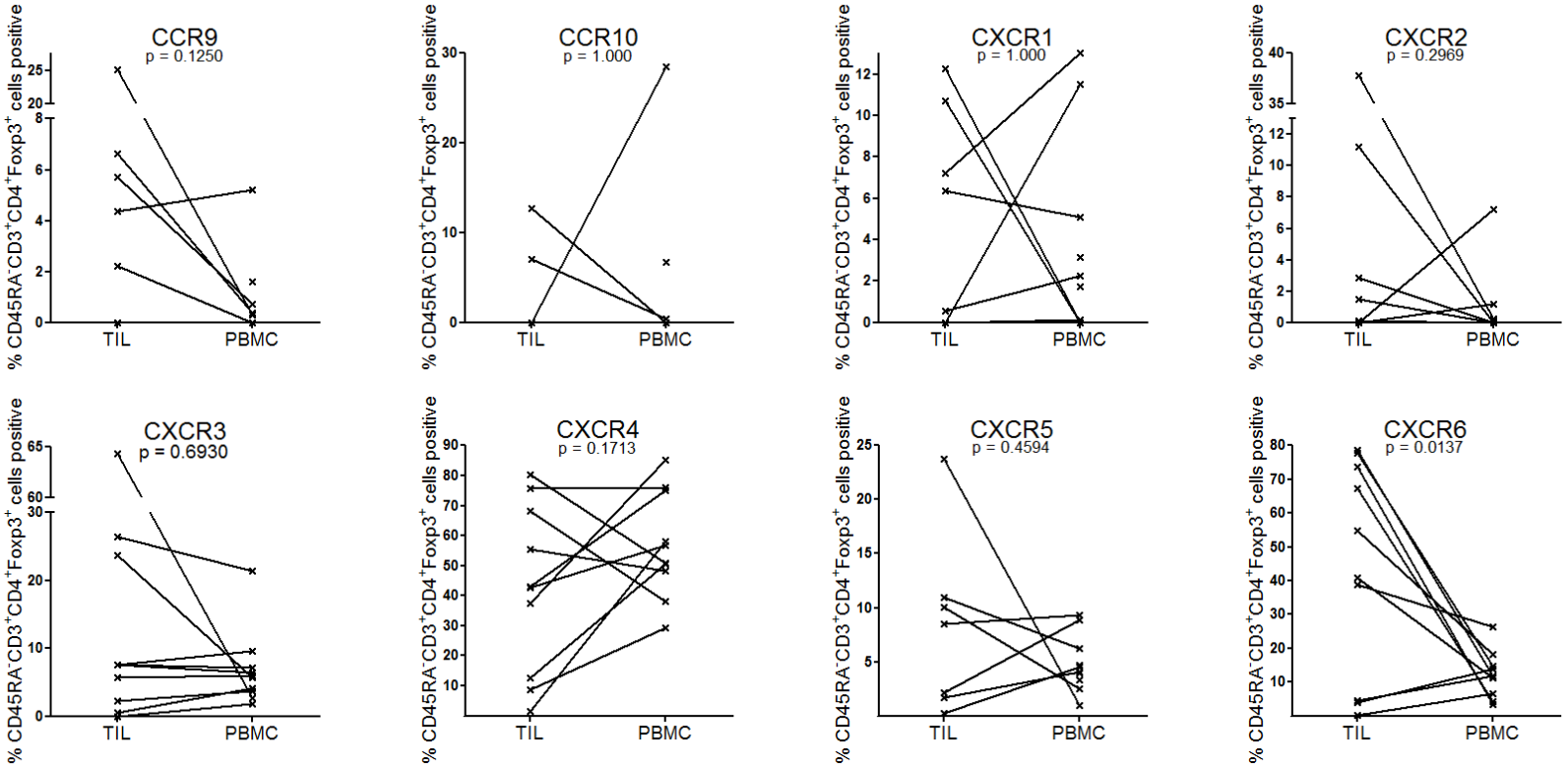
A greater proportion of putative Tregs in TIL than in PBMC expressed the receptors CCR1 ($p = 0.0156$), CXCR6 ($p = 0.0137$) and CXCR7 ($p = 0.0156$) (**Figure 23**).

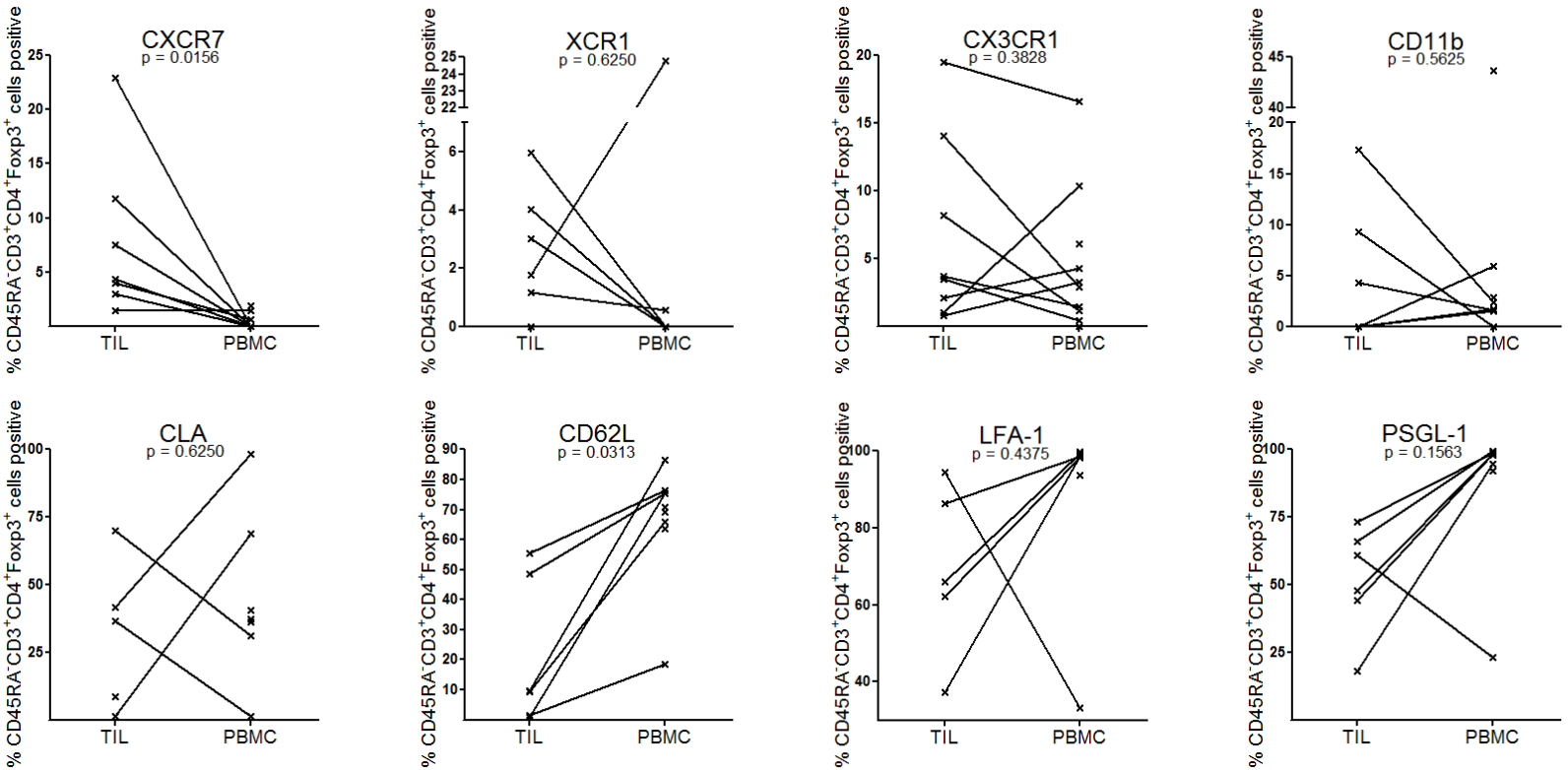
In common with the total TIL T cell population, compared to PBMC Foxp3$^+$ TIL express significantly higher levels of CCR1 ($p = 0.0313$), CCR5 ($p = <0.0001$), CXCR3 ($p = 0.0078$) and CXCR6 ($p = 0.0020$). Interestingly they also express significantly higher levels of CCR6 ($p = 0.0101$) CXCR5 ($p = 0.0313$) and CX3CR1 ($p = 0.0156$) (**Figure 24**).

These results showing enrichment of certain receptors on Foxp3$^+$ TIL give an indication of the chemokine receptors Tregs use to enter RCC. However, as before, receptors CCR1, CXCR5, CXCR7 and CX3CR1 are only expressed on a very small proportion of the putative Tregs (**Figure 23**), and therefore I did not pursue these further. CCR6 was of particular interest as I only saw enrichment on the Foxp3$^+$ subset of TIL and it was expressed by a significant proportion of cells, suggesting Tregs may have a separate mechanism of recruitment to the tumour via this receptor.

CD62L was expressed on a lower proportion of Foxp3$^+$ TIL than Foxp3$^+$ PBMC, which is unsurprising as it is a selectin usually involved in homing to lymph nodes.


CCR1
p = 0.0156
CCR2
p = 0.1644
CCR3
p = 0.2017
CCR4
p = 0.0059
CCR5
p = 0.1484
CCR6
p = 0.3132
CCR7
p = 0.0645
CCR8
p = 0.1875
% CD45RA-CD3+CD4+Foxp3+ cells positive
TIL
PBMC


CCR9
p = 0.1250
CCR10
p = 1.000
CXCR1
p = 1.000
CXCR2
p = 0.2969
CXCR3
p = 0.6930
CXCR4
p = 0.1713
CXCR5
p = 0.4594
CXCR6
p = 0.0137
% CD45RA-CD3+CD4+Foxp3+ cells positive
TIL
PBMC


CXCR7
p = 0.0156
XCR1
p = 0.6250
CX3CR1
p = 0.3828
CD11b
p = 0.5625
CLA
p = 0.6250
CD62L
p = 0.0313
LFA-1
p = 0.4375
PSGL-1
p = 0.1563
% CD45RA-CD3+CD4+Foxp3+ cells positive
% CD45RA-CD3+CD4+Foxp3+ cells pgsitive
TIL
PBMC

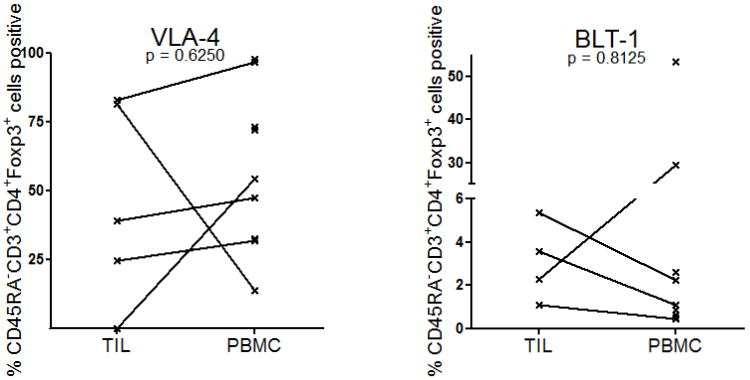
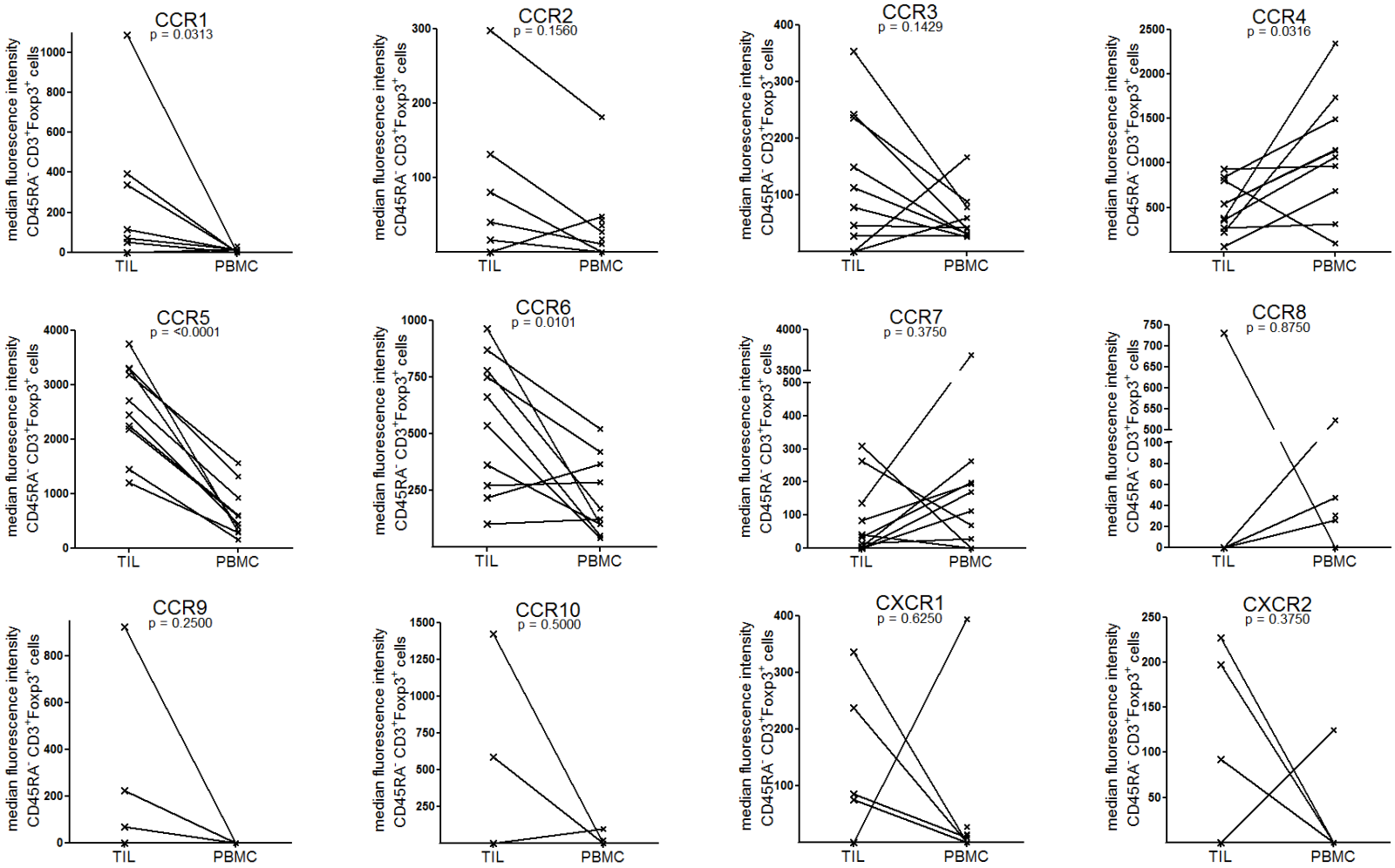
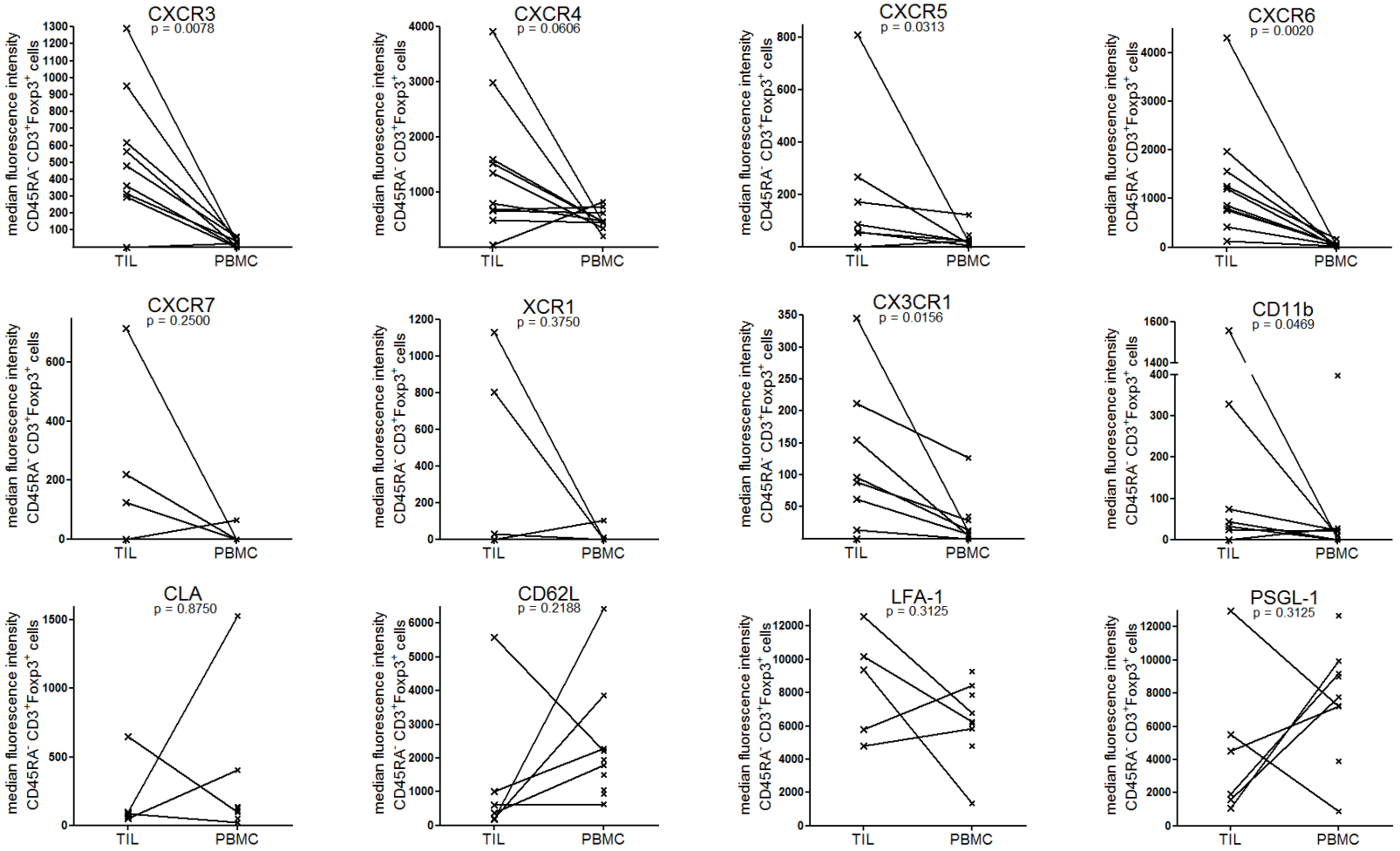


Figure 23: Paired analysis of the percentage of $CD4^+Foxp3^+$ T cells from TIL and PBMC expressing chemokine receptors and adhesion molecules

RCC TIL and PBMC were stained with antibodies to all 19 chemokine receptors and 7 adhesion molecules. Graphs display the percentage of viable $CD3^+CD45RA^-CD4^+Foxp3^+$ cells positive for each marker. Results are corrected for non-specific staining using concentration matched isotype controls. Significance calculated using a Wilcoxon matched pairs test.


CCR1
p = 0.0313
CCR2
p = 0.1560
CCR3
p = 0.1429
CCR4
p = 0.0316
CCR5
p = <0.0001
CCR6
p = 0.0101
CCR7
p = 0.3750
CCR8
p = 0.8750
CCR9
p = 0.2500
CCR10
p = 0.5000
CXCR1
p = 0.6250
CXCR2
p = 0.3750
median fluorescence intensity CD45RA- CD3+Foxp3+ cells
TIL
PBMC


CXCR3
p = 0.0078
CXCR4
p = 0.0606
CXCR5
p = 0.0313
CXCR6
p = 0.0020
CXCR7
p = 0.2500
XCR1
p = 0.3750
CX3CR1
p = 0.0156
CD11b
p = 0.0469
CLA
p = 0.8750
CD62L
p = 0.2188
LFA-1
p = 0.3125
PSGL-1
p = 0.3125
median fluorescence intensity CD45RA- CD3+Foxp3+ cells
TIL
PBMC

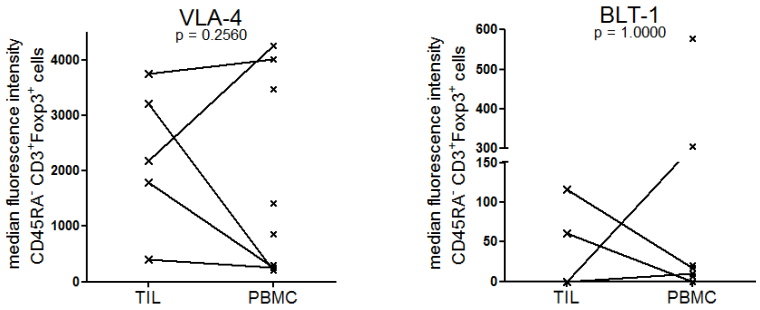


Figure 24: Paired analysis of the median fluorescence intensity of staining of $CD4^{+}Foxp3^{+}$ T cells from TIL and PBMC for chemokine receptors and adhesion molecules

RCC TIL and PBMC were stained with antibodies to all 19 chemokine receptors and 7 adhesion molecules. Graphs display the MFI of staining for each marker on viable $CD3^{+}CD45RA^{-}CD4^{+}Foxp3^{+}$ cells. Results are corrected for non-specific staining using concentration matched isotype controls. Significance calculated using a Wilcoxon matched pairs test.

3.5. Location of chemokine receptor positive lymphocytes in RCC

I used immunohistochemistry to stain paraffin embedded RCC tissue for CCR5, CCR6, CXCR3 and CXCR6 to verify their expression by lymphocytes in the tumour and to examine the distribution of receptor positive cells. Positive staining was compared to concentration matched isotype controls.

I found positive staining for CCR5 on lymphocytes in nine out of the ten samples studied; I could not identify any lymphocytes in the CCR5 negative sample. The positively stained lymphocytes were associated with vessels in the fibrous stroma. Interestingly some vessels, both in the fibrous area and within the tumour, also stained positively for CCR5, and tumour cells stained weakly in 6 out of 10 samples (**Figure 22**).

CCR6 also stained a proportion of lymphocytes in all samples where lymphocytes were identifiably present. Again the majority of receptor positive lymphocytes were in the fibrous regions of the tissue, however I observed CCR6 positive lymphocytes in contact with tumour cells in half the samples studied. CCR6 also stained areas of tumour cells and some vessels in half of the samples (**Figure 22**).

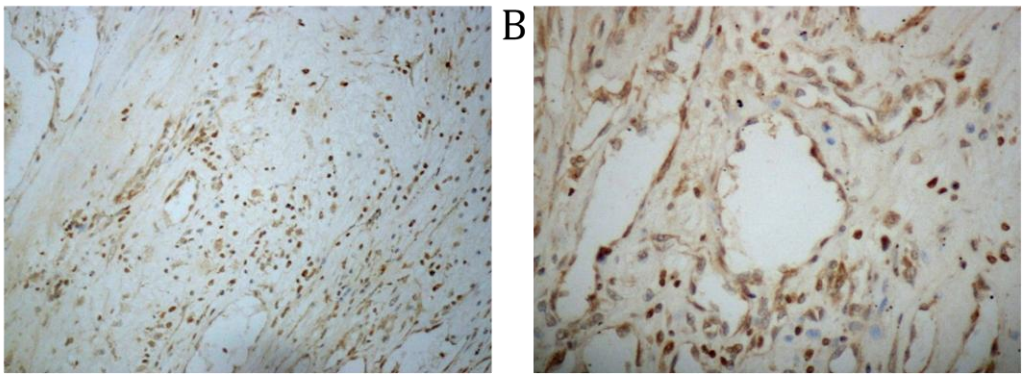
The CXCR3 antibody appeared to exclusively stain lymphocytes and a large proportion of lymphocytes in the stroma were CXCR3 positive. CXCR3 positive cells were also seen in contact with tumour cells, with the greatest levels of cell infiltration in the most vascularised areas (**Figure 22**).

CXCR6 also stained lymphocytes, and in common with CCR5 was most commonly seen on lymphocytes in the fibrous areas, with only a small minority of lymphocytes within the tumour staining positively. In addition the tumour cells stained weakly for the receptor (**Figure 22**)

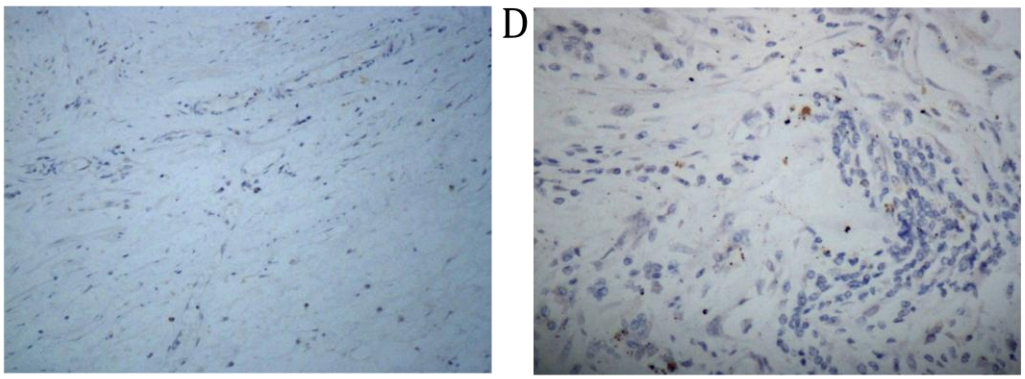
CCR5

X20 X40

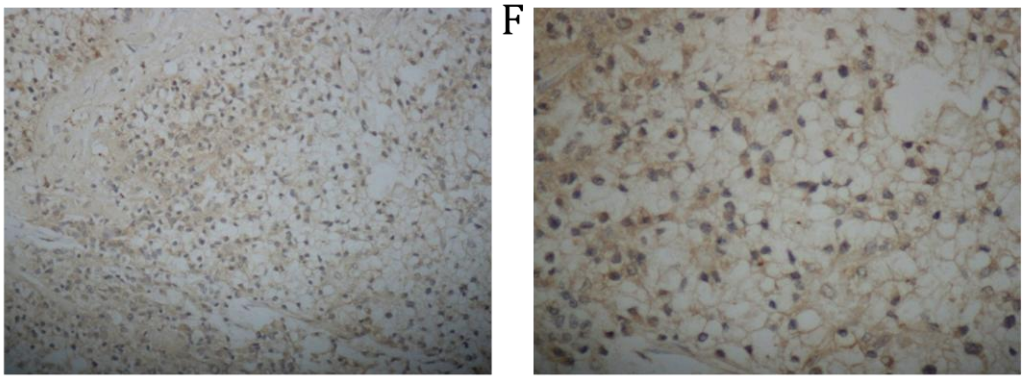
A B



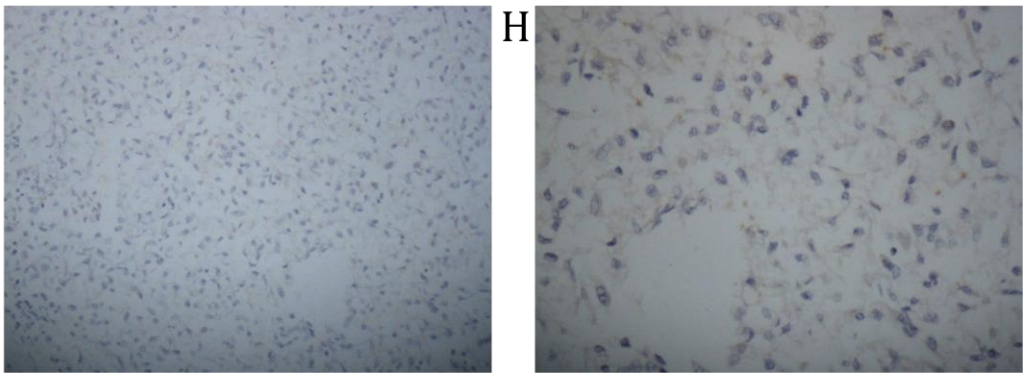
C D



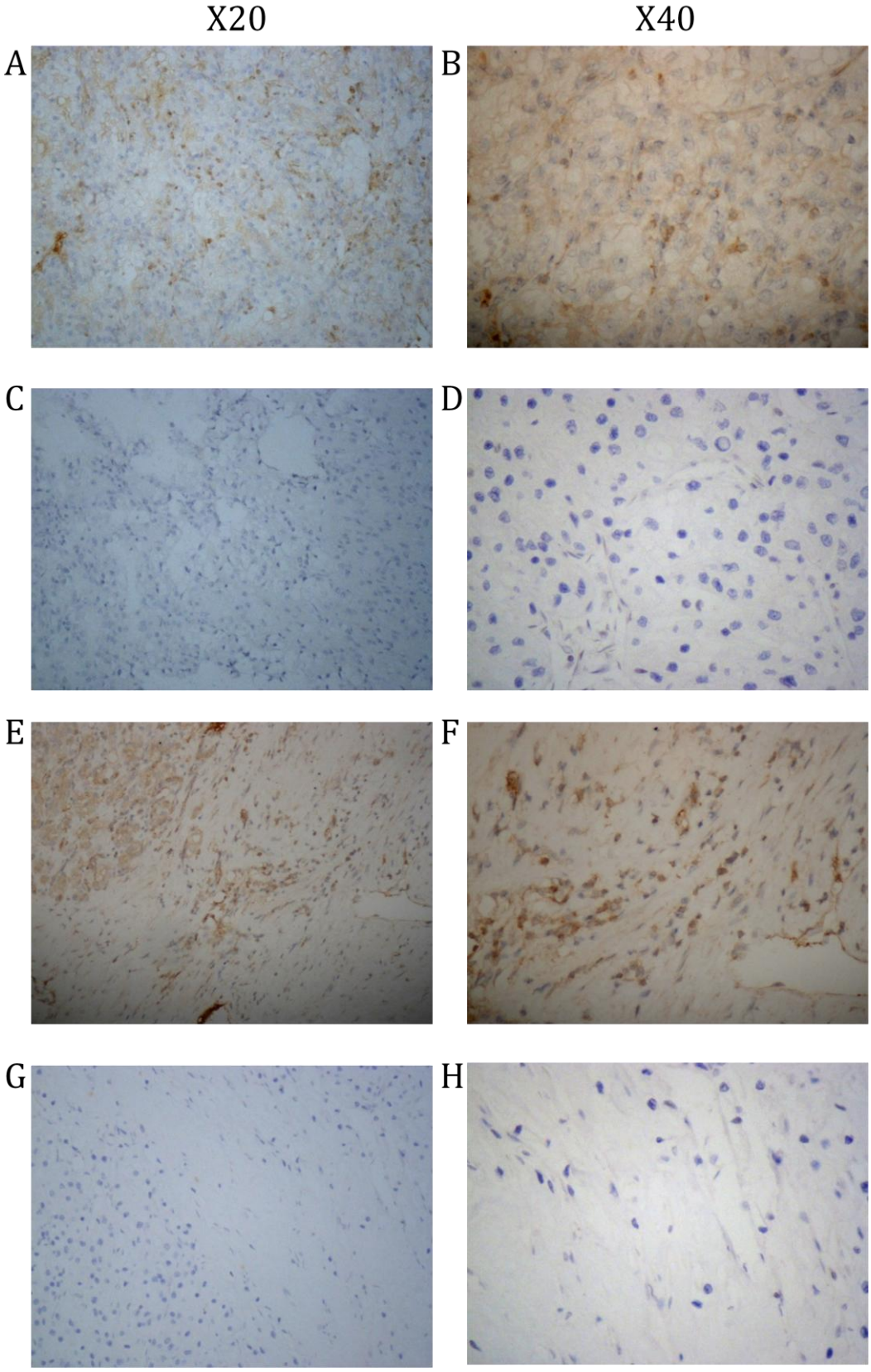
E F



G H



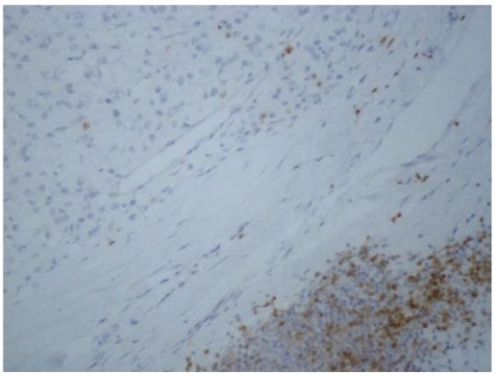
CCR6

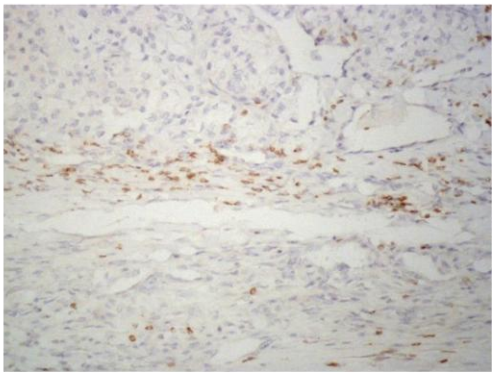


CXCR3

X20 X40

A B





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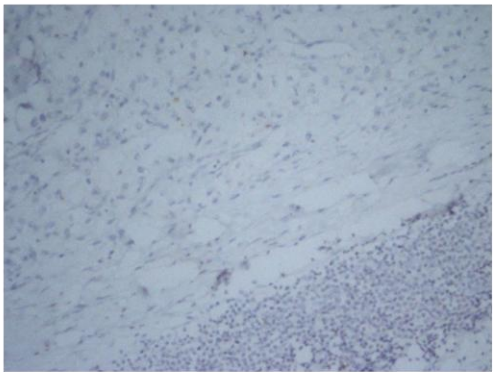
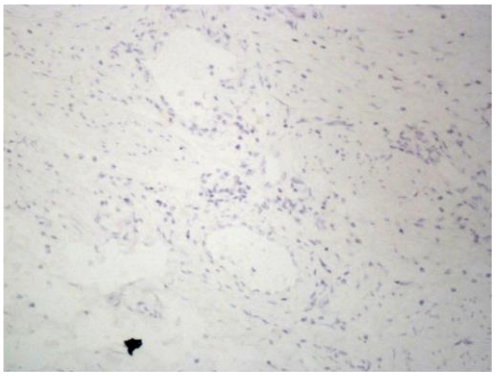
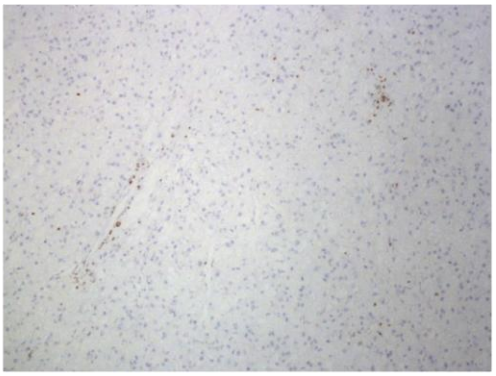
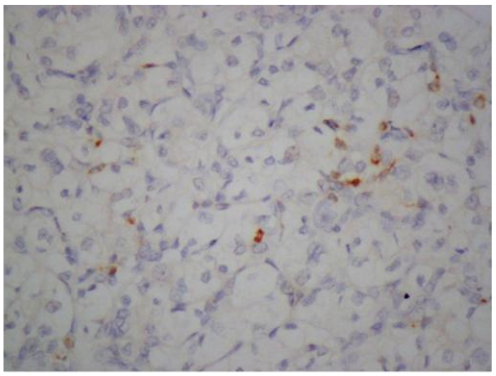


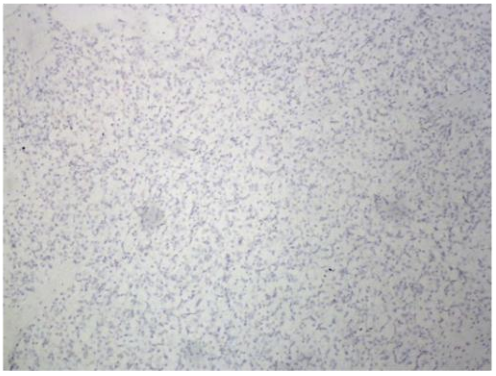


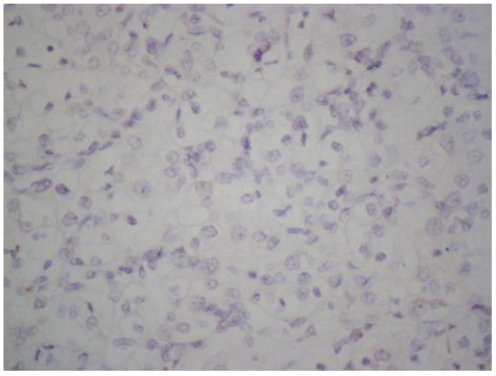
E F





G H

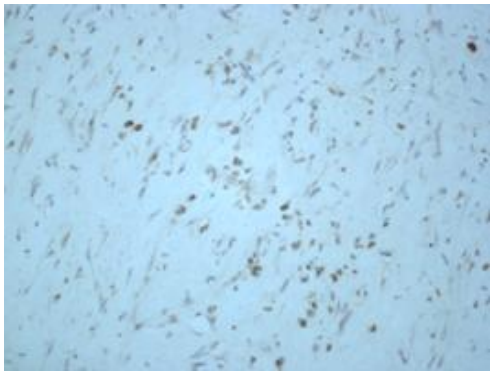


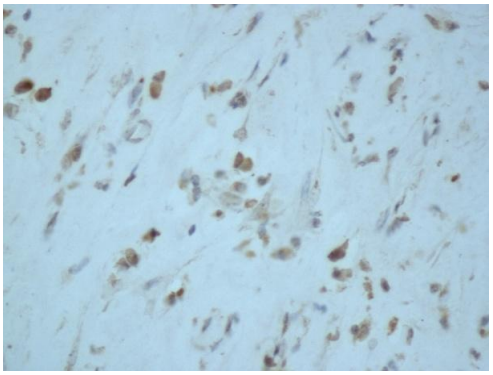


CXCR6

X20 X40

A B





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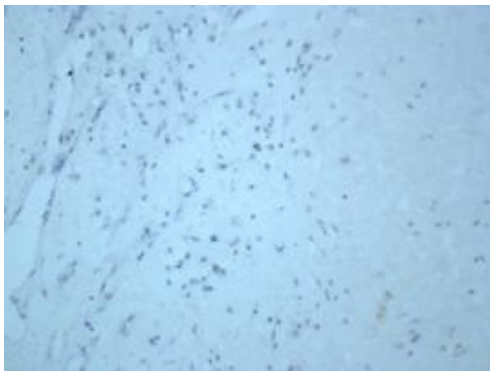
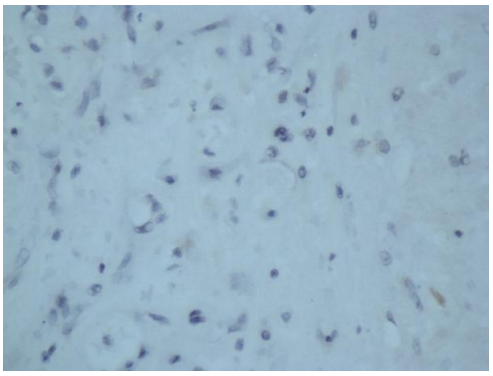
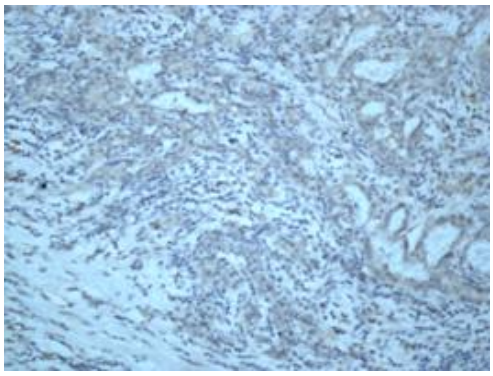
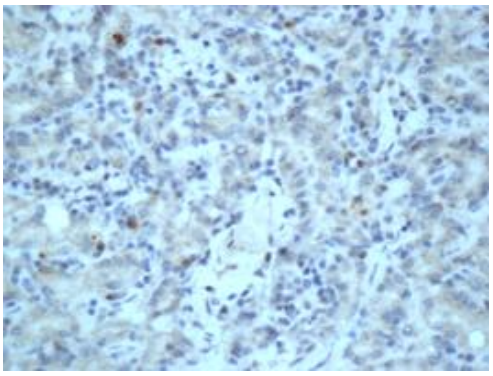


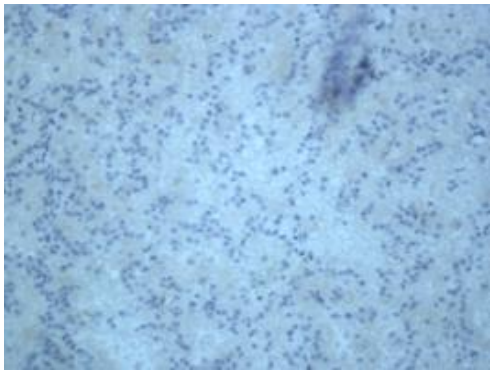


E F





G H



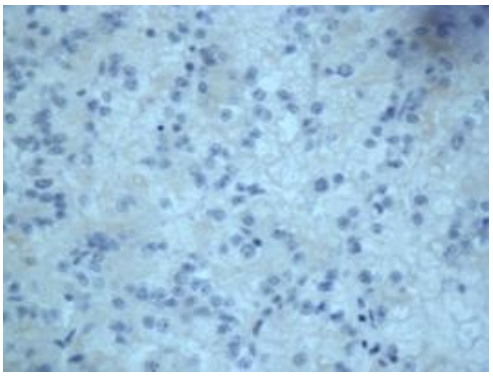


Figure 25: Expression of selected chemokine receptors in RCC tissue

5μm paraffin sections from RCC tumours were stained with antibodies to CCR5, CCR6, CXCR3 or CXCR6. Photos are representative of 10 RCC cases. For each receptor figures A, B, E and F show staining by antibodies to the chemokine receptors, and C, D, G and H show the corresponding isotype controls.

3.6. Chemokine expression in RCC

In order for a T cell to home to RCC via a specific chemokine receptor, the ligand for that receptor must be present at the tumour site. Immunohistochemical analysis was used to confirm the presence of the chemokine ligands for the receptors CCR5, CCR6, CXCR3 and CXCR6.

I stained for CCL4 and CCL5, both ligands for CCR5 and found both to be expressed by lymphocytes. CCL4 was expressed by lymphocytes both within the tumour and in fibrous stromal areas. CCL5 was only expressed by lymphocytes in fibrous areas, however it was also expressed occasionally on vessels in 3 of the 10 samples, both in the stroma and within the tumour. Tumour cells also stained positively for CCL5 (**Figure 26**).

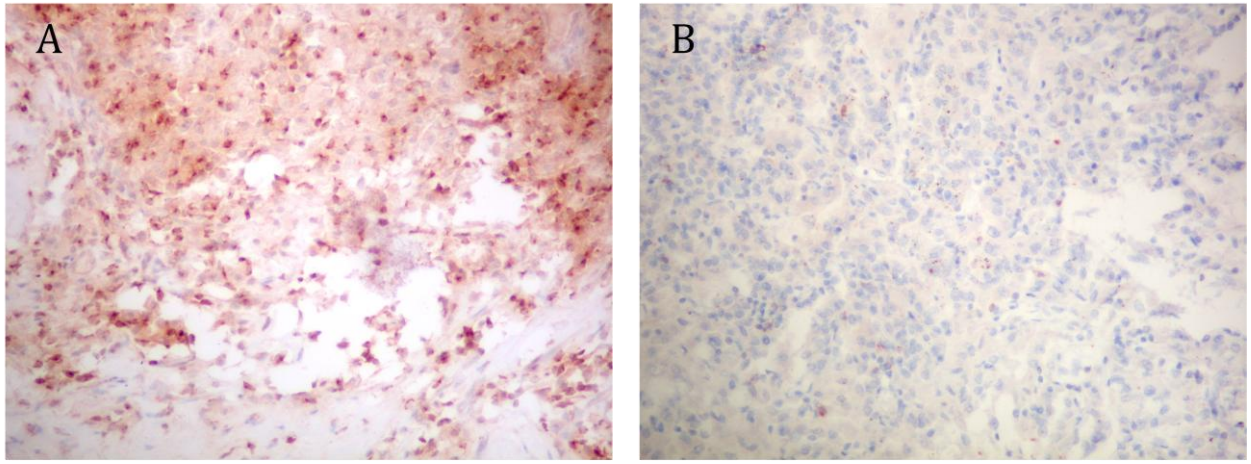
CCL20 is the only ligand for CCR6. Of ten samples stained for this chemokine only 3 stained strongly, with CCL20 being expressed on lymphocytes in the stroma. In the other samples some staining was seen on tumour cells and vessels, however the staining was extremely weak (**Figure 26**).

The ligands of CXCR3 are CXCL9, 10 and 11. I found CXCL9 to be expressed by tumour cells in all samples analysed. It was also expressed by lymphocytes both within the tumour and in fibrous regions, and by vessels in the stroma. CXCL10 was less frequently seen, with capillary-like cells staining within the tumour nests and occasional lymphocytes and tumour cells staining positively. CXCL11 was expressed most

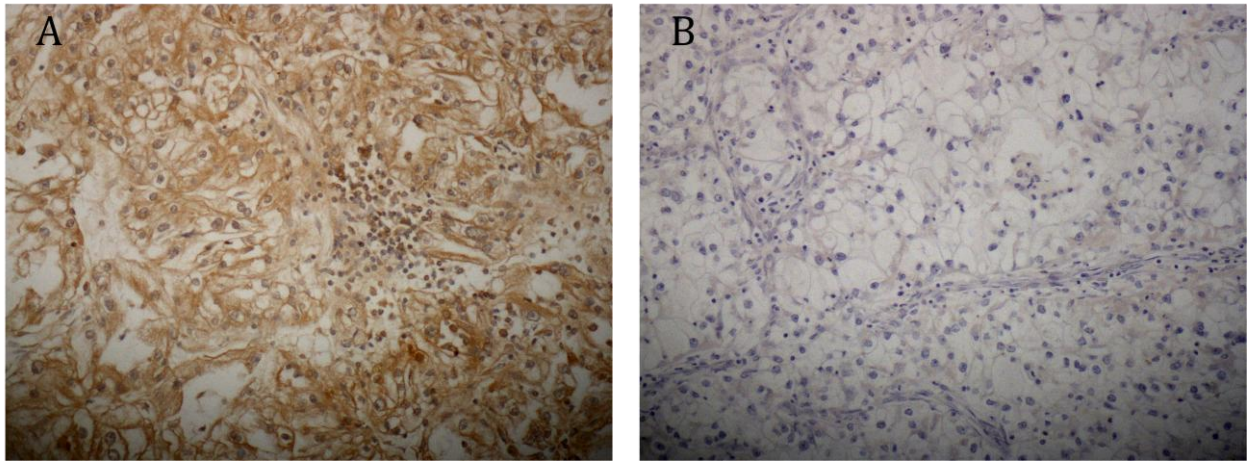
frequently by tumour cells. However in half of the samples it stained vessels within the stroma, although vessels within the tumour were negative (**Figure 26**).

The unique ligand for CXCR6 is CXCL16. I found CXCL16 to be expressed by tumour cells and vessels in the stroma. It was also seen on lymphocytes in the stroma (**Figure 26**).

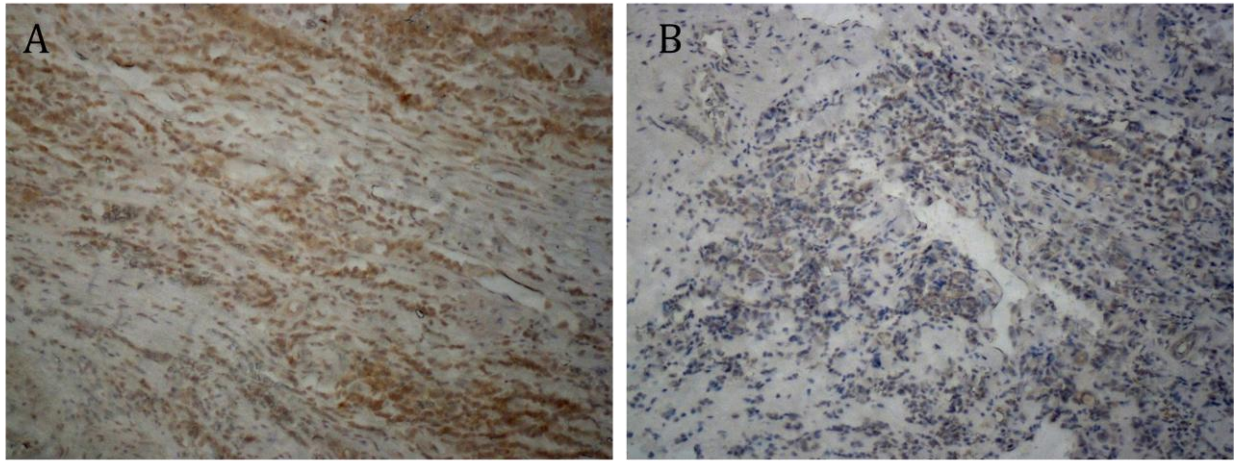
CCL4 X20



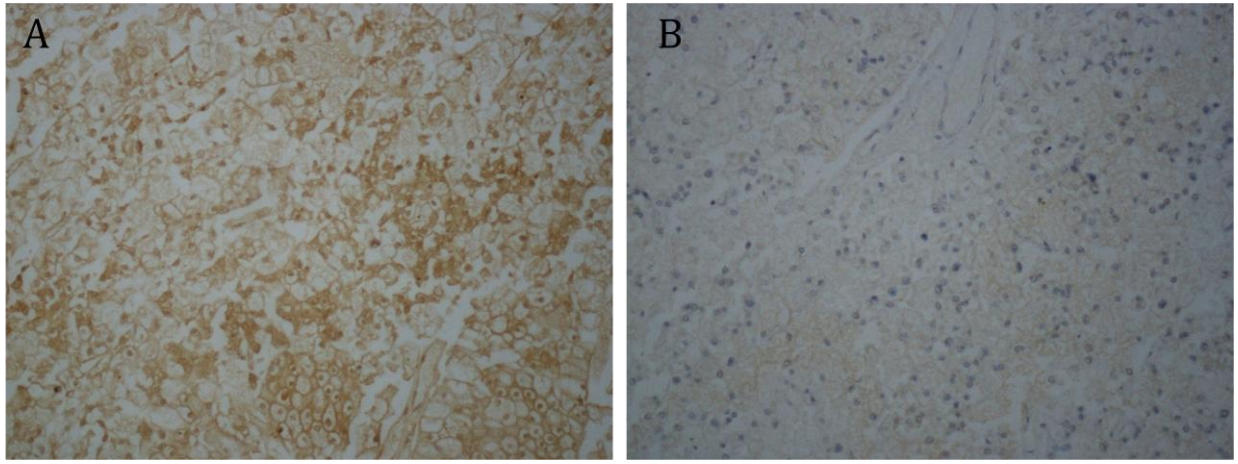
CCL5 X20



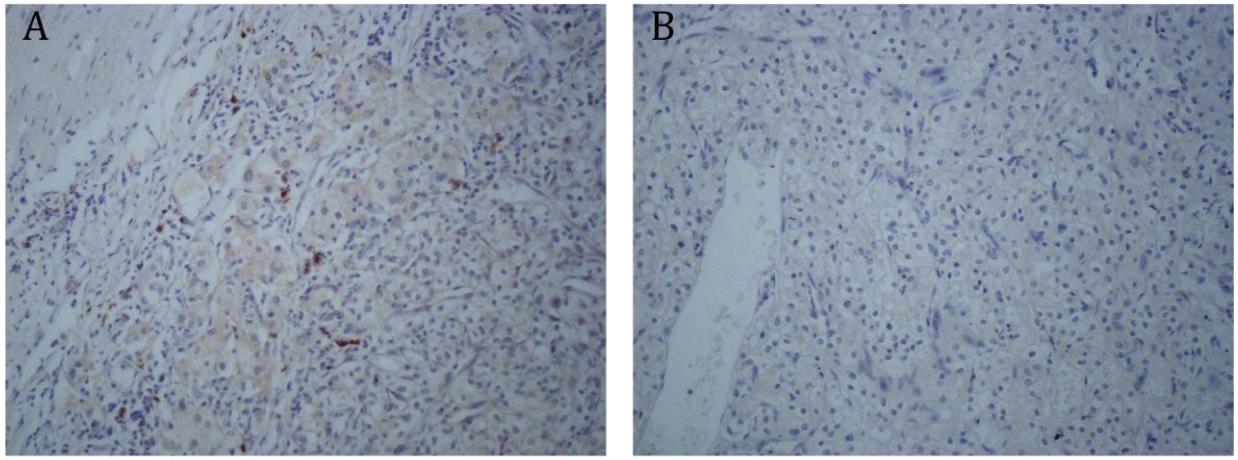
CCL20 X20



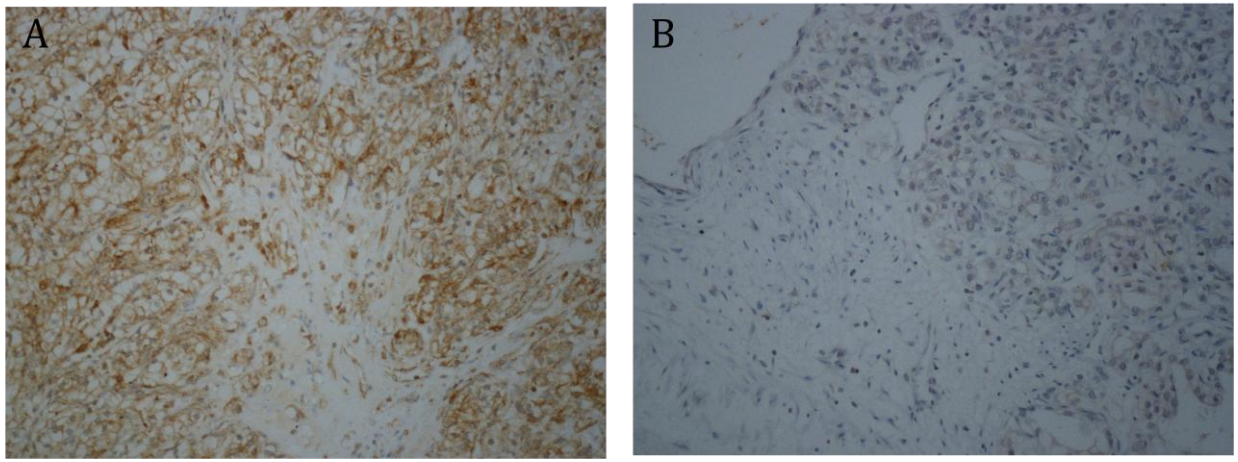
CXCL9 X20



CXCL10 X20



CXCL11 X20



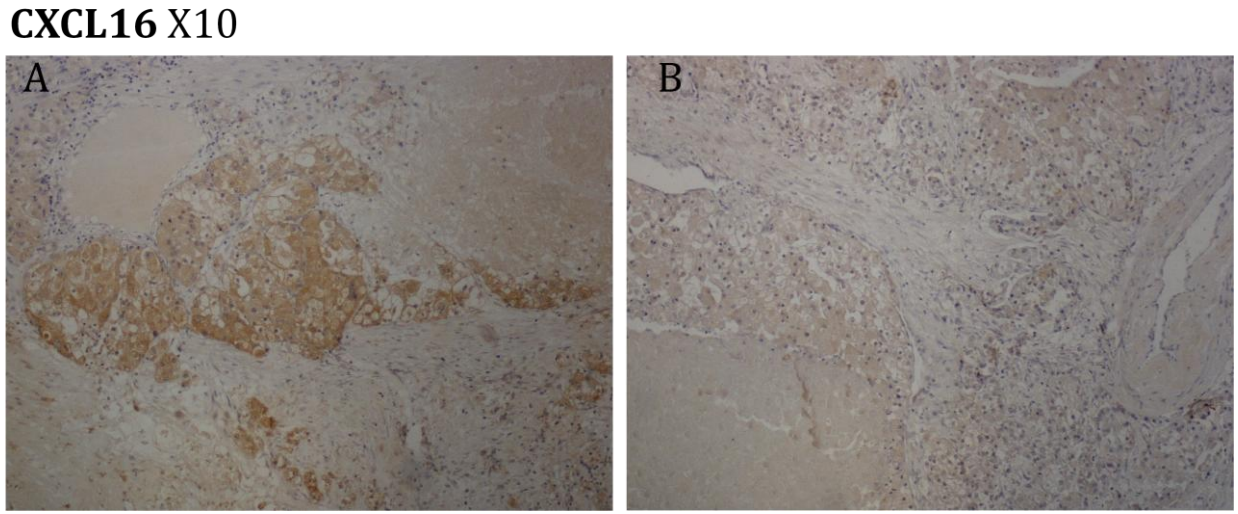


Figure 26: Expression of selected chemokines in RCC tissue

5µm paraffin sections from RCC tumours were stained with antibodies to CCL4, CCL5, CCL20, CXCL9, CXCL10, CXCL11 or CXCL16. Photos are representative of 10 RCC cases. For each receptor figure A shows staining by antibodies to the chemokine receptors, and B shows the corresponding isotype controls.

3.7. Chemokine receptors can induce migration to their ligands

Having shown that CCR5, CXCR3 and CXCR6 are enriched on TIL compared to PBMC and that the corresponding ligands are expressed in the tumour tissue, I used migration assays to determine whether the chemokine receptors on TIL are functional.

Initially I titrated the concentration of chemokine needed to induce maximal migration and determined 10nM to be the optimum concentration for CCL4, CCL5 and the CXCR3 ligands (CXCL9, 10 and 11). Migratory activity with CXCL16 was greatest at 20nM. PBMC from healthy donors were used to determine the optimum concentration for CCL4, CCL5 and CXCL9-11. RCC TIL were used to titrate CXCL16, as PBMC do not express the CXCL16 receptor, CXCR6.

The CCR5 ligand CCL5 elicited the strongest migration index (2.3 ± 0.74), but reproducible responses were also seen to CCL4 (1.6 ± 0.67), the combination of CXCR3 ligands CXCL9, 10 and 11 (1.7 ± 0.46) and the CXCR6 ligand CXCL16 (1.5 ±0.52) (**Figure 27**).

As 70% of TIL express more than one of the receptors being studied, I performed migration assays on 6 samples using mixtures of chemokines for multiple chemokine receptors, and compared levels of migration to assays using single chemokine receptor ligands performed in parallel. Combinations of ligands for CCR5 and CXCR6, CCR5 and CXCR3 and CXCR3 and CXCR6 did not significantly increase migration compared to

ligands to individual receptors. However combining the ligands of all three receptors of interest increased migration compared to using ligands of individual receptors or pairs of receptors (**Figure 28**). However, the variation in data between different experiments was large and therefore further study would be required to verify this result.

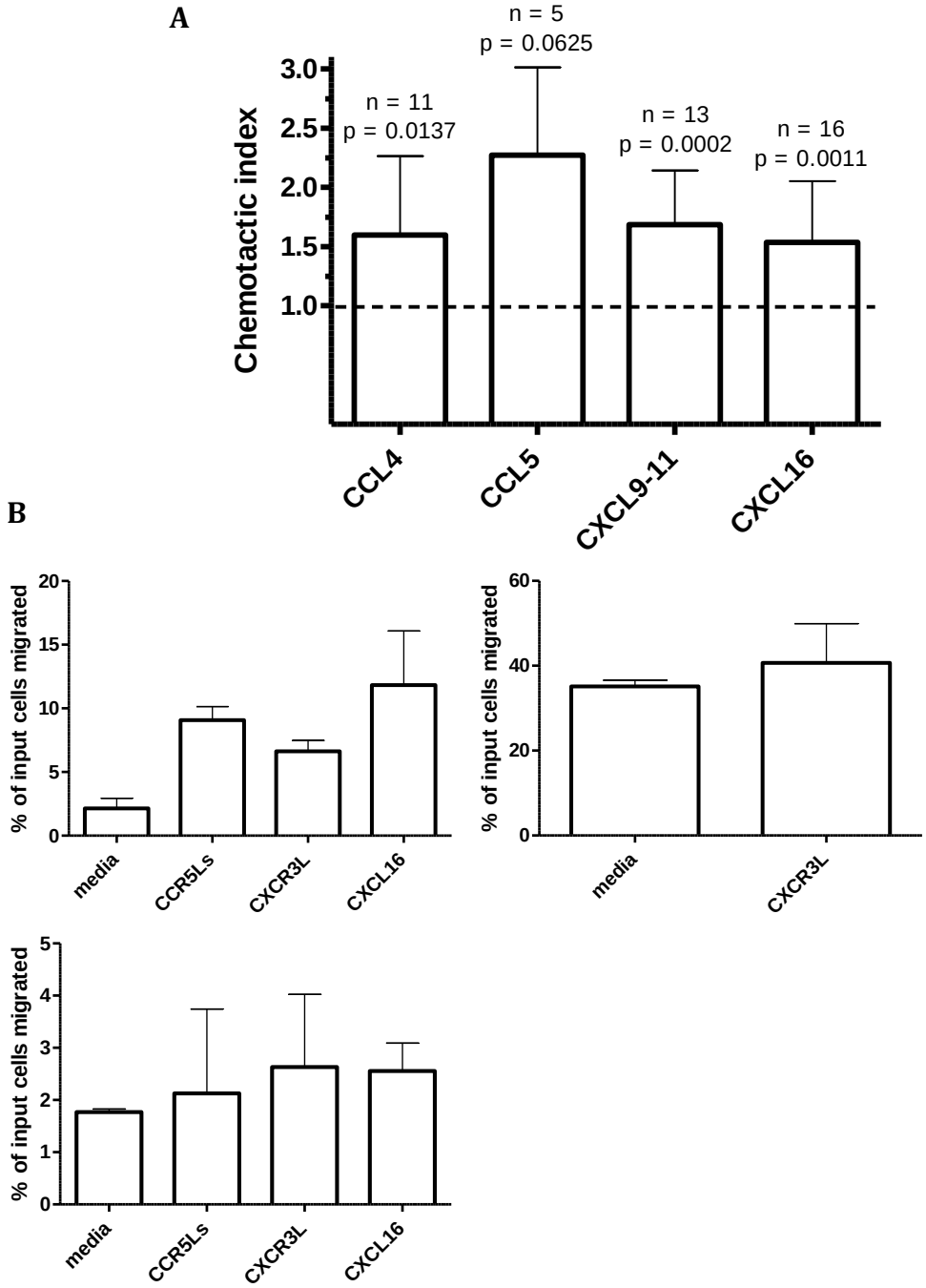


Figure 27: Functional test of CCR5, CXCR3 and CXCR6 on RCC TIL.

Infiltrating lymphocytes isolated from RCC tissue were placed in the top chamber of a transwell filter with the indicated chemokine(s) or medium alone placed in the bottom chamber. Numbers of $CD3^+$ T cells that migrated through the transwell were counted and a migration index measured by dividing the number of cells migrating in response to chemokine by the number that migrated in response to medium alone. Results shown represent mean migration index (+ SD) using TIL from multiple RCC cases as indicated. P values were calculated using the Wilcoxon signed rank test and indicate a significant increase in migratory activity in response to the chemokine indicated compared to media alone (A). The percentage of the cells inputted that migrated to chemokine or media was also calculated. Data from only 3 experiments are shown, as owing to low cell numbers for most samples I was not able to reserve enough cells to accurately measure the number of cells input Results show the median of each triplicate (+IQR) (B).

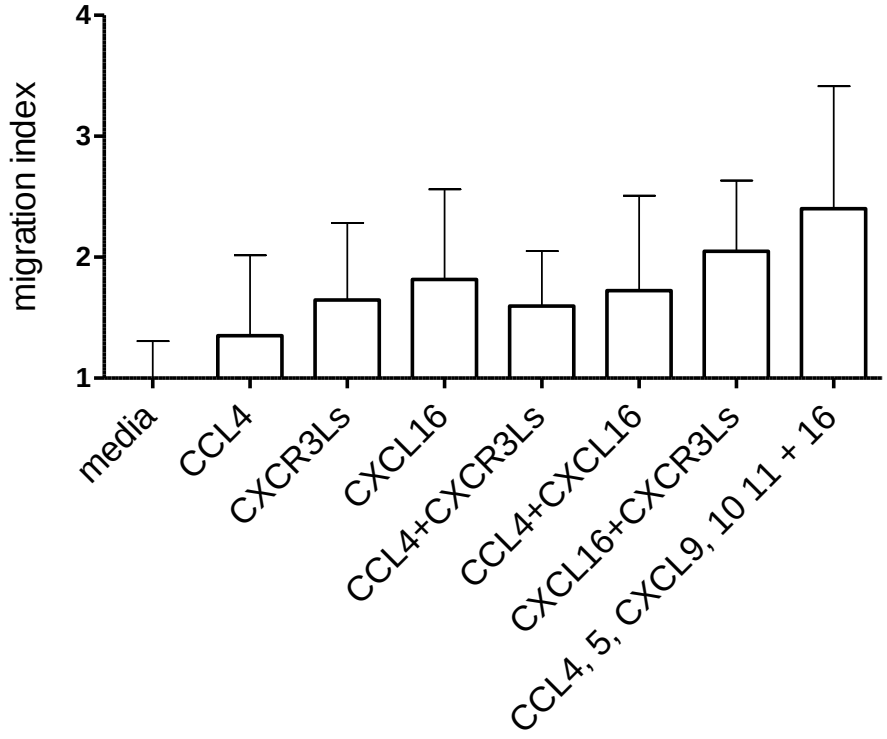


Figure 28: Migration of TIL to ligands for individual and combinations of receptors

Infiltrating lymphocytes isolated from RCC tissue were placed in the top chamber of a transwell filter with the indicated chemokine(s) or medium alone placed in the bottom chamber. Numbers of $CD3^+$ T cells that migrated through the transwell were counted and a migration index measured by dividing the number of cells migrating in response to chemokine by the number that migrated in response to medium alone. Results shown represent mean migration index of 7 RCC TIL samples (+ SD).

3.8. Discussion

This chapter described an investigation into which recruitment mechanisms T cells utilise to home to renal cell carcinomas, and which T cell subsets are present at the tumour site.

Whilst it has been shown that chemokine receptors can be internalised after engaging their ligands (Neel et al. 2005;Unutmaz et al. 2000), resulting in decreased surface expression of the receptor, studies investigating the roles of chemokine receptors in homing have demonstrated that overall, expression of a receptor required for homing to a tissue is enriched on cells within the tissue. In ovarian carcinoma Treg cells ubiquitously express CCR4, and blocking its ligand, CCL22, in *in vivo* models decreased Treg migration into tumour tissue. The Tregs also expressed CCR7 and migrated towards CCL19 and 21 *in vitro* (Curiel et al. 2004). It has also been shown that in tumour models where infiltrating T cells express CCR5, the addition of anti-CCR5 reduced accumulation of these cells in the tumours (Uekusa et al. 2002). Similar correlations between accumulation of receptor positive T cells and requirement of those receptors for homing to those tissues have been shown for the skin (Wang et al. 2010b), and gut (Stenstad et al. 2006). Therefore I examined differences in chemokine receptor expression between T cells in TIL and matched blood, as an increase in expression of chemokine receptors in TIL would indicate they had been recruited via those receptors.

It has been established in the literature (and I have also observed) that compared to RCC TIL, PBMC from RCC patients contain a larger proportion of naive T cells. One paper looked at CD45RO expression and found expression was significantly greater in TIL than PBMC. Another study used CD45RA and CCR7 to define memory phenotypes, and showed the percentage of naive cells in TIL was lower than in PBMC, for both CD4s and CD8s. In contrast T_{EMs} were enriched within the TIL (Attig et al. 2009). Naive T cells are more likely to express CCR7 and CXCR4, and express very low levels of the inflammatory chemokine receptors such as CCR5, CXCR3 and CXCR6, compared to activated, memory T cells (Bleul et al. 1997;Campbell et al. 1998a;Gunn et al. 1998a;Qin et al. 1998;Unutmaz et al. 2000). Therefore to ensure I was comparing equivalent populations, and not biasing the results, when looking at chemokine receptor expression in peripheral blood I only looked at the $CD45RA^-$ population. Although the memory CD8 T cell subset T_{EMRA} is $CD45RA^+$ and so would be excluded from this analysis, the low percentage of $CD45RA^+$ T cells in TIL suggests this T cell subset does not commonly infiltrate RCC.

Initially the scope of my flow cytometry assays were limited by the available equipment, and so preliminary investigations used 4-colour flow cytometry to ascertain chemokine receptor and adhesion molecule expression on CD4 and CD8 T cells from RCC TIL. Later, the availability of the BD LSRII cytometer permitted staining for a wider range of markers, including CD45RA, meaning I could accurately compare TIL and matched PBMC samples, as I was able to exclude naive T cells from PBMC which could skew the results. Both methods showed T cells from TIL frequently express the chemokine

receptors CCR5, CXCR3, CXCR4 and CXCR6. The increased sensitivity of the LSRII also showed TIL express high levels of these receptors, as measured by median fluorescence intensity, and that CCR6 was also expressed by >10% of TIL.

I found that CCR5, CXCR3 and CXCR6 were expressed on a significantly greater proportion of T cells from TIL than on those from PBMC. When I examined the MFI of chemokine receptor staining, which gives an indication of receptor density on the cell surface, I found CCR5, CXCR3 and CXCR6 were expressed at significantly higher levels on T cells from TIL than from PBMC. This suggests that the level of chemokine receptor expression may be significant in determining whether or not a T cell can migrate into RCC. There is limited evidence in the literature that the level of chemokine receptor expression affects trafficking. A study on monocytes showed increased CCR2 mRNA expression enhanced migration to CCL2 (Han et al. 1999). Another study looking at Jurkat cells found those expressing more CCR5 molecules migrated better to its ligand, and that this migration was blocked by CCR5 antibody (Desmetz et al. 2007) Also after CXCR6 transfection the cells with the highest levels of the receptor were most responsive to CXCL16, although this was measured in terms of induction of proliferation (Darash-Yahana et al. 2009). Increasing levels of CCR2 mRNA, in response to incubation of T cells with IL-2, correlated with in increased chemotaxis to CCL2. Increased levels of CCR1 mRNA also correlated with an increased chemotactic response to CCL5, however multiple chemokine receptors migrate to this ligand, which were not studied by this paper (Loetscher et al. 1996).

I also showed a significantly greater proportion of TIL expressed more than one of the chemokine receptors of interest compared to PBMC, and there was a reduction in the proportion of single positive $CXCR3^+$ and $CXCR6^+$ T cells in TIL compared to PBMC. This suggests a multi-step mechanism may be required for T cell homing to RCC, and expression of just one of the chemokine receptors may be insufficient for T cell migration into the tumour tissue. The ability of neutrophils to migrate sequentially to different chemokine stimuli has been demonstrated *in vitro* (Foxman et al. 1997), and more recently a multi-step mechanism of T cell recruitment has also been suggested in diseased liver tissue, where CXCR3 mediates recruitment through endothelium and CCR4 migration within the tissue to sites of inflammation (Oo et al. 2010). It is possible in RCC that only one or two receptors are needed for T cells to enter through vessels, and other receptors are involved in precise localisation or retention of the T cells within the tissue. As CXCL16 only mediated a low level of migration, and is a trans-membrane receptor, there has been suggestion of it functioning in adhesion (Shimaoka et al. 2004). Therefore one hypothesis could be that CXCR3 and CCR5 may play a role in cell migration into the tumour, and CXCR6 in cell adhesion and retention at the tumour site.

Only 13 ± 8.3 % of TIL co-expressed all three receptors though, suggesting a maximum of two of these receptors is necessary for migration into RCC, indicating some redundancy in the mechanism of T cell homing. Interestingly, whilst only a negligible proportion of TIL expressed either CXCR3 or CXCR6 alone, 10 ± 18 % of TIL expressed CCR5 alone, suggesting of the three receptors CCR5 might be capable of facilitating T cell migration into RCC by itself.

I also examined the expression of adhesion molecules on TIL and PBMC, however the antibodies that were available to us were not conformation dependent, and so did not give an indication of whether the integrins investigated were activated and able to function. I saw no significant differences in adhesion molecule expression between TIL and PBMC.

The predominance of CCR5, CXCR3 and CXCR6 suggests the infiltrating T cells have a memory, Th1 phenotype, which is desirable as there are some indications that those subsets are more likely to have anti-tumour activity (D'Ambrosio et al. 1998;Ito et al. 1999;Kim et al. 2001a;Kondo et al. 2006;Loetscher et al. 1998b;Soleimani et al. 2009). Expression of all these receptors has been demonstrated on Th2 polarised cells in diseased tissue (psoriatic arthritis) (Kim et al. 2001b), however they are more common on Th1 cells and my cytokine staining (see section 4.2.1, p157) suggests a significant proportion of TIL are type 1 polarised. CXCR3 has also been demonstrated on Th17 cells, however my studies indicate these only constitute a tiny proportion of the TIL population (see section 5.2 p214).

Perhaps it is unsurprising that CCR5, CXCR3 and CXCR6 are expressed together as their expression, along with CCR6, can be induced by the same cytokines— IL-2 and IL-15, and in that way they are co-ordinately regulated (Unutmaz et al. 2000). CXCR3 is also induced by IL-2 (Loetscher et al. 1998a).

When I examined which T cell subsets had infiltrated RCC, it became apparent a sizeable proportion of CD4 T cells had a regulatory T cell phenotype ($Foxp3^{+}CD25^{hi}CD127^{lo}$), and that the proportion of Tregs present correlated with the Fuhrman grade of the tumour. This supports data found by other groups. One study found levels of $CD4^{+}Foxp3^{+}$ cells to be higher in all the RCC tissue samples they tested when compared to levels in peripheral blood, and that levels of Tregs in peripheral blood correlated with survival (Griffiths et al. 2007). Another study has also found an increase of $Foxp3^{+}CD4^{+}CD25^{+}$ cells in RCC TIL compared to PBMC, and found that depletion of these cells increased the proliferative capacity of $CD4^{+}CD25^{-}$ cells, and their ability to secrete IFN-γ following an MLR stimulation. Higher frequency of the Tregs correlated with grade and stage of disease and a higher risk of tumour recurrence (Liotta et al. 2010b). Furthermore studies of patients treated with IL-2 have found patients who responded to treatment had lower increases in Treg levels and a swifter reduction in Treg numbers post-treatment, responded best and had better prognoses (Cesana et al. 2006;Jensen et al. 2009).

In contrast another group detected $CD4^{+}CD25^{+}Foxp3^{+}$ cells in only a quarter of the 170 tumours studied, at a mean of 4.1% of CD4 T cells. The presence of these cells did not correlate with an increased risk of death from RCC, whereas there was such a correlation with the number of $CD4^{+}CD25^{+}Foxp3^{-}$ cells. This T cell subset was detected within 84.1% of tumours studied at a mean percentage of 12.1% of CD4 T cells (Siddiqui et al. 2007). However these experiments were performed using immunohistochemistry

which may be less sensitive than flow cytometry. It is interesting to speculate that these Foxp3$^-$ CD4 T cells could be Tr1s which, as described earlier, also have a regulatory function.

In light of the potential impact of these Tregs, I was interested in how they were being recruited into RCC. I found their homing phenotype was similar to that of the whole T cell population, with the addition that CCR6 was also more highly expressed on Tregs in TIL than in PBMC. This may explain the significant difference between CD4 and CD8 T cells in terms of the proportion and level of CCR6 expression, as the Tregs are within the CD4 subset. CCR6 has been found on PBMC Tregs in healthy donors and has been demonstrated to be expressed by Tregs in breast cancer and hepatocellular carcinoma tumours (Chen et al. 2011;Xu et al. 2010;Yamazaki et al. 2008). CCR6 is also associated with the pro-inflammatory Th17 cell subset (Yamazaki et al. 2008). However my preliminary investigations suggest only very low levels of Th17s (<1%) are present in RCC (see section 5.2, p214). Interestingly other chemokine receptors commonly found on Tregs in PBMC and other diseased tissues, such as CCR4 and CCR7 (Hirahara et al. 2006;Iellem et al. 2001;Oo et al. 2010;Tosello et al. 2008), were absent from Tregs in TIL suggesting Tregs may home to RCC tumours via an RCC-specific mechanism. That is, they may use different chemokine receptors to migrate to RCC, compared with the receptors Tregs use to migrate to other sites of disease. In addition a higher proportion of Tregs expressed CXCR3 than the whole T cell population, and Tregs expressed higher levels of CCR5 and CXCR6, suggesting that while they share these receptors with effector T cells, they may be more efficient at migrating to their ligands. Due to restrictive cell

numbers and sample availability I was unable to examine co-expression of CCR6 with the other receptors of interest, however in common with the other receptors IL-2 and IL-15 increase its expression, suggesting co-expression is likely, however this has not been investigated in the Treg subset (Unutmaz et al. 2000).

It was important to determine whether the chemokine ligands of CCR5, CCR6, CXCR3 and CXCR6 are expressed in RCC, as without them the receptors would not function in T cell migration to the tumour. Ligands to all the receptors were found to be present to varying degrees.

My results corroborate previous reports to some extent. RT-PCR studies have shown RNA levels for CCL4, CCL5, CXCL9, 10 and 11 are significantly higher in tumour than normal tissue (Kondo et al. 2004;Romero et al. 2006).

The expression of CXCL9 and 11 has also been demonstrated in the literature by immunohistochemistry, where the former was positive on capillaries and lymphocytes and the latter on pericytes associated with vascular smooth muscle cells (Suyama et al. 2005b). In contrast I saw no staining of CXCL9 or 11 on vasculature within the tumour. In my hands CXCL9 stained lymphocytes (in common with the published study) and tumour cells. CXCL11 also stained tumour cells. I did see both chemokines on vessels, but only those within the stroma, not within the tumour nests. The reason for these

differences is uncertain, however it is unclear which antigen retrieval method Suyama T et al. used, and in my hands EDTA buffer resulted in a very high background. Without this knowledge and the pictures of the corresponding isotype controls I cannot discount background staining accounting for some of their positive results.

In agreement with my data, an immunohistochemical study of 104 RCC tumour samples found CXCL16 to be present in 86% of samples and it exhibited diffuse staining throughout the cytoplasm (Gutwein et al. 2009). CCL20 has also previously been demonstrated to be expressed in the cytoplasm of RCC cells (Middel et al. 2010). In my experiments I saw weak staining of tumour cells in 8 out of 11 samples, however the lymphocytes in the stroma stained more strongly. The previous study used a tyramide amplification system, so perhaps my assay was not sensitive enough. However the staining of my positive control, tonsil, was strong, suggesting at least in comparison that tissue expression in RCC is weak. It is also unclear how many samples the other study looked at.

Despite CCL4 being expressed by lymphocytes within the tumour and some tumour cells expressing CCL5, $CCR5^+$ T cells didn't infiltrate the tumour nests. To my knowledge this is the first time the expression of CCR5 in RCC has been examined by IHC. CXCR6 expression by tumour cells has previously been demonstrated (Gutwein et al. 2009). I saw weak staining of tumour cells, but also observed lymphocytes in the stromal areas to be positive. However despite tumour cell expression of the ligand, very few $CXCR6^+$ T

cells appeared to infiltrate the tumour. CXCR3$^+$ T cells infiltrated the tumour nests more effectively, perhaps due to strong expression of CXCL9 and 11 on the tumour cells, and the expression of CXCL10 by on some capillaries within the tumour. Previous studies have also shown CXCR3$^+$ lymphocytes within RCC by IHC (Kondo et al. 2006), within the tumour and at peri-tumoural areas (Suyama et al. 2005a). CCR6$^+$ T cells also infiltrated the tumour, despite weak expression of the CCR6 ligand, CCL20. The presence CCR6$^+$ dendritic-like cells have previously been suggested within RCC tumours (Middel et al. 2010). I observed lymphocyte-like cells to stain positively both within the tumour and in the fibrous regions. They appeared to have lymphocyte morphology but without dual-staining with a T cell marker I could not definitively determine the cell type. I did not investigate co-expression of CCR6 with other receptors but perhaps it works in concert with another chemokine receptor to aid infiltration of cells despite possible low levels of its ligand.

In addition to CXCR6, I also observed CCR5 and CCR6 expression by tumour cells. CCR6 expression has previously been demonstrated by PCR on RCC tumour cells in short term culture, however the same study found no CCR5 expression (Johrer et al. 2005). However CCR5 expression has been suggested in oral cancer, and prostate cancer (Chuang et al. 2009;Vaday et al. 2006). These chemokine receptors could be involved in tumour metastasis. Knockdown of CCR5 inhibits the metastatic potential of breast cancer cells in mouse studies (Karnoub et al. 2007), and a CCR5 antagonist is currently being investigated to limit metastasis of prostate cancer (Zhang et al. 2010). There are also indications CCR6 and CCL20 mediate metastasis to the liver in colorectal cancer

(Ghadjar et al. 2006;Rubie et al. 2006). In contrast, knocking down CXCL16 increased rather than decreased migration of cells from the RCC line ACHN3. The migration inhibitory effect of CXCL16 is presumably carried out by the membrane bound form as soluble CXCL16 increased cell migration, suggesting that the soluble form of the ligand affects metastasis in a similar way to other chemokines (Gutwein et al. 2009). Unexpectedly, some vessels in RCC appeared to express CCR5 and CCR6. There is evidence that human endothelial cells can express some chemokine receptors, such as CXCR2, CXCR3 and CXCR4 (Mestas et al. 2005;Strieter et al. 2005), but literature on CCR5 and CCR6 expression is rare. However CCR5 expression has been reported on brain, coronary, appendix, mesenteric lymph node, dermal, umbilical vein and saphenous vein endothelia, and CCR6 on dermal, lung microvascular and saphenous vein endothelia, and on capillaries in normal renal tissue (Berger et al. 1999;Crola Da et al. 2009;Hillyer et al. 2003;Welsh-Bacic et al. 2011). The vasculature in RCC is abnormal which may account for aberrant expression of receptors in some samples.

The predominance of lymphocyte aggregates within stroma has also been observed in colorectal cancer (Musha et al. 2005), seminomas (Bell et al. 1987), sertoli cell tumours (Henley et al. 2002) and merkel cell carcinomas (Ciudad et al. 2010). I observed that the vessels in the stroma expressed more chemokines that vessels within the tumour, which may account for T cells being able to migrate to fibrous areas. The abundance of T cells in the fibrous regions might also be due to successful killing of tumour cells in those areas, resulting in the formation of stromal areas. Presumably the surrounding tumour has evolved to better evade the immune response.

Previous studies on the homing receptor profile of RCC TIL are limited, with very small panels of receptors and T cell markers. One study investigated expression of CCR4, CCR5 and CXCR3 by flow on 24 matched $CD4^+$ TIL and PBMC (without stating the use of isotype controls), and found the frequency of CCR5 and CXCR3 to be increased in TIL compared to PBMC, and CCR4 expression to be decreased. Interestingly their data suggests the expression of CCR5 and CXCR3 decreases with tumour stage, whereas CCR4 expression on TIL increases (Cozar et al. 2005). However looking at the whole T cell population I did not see a correlation between chemokine receptor and stage. Another study showed that in 16 out of 21 RCC samples, CXCR3 RNA was expressed, compared to 2 out of 21 normal tissue samples. IHC demonstrated that this CXCR3 was expressed by lymphocytes (as well as tumour cells and macrophages) within the tumour and in the peri-tumoural area (Suyama et al. 2005a). A more comprehensive study of 33 RCC samples and 20 matched PBMC, examining expression of 4 chemokine receptors by flow cytometry, reported an increase in $CCR5^+$ and $CXCR3^+$ CD4 and CD8 T cells in TIL, compared to PBMC. However again isotype controls were not described and naive T cells were not excluded from the data (Attig et al. 2009). Finally a fourth study showed enrichment of $CCR5^+$ and $CXCR3^+$ CD8 and CD4 T cells in RCC compared to PBMC, however it is unclear whether the blood and TIL samples were matched and whether isotype controls were used (Kondo et al. 2006). Therefore my data support previous findings that RCC TIL express CCR5 and CXCR3, but also improve the quality of the evidence by examining the possibility that any of the known chemokine receptors could be involved in T cell recruitment to RCC. This study is also the first to avoid potential

bias by excluding naive cells from the analysis, which would otherwise skew the apparent chemokine receptor profile of PBMC.

I was also the first to test whether the chemokine receptors on TIL are able to function and induce migration in response to their ligands. The transwell assay I used is artificial in that it only measures response to the chemokine, whereas in physiological situations cell migration involves interactions between molecules expressed on an HEV and selectins and integrins, as well as chemokine receptors. However the movement I measured in response to the chemokine ligands illustrates that the chemokine receptors are capable of inducing downstream signalling pathways resulting in cell movement, and therefore if the TIL encounter their ligands *in vivo* they are likely to migrate towards them.

As I found the majority of T cells to express multiple chemokine receptors, I investigated the effect of multiple ligands on their migration. While I saw a slight increase in movement when using ligands for CCR5, CXCR3 and CXCR6 in the same well, the effect was small and there was large variation. Further work would be required to confirm this finding.

4. Function of TIL

4.1. Introduction

I established in chapter 3 that T cells, including those of a regulatory phenotype, infiltrate RCC. There is evidence to suggest RCC-specific T cells can be present in cancer patients, and that they can recognise and kill tumour cells *in vitro* as discussed in section 1.4.3. Evidently, however, these T cells are not sufficient for eliminating the established tumour. Chapter 4 aims to elucidate why this might be.

It is unclear precisely why T cells in cancer patients are unable to kill the tumour cells. The T cells may have been rendered anergic and dysfunctional by tumour-derived factors, or by interactions with other cells types recruited to the tumour, such as Tregs.

In order to determine the degree to which effector T cells have been made unresponsive *in situ* I compared the function of TIL and matched PBMC using various parameters. I also further investigated the functional phenotype of infiltrating Tregs to determine whether they may have a role in effector T cell suppression. Finally I also performed some preliminary studies on the effects of the inhibitory molecule PD-1 on T cell function.

Unless otherwise stated, throughout this chapter 'TIL' and 'PBMC' refer to the T cell compartment within the respective populations. All significances were calculated using Wilcoxon matched pairs tests.

4.2. Effector T cells from RCC TIL are able to function *ex vivo*

I investigated the function of TIL isolated from RCC tumours, without any *in vitro* culture or expansion. I looked at both their cytokine expression in response to stimulation, and surface markers that give an indication of the functional status of T cells.

4.2.1. Effector cytokine production by TIL

The cytokines interleukin-2 (IL-2), interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNFα) are association with type 1 T cell immune responses. Therefore I looked at their expression by TIL post-stimulation to get an indication of whether T cells from TIL retained the same functional capacity as those in PBMC, or to what extent they had been anergised in the tumour environment.

TIL and matched PBMC were stimulated with PMA/ionomycin and subsequently analysed by intracellular cytokine staining to determine their capacity to produce effector cytokines. TIL showed no defect in production of any of the three cytokines in comparison to PBMC (**Figure 26**). In fact, a significantly greater proportion of $CD4^+$ TIL than $CD4^+$ PBMC were able to produce IFN-γ ($p = 0.0280$) and IL-2 ($p = 0.0003$). As expected, in both TIL and PBMC $CD4^+$ T cells were more likely to produce IL-2 than $CD8^+$

T cells (p-values of 0.0003 and 0.0008 respectively) (Croft et al. 1994). Interestingly, IFN-γ production by CD4 T cells was greater in TIL than PBMC, whereas CD8 T cells from these two sites showed the same level of IFN-γ production. The capacity of TIL to produce cytokines in response to stimuli suggests the cells are not irreversibly anergised or inherently defective and that their function can be rescued (**Figure 26**). A table describing effector cytokine production by TIL compared to the Fuhrman grade of the sample and subtype of RCC can be found in Appendix B

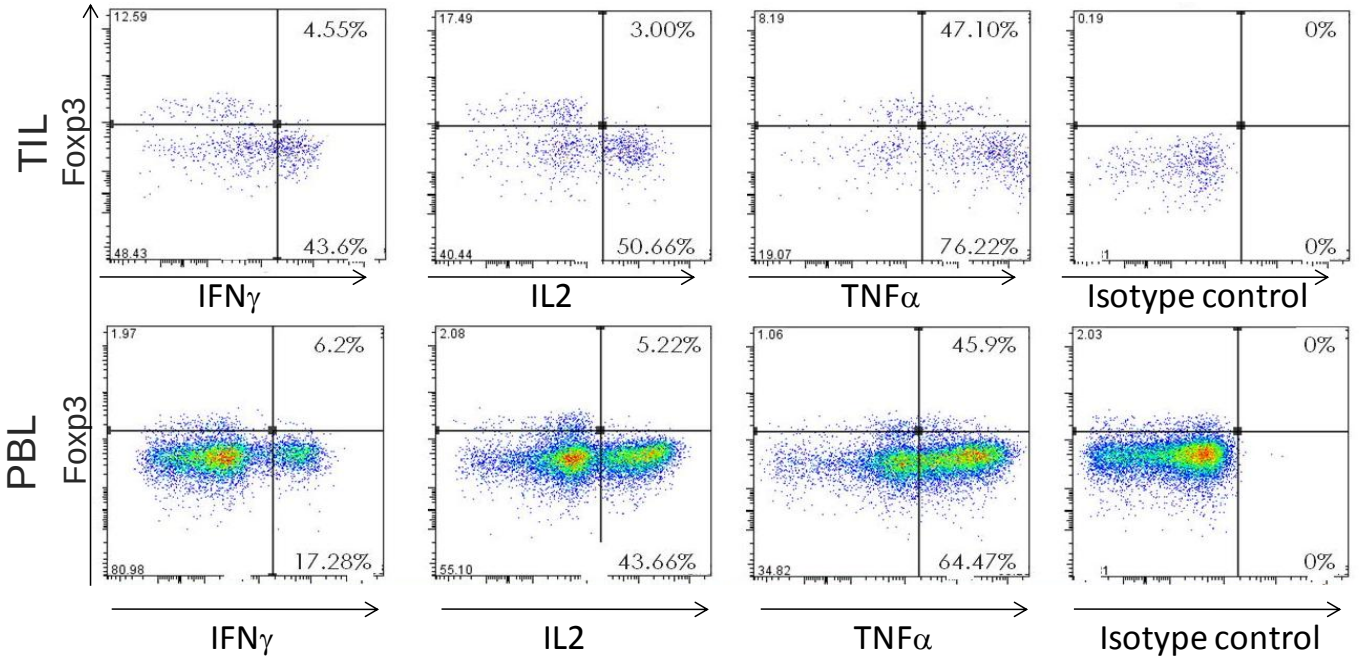


Figure 29: Gating strategy for defining effector cytokine producing cells

TIL and PBMC were stimulated with PMA/ionomycin, and the percentage of CD4 and CD8 T cells producing effector cytokine was analysed by flow cytometry. The diagram indicates how isotype controls were used to define positive staining. In this representative plot the cells are gated on CD4. The cells have also been co-stained for Foxp3, and the same assays were used to determine cytokine production by Tregs (see section 4.3.2 p176)

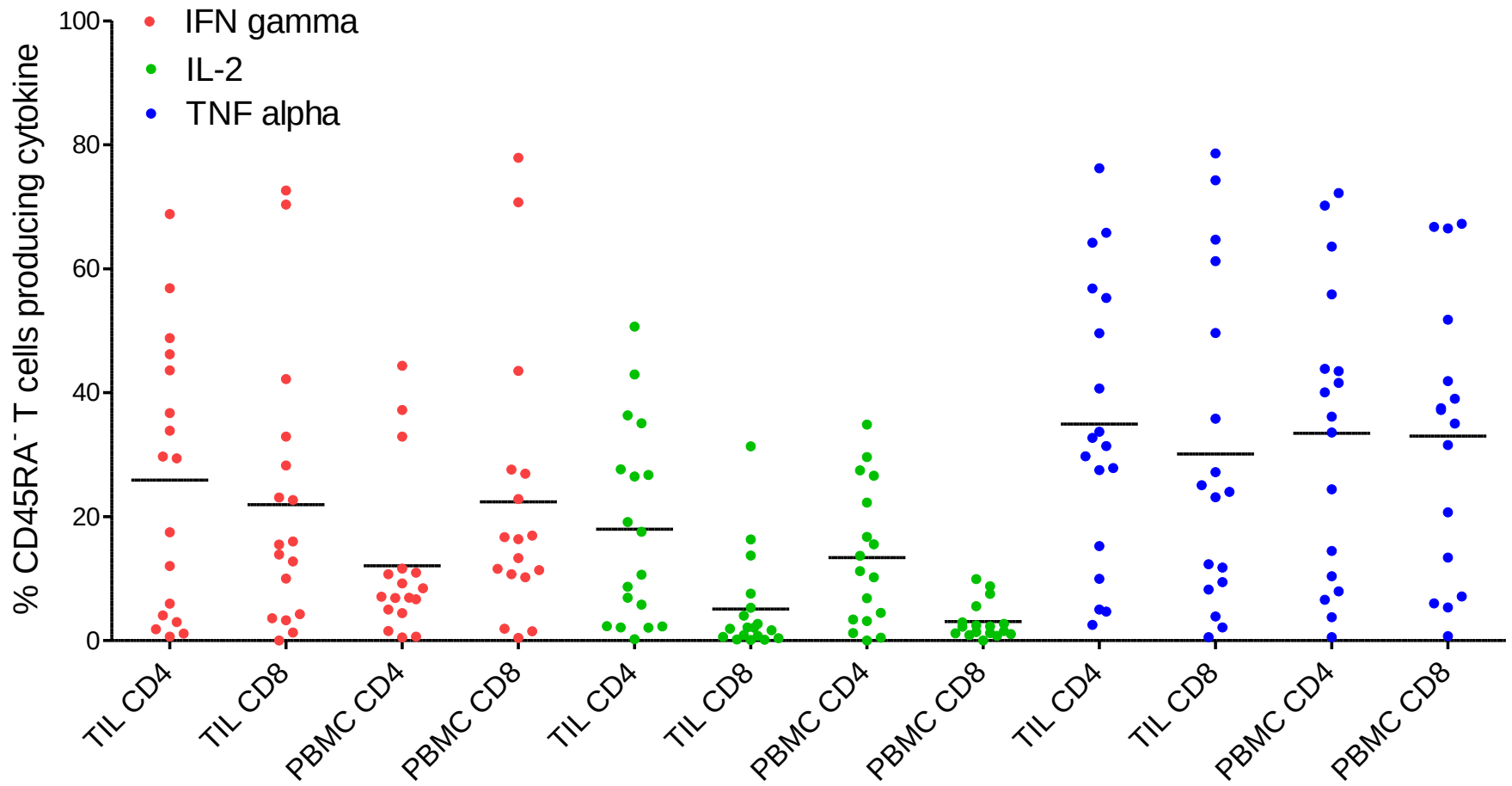


Figure 30: Effector cytokine production by TIL and PBMC

TIL and PBMC were stimulated with PMA/ionomycin, and the percentage of CD4 and CD8 T cells producing effector cytokine was analysed by flow cytometry

4.2.2. CD107a, a marker of degranulation, is expressed on TIL

CD107a is mobilised to the cell surface when degranulation of T cells takes place in response to stimulation (Betts et al. 2003). I therefore examined its cell-surface expression as a surrogate to detect cytotoxic T cell effector function.

CD8 T cells are more commonly cytotoxic, and in TIL significantly more CD8 T cells expressed CD107a than CD4 T cells. This was true both before and after PMA/Ionomycin stimulation, however the difference was more marked in un-stimulated cells (**Figure 31**A). Un-stimulated CD8 T cells from PBMC also had a significantly greater proportion of $CD107a^+$ cells than their $CD4^+$ counterparts (**Figure 31**A). Un-stimulated $CD107a^+$ CD4 T cells in both TIL and PBMC represented a small minority of the population, and this subset increased significantly upon stimulation (**Figure 31**A).

Both CD4 and CD8 T cell subsets in un-stimulated TIL had significantly larger subsets of $CD107a^+$ cells than the same subsets in un-stimulated PBMC, perhaps due to degranulation in response to antigen recognition at the tumour site ($p = 0.0010$ for CD4 T cells and $p = 0.0068$ for CD8 T cells) (**Figure 31**B). However the MFI of the staining on un-stimulated cells was weak (data not shown).

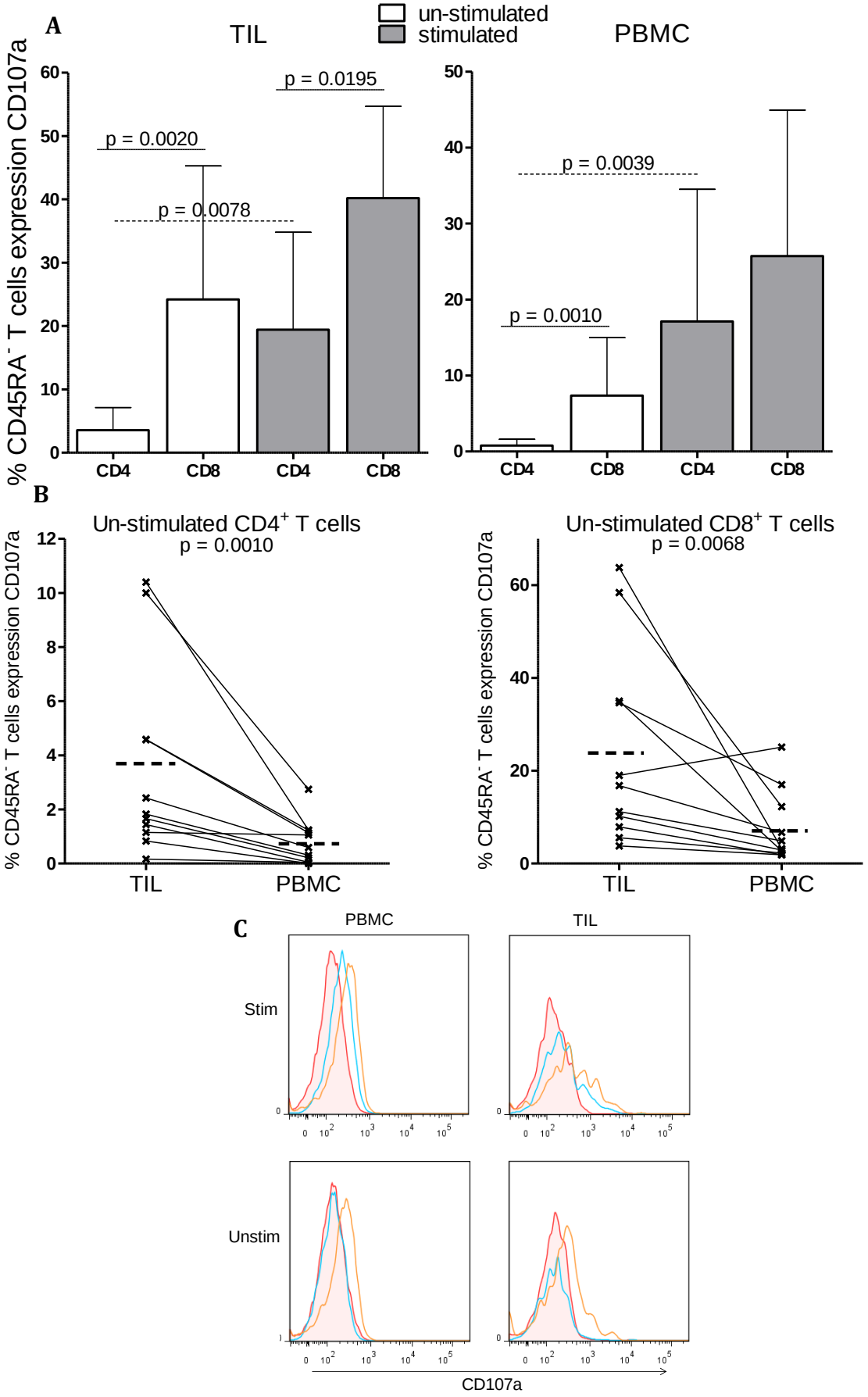

A
TIL
un-stimulated
stimulated
PBMC
% CD45RA- T cells expression CD107a
p = 0.0020
p = 0.0078
p = 0.0195
p = 0.0039
p = 0.0010
CD4
CD8
CD4
CD8
CD4
CD8
CD4
CD8
B
Un-stimulated CD4+ T cells
p = 0.0010
Un-stimulated CD8+ T cells
p = 0.0068
% CD45RA- T cells expression CD107a
TIL
PBMC
TIL
PBMC
C
PBMC
TIL
Stim
Unstim
CD107a

Figure 31: CD107a expression on TIL and PBMC

TIL and PBMC were stained for CD107a and analysed by flow cytometry. The percentage of CD4 and CD8 T cells expressing CD107a both with and without stimulation by PMA/ionomycin was determined (A) for stimulated data n = 9, for un-stimulated data n = 11. Data represents the mean percentage of cells expressing CD107a (+SD). CD107a expression in un-stimulated CD4 and CD8 T cells from matched TIL and PBMC was compared (B). All significance values were calculated using a Wilcoxon matched pairs test. Representative plots showing CD107a staining on CD8 T cells (orange) and CD4 T cells (blue) compared with an isotype control (shaded red) (C).

4.2.3. Expression of CD27 and CTLA-4 on TIL and PBMC

CD27 is upregulated on T cells after activation, and lost after repeated antigen stimulation when T cells enter a late stage of differentiation (De et al. 1992;Hintzen et al. 1993). Therefore I investigated its expression to gain insight into the differentiation status of the T cells in tumour and peripheral blood. In both TIL and PBMC it was expressed by a mean of over 50% of all $CD45RA^-$ T cells, however the percentage of CD4 T cells from TIL expressing the marker was significantly reduced compared to PBMC (**Figure 32**)

There was no difference in CD27 expression between CD8 T cells from TIL and PBMC (data not shown)

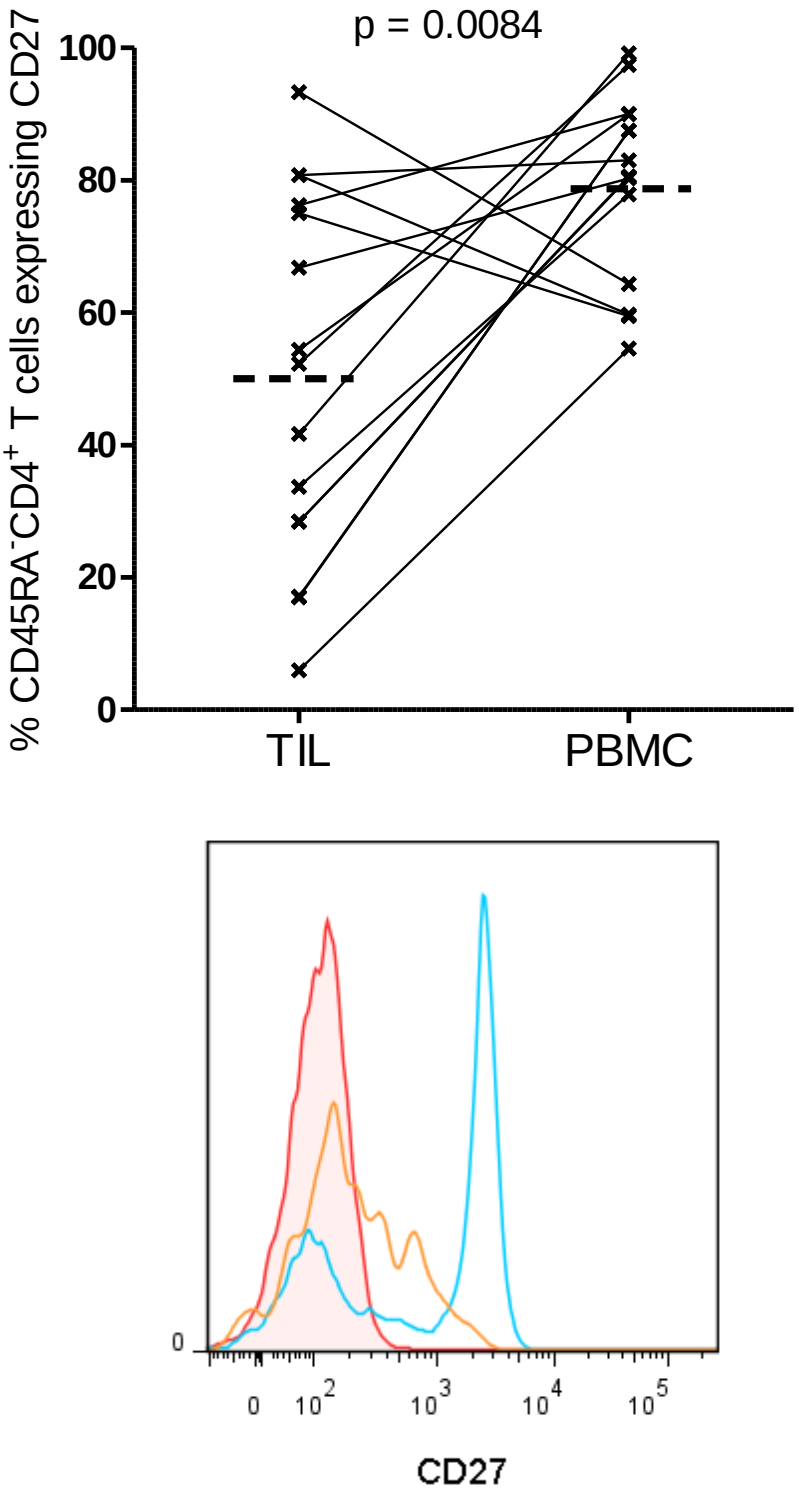


Figure 32: CD27 expression by CD4 TIL and PBMC

The expression of CD27 on CD4 T cells from TIL and PBMC was analysed by flow cytometry. Significance was calculated using a Wilcoxon matched pairs test (A). Representative plot showing CD27 staining on $CD4^+$ T cells from PBMC (blue), TIL (yellow) compared to an isotype control (shaded red) (B).

CTLA-4 is also upregulated upon T cell activation, however its expression is associated with subsequent suppression of the immune response (Walunas et al. 1994). I looked at the percentage of cells expressing CTLA-4 in both un-stimulated TIL and PBMC, and in the same samples after incubation with the stimuli PMA/ionomycin. Post-stimulation there was little difference in CTLA-4 expression between CD4 and CD8 T cells, and between TIL and PBMC (**Figure 33**A). The T cells from TIL did not significantly up-regulate CTLA-4 upon stimulation, whereas the proportion of CD4 T cells from PBMC expressing CTLA-4 significantly increased after stimulation ($p = 0.0195$). This pattern was also evident in the CD8 PBMC T cells ($p = 0.0419$) (**Figure 33**A). This may suggest TIL have already reached their maximum levels of CTLA-4 expression.

Both the CD4 and CD8 un-stimulated T cells from TIL displayed a significantly higher frequency of CTLA-4 expression than their counterparts in matched PBMC (**Figure 33**B). This indicates they may have been activated at the tumour site.

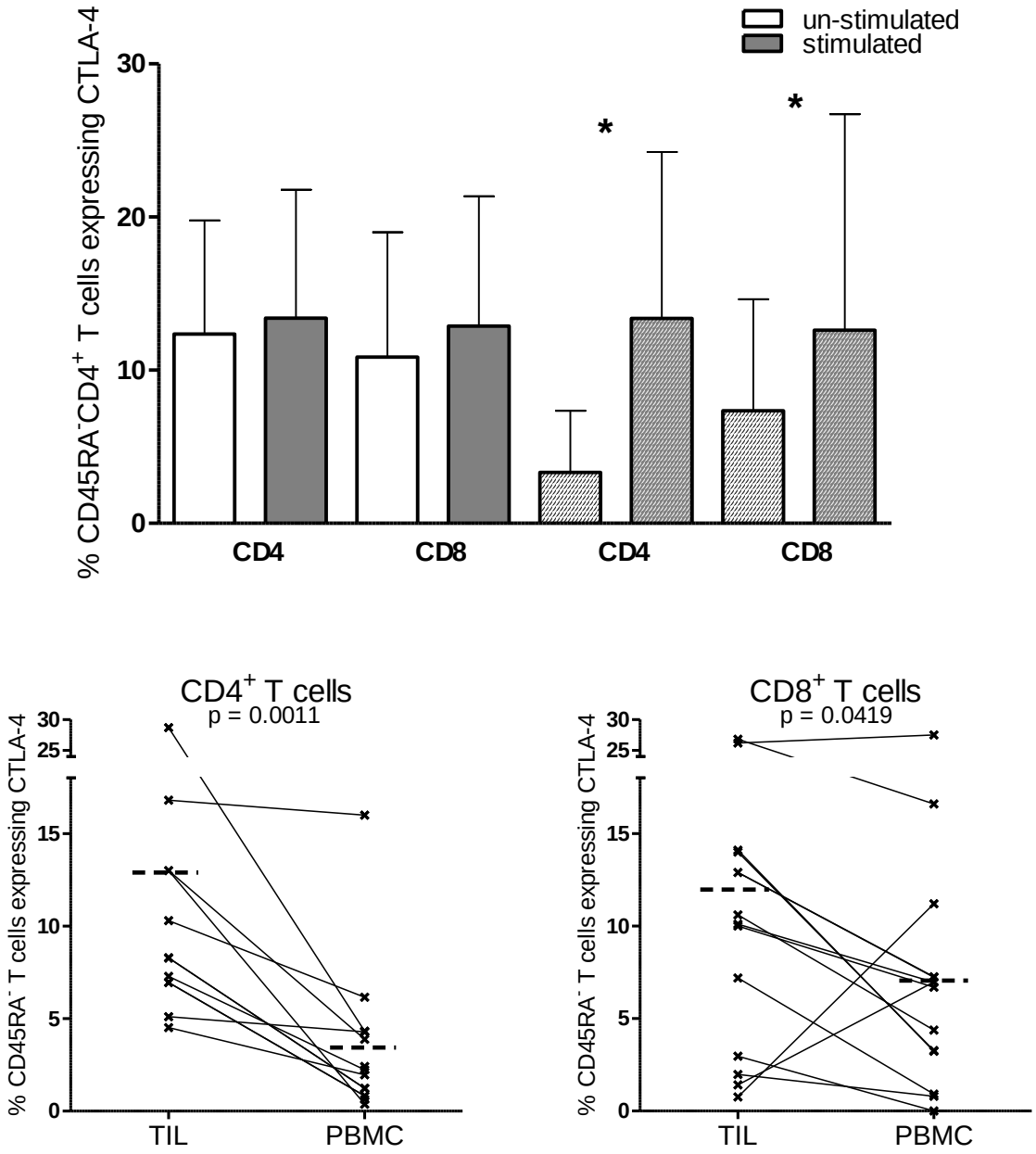


Figure 33: CTLA-4 expression by TIL and PBMC

CTLA-4 expression was measured on TIL and PBMC that were either stimulated with PMA/ionomycin, or left un-stimulated. Results were analysed by flow cytometry. Solid bars represent TIL, lined bars represent PBMC. * indicates p = <0.05, ** indicates p = <0.01 (A). CTLA-4 expression on un-stimulated CD4 and CD8 T cells from TIL and PBMC was compared (B). Significance was calculated using Wilcoxon matched pairs tests.

4.2.4. PD-1 is expressed more frequently on TIL than PBMC

PD-1 is upregulated upon T cell activation, however if it then binds either PD-L1 or PD-L2 (its ligands), T cell function becomes impaired and T cells become anergised (Barber et al. 2006;Freeman et al. 2000;Latchman et al. 2001). As the expression of PD-L1 has been reported in RCC (Blank et al. 2006;Thompson et al. 2004;Thompson et al. 2006), I investigated PD-1 expression on TIL and matched PBMC.

In both TIL and PBMC a significantly higher proportion of CD8 T cells than CD4 T cells expressed PD-1 (**Figure 34**a). T cells from both subsets of TIL included a greater proportion of PD-1^{+} cells than those in PBMC. (Figure 34b). As with CD107a this difference between TIL and matched blood may be due to TIL being activated at the tumour site. However the expression of PD-1 may indicate an anergised phenotype arising from repeated antigen stimulation. I was able to confirm the expression of PD-1 by RCC infiltrate using IHC (**Figure 35**).

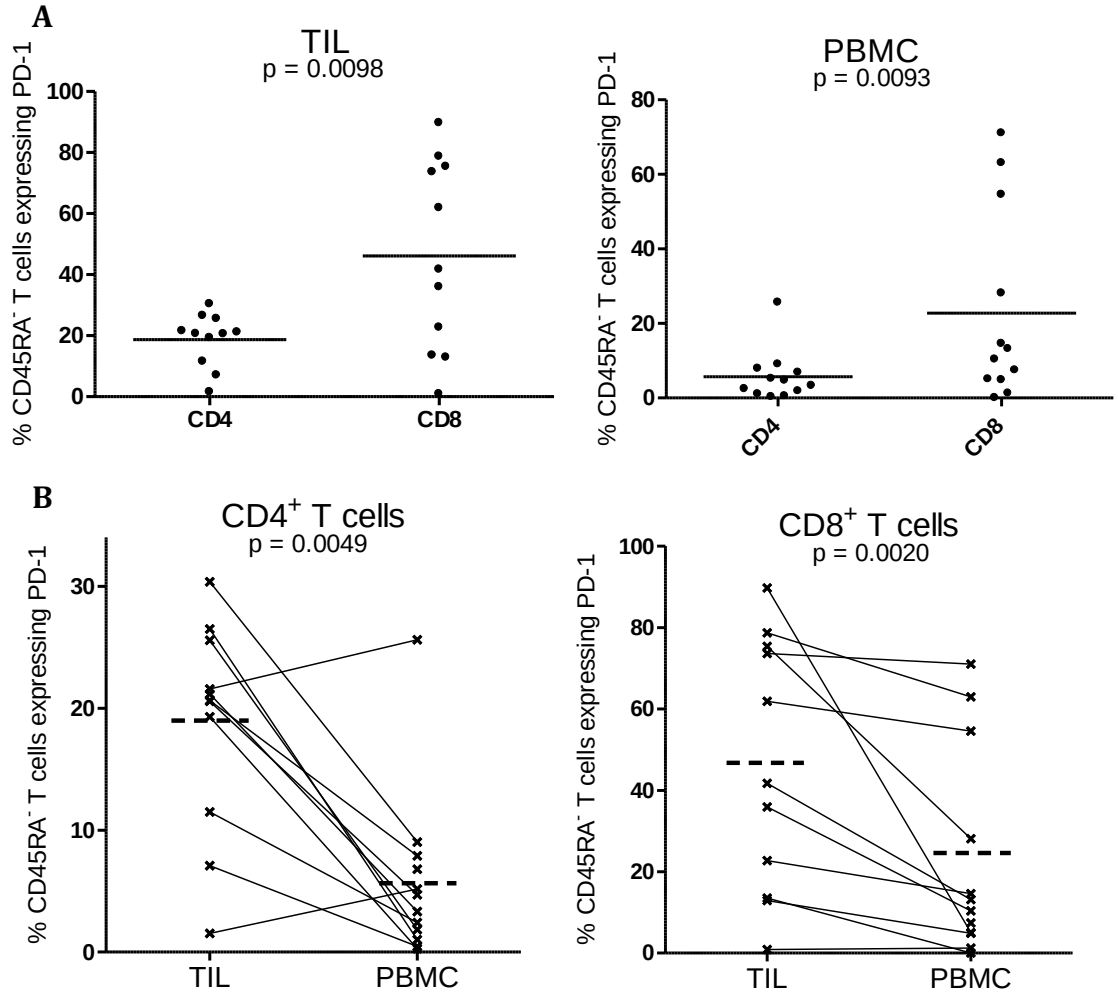


Figure 34: Expression on PD-1 on TIL and PBMC

The expression of PD-1 was compared on CD4 and CD8 T cells from both TIL and PBMC (A). Expression was also compared on CD4 T cells from TIL and PBMC, and CD8 T cells from TIL and PBMC (B). Significance was calculated using Wilcoxon matched pairs tests.

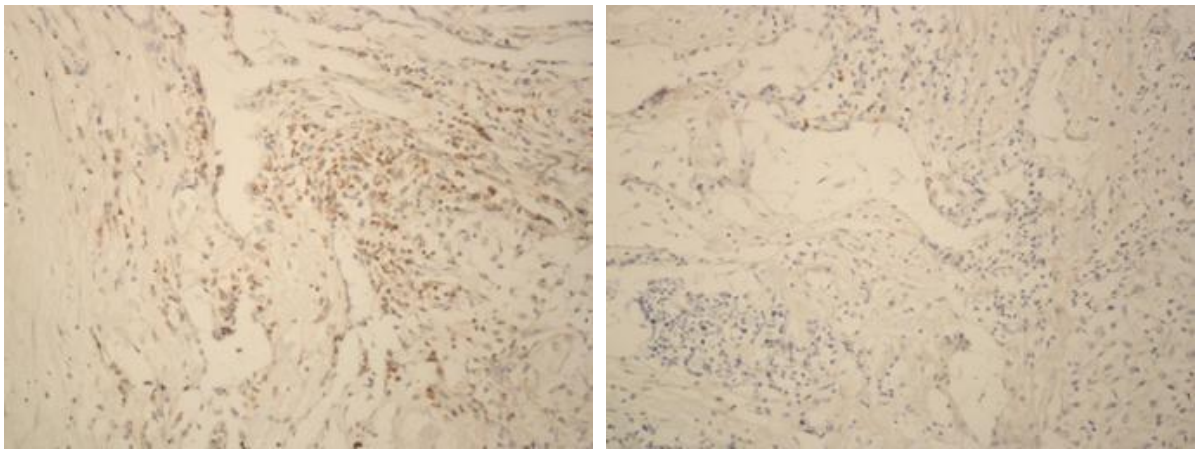


Figure 35: PD-1 expression in RCC tissue

5µm paraffin sections from RCC tumours were stained with an antibody to PD-1(left) or a concentration matched isotype control (right). Photos are representative of 10 RCC cases

4.2.4.1. Phenotype and function of PD-1⁺ T cells

To further investigate the functional status of PD-1⁺ TIL I compared expression of the functional markers HLA-DR, CD127, CD25, CD27, Ki67, CTLA-4 and CD107a on PD-1⁺ and PD-1⁻ memory T cells from TIL and matched PBMC. While there were no significant differences between the phenotypes of PD1⁺ cells in TIL and PBMC (data not shown), I did find distinctions between PD-1⁺ and PD-1⁻ TIL. HLA-DR was expressed by a significantly larger proportion of PD-1⁺ cells than PD-1⁻ cells ($p = 0.0313$), whereas CD25 was found more frequently on the PD-1⁻ subset ($p = 0.0313$) (). I also saw increased numbers of CD27⁺, Ki67⁺ and CD107a⁺ cells, and decreased numbers of CD127⁺ cells within the PD-1⁺ TIL subset, although the differences did not reach significance (**Figure 36**). With the exception of CD27, PD-1⁺ and PD-1⁻ PBMC followed a similar pattern (data not shown).

To determine whether PD-1⁺ cells were dysfunctional in terms of cytotoxic capacity or effector cytokine production, cells were stimulated with anti-CD3 and anti-CD28 beads, before intracellular staining was carried out for IL-2, IFN-γ, perforin and granzyme B. In terms of cytokine production, only CD4 T cells with very high levels of PD-1 appeared to have an impaired ability to produce IL-2 and IFN-γ, but this did not reach significance, whereas CD8 T cell cytokine production did not appear to be affected by PD-1 expression (**Figure 37**B). However a significantly greater proportion of PD-1^{hi} CD4 T cells expressed granzyme B than their PD-1⁻ counterparts (**Figure 37**C), and again this pattern was not mirrored in the CD8 T cell population. Expression of PD-1 by CD8 T cells did significantly decrease the proportion of perforin-expressing cells (**Figure 37**C). No

significant differences in perforin expression by CD4 T cells were seen but perforin expression by all CD4 T cells was extremely low.

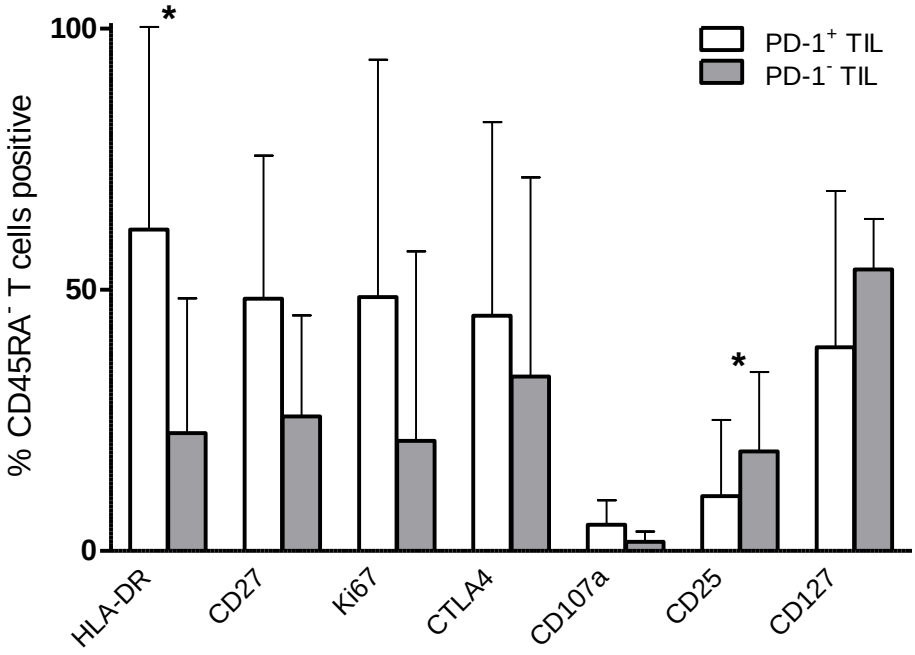


Figure 36: Expression of various functional markers on PD-1$^+$ and PD-1$^-$ TIL

TIL were co-stained for PD-1 and HLA-DR, CD27, Ki67, CTLA-4, CD107a, CD25 or CD127. For HLA-DR, CD127, CD25 and CD27 n = 6, for Ki67n = 4 and for CD107a and CTLA-4 n = 3. Data represent the mean percentage of cells positive for CTLA-4 (+SD). * indicates a p = <0.05. Significant calculated using Wilcoxon matched pairs tests.

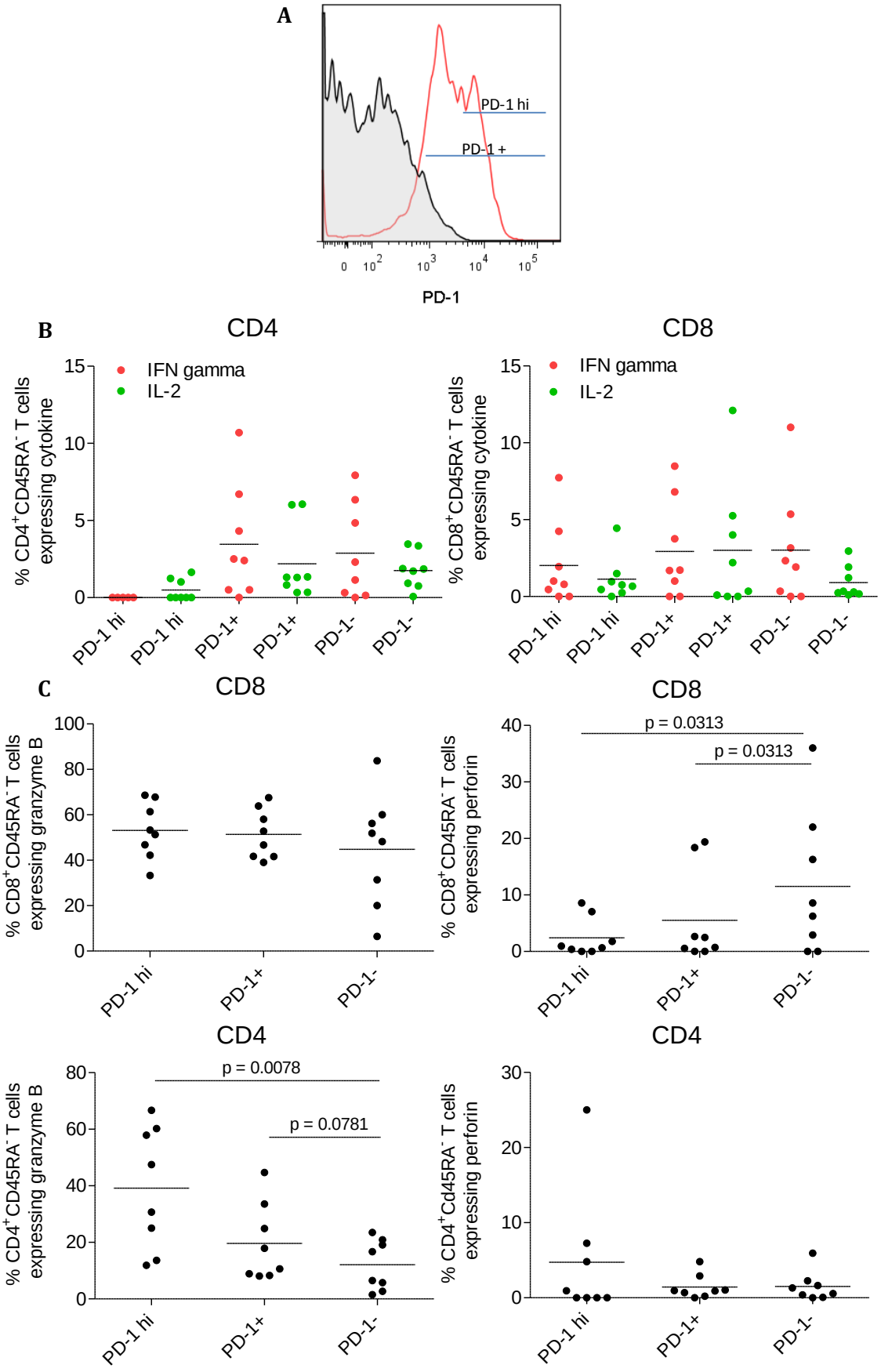

A
PD-1 hi
PD-1 +
PD-1
B
CD4
CD8
IFN gamma
IL-2
% CD4+CD45RA- T cells expressing cytokine
% CD8+CD45RA- T cells expressing cytokine
PD-1 hi
PD-1+
PD-1-
C
CD8
% CD8+CD45RA- T cells expressing granzyme B
% CD8+CD45RA- T cells expressing perforin
p = 0.0313
CD4
% CD4+CD45RA- T cells expressing granzyme B
% CD4+Cd45RA- T cells expressing perforin
p = 0.0078
p = 0.0781

Figure 37: Cytokine production, granzyme B and perforin expression of PD-1^{+} and PD-1^{-} TIL

T cells were stimulated with αCD3 and αCD28 beads before being stained and gated on PD-1 expression (A). Cells were co-stained with IL-2, IFN-γ (B), granzyme B or perforin (C). Significance was calculated using Wilcoxon matched pairs tests.

4.3. Function of Foxp3$^+$ T cells *in vitro*

In section 3.4 I found that there were increased levels of Foxp3$^+$CD4$^+$ T cells in TIL compared to PBMC, and that they have the CD25^{hi}CD127^{lo} phenotype typical of classic regulatory T cells. I then sought to perform further phenotypic and functional analysis to confirm these cells have a regulatory role and to establish their mechanism of function at the tumour site.

4.3.1. Attempt at proliferation assays

The most common method of proving Treg function is to perform a proliferation assay and look at how Tregs affect the proliferation of responder cells. Initially I attempted to isolate Tregs from limited numbers of TIL using regulatory T cell isolation kits. However the purity and yield were insufficient to permit proliferation assays.

4.3.2. Effector cytokine production by Foxp3$^+$ T cells

In order to investigate the function of Foxp3$^+$ TIL, I looked at cytokine production by those cells compared to the general T cell cohort. Effector T cells produce IFN-γ, IL-2 and TNFα upon stimulation (see section 4.2.1, p157), whereas Tregs are characterised by their anergy and lack of cytokine production (Ahmadzadeh et al. 2008;Kryczek et al. 2009b;Wolf et al. 2003).

Not only did a significantly smaller proportion of Tregs compared to effector CD4 T cells produce the cytokines IFN-γ ($p = 0.0006$), IL-2 ($p = 0.0008$) and TNFα ($p = 0.0045$), but the difference between Treg and effector T cell cytokine production was more marked in TIL than PBMC (**Figure 38**).

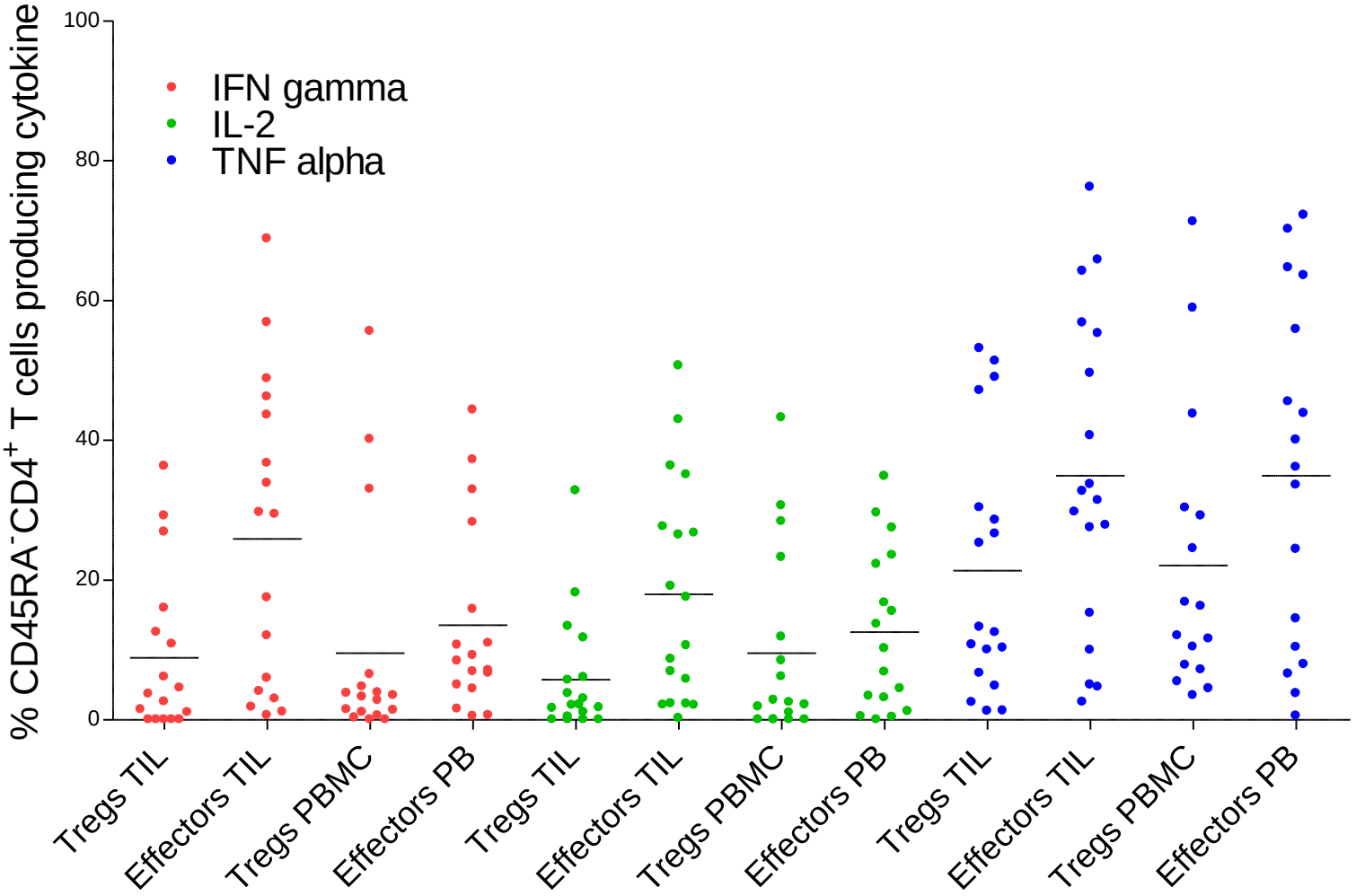

% CD45RA-CD4+ T cells producing cytokine
100
80
60
40
20
0
IFN gamma
IL-2
TNF alpha
Tregs TIL
Effectors TIL
Tregs PBMC
Effectors PB
Tregs TIL
Effectors TIL
Tregs PBMC
Effectors PB
Tregs TIL
Effectors TIL
Tregs PBMC
Effectors PB

Figure 38: Effector cytokine production by Foxp3^{+} and Foxp3^{-} TIL

TIL and PBMC were stimulated with PMA/ionomycin, and the percentage of Foxp3^{+} (Treg) and Foxp3^{-} (effector) CD4 TIL producing effector cytokine was analysed by flow cytometry. For a representative plot see **Figure 29** p159.

4.3.3. Putative Tregs express CTLA-4

CTLA-4 is expressed by Tregs and constitutes a mechanism by which they can suppress T cell function (Qureshi et al. 2011;Tivol et al. 1995;Waterhouse et al. 1995). I found the $Foxp3^{+}CD4$ T cells in TIL contained significantly more CTLA-4^{+} cells and expressed the marker at significantly higher levels than $Foxp3^{-}CD4$ T cells (**Figure 39**A). Comparing $Foxp3^{+}CD4$ T cells in TIL with their counterparts in PBMC showed that CTLA-4 was expressed on a greater proportion of those in TIL and expression of the marker in TIL Tregs was increased compared to PBMC when measuring MFI. (**Figure 39**B). The high levels of CTLA-4^{+} on the $Foxp3^{+}$ population is suggestive of a regulatory phenotype.

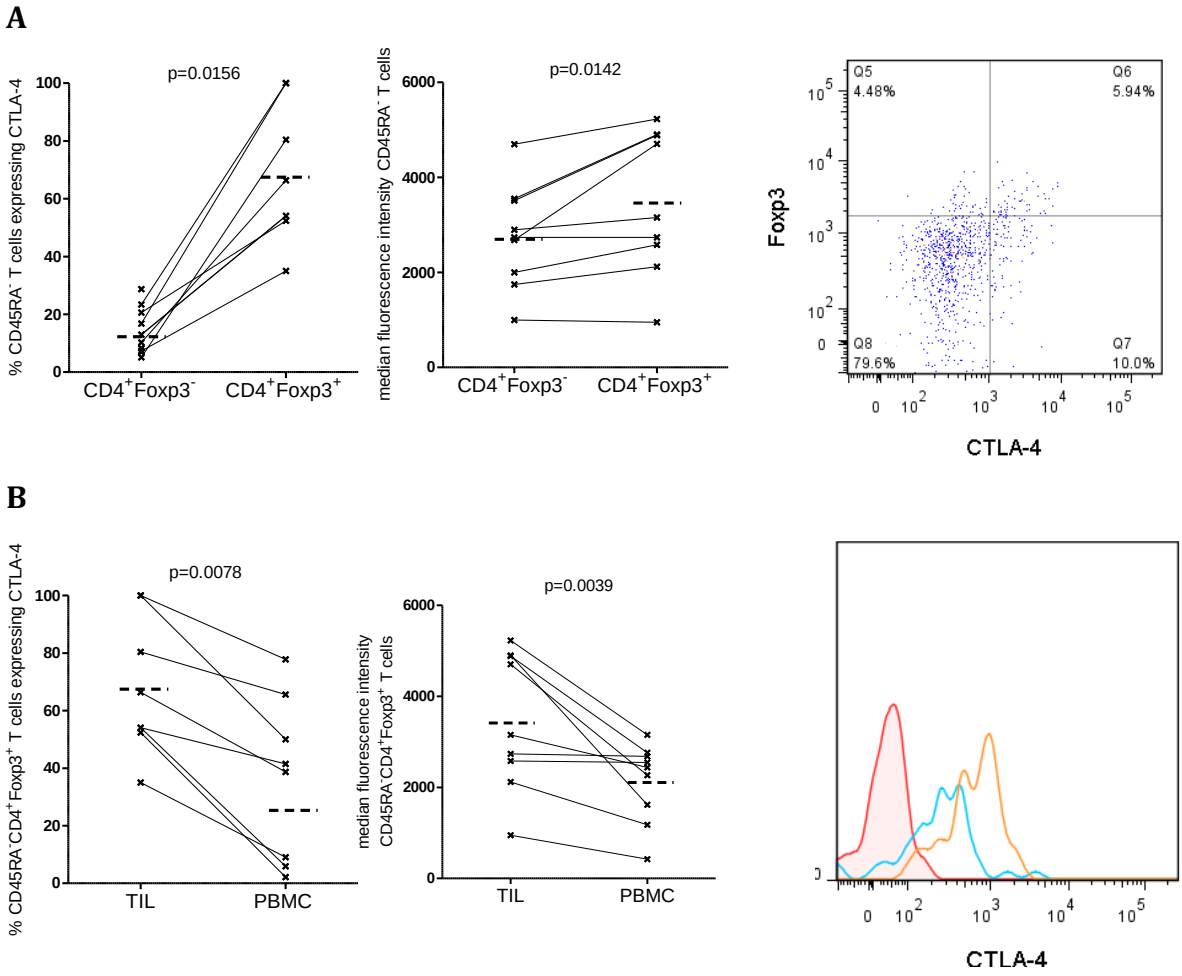


Figure 39: CTLA-4 expression on Foxp3⁺ and Foxp3⁻ T cells from TIL and PBMC

RCC TIL were stained co-stained for CTLA-4 and Foxp3. Graphs display the percentage of T cells positive for each marker and the median fluorescence intensity of the staining. CTLA-4 staining on Foxp3⁺ and Foxp3⁻ T cells from TIL was compared and a representative plot gated on CD4⁺ T cell is shown (A). Staining on Foxp3⁺ T cells from TIL and PBMC was compared and a representative plot showing CD4⁺Foxp3⁺ T cells from PBMC (blue) and TIL (orange) compared to an isotype control (shaded red) is shown (B). Significance was calculated using a Wilcoxon matched pairs test

4.3.4. Expression of PD-1, CD107a and CD27 on Tregs in RCC

PD-1 and CD107a expression are not generally associated with Tregs. However I saw expression of both PD-1 (39 ± 15 %) and CD107a (4.4 ± 3.6 %) on a proportion of $Foxp3^+$ CD4 T cells from TIL. (**Figure 40**A and B).

The percentage of $Foxp3^+CD4^+$ cells expressing PD-1 in TIL was greater than the proportion of $Foxp3^-CD4^+$ cells expressing this marker and it was also greater than the percentage of $Foxp3^+CD4^+$ cells from PBMC expressing PD-1 (**Figure 40**A). The putative Tregs from TIL were also more likely to express CD107a than those from PBMC (**Figure 40**B), however there was no significant difference between CD107a expression on $Foxp3^+$ and $Foxp3^-$ CD4 T cells from TIL (data not shown). The expression of these markers suggests additional mechanisms by which Tregs might carry out their function.

The expression of CD27 varies between different functional subsets of T cells. I found a large proportion (79 ± 29 %) of $Foxp3^+$ TIL expressed CD27, which was significantly greater than the percentage of $Foxp3^-$ TIL (50 ± 28 %) (**Figure 40**C). Expression of CD27 on $Foxp3^+$ cells from TIL and PBMC was similar (data not shown).

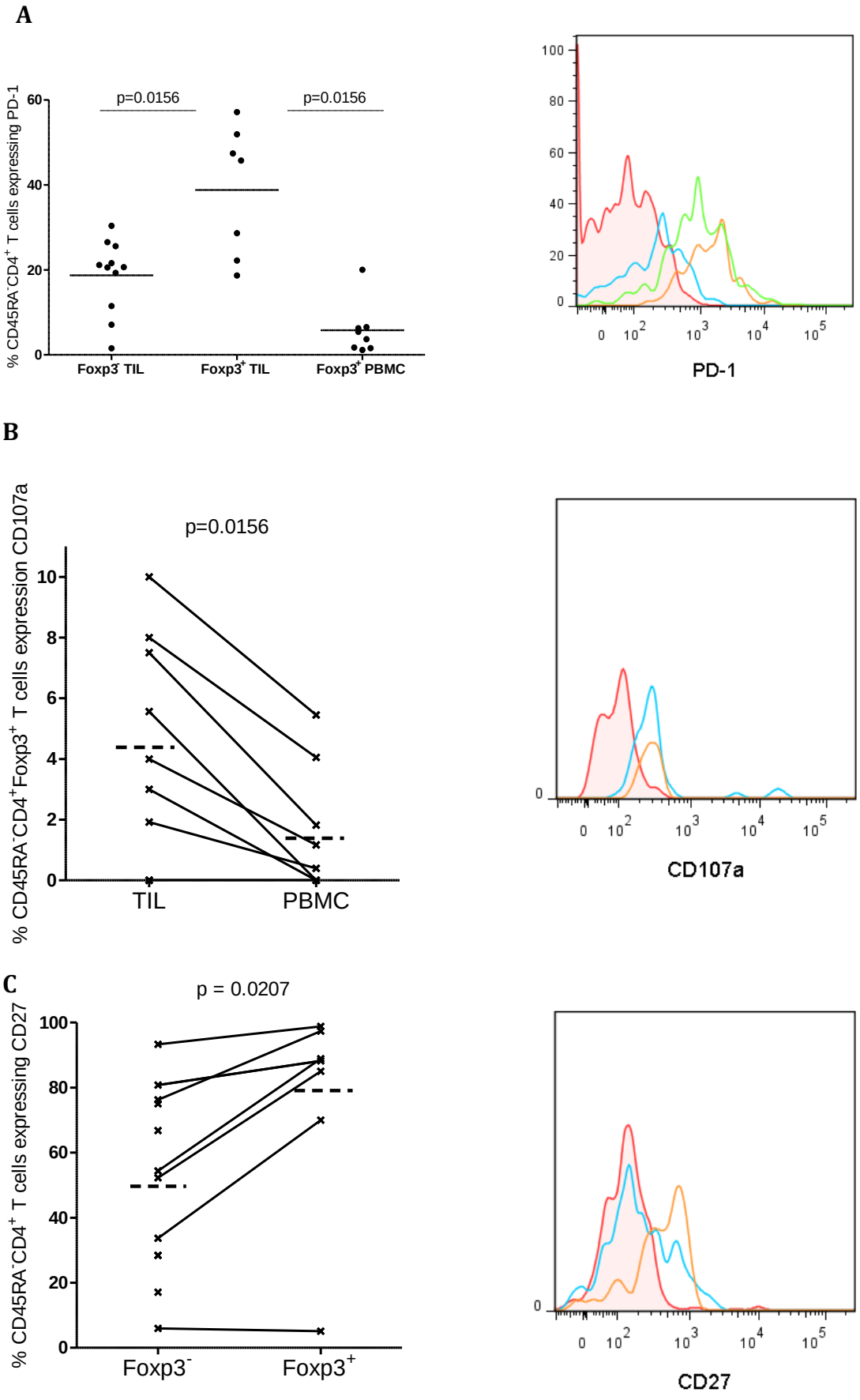

A
p=0.0156
p=0.0156
% CD45RA-CD4+ T cells expressing PD-1
Foxp3- TIL
Foxp3+ TIL
Foxp3+ PBMC
PD-1
B
p=0.0156
% CD45RA-CD4+Foxp3+ T cells expression CD107a
TIL
PBMC
CD107a
C
p = 0.0207
% CD45RA-CD4+ T cells expressing CD27
Foxp3-
Foxp3+
CD27

Figure 40: Expression of PD-1, CD107a and CD27 on Foxp3$^+$ and Foxp3$^-$ T cells

TIL and PBMC were co-stained for Foxp3 and PD-1 and the expression of PD-1 compared on Foxp3$^+$ and Foxp3$^-$ T cells. Representative plot shows PD-1 staining on PBMC (blue), CD4$^+$Foxp3$^-$ T cells (green) and CD4$^+$Foxp3$^+$ T cells (orange) compared to an isotype control (shaded red) (A). Foxp3$^+$ T cells from TIL and PBMC were stained for CD107a and the proportions positive for the marker compared. The plot shows CD107a staining on CD4$^+$Foxp3$^+$ T cells from TIL (blue) and PBMC (orange) compared to an isotype control (shaded red) (B). Foxp3$^+$ and Foxp3$^-$ TIL were stained for CD27 and compared. The plot shows CD4$^+$Foxp3$^-$ T cells (blue) and CD4$^+$Foxp3$^+$ T cells (orange) from TIL compared to an isotype control (shaded red) (C). Significance was calculated using a Wilcoxon matched pairs test.

4.3.5. The ratio of the percentage of Tregs in TIL: Tregs in PBMC correlates with function of TIL

I investigated whether there was a correlation between the markers of T cell function discussed above and the percentage of Tregs present in the TIL, to determine whether the Tregs were having a detectable effect on TIL function. Whereas I found no correlations between these markers and the proportion of Tregs in TIL, I did find that the ratio of Tregs in TIL: PBMC inversely correlated with effector cytokine production by $Foxp3^-$ T cells. For $CD4^+$ T cells, the correlation coefficient looking at IFN-γ expression was -0.5679, p = 0.0272. The correlation for IL-2 approached significance: r = -0.4735, p = 0.0639, and for TNFα r = -0.5794, p = 0.0187 (**Figure 41**). For $CD8^+$ cells the correlation was less clear, with IFN-γ and TNFα not quite reaching significance (r = -0.5071, p = 0.0537 and r = -0.4571, p = 0.0867) (**Figure 42**) and IL-2 expression did not correlate (data not shown). These data suggest it is an enrichment of Tregs in TIL compared to PBMC that affects effector T cell function, rather than simply the proportion of Tregs present in the TIL.

In addition, there was a inverse correlation between the level of CTLA-4 expression on effector T cells and the ratio of Tregs in TIL:PBMC. For $CD4^+$ T cells the correlation coefficient was -0.7857, p = 0.0480, and for $CD8^+$ T cells it was -0.8571, p = 0.0238 (**Figure 43**).

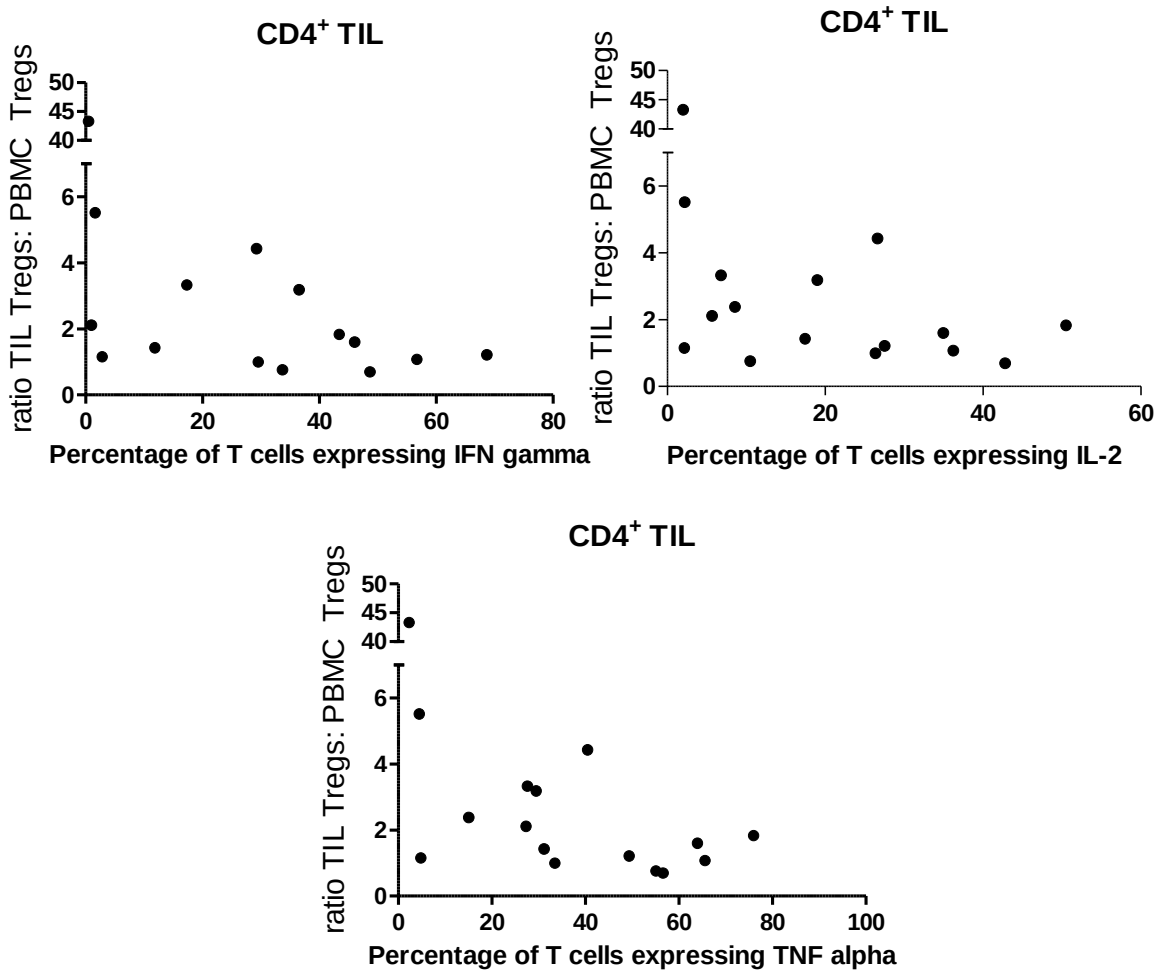


Figure 41: Correlations between the ratio of Tregs in TIL: PBMC and CD4 T cell effector cytokine production

Effector cytokine production by Foxp3$^-$ CD4 TIL was measured by intracellular cytokine staining and compared to the ratio of the percentage of Tregs in TIL: percentage of Tregs in PBMC. Correlation was calculated using the nonparametric Spearman correlation coefficient.

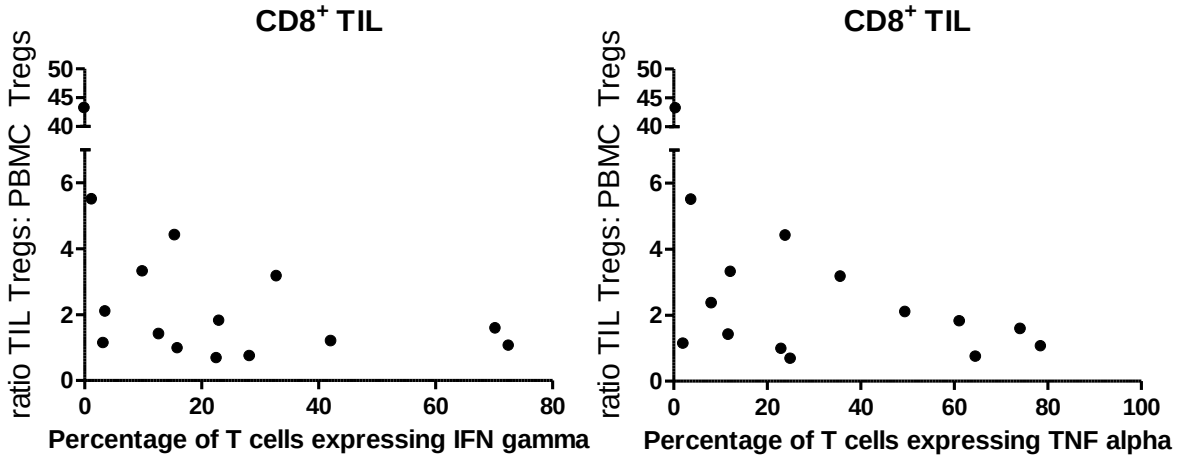


Figure 42: Correlations between the ratio of Tregs in TIL: PBMC and CD8 T cell effector cytokine production

Effector cytokine production by Foxp3$^-$ CD8 TIL was measured by intracellular cytokine staining and compared to the ratio of the percentage of Tregs in TIL: percentage of Tregs in PBMC. Correlation was calculated using the nonparametric Spearman correlation coefficient.

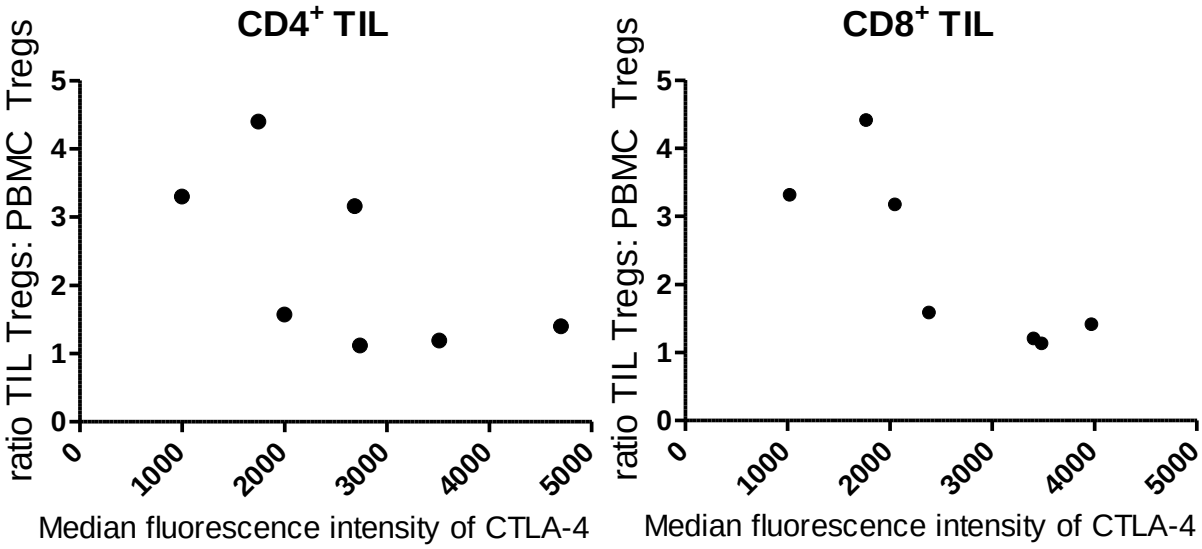


Figure 43: Correlations between the ratio of Tregs in TIL: PBMC and CTLA-4 expression

CTLA-4 expression by Foxp3$^-$ CD4 and CD8 TIL was measured by flow cytometry and compared to the ratio of the percentage of Tregs in TIL: percentage of Tregs in PBMC. Correlation was calculated using the nonparametric Spearman correlation coefficient.

4.4. Discussion

In the previous chapter I confirmed the presence of T cells in RCC, and examined their homing receptor profile to elucidate the mechanisms of their recruitment. In this chapter I determined the different T cell subsets that make up the TIL population, and investigated their functional capacities.

Although there has been some suggestion in the literature that $CD8^+$ TIL from RCC are defective in function (Kudoh et al. 1997;Van den Hove et al. 1997a), my data suggest both CD4 and CD8 T cells from TIL are just as able, or in the case of CD4 T cells and IFN-γ and IL-2, more able, to produce effector cytokines, as their counterparts in peripheral blood. This discrepancy between TIL and PBMC could be due to the increased proportion of T_{EMs} compared to T_{CMs} in TIL as opposed to PBMC, as T_{EMs} produce effector cytokine more efficiently upon stimulation (Sallusto et al. 2004).

PMA/ionomycin function by bypassing the need for signalling through the TCR by directly increasing the concentration of Ca^{2+} in the cell cytoplasm and activating protein kinase C (Truneh et al. 1985). Since this is a strong stimulus for T cells (Kay 1991), it could have masked defects in TIL cytokine production. However, another group have looked at cytokine secretion by RCC TIL in response to stimulation with anti-CD3 and anti-CD28 antibodies, which more closely mimic natural T cell activation. Although the proportion of cells producing cytokine was smaller, the T cells again demonstrated equivalent or increased capacity to produce TNFα, IL-2 and IFN-γ compared to RCC

patients' and healthy donors' PBMC (Attig et al. 2009). Further evidence that cytokine production by RCC TIL is not compromised has been reported using ELISA (rather than intracellular cytokine staining) following stimulation with anti CD3 with or without PMA (Angevin et al. 1997;Van den Hove et al. 1997a). A study looking at mRNA expression of cytokines found TIL to have significantly higher levels of IFN-γ mRNA than matched PBMC, however the $CD8^+$ TIL had significantly less TNFα mRNA than the PBMC (Elsasser-Beile et al. 2000).

Animal models have shown IL-2, TNF-α and IFN-γ expression promotes an anti-tumour immune response (Gansbacher et al. 1990a;Gansbacher et al. 1990b;Lasek et al. 2000). In addition, as discussed previously (see section 3.8, p143) a type 1 immune bias within the tumour infiltrate is associated with a favourable immune response. Therefore these data suggest the TIL are capable of a Type 1 response, that should promote anti-tumour immunity.

Both CD4 and CD8 T-cells are needed for an effective anti-tumour response (Hung et al. 1998), however previous studies have suggested tumour-infiltrating CD8 T cells in RCC lack cytolytic activity (Kudoh et al. 1997;Van den Hove et al. 1997a). I found that CD8 T cells from TIL express CD107a, a marker of degranulation (Betts et al. 2003), suggesting they have degranulated prior to isolation, i.e. *in vivo*. There was also an enrichment of $CD4^+CD107a^+$ T cells in TIL compared to PBMC. This suggests a small proportion of the CD4 T cell subset has also degranulated. Low levels of $CD4^+$ CTLs have been

demonstrated in healthy and diseased individuals and although various mechanisms of action have been postulated, they appear at least in some settings to work by releasing cytotoxic granules, and hence can be detected by looking at CD107a mobilisation (Appay et al. 2002;Zheng et al. 2007). Indeed, mouse models have shown that cytotoxic CD4 T cells can kill melanoma cells in a granzyme B-dependent manner (Quezada et al. 2010). Attig et al also reported CD107a expressed on both $CD4^+$ and $CD8^+$ RCC TIL following stimulation with PMA/ionomycin (Attig et al. 2009).

There is some suggestion that $CD107a^+$ T cells from TIL, such as those I have observed in RCC, represent a tumour antigen-specific population. One group used CD107a expression to accurately identify tumour-reactive T cells post vaccination in melanoma patients (Rubio et al. 2003). It has since been shown to be upregulated on peptide specific T cells in patients that responded to vaccination for chronic lymphatic leukaemia (Giannopoulos et al. 2010). In colon cancer a significantly higher (albeit small) proportion of CD8 TIL express CD107a, compared to CD8 cells from normal mucosa. Higher proportions of $CD107a^+$ CD8 T cells correlated with the presence of tumour antigen reactive T cells in the TIL (Koch et al. 2006). Thus it is possible that the presence of CD107a on a significant proportion of CD8 and CD4 TIL in RCC indicates that these T cells are specific for antigens expressed at the tumour site and have been activated upon antigen encounter, yet, clearly, this has not prevented outgrowth of the tumour.

Turning to CTLA-4 expression, my data show that this increased in PBMC following PMA/ionomycin stimulation to a level equivalent to that of un-stimulated TIL, whereas TIL did not increase CTLA-4 expression post-stimulation, suggesting CTLA-4 expression on TIL had already reached a maximum level within the tumour environment. Expression of CTLA-4 is further evidence that the TIL may have seen antigen in the tumour as CTLA-4 is known to be up-regulated upon activation (Freeman et al. 1992;Walunas et al. 1994). Additional evidence that tumour-infiltrating T cells have been activated *in vivo* comes from my CD27 expression studies where fewer CD4 T cells from TIL compared with PBMC expressed this marker. The loss of CD27 expression in $CD45RA^-$ $CD4^+$ T cells is associated with chronic antigen stimulation (De et al. 1992). A $CD27^-$ $CD45RA^-$ $CD4^+$ T cell phenotype also correlates with CD4 T cell cytotoxic potential (Appay et al. 2002;Duvall et al. 2008). Therefore it is possible that $CD27^-$ $CD4^+$ TIL represent cytotoxic T cells that have repeatedly encountered their target antigen. Notably, without CD27, these T cells would not be subject to apoptosis following encounter with $CD70^+$ tumour cells, a proposed mechanism by which RCC evades the immune system (Diegmann et al. 2006).

PD-1 is also upregulated on T cells post-activation and expression increases during chronic antigen stimulation (Barber et al. 2006). Un-stimulated T cells expressed PD-1 *ex vivo*, suggesting they may have seen their antigen at the tumour site. However if PD-1 is ligated by either of its ligands, PD-L1 or PD-L2, T cell function is attenuated and cell survival pathways are inhibited (Bennett et al. 2003;Chemnitz et al. 2004;Freeman et al. 2000;Latchman et al. 2001). In RCC patients PD-1 is expressed by a greater proportion

of CD4 and CD8 TIL than their counterparts in PBMC. This could indicate they have been activated and therefore have encountered their antigen at the tumour site. In support of this a study in melanoma has confirmed antigen-specific cells are more likely to be PD-1$^+$ (Ahmadzadeh et al. 2009). However, numerous studies have shown expression of PD-L1 on RCC tumours, and therefore PD-1$^+$ T cells in RCC are likely to have impaired function and an exhausted phenotype. One study found 66% of RCC tumours (n=196) stained positively for B7-H1 (PD-L1, CD274) by IHC, and in 37.2% of cases, more than 10% of the tumour tissue expressed this marker (Thompson et al. 2004). This is supported by a paper demonstrating PD-L1 expression on RCC and melanoma (Blank et al. 2006). Another study of 306 patients found 23.9% to have positive staining, and that these patients were almost four times more likely to die from RCC (univariate analysis) (Thompson et al. 2006). As the interaction between PD-L1 and its receptor, PD-1, is associated with cell exhaustion and anergy, its presence in the tumour could be a mechanism of immune suppression (Freeman et al. 2000;Ishida et al. 1992). In support of my data, a study of 136 patients using IHC found PD-1 was present on TIL in 56.6% of tumours. Presence of PD-1$^+$ lymphocytes was associated with poor outcome (Thompson et al. 2007).

When I characterised the PD-1$^+$ T cells further I found that compared to PD-1$^-$ T cells a greater proportion were HLA-DR$^+$ and CD25$^-$. They also appeared more likely to be CD27$^+$, Ki67$^+$, CTLA4$^+$ and CD107a$^+$ cells, and CD127$^-$. A similar phenotype on PD-1$^+$ TIL was seen in melanoma by Ahmadzadeh et al. who found PD-1$^+$ TIL to be HLA-DR$^+$, CD127$^-$, CTLA-4$^+$, CD27$^+$ and Ki67$^+$, and suggested this is indicative of an exhausted

phenotype (Ahmadzadeh et al. 2009). Expression of Ki67, CTLA4 and CD107a suggest these cells have been activated previously and are proliferating, and the expression of CD107a suggests they may even have been capable of cytotoxicity (Betts et al. 2003;Linsley et al. 1992;Malizia et al. 1985). However lack of CD127 is associated with impaired T cell function (Lang et al. 2005), and reduced CD25 combined with increased CD27 expression suggests the T cells have not been in contact with IL-2, as exposure to this cytokine results in the opposite phenotype (Ahmadzadeh et al. 2009;Huang et al. 2006;Sereti et al. 2000). This is not surprising, as the presence of IL-2 can overcome PD-1 mediated suppression and down-regulate its expression (Carter et al. 2002;Inozume et al. 2010), therefore high PD-1 expression is more likely where IL-2 levels are limited.

Stimulated PD-1^{hi} CD4 T cells appeared deficient in IL-2 and IFN-γ production compared to cells with lower levels of PD-1. However the pattern did not reach significance, and no effect was seen in the CD8 T cells, contrasting with results described previously looking at CD8 T cells in murine viral infections (Jin et al. 2010;Zelinskyy et al. 2011). This discrepancy could be due to the lack of sensitivity in my assay due to low overall cytokine production by the T cells, presumably due to the stimulation method. I have demonstrated that PMA/ionomycin stimulation can result in good effector cytokine production by TIL, however I deemed it unsuitable for use in this assay as it bypasses the early T cell signalling events that are thought to be important in PD-1 mediated inhibition (Wang et al. 2011b). Alternatively it could be that in the absence of PD-1 ligands, which may not have been present in the TIL used, stimulation overcame previous PD-1 mediated suppression.

Previous studies have shown that blocking the PD-1:PD-L1 interaction can increase cytotoxicity of T cells (Lukens et al. 2008;Phares et al. 2010). Nonetheless, PD-1 expression has been shown to positively correlate with expression of the cytotoxic molecule granzyme B in CD8 T cells in viral infections in mice (Jin et al. 2010;Zelinskyy et al. 2011). I found CD4 T cells with high expression of PD-1 to have greater expression of granzyme B. Cytotoxic CD4 T cells are thought to be an antigen experienced population and their presence is seen most frequently in chronic diseases (Appay 2004). Therefore it could be expected that PD-1 up-regulation and CD4 cytotoxicity would correlate. Perforin expression by CD8 T cells appeared impaired in PD-1 expressing cells, whereas granzyme B did not. Perforin is a key mediator of cytotoxicity and therefore this could indicate these T cells are suppressed. The presence of granzyme B does not necessarily contradict this hypothesis, as it has been suggested in the literature that high granzyme B expression can be a marker of exhaustion in CD8 T cells, and it is the loss of perforin which affects their cytotoxic capacity (Takamura et al. 2010;Wherry et al. 2007).

Despite my evidence that TIL display markers of activation *ex vivo* and can release effector cytokines in response to stimulation, spontaneous regression of RCC tumours in the clinic is rare, indicating the immune system is not able to kill the tumour. The tumour could be resistant to the cytotoxic mechanisms employed by the T cells, or alternatively could be causing suppression of the function of the T cells. The PD-1: ligand interaction may account for this, however I also found enrichment of $Foxp3^+$ Tregs within the TIL, which may suppress a tumour-specific immune response.

It has been shown that an infiltrate with a large proportion of Tregs may be detrimental to RCC patient survival (Griffiths et al. 2007). Treg control of the immune response has been shown to play roles in suppressing autoimmune and inflammatory immune responses (Behrens et al. 2007;Sakaguchi et al. 1995;Takahashi et al. 2006). They may also have a negative effect in cancer patients by suppressing anti-tumour immunity. In section 3.4 I found cells with a Treg-like phenotype ($CD3^+CD4^+CD25^{hi}CD127^{lo}Foxp3^+$) to be enriched at the tumour site compared to matched PBMC. I next wanted to investigate these cells' capacity to suppress an immune response. Due to low numbers of TIL I was unable to perform conventional suppression assays using these cells, so instead I analysed their effector cytokine profile following PMA/Ionomycin stimulation; an approach recently demonstrated to identify Tregs in melanoma (Ahmadzadeh et al. 2008). I found that the putative Tregs from TIL produced very little IFN-γ, IL-2 or TNFα, in contrast to the $Foxp3^-$ T cell population. This functional profile is consistent with that described for Tregs in the literature (Ahmadzadeh et al. 2008;Kryczek et al. 2009b), and with the $Foxp3^+$ cells in PBMC from RCC and healthy donors, where we could also confirm suppressive function using conventional assays of T cell proliferation (data not shown).

The putative Tregs expressed CTLA-4 at higher frequencies and levels than $Foxp3^-$ TIL, and therefore may suppress an immune response via this receptor. CTLA-4 has been described as an immunoregulatory molecule which is vital in preventing autoimmune disease (Tivol et al. 1995;Waterhouse et al. 1995), and which may contribute to regulatory T cell suppression of T cell function by stripping APCs of their co-stimulatory

molecules, CD80 and CD86 (Qureshi et al. 2011). Although all activated T cells can transiently express CTLA-4, Tregs constitutively express high levels of the molecule (Dieckmann et al. 2001;Takahashi et al. 2000;Tang et al. 2004). Therefore their increased CTLA-4 expression compared to $Foxp3^-$ T cells suggests the $Foxp3^+$ TIL are true Tregs. If CTLA-4 is one of the main mechanisms Tregs use to suppress effector T cells, it may be possible to reverse this suppression using treatments currently under investigation. Trials using an antibody to block CTLA-4 have shown some success in a phase II trial with RCC patients. However, as perhaps would be expected, patients with better responses also suffered severe autoimmune reactions (Beck et al. 2006;Yang et al. 2007).

A higher percentage of $Foxp3^+$ CD4 T cells expressed CD27 compared to $Foxp3^-$ $CD4^+$ T cells. Previous studies have demonstrated CD27 defines a subset of Tregs with greater suppressive capacity and higher Foxp3 expression (Grossman et al. 2004;Ruprecht et al. 2005), again indicating this cell subset has a regulatory function.

I found over a third of Tregs in TIL expressed PD-1, and in common with studies on mouse models of GVHD and human melanoma and hepatitis C virus patients, I found an increased PD-1 expression on Tregs compared to $Foxp3^-$ cells (Franceschini et al. 2009;Kitazawa et al. 2007;Wang et al. 2009). Using cells from melanoma patients it was shown that blocking PD-1 on Tregs before adding them to a suppression assay decreased their capacity to suppress the proliferation of melanoma-antigen-specific CD8

T cells, and down-regulated their Foxp3 expression (Wang et al. 2009). In addition, in the mouse model of GVHD, blocking PD-L1 in wild type mice increased disease levels to those seen in Treg-depleted mice, and adding a PD-L1 block to Treg-depleted mice did not further increase disease. This suggests Tregs require the PD-1:PD-L1 interaction for suppression of GVHD. However it is unclear whether this interaction has a direct effect on regulatory T cells or whether the interaction between Tregs and APCs modulates APC function in a PD-L1 dependent manner (Kitazawa et al. 2007). On the contrary, studies in hepatitis C infection have shown blocking PD-L1 during antigenic stimulation of Tregs increases Treg proliferation and thereby enhances Treg-mediated immune suppression (Franceschini et al. 2009). Further study is required to determine what effect PD-1 expression on Tregs from RCC has on their suppressive function, as this will have implications for any potential therapeutic approaches that seek to block PD-1:ligand interactions.

A subset of Tregs expressed the CD107a marker of degranulation, and although the proportion of $CD107a^+$ Tregs was small, it was nonetheless significantly greater than the proportion of $Foxp3^+$ cells in PBMC that expressed this marker. There is evidence in the literature that Tregs can mediate suppression via perforin or granzyme induced cytotoxicity (Grossman et al. 2004), however this would seemingly only be relevant for a minority of the Treg population in RCC.

Although it might have been anticipated that lack of effector function in TIL would correlate with the frequency of infiltrating Tregs, our data did not support this. However, we did find a correlation between both lack of effector cytokine production and CTLA-4 expression with an increased ratio of Tregs in TIL:Tregs in matched PBMC.

The reason for this correlation is unclear, but the pattern suggests that it is a relative enrichment rather than simply a high proportion of Tregs that determines their effect on T cell function. Perhaps the enrichment is due to preferential recruitment of a more suppressive Treg subset by the tumour, or induction of Tregs at the tumour site which are more potent than nTregs. Further phenotypic analysis to determine whether there are any functional differences between Tregs in tumours where they are enriched compared to where there is no enrichment, may shed light on the mechanisms of this phenomenon.

5. Rare subsets in TIL

In addition to the effector and regulatory CD4 and CD8 T cells already investigated in the previous chapters, additional T cell subsets may be present in the tumour and have the ability to influence tumour growth. Tr1s are another subset of regulatory CD4 T cell which are thought to play a role in squamous cell carcinoma of the head and neck (Badoual et al. 2006). Th17s, on the other hand, are pro-inflammatory cells that may either promote or inhibit tumour growth (Kryczek et al. 2009a). There is little in the literature on the relative proportions and contributions of these cell subsets in RCC, so this work investigated their presence within the tumours. I also observed a significant proportion of $CD4^+CD8^+$ T cells within a subset of RCC tumours. I therefore also explored their potential function in RCC, and examined their homing phenotype to gain insight into how they may have migrated into the tumour.

Unless otherwise stated, throughout this chapter 'TIL' and 'PBMC' refer to the T cell compartment within the respective populations. All significances were calculated using Wilcoxon matched pairs tests.

5.1. Double positive $CD4^+CD8^+$ T cells in TIL

While analysing my flow cytometry data I observed that in a proportion of samples, a substantial population of $CD4^+CD8^+$, 'double positive' (DP) T cells were present. According to the literature DP T cells constitute 1-3% of human PBMC (Blue et al. 1985;Patel et al. 1989), and I saw similar proportions in the PBMC samples I studied

from RCC patients. I therefore defined DP^{hi} samples as those with 5% or more of their T cells expressing both CD4 and CD8.

I found that 35% of RCC TIL samples studied were DP^{hi}, with DPs consisting of from 5.7%-39.7% (median 11%) of T cells, whereas in matched PBMC consistently fewer than 5% of T cells expressed both CD4 and CD8 (**Figure 44**A and B). Flow cytometry showed the DP TIL displayed the same forward scatter as single positive (SP) T cells, and did not have an altered pulse width. They were therefore not an artefact of two SP T cells sticking together. The DP cells expressed high levels of CD8, however the MFI for CD4 staining was lower than that of CD4 SP T cells. Therefore the DPs in RCC are $CD4^{dim}CD8^{bright}$ (**Figure 44**C).

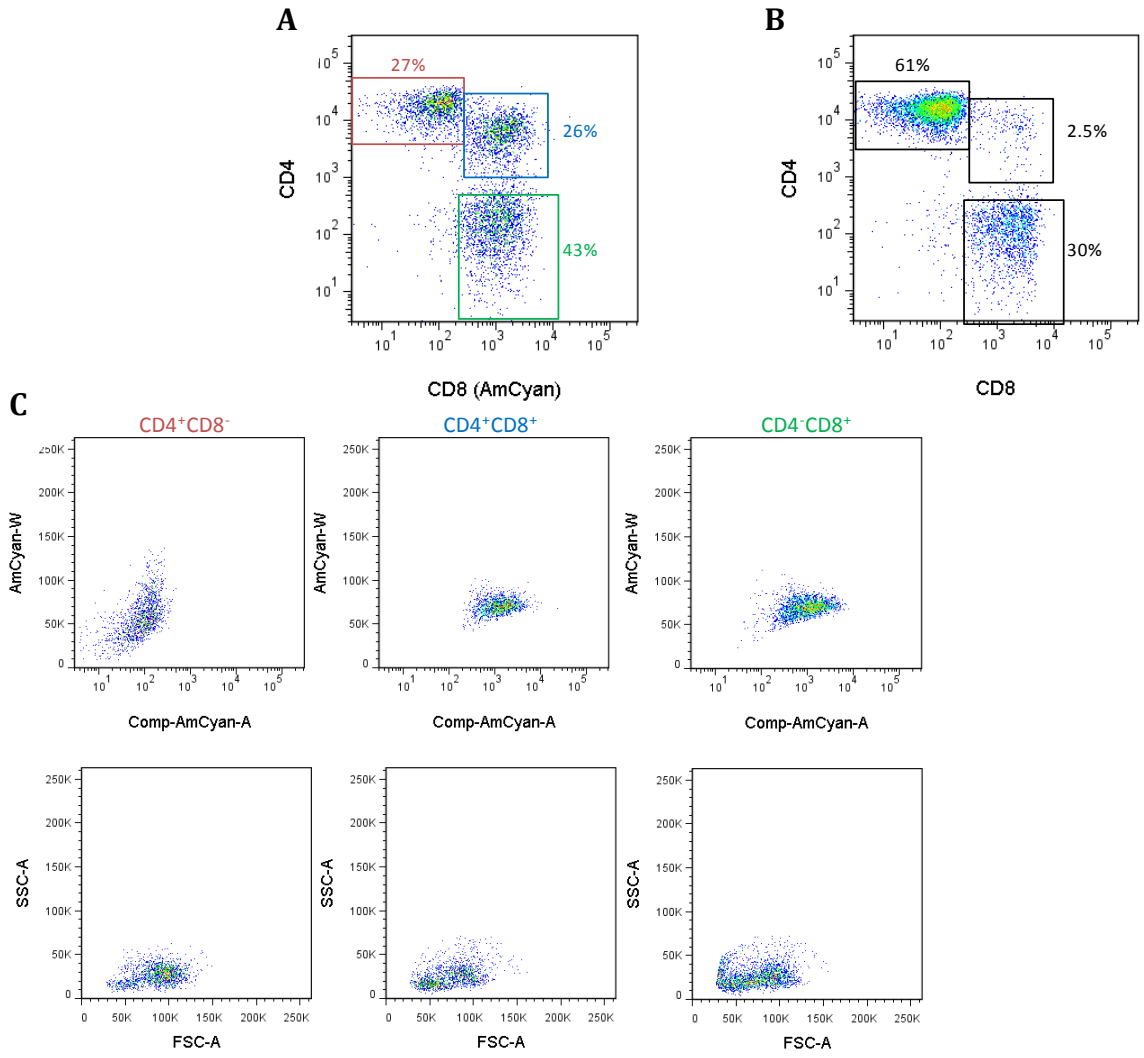


Figure 44: Double positive T cells are found in RCC TIL

Double positive T cells were found in 35% of TIL samples and had a $CD4^{dim}CD8^{bright}$ phenotype (A). Matched PBMC were not enriched with DP T cells (B). The DPs had similar pulse widths and forward scatter(FSC):side scatter(SSC) profiles to single positive T cells, indicating they are not doublets (C). Pictures representative of 18 cases.

5.1.1. Homing phenotype of DP TIL

I went on to look at the homing phenotype of the DPs to determine if they use the same mechanisms as single positive T cells to enter the tumour, or whether they have a unique homing phenotype. I compared double positive T cells to single positive T cells from the same sample to ensure any differences were not due to variations in individual's immune and tumour biology.

Data analysed during both the initial screen of TIL using the Coulter XL cytometer, and the later LSRII analysis, were used to maximise the sample size. I did not find any significant differences in the homing phenotype of DP and SP T cells, suggesting they are recruited to the tumour via the same mechanisms (**Figure 45**).

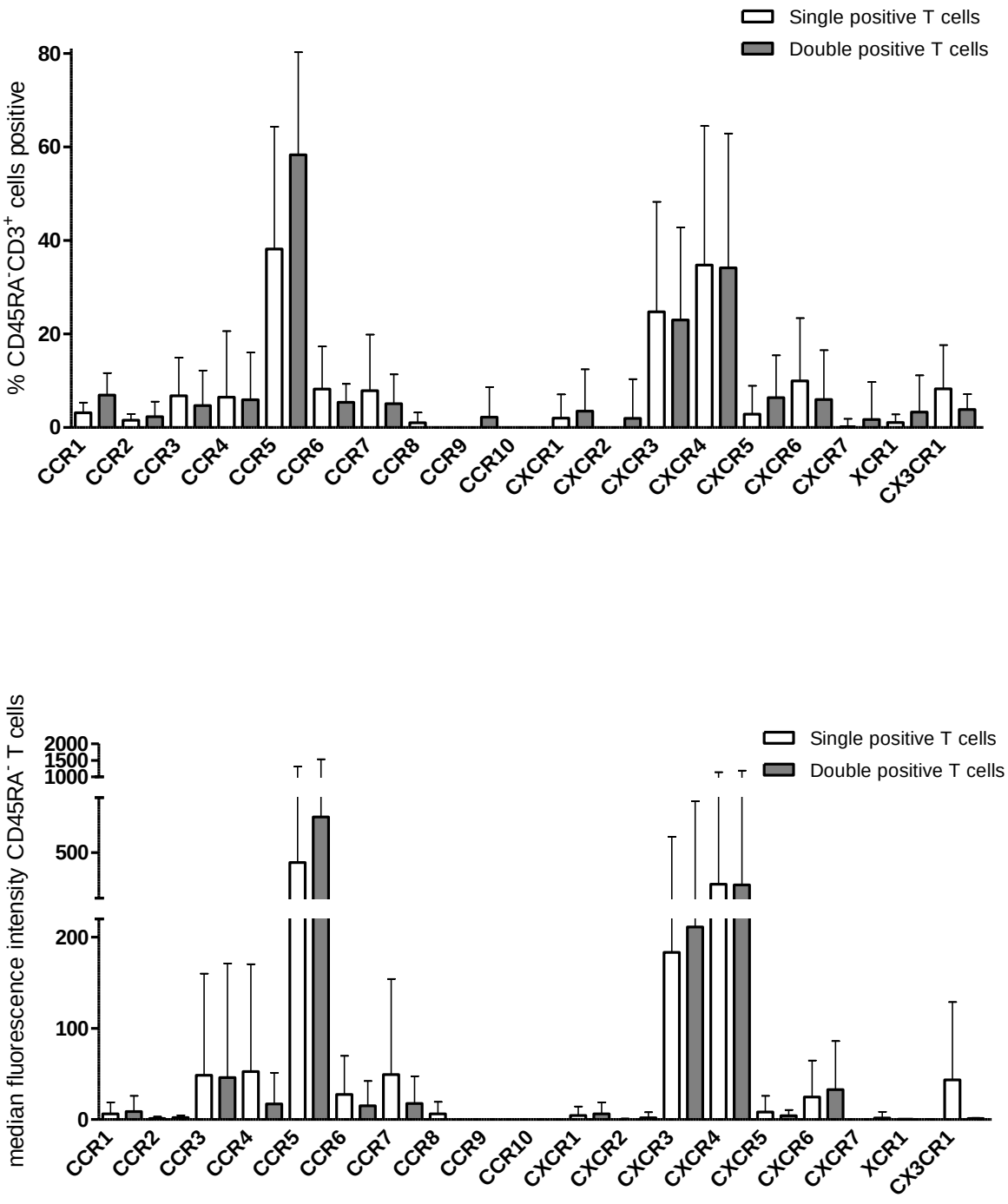


Figure 45: Comparison of the homing phenotype of SP ($CD4^+CD8^-$ and $CD4^-CD8^+$ T cells) and DP RCC TIL

RCC TIL samples were stained with antibodies specific for 19 chemokine receptors. Graphs display the percentage of T cells positive for each marker and the median fluorescence intensity of the staining, both corrected for non-specific staining using concentration matched isotype controls. The number of RCC cases stained was as follows: CCR3, 4, 5, 6, 7, CXCR3, 4, 6, n = 6, CCR1, 2, 8, 9, CXCR1, 2, 5, 7, n = 5, CCR10, XCR1 and CX3CR1, n = 4.

5.1.2. DP effector cytokine production

The literature on the function of double positive T cells is far from consistent, therefore it was unclear how the DPs in RCC TIL would function in response to stimulus. I stimulated the T cells with PMA/ionomycin and found that compared to SP CD4 T cells, production of effector cytokines was significantly lower: IFN-γ ($p = 0.0456$), IL-2 ($p = 0.0078$) and TNFα ($p = 0.0078$). CD8 SP T cells expressed similar levels of cytokine to DPs, with only IFN-γ expression being greater in SP cells and approaching significance ($p = 0.0625$) (**Figure 46**).

Interestingly, in samples containing DP T cells, fewer SP T cells produced cytokine, than SP T cells from samples without DPs (**Figure 47**). However the differences did not reach significance.

These data suggest DPs are most similar to CD8 SP T cells in their effector cytokine profile. They also indicate that samples with high levels of DPs have TIL that are less functional.

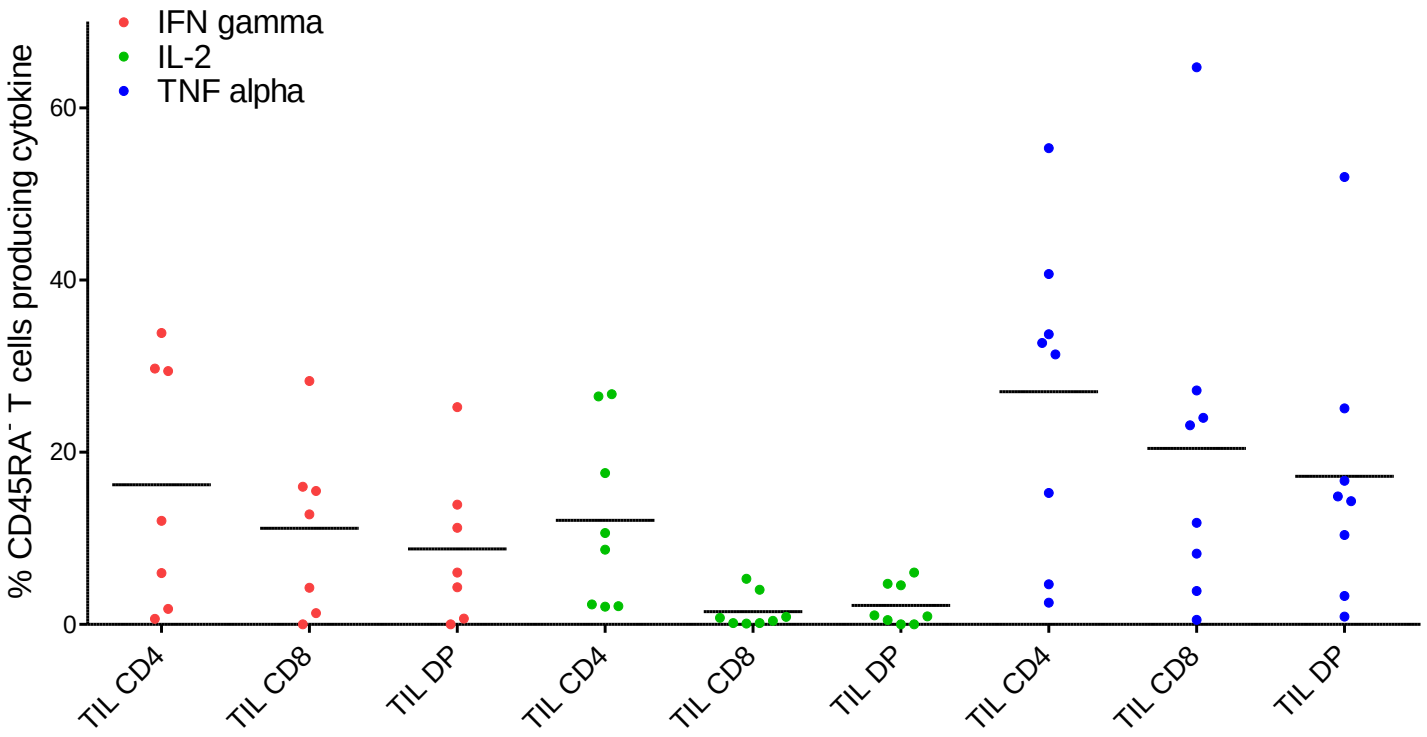


Figure 46: Effector cytokine production by SP and DP TIL

DP and SP TIL were stimulated with PMA/ionomycin for 5 hours, and the percentage of cells producing effector cytokines was measured using intracellular cytokine staining.

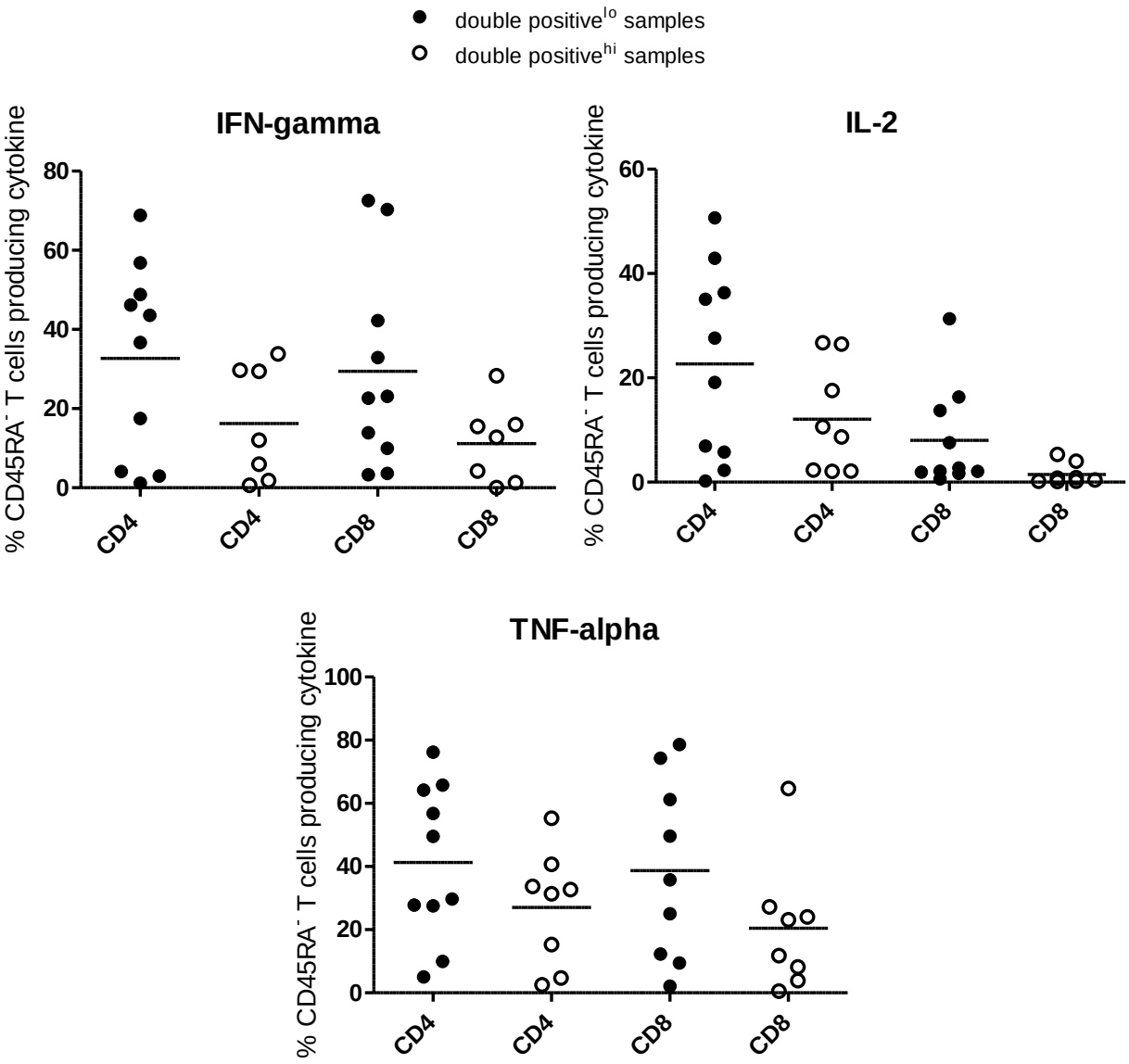


Figure 47: Effector cytokine production by SP cells from DP^{hi} and DP^{lo} samples

The effector cytokine production by single positive CD4 or CD8 T cells from samples with <5% T cells with a DP phenotype (filled circles) or >5% T cells with a DP phenotype (open circles), was measured by intracellular cytokine staining.

5.1.3. CD107a expression by DP TIL

To investigate whether the DP T cells were cytotoxic and had previously undergone degranulation, I looked at their CD107a expression *ex vivo*. The DP T cells had similar expression of CD107a to single positive CD8 T cells, but a greater proportion of DP compared to CD4 SP T cells were $CD107a^+$ (30 ± 23 % vs. 5.2 ± 4.9 %) (**Figure 48**). However, this did not reach significance ($p = 0.0625$), probably due to the small sample size ($n = 5$). Therefore the DPs appear to have a similar cytotoxic capacity to CD8 SP T cells.

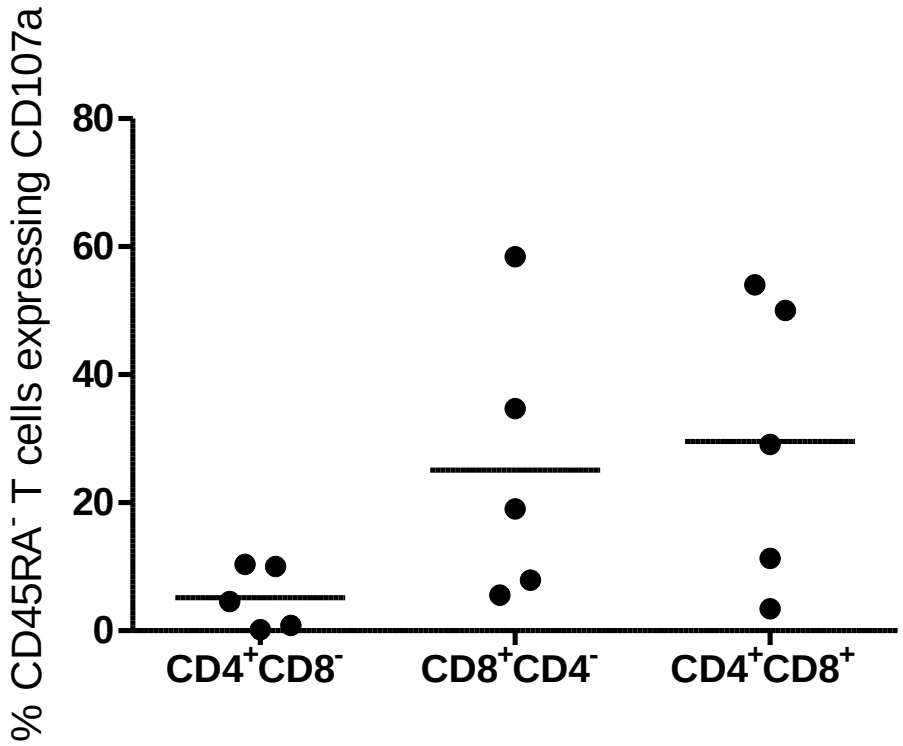


Figure 48: CD107a expression by SP and DP TIL

TIL were stained with an antibody to CD107a and the percentage of cells expressing the marker was analysed using flow cytometry.

5.1.4. PD-1 and CTLA-4 expression by DP TIL

As the double positive T cells were CD107a$^+$, I inferred they may have been activated *in vivo* prior to isolation. If so, I would also expect them to express PD-1 and CTLA-4.

The vast majority of DP T cells expressed PD-1 (87 ± 12 %), and the mean proportion of PD-1$^+$ cells was greater in the DP subset than in both the single positive CD8 subset (69 ± 18 %) and the single positive CD4 subset (38 ± 14 %) (**Figure 49**A and C). Neither of the differences reached significance (the p-value was 0.0625 for both comparisons) but again, this could be due to the small sample size (n = 5). Again, the DP T cells appear most similar to CD8 rather than CD4 SP cells.

Intriguingly, SP CD8 T cells from DP^{hi} samples contained a significantly greater proportion of PD-1$^+$ cells than CD8 T cells from samples without a double positive T cell population (**Figure 49**B). This also indicates DP^{hi} samples are more immunosuppressed.

DP T cells expressed slightly higher levels of CTLA-4 than single positive T cells (**Figure 49**D), which may indicate a greater degree of activation.

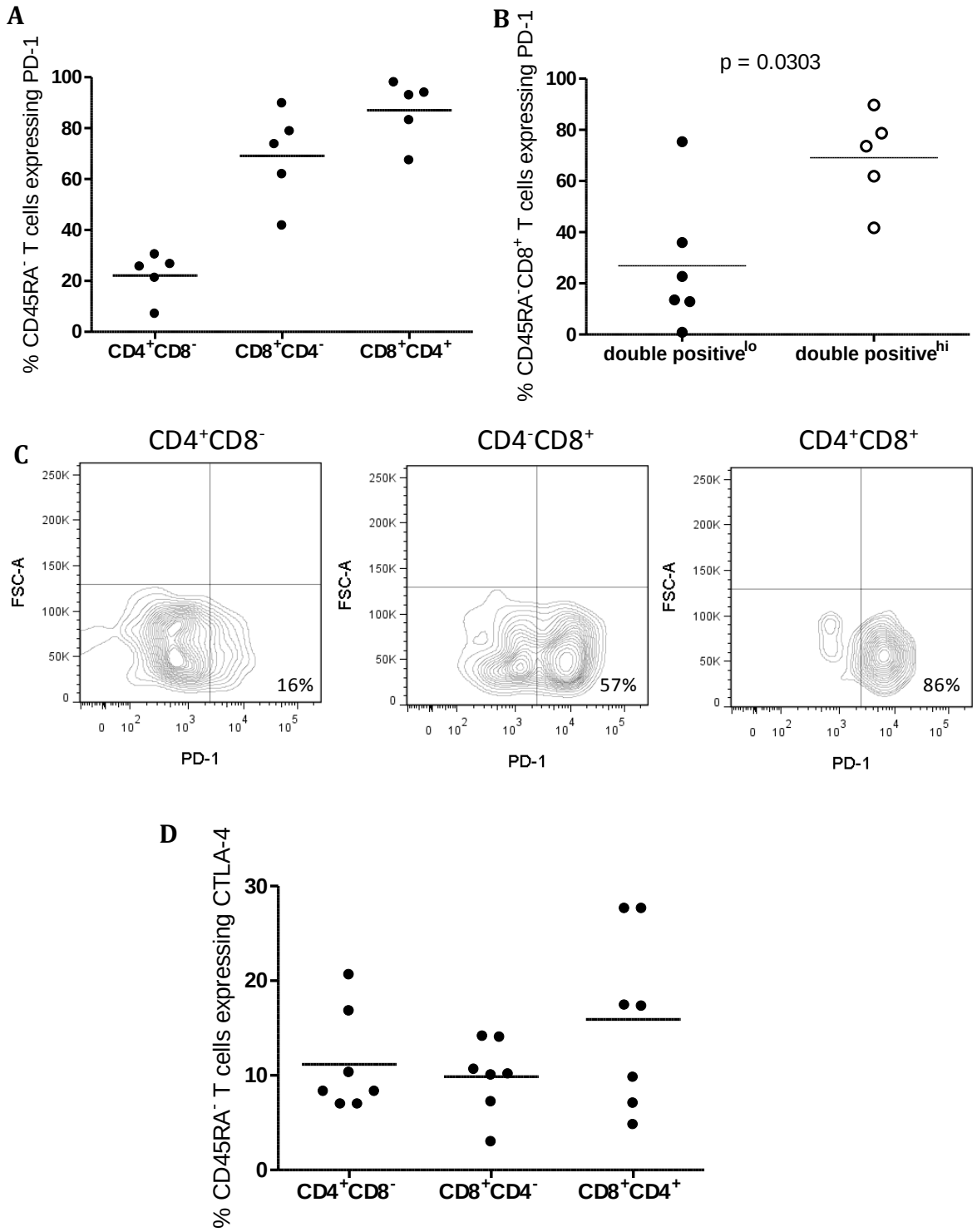


Figure 49: Comparison of PD-1 and CTLA-4 expression on SP and DP TIL

TIL were stained with antibodies to PD-1 and expression on SP and DP subsets was compared using flow cytometry (A). PD-1 expression on single positive T cells from samples with <5% T cells with a DP phenotype (filled circles) or >5% T cells with a DP phenotype (open circles), was analysed. Significance was calculated using a Wilcoxon matched pairs test (B). The proportion of PD-1$^+$ DPs was higher than that for CD4 and CD8 SP cells (C) Expression of CTLA-4 on SP and DP TIL was measured by flow cytometry (D).

5.1.5. CD27 expression by DP TIL

As discussed in chapter 4, CD27 expression gives an insight into the differentiation status of T cells. DP T cells from TIL expressed similar levels of CD27 to single positive T cells (**Figure 50**)

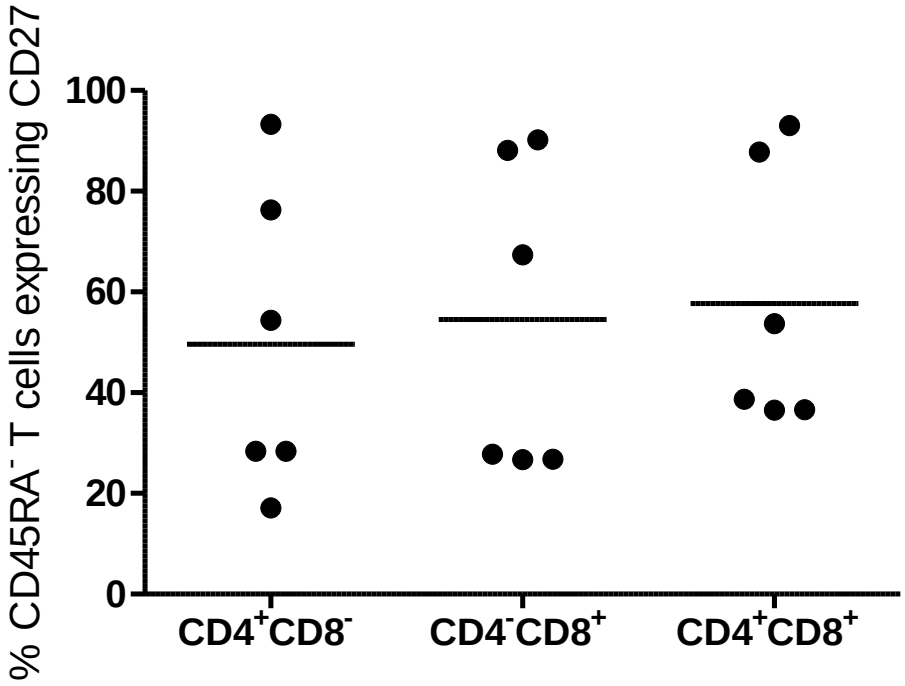


Figure 50: CD27 expression by SP and DP TIL

TIL were stained with an antibody to CD27 and expression on SP and DP TIL was analysed by flow cytometry.

5.2. Th17s do not represent a significant proportion of TIL

Th17 T cells are a subset of CD4 T cells characterized by their expression of the cytokine IL-17. Their function and contribution in tumour immunology is currently the subject of much debate.

Using intracellular cytokine staining I looked at IL-17 production by TIL and PBMC subsets with and without stimulation by PMA/ionomycin. There were no significant differences between any of the subsets tested, and only small proportions of T cells expressed the cytokine (**Figure 51**), with the highest mean expression for any subset being 3.6 ± 5.9 % (stimulated Tregs from PBMC), and even for this T cell population, half the RCC samples tested contained no IL-17-producing cells. Due to the low numbers of IL-17 producing cells in my samples, further study of these cells was impossible.

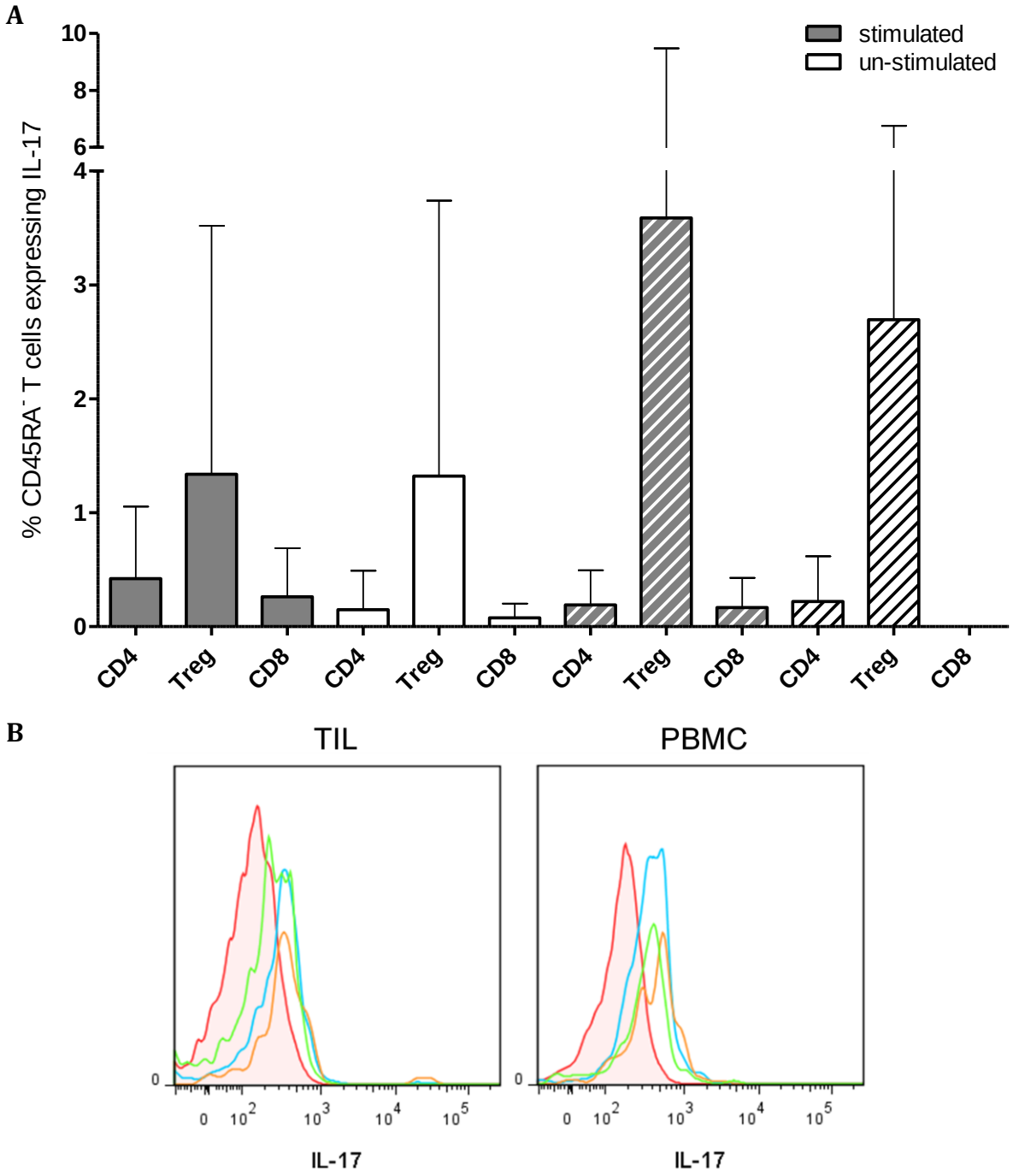


Figure 51: IL-17 expression by T cell subsets in TIL and PBMC, before and after stimulation

IL-17 expression was measured on both TIL and PBMC that had either been stimulated with PMA/ionomycin, or left un-stimulated. Results were analysed by flow cytometry. solid bars represent TIL, lined bars represent PBMC. For stimulated CD4 TIL, n = 11, for stimulated CD8 TIL and un-stimulated CD4 TIL, n = 8, for all other populations, n = 6. Results show mean percentage of cells expression IL-17 (+SD). (A). Flow plots show IL-17 staining on stimulated CD4 (blue) CD8 (green) and regulatory (orange) T cells compared to an isotype control (shaded red).

5.3. IL-10-producing T cells in RCC

IL-10 is an immunosuppressive cytokine which can inhibit the function of immune cells, including T cells. $Foxp3^+$ Tregs in the intestine produce IL-10 and this appears to be a mechanism by which they suppress effector responses (Maynard et al. 2007). However there are other $CD4^+$ suppressor T cells, known as Tr1s, that also produce IL10 but do not express Foxp3 (Groux et al. 1997;Levings et al. 2005). CD8 T cells can also be suppressive, and it has been shown that CD8 Tregs can act in an IL-10 dependent manner (Filaci et al. 2007). We therefore investigated IL-10 expression by the T cells infiltrating RCC.

I found that un-stimulated Tregs contain significantly more IL-10 producing cells than $Foxp3^-$ CD4 T cells ($p = 0.0313$), however the mean percentage of Tregs producing IL-10 was only 5.3 ± 4.2 %, therefore it is unlikely this is the dominant mechanism of suppression (**Figure 52**A).

Considering the $Foxp3^-$ population, a minority of CD4 and CD8 TIL also produced IL-10 (**Figure 52**B). CD4 TIL were the only subset which showed an increase in the proportion of cells producing IL-10 after stimulation, which approached significance ($p = 0.0547$). These $CD4^+Foxp3^-IL\text{-}10^+$ T cells do not express high levels of CD25 or IL-2, which is consistent with them being Tr1 cells (Groux et al. 1997;Levings et al. 2005) (**Figure 52**C).

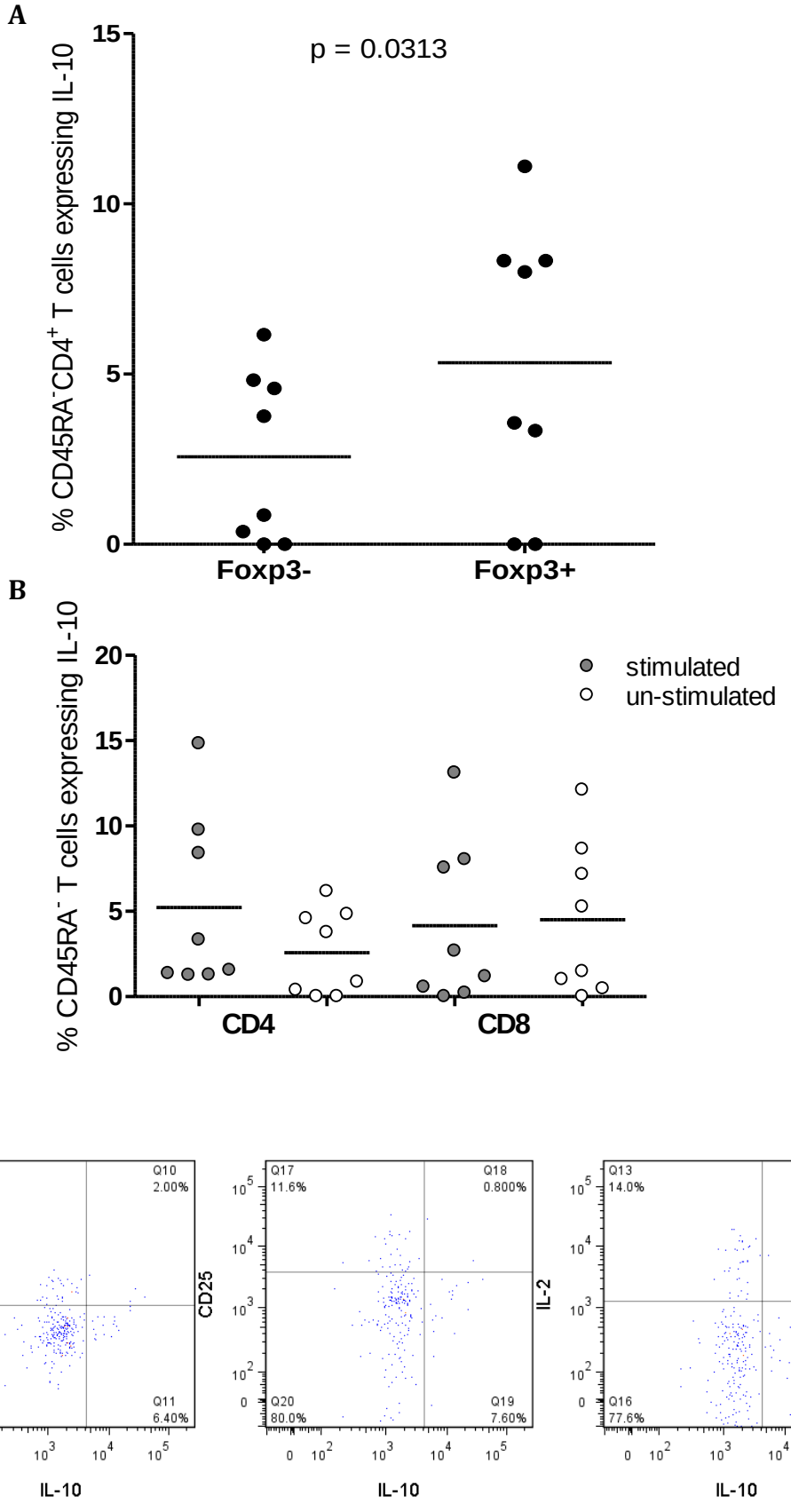

A
p = 0.0313
% CD45RA-CD4+ T cells expressing IL-10
15
10
5
0
Foxp3-
Foxp3+
B
% CD45RA- T cells expressing IL-10
20
15
10
5
0
stimulated
un-stimulated
CD4
CD8
C
Foxp3
IL-10
Q9
12.4%
Q10
2.00%
Q12
79.2%
Q11
6.40%
CD25
Q17
11.6%
Q18
0.800%
Q20
80.0%
Q19
7.60%
IL-2
Q13
14.0%
Q14
0.800%
Q16
77.6%
Q15
7.60%

Figure 52: IL-10 expression by TIL subsets

IL-10 expression by subsets of TIL was measured by intracellular cytokine staining. IL-10 expression by $Foxp3^-$ and $Foxp3^+$ CD4 TIL was compared (A) and significance calculated using a Wilcoxon matched pairs test. IL-10 expression was also analysed in $Foxp3^-$ TIL before and after stimulation with PMA/ionomycin (B). The majority of IL-10 expressing cells were $Foxp3^-$, $CD25^-$ and $IL\text{-}2^-$ (C), plot is representative of 5 experiments.

5.4. Discussion

While studying the properties of the TIL population I observed a third of RCC TIL samples contained a significant proportion of $CD4^+CD8^+$ double positive T cells. This chapter described my investigation of their recruitment and function. I also looked for the presence of other subsets of T cells in TIL, namely Th17s and IL-10 producing T cells.

Double positive lymphocytes make up around 1-3% of human peripheral blood T cells (Blue et al. 1985;Patel et al. 1989;Prince et al. 1994). The levels of DP cells in peripheral blood increase with age(Laux et al. 2000), although this only occurs in a proportion of the elderly (Ghia et al. 2007). I found a third of RCC TIL samples had high levels of DP T cells (>5% T cells), whereas levels in matched PBMC were consistently below 5%, suggesting the increased numbers in TIL were not due to the age of my patients. My data indicates the cells are not doublets, and others have demonstrated mRNA for both CD4 and CD8 in DP T cells from healthy donors (Sullivan et al. 2001).

In rare cases double positive T cells can be significantly increased in people with normal health (Kay et al. 1990;Tonutti et al. 1994). More frequently they have been reported to be elevated in certain diseases. Patients with multiple sclerosis, atopic dermatitis and systemic sclerosis have higher proportions of DP T cells in their peripheral blood or site of disease (Bang et al. 2001;Munschauer et al. 1993;Parel et al. 2007). *In vitro* infection of T cells with HTLV-1 induces a DP phenotype, transient increases in some infectious mononucleosis patients have been observed, and DPs specific for HIV in AIDs patients

have also been reported (Howe et al. 2009;Macchi et al. 1993;Ortolani et al. 1993). Elevated levels DP T cells have also been reported in nodular lymphocyte predominant Hodgkin lymphoma, T cell large granular lymphocyte leukaemia, melanoma and breast cancer (Desfrancois et al. 2009;Desfrancois et al. 2010;Karasawa et al. 2003;Rahemtullah et al. 2006).

Increased levels of DPs have also been reported in RCC. Porta et al. found a significant increase in the number of circulating DPs in RCC patients compared to age and gender matched healthy controls (Porta et al. 2007), and Van den Hove et al. reported the presence of DPs in RCC TIL (Van den Hove et al. 1997b).

The role of double positive T cells in health and disease is unclear. Studies where phenotyping has been performed found DPs to be memory T cells (Bang et al. 2001;Desfrancois et al. 2009;Nascimbeni et al. 2004;Prince et al. 1994;Rahemtullah et al. 2006;Tonutti et al. 1994;Van den Hove et al. 1997b;Weiss et al. 1998) and to express the α and β TCR chains rather than the γδ TCR (Bang et al. 2001;Desfrancois et al. 2009;Ortolani et al. 1993;Prince et al. 1994;Tonutti et al. 1994;Van den Hove et al. 1997b;Weiss et al. 1998). I examined the cytokine production of DP TIL in response to PMA/ionomycin stimulation and found DPs to be similar to CD8 SP T cells in their cytokine profile, with the exception that they may be less able to produce IFN-γ. DPs appeared deficient in effector cytokine production compared to CD4 SP T cells. I do not know whether the DP positive T cells arise from CD8 SPs gaining CD4, or vice versa,

although the DPs had more phenotypic similarities with CD8 SP T cells than CD4s. My cytokine data contrasts with that on DP cytokine production in healthy donors, breast cancer and an HIV case study, which all reported no reduction in ability to produce IFN-γ and TNFα compared to SP CD4 and CD8 T cells (Desfrancois et al. 2009;Nascimbeni et al. 2004;Weiss et al. 1998), however my data are in agreement with the only other study to address this in RCC where van den Hove et al. reported preliminary data indicating reduced cytokine production by DP cells (Van den Hove et al. 1997b). Interestingly the study on healthy donors and an HIV patient found $CD4^{bright}CD8^{dim}$ DPs, which may have a different functional profile. The DPs studied in melanoma and breast cancer were, like the ones observed in my experiments, $CD4^{dim}CD8^{bright}$, however they had been expanded by phytohaemagglutinin (PHA) treatment prior to their use in functional assays. PHA has been shown to induce CD4 expression on CD8 T cells, and therefore the cells they were studying may have been an artefact of the culture conditions (Sullivan et al. 2001).

Van den Hove's analysis of DPs in RCC TIL found $CD4^{dim}CD8^{bright}$ DPs to be present in 15/27 RCC samples studied (all the positive samples were clear cell) and $CD4^{bright}CD8^{dim}$ in 8/27 samples. They were also able to show the $CD4^{dim}CD8^{bright}$ cells express both the CD8 α and β chains. The antibody I used only binds the α chain of CD8, but Van den Hove's results suggest the DPs in my TIL samples are likely to be CD8αβ.

To further examine these cells' function I investigated their expression of CD107a, PD-1, CTLA-4 and CD27. There were no differences in CTLA-4 and CD27 expression between

SP and DP T cells, the latter in contrast to the DPs in Nascimbeni et al.'s study which found reduced CD27 on DPs compared to SPs (Nascimbeni et al. 2004). The CD107a expression on DP TIL *ex vivo* was similar to that of CD8 SP T cells, suggesting they are just as able to degranulate. This result suggests in terms of cytoxicity, the DPs behave similarly to those reported in breast cancer (Desfrancois et al. 2009), where CD107a, perforin and granzyme expression were demonstrated to be at similar levels to those seen in SP CD8 T cells. To my knowledge there is no literature on PD-1 expression by DP T cells. In RCC TIL the majority of DP T cells express PD-1, and the proportion of $PD\text{-}1^{+}$ cells was greater than that in both SP subsets.

Combined, these data suggest the DP T cells in RCC have been activated to the same degree as SP T cells, as they express similar or higher levels of CTLA-4, PD-1 and CD27. The CD107a and cytokine data suggests they have a similar function to CD8 SP T cells. The literature indicates $CD4^{dim}CD8^{bright}$ DPs arise from stimulated CD8 T cells, and therefore in RCC TIL may be an indication that a T cell response has taken place at the tumour site (Sullivan et al. 2001).

As levels of DP T cells increase with age, are more likely to be have a restricted repertoire of T cell receptor beta variable regions (TRBV) and therefore be clonal, and in healthy donors have been shown to be frequently specific for viral antigens, it could be hypothesised that they arise from chronic antigen stimulation (Ghia et al. 2007;Laux et al. 2000;Nascimbeni et al. 2004). I found that in DP^{hi} samples, a higher percentage of SP

CD8 T cells expressed PD-1 than in DP^{lo} samples. I could speculate that DPs occur in samples where T cells have repeatedly come into contact with antigen, and hence would be found in samples with a greater number of $PD\text{-}1^{+}$ T cells. The DPs themselves also had increased PD-1 expression compared to SP T cells, suggesting they have had greater chronic exposure to antigen and may therefore be exhausted. This may also explain their lack of effector cytokine production, compared to DPs isolated from other patient types described in the literature.

I investigated the homing phenotype of DP T cells to determine if they could migrate into RCC by a unique mechanism. I didn't find any significant differences between SP and DP cells' homing phenotype, suggesting they are recruited via the same receptors. It has been reported that in healthy donors greater proportions of DPs express CXCR3 compared to SP T cells (Nascimbeni et al. 2004), but I did not see this in my assays.

Th17s, a CD4 T cell subset characterised by expression of IL-17, play a role in inflammatory disease. However, as discussed in section 1.3.6.4 p31, their contribution to anti-tumour immunity is controversial.

The contribution of Th17s to anti-tumour immunity in RCC is equally unclear. My studies indicate that pre- and post-stimulation levels of Th17s were extremely low in RCC. There are very few published studies on Th17s in RCC, but one study reported that IL-17 released from Th17-like T cells caused RCC lines to increase IL-8 secretion.

Preliminary data also suggested that RCC tumours with higher levels of IL-17-producing T cells have greater levels of IL-8 mRNA expression, therefore Th17s within the tumour may alter the immune response (Inozume et al. 2009). In agreement with our data, the paper found levels of IL-17 producing cells to be around 1-3.5% of total $CD3^+$ TIL after PMA/ionomycin stimulation. Another paper reported very low numbers of IL-17 producing $CD4^+$ TIL, but not an enrichment of IL-17 producing cells compared to PBMC (Attig et al. 2009). While I cannot exclude them having an effect on tumour immunity, the numbers of Th17s in RCC precluded further study on the phenotype and function of this cell subset.

IL-10 is a cytokine that can suppress immune responses by inhibiting T cell proliferation and cytokine release (Del et al. 1993;Groux et al. 1997). $Foxp3^+$ IL-10 producing Tregs have been described in bowel disease and head and neck squamous cell carcinoma (Maynard et al. 2007;Strauss et al. 2007;Uhlig et al. 2006). However, a study of TIL from six other types of solid cancers, including RCC, concluded neither $Foxp3^+$ Tregs, nor $Foxp3^-$ Tr1 cells from TIL produce IL-10 *ex vivo* (Kryczek et al. 2009b). While I did find very small percentages of $CD4^+Foxp3^+$, $CD4^+Foxp3^-$ and $CD8^+$ T cells produce IL-10, in common with other studies (Attig et al. 2009;Kryczek et al. 2009b), the percentages were very small and therefore these cells are unlikely to be dominant suppressive populations in TIL.

6. Concluding Discussion

Harnessing the power of T cells shows promise as a treatment for renal cell carcinoma, but to date significant clinical responses have eluded scientists and clinicians. One reason for this may be insufficient tumour infiltration by anti-tumour T cells and so this study aimed to elucidate the receptors required for T cell migration into RCC. Another possibility is that immunosuppressive mechanisms within the tumour environment prevent anti-tumour T cell function. Therefore the study also examined the suppressive Treg subset within RCC and the functional capacity of TIL within this tumour.

Previous studies have found RCC TIL express CCR5 and CXCR3 (Attig et al. 2009;Cozar et al. 2005;Kondo et al. 2006;Suyama et al. 2005a). We expanded current knowledge by examining the expression of the entire repertoire of chemokine receptors on TIL and matched PBMC. Furthermore, since TIL are almost entirely $CD45RA^-$ memory cells, in contrast to previous studies we restricted our analysis to $CD45RA^-$ memory cells in both the tumour and blood. In this way we avoided any bias due to the large proportion of naive T cells carried in the blood that are known to display a different chemokine receptor phenotype to memory T cells. Our work confirmed expression of CCR5 and CXCR3 by infiltrating CD4 and CD8 T cells, and also found CXCR6 to be enriched on these populations. We also examined the homing phenotype of two additional T cell subsets which make up a significant proportion of TIL: Tregs and double positive T cells. The homing phenotype of these cells was similar to that of the whole T cell population, suggesting they are recruited via similar mechanisms. However Tregs also expressed CCR6, which may represent a unique homing mechanism for these cells.

Data on the chemokine receptors required for T cell homing to RCC will aid the development of T cell therapies for RCC. For example, it should help determine whether anti-tumour T cells generated through vaccination carry an appropriate tumour-targeting phenotype. Also, using cytokines such as IL-2 and IL-15, it should be possible to induce this homing phenotype *in vitro* on T cells used for adoptive therapy (Loetscher et al. 1998a;Unutmaz et al. 2000). Alternatively it may be desirable to block receptor function to prevent the infiltration of Tregs. Blocking CCR5, CXCR3 or CXCR6, may block the infiltration of beneficial effector T cells, so the most advantageous strategy would be to block the CCR6:CCL20 interaction.

The work described in this thesis to analyse T cell homing into tumours is an important first step to fully characterising the mechanisms involved. However, there are limitations to the approach. Most notably it is not possible to fully determine whether T cells used these receptors to migrate into the tumour, whether the receptors function in retaining the T cells at the site, or whether their expression is in fact induced at the tumour site. Nevertheless, demonstrating the ability of infiltrating T cells to migrate in response to the relevant ligands suggests that these cells at least have the capacity to be recruited via those receptors. We were also able to demonstrate the presence of the appropriate ligands within RCC tissue which is consistent with a role for CCR5, CCR6, CXCR3 and CXCR6 in T cell recruitment. Although cytokines could have upregulated expression of these chemokine receptors in the tumour microenvironment, the $CD25^{-}$ $CD27^{+}PD\text{-}1^{+}$ phenotype that we observed for many TIL suggests IL-2 is limited (Ahmadzadeh et al. 2009), so the chemokine receptors are not likely to be induced via

IL-2 stimulation. Studies of mRNA present in RCC also suggest there is little IL-2 present (Olive et al. 1998;Wang et al. 1995). IL-15 can induce expression of Th1 chemokine receptors, however in RCC it has only been demonstrated to be expressed in its membrane bound form (Wittnebel et al. 2007), and it is unclear whether this form is capable of inducing chemokine receptor expression. In addition, E. Maher's work using a microarray found IL-2 and IL-15 are not up-regulated in RCC compared to normal kidney tissue (personal communication, 2010) Now that we can hypothesise which receptors are relevant, studies in animals such as the RENCA model (Murphy and Hrushesky 1973) could confirm their role in T cell recruitment to RCC.

Of all the chemokines studied, CXCL9 was expressed most consistently by tumour cells, and may therefore explain the increased tumour infiltration of $CXCR3^+$ T cells. Nevertheless most T cells were seen on the periphery of the tumour rather than within tumour nests suggesting that T cell migration into the tumour tissue may be restricted. This might be explained by the observation that although many of the relevant chemokines were expressed on vessels within the fibrous areas, they were generally not expressed on vessels within the tumour nests. A very recent paper described the nitration of the chemokine CCL2 by the reactive nitrogen species that are present in many human tumours, and the inverse correlation between nitration of proteins in tumours and T cell infiltration. *In vitro* and *in vivo* work also demonstrated T cells have reduced ability to migrate to nitrated CCL2, and that blocking CCL2 nitration increased T cell infiltration into tumours (Molon et al. 2011). If this is applicable to RCC tumours and other chemokines, it could be hypothesised that chemokine nitration accounts for the

lack of T cell migration into the tumour nests. The inability of most T cells to infiltrate the tumour nests is relevant for immune therapy since T cell-mediated lysis of the malignant cells is likely to require cell-cell contact.

In contrast to other T cell subsets, our preliminary observations suggested that $Foxp3^+$ Tregs are capable of infiltrating the tumour nests, although a larger number of samples would be required to confirm this. This could be due to their additional expression of CCR6. DCs expressing CCR6 also infiltrate tumour nests, whereas $CCR6^-$ DCs are found at the tumour edge (Middel et al. 2010). Another possibility is that CD4 effector cells differentiate into a Treg phenotype upon migration into the tumour nest. However recent work has shown that at least in RCC patients' PBMC, $Foxp3^+$ cells co-express the transcription factor Helios, which is thought to be a marker of nTregs (Elkord et al. 2011).

The receptors CCR5, CCR6, CXCR3 and CXCR6 are frequently associated with trafficking to areas of inflammation (Luster et al. 2005), and therefore it appears RCC is mimicking an inflammatory disease. Tumours often up-regulate inflammatory cytokines and chemokines as the resulting signalling facilitates cell proliferation and survival. It is possible that the TIL themselves promote this inflammatory environment. Both their chemokine receptor profile and effector cytokine production on stimulation suggest they are type 1 T cells. Therefore if they are stimulated within the tumour they will release IFN-γ, which will in turn up-regulate the chemokines CXCL9, 10 and 11, thereby

recruiting more type 1 T cells and promoting further inflammation. This would be advantageous to the patient if the T cells were able to function and destroy the tumour, but in RCC this does not appear to be the case.

There have been reports that T cells in cancer are often dysfunctional and that in RCC CD8 T cells in particular in RCC are defective (Kudoh et al. 1997;Van den Hove et al. 1997a). However we found that, at least in response to a strong stimulus, the infiltrating T cells were capable of producing effector cytokines. Staining TIL *ex vivo* also showed they had upregulated CD107a, CTLA-4 and PD-1, all associated with antigen recognition and activation. Thus it appears that T cells entering the tumour are partly activated, possibly through antigen recognition, yet clearly they are incapable of completely destroying the malignant cells. In other disease settings, if the target antigen persists, T cells eventually become exhausted, possibly to prevent uncontrolled T cell stimulation resulting in massive lymphoproliferation and immune pathology by cytotoxicity. In the context of cancer, however, this exhaustion would allow the tumour to escape immune control. The levels of PD-1 seen on CD8 and DP TIL were high, consistent with an exhausted phenotype. PD-1 ligands are expressed at the tumour site (Thompson et al. 2006) and are therefore likely to suppress the function of PD-1$^+$ T cells. Although in RCC TIL PD-1 only appeared to negatively affect perforin expression, these assays were not done in the presence of a defined amount of PD-1 ligands, without which T cell function could have been rescued by the stimulus. Therefore further work is necessary to determine the true extent of PD-1 mediated suppression in RCC. Overall our data

suggest one of the ways the malignant cells in RCC escape immune-mediated destruction is by suppressing T cell function and inducing an exhausted phenotype.

The expression of PD-1, as with the expression of CTLA-4 by non-Tregs, also suggests there may be antigen-specific T cells amongst the infiltrate. Not all the T cells will be antigen specific as the activation of T cells can release chemokines that attract other effector memories T cells. It is unclear as to what antigen these cells may recognise, but by isolating T cells based on their PD-1 or CTLA-4 expression, could potential enrich for an anti-tumour population. The enrichment of PD-1 on double positive T cells also suggests double positive high samples potentially have more antigen-specific cells. Therefore if further study confirms PD-1^{hi} cells are more likely to be antigen specific, the presence of double positive T cells could be used as a prognositic or predictive marker, for patients more likely to benefit from an immune-boosting therapy.

Therefore therapies aimed at restoring the function of such exhausted cells (for example the use of PD1-blocking antibodies (Barber et al. 2006)) may be beneficial in this disease. Encouragingly, a phase II study using MDX-1106 (an anti-PD-1 antibody) to treat patients with treatment-refractory solid tumours, including RCC, found it had clinical efficacy against RCC and melanoma. One patient with RCC achieved a partial response that lasted at least five months (Brahmer et al. 2009). Further trials are planned, but my data supports the rationale for pursuing this line of treatment.

Interestingly the administration of IL-2 to RCC patients is the only therapy that can cure late stage disseminated disease. IL-2 has been shown to counteract PD-1-mediated exhaustion (Inozume et al. 2009). It can also substitute for insufficient help from CD4 T cells (Fearon et al. 1990) thereby improving the cytotoxic response. It could also offset the IL-2-depleting effects of Tregs which are thought to act as an IL-2 sink (Pandiyan et al. 2007). Therefore it is unsurprising that therapeutic treatment with IL-2, which has the potential to reverse all these inhibitory effects, can produce good results. Finally, IL-2 can up-regulate the chemokine receptors that our data indicate are important for T cell recruitment to RCC (Loetscher et al. 1998a;Unutmaz et al. 2000), thereby improving T cell infiltration into the tumour.

However, IL-2 therapy only works in a small subset of patients. Work is now being done on the use of IL-21 instead of IL-2. IL-21 is from the same family of cytokines as IL-2 and uses the same common γ chain receptor subunit. Encouragingly, in a study on a mouse model of thymoma IL-21 was more effective at producing a long-lasting CD8 T cell response resulting in tumour-free survival (Moroz et al. 2004). IL-21 has already gone through a phase I trial in RCC where it produced some objective responses and stable disease (Thompson et al. 2008).

Another reason for the inability of TIL to clear the tumour could be additional suppressive mechanisms employed by Tregs. We and others have found Tregs to be enriched in RCC, and we have demonstrated their numbers correlate with tumour grade.

Furthermore, others have shown their presence predicts a worse prognosis (Griffiths et al. 2007). We found that a greater enrichment of Tregs in TIL compared to PBMC correlated with both decreased cytokine production by infiltrating T cells upon stimulation *in vitro* and decreased expression *ex vivo* of CTLA-4 (a marker normally up-regulated on effector T cells following antigen recognition). In contrast, CTLA-4 was expressed at high levels on the infiltrating Tregs, where it may impair T cell priming by inhibiting co-stimulation by DCs (Qureshi et al. 2011). Infiltrating Tregs also express PD-1 which may interact with its ligand on DCs, further suppressing their capacity to stimulate T cells (Kitazawa et al. 2007).

IL-10 is produced by gut-resident Tregs (Annacker et al. 2003) and can induce an exhausted or Tr1 T cell phenotype (Brooks et al. 2006;Levings et al. 2005). We only detected expression of IL-10 by a small proportion of infiltrating RCC Tregs and Tr1 cells. However IL-10 is detectable in RCC tumours (Knoefel et al. 1997), and therefore its production by Tregs, Tr1s or another cell type may yet be influencing T cell function.

The results described above suggest there may be multiple mechanisms by which the tumour prevents immune function. Therefore a combination of therapies may be beneficial. The use of tumour specific TIL with the correct homing phenotype might be more beneficial if combined with an approach to increase T cell migration into the tumour nests. Further work is needed to determine why T cells remain on the periphery of the tumour, but the abnormal vasculature seen in RCC may be having an effect. TKIs

have been shown to normalise vasculature, which could improve T cell infiltration as other anti-angiogenic agents have been shown to increase adhesion molecule expression on endothelium and enhance T cell infiltration in a mouse melanoma model (Dirkx et al. 2006;Shrimali et al. 2010). Therefore combining ACT or other immunotherapy with TKI administration may improve response rates. However even if the T cells infiltrate the tumour, expression of PD-L1 and the presence of Tregs that we have seen in RCC may suppress a response. Early investigations using anti-CTLA-4 in RCC suggest this may aid tumour regression (Beck et al. 2006;Yang et al. 2007), and anti-PD-1/PD-L1 improves responses in mouse models of melanoma (Sierro et al. 2011). Our data supports the rationale for using these agents.

The presence of double positive T cells in RCC was unexpected and the current literature is unclear on their precise function. Our data suggest that in RCC they have an exhausted phenotype, with very high levels of PD-1 expression and reduced effector cytokine production. Notably single positive T cells from samples with high levels of DPs are also deficient in function. DP T cells are known to arise in chronic diseases, and this combined with their exhausted phenotype supports the hypothesis that RCC is mimicking a chronic inflammatory disease or viral infection. Therefore modulating the immune system to reactivate exhausted T cells may be all that is required for tumour regression, as the expression of activation markers on T cells directly *ex vivo*, combined with evidence from previous studies, suggest that tumour specific TIL do exist.

In conclusion we have found effector (CD4 and CD8), regulatory and double positive T cell subsets within RCC tissue. All the subsets appear to use the chemokine receptors CCR5, CXCR3 and CXCR6 to migrate into the tumour, with Tregs using an additional receptor, CCR6. Despite the effector T cells having the ability to produce effector cytokines *ex vivo*, they do not kill the tumour in RCC patients. Our results suggest the enrichment of Tregs at the tumour site and engagement of PD-1 ligands by effector T cells, may account for the lack of an anti-tumour response *in vivo.*

7. Future work

We have shown CCR5, CCR6 CXCR3 and CXCR6 are enriched on subsets of TIL. Further work is necessary to fully elucidate their roles in T cell homing. Due to low cell numbers we were unable to use migration assays to determine whether Tregs could migrate towards the CCR6 ligand CCL20. Larger samples may permit this and confirm the CCR6 receptor is capable of functioning when expressed by Tregs. Flow based assays looking at adhesion and transmigration of cells across an endothelial layer would be beneficial as they more accurately mimic *in vivo* cell recruitment. Selective blocking of receptors and adhesion molecules would show whether the chemokine receptors are involved in integrin-mediated adhesion and transmigration, or potentially have another role such as guiding T cells once they are within the tissue, or functioning as adhesion molecules in their own right.

In order to definitively prove the receptors are capable of facilitating T cell migration to RCC an *in vivo* model would be required. RENCA is a mouse renal cell carcinoma which has frequently been used as an *in vivo* model of RCC. In common with human RCC RENCA has infiltrating T cells and responds to IL-2 therapy. Studies on the mechanisms by which cytokines cause tumour regression have already shown that $CXCR3^{-/-}$ mice do not respond to IL-2 therapy. IL-2 increases CXCR3 expression on T cells, and their infiltration into the tumour is enhanced when CXCL9 is injected intra-tumourally (Pan et al. 2006). In addition tumour regression in the RENCA model caused by IL-12 administration is dependent on CXCL9 and CXCL10 and blocking these chemokines reduced T cell infiltration of the tumour (Tannenbaum et al. 1998). It is likely that, at

least in terms of the CXCR3:ligand axis, RENCA mimics human tumours, as patients treated with IL-12 also have increased levels of CXCL9 and CXCL10 and increased T cell infiltration of their tumours (Bukowski et al. 1999). These experiments demonstrate the involvement of CXCR3 and its ligands in T cell recruitment to RCC *in vivo*. RENCA lung metastases have been shown to express CCL3 and CCL4 and T cells expressing CCR5 have been shown to infiltrate them (Wu et al. 2008). Assuming the metastases have a similar profile to the primary tumour, this again suggests a strong similarity between murine and human RCC and may allow studies in RENCA to confirm the involvement of CCR5 in T cell trafficking. Experiments in RENCA using blocking antibodies and intra-tumour ligand injection, or chemokine and chemokine receptor knockout mice, could confirm the involvement of other chemokine receptors, especially as it is possible to track infused cells in this model.

Another approach could be to use a xenograft, as these have been shown to maintain a similar morphology to the tumours in RCC patients (Wang et al. 2010a). In addition patient T cells can then be used in the mouse model to study their function.

These experiments are particularly desirable in the light of the limitation of our assays in that we could not use the Bonferroni correction on our data due to the large number of comparisons that were being made. This work has already gone some way to test the hypotheses regarding which receptors are involved in T cell infiltration of RCC by examining tumour tissue for the relevant ligands and demonstrating the infiltration of

receptor positive lymphocytes by IHC. However *in vivo* work would give further proof of the involvement of these receptors migration to the tumour.

Ultimately however, the most relevant setting in which to study T cell migration into RCC is within the context of an adoptive T cell therapy trial, where T cells prepared for infusion could be analysed for their homing phenotype (or be manipulated to express a given phenotype), labelled (for example with Indium111 (Meidenbauer et al. 2003)) and then tracked in vivo. If tumour biopsy material is then taken post-infusion, it would be possible to check for the presence of the infused cells at this site, as well as determining their functional status.

It would be interesting to further investigate the involvement of Tregs within RCC. If further samples corroborated the preliminary data that Tregs infiltrate tumour nests more effectively than effector T cells, elucidating the mechanisms by which they do this could help both block Treg recruitment and enhance the infiltration of beneficial T cells.

Further studies using blocking antibodies and transwells could determine which mechanisms the Tregs are using in immune suppression and whether they involve cell-cell contact. These observations could also be confirmed using animal models. Blocking the PD-1:ligand interaction when stimulating TIL in the presence of PD-L1 expressing RCC cells could also more accurately determine the degree of T cell suppression or exhaustion conferred by a PD-1:PD-L1 interaction in the tumour.

Of course, it is not only T cells that infiltrate RCC and have an effect on the tumour environment. NK cells are enriched in RCC TIL compared to RCC patients' PBMC, and compared to TIL from colorectal and breast cancer (Cozar et al. 2005), and have cytotoxic potential in freshly isolated TIL (Schleypen et al. 2003). However their mechanism of homing to RCC is unknown and understanding how to improve their infiltration and anti-tumour activity could benefit patients. Macrophages and myeloid derived suppressor cells (MDSCs) also migrate to RCC and have a deleterious effect as they inhibit effector T cell function (Daurkin et al. 2011;Ochoa et al. 2007). Therefore preventing their recruitment could augment the anti-tumour response. Dendritic cells also infiltrate the tumour, however they are thought to be unable to mature properly at the tumour site (Figel et al. 2011;Gigante et al. 2009;Middel et al. 2010). Improving DC maturation and function may inhibit the down-regulation of effector T cell function seen when immature DCs interact with T cells.

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Appendix A. Correlation calculations performed

Table 1. Correlation of the percentage of TIL expressing chemokine receptors and adhesion molecules, with the Fuhrman grade of the tumour

	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CCR10	CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	CXCR6	CXCR7	XCR1	CX3CR1
r	0.359	0.549	-0.234	-0.176	0.480	0.337	0.091	0.271	-0.090	-0.075	-0.579	-0.692	0.143	0.078	0.310	0.247	-0.392	-0.099	-0.496
p-value	0.430	0.259	0.516	0.627	0.160	0.341	0.802	0.516	0.847	0.874	0.228	0.085	0.694	0.831	0.499	0.492	0.385	0.833	0.212

	cd11B/MAC1	CLA	CD62L	LFA-1	PSGL-1	VLA-4	BL-1
r	0.179	-0.894	0.123	-0.112	0.031	0.738	0.000
p-value	0.701	0.061	0.816	0.858	0.954	0.155	1.000

Table 2. Correlation calculations performed looking at the correlation between TIL phenotype and clinical characteristics, percentage of Tregs infiltrating tumour samples and the ratio of the percentage of Tregs in TIL: percentage of Tregs in PBMC

Correlation with Fuhrman Grade, r (p-value)		Correlation with % Tregs, r (p-value)		Correlation with ratio % Tregs in TIL: % Tregs in PBMC, r (p-value)	
% DPs	-0.2849 (0.2678)	CD107a CD8	0.03571 (0.9635)	CD107a CD4	0.0 (1.0365)
% Tregs	**0.7096 (0.0097)**	CD27 CD4	**0.7748 (0.0480)**	CD107a CD8	-0.2143 (0.6615)
CCR5	0.3233 (0.0761)	CD27 CD8	0.5943 (0.1667)	CD27 CD4	0.2523 (0.5943)
CCR6	-0.03945 (0.8360)	CTLA-4 CD4	0.7208 (0.0881)	CD27 CD8	0.1802(0.7131)
CD107a CD4	0.2058 (0.6615)	CTLA-4 CD8	0.3964 (0.3956)	CTLA-4 CD4	**-0.7857 (0.0480)**
CD107a CD8	0.05614 (0.9063)	CTLA-4 Treg	0.3964 (0.3956)	CTLA-4 CD8	**-0.8571 (0.0238)**
CD27 CD4	0.7094 (0.0576)	IFNg CD4	-0.174 (0.0520)	CTLA-4 Treg	0.5946 (0.1667)
CD27 CD8	0.2518 (0.5364)	IFNg CD8	-0.1794 (0.5061)	IFNg CD4	**-0.5679 (0.0272)**
CD27 Treg	0.1147 (0.9500)	IL-2 CD4	0.03922 (0.8812)	IFNg CD8	-0.5017 (0.0537)
CTLA-4 CD4	0.4619 (0.1786)	IL-2 CD8	0.009804 (0.9702)	IL-2 CD4	-0.4735 (0.0639)
CTLA-4 CD8	-0.1530 (0.6821)	PD-1 CD4	-0.6571 (0.1750)	IL-2 CD8	-0.3412 (0.1959)
CTLA-4 Treg	0.0 (1.0000)	PD-1 CD8	-0.5429 (0.2972)	PD-1 CD4	-0.3143 (0.5639)

Correlation with Fuhrman Grade, r (p-value)		Correlation with % Tregs, r (p-value)		Correlation with ratio % Tregs in TIL: % Tregs in PBMC, r (p-value)	
CXCR3	0.3265 (0.0682)	PD-1 Treg	-0.4286 (0.4194)	PD-1 CD8	-0.1429 (0.8028)
CXCR6	0.4560 (0.4103)	TNFa CD4	-0.2206 (0.3949)	PD-1 Treg	-0.5429 (0.2972)
IFNg CD4	0.122 (0.706)	TNFa CD8	-0.2824 (0.2893)	TNFa CD4	**-0.5794 (0.0187)**
IFNg CD8	0.118 (0.714)			TNFa CD8	-0.4571 (0.0867)
IL-2 CD4	0.2994 (0.3445)				
IL-2 CD8	0.3991 (0.1987)				
PD-1 CD4	0.09356 (0.8397)				
PD-1 CD8	-0.1684 (0.7131)				
PD-1 Treg	-0.775 (0.333)				
TNFa CD4	-0.02217 (0.9455)				
TNFa CD8	-0.2439 (0.4449)				

Appendix B. Correlation of lab findings with grade.

Table 1. Chemokine receptor expression on T cells from RCC TIL samples, and clinical characteristics

Sample characteristics		Percentage of CD45RA⁻LIVE T cells expression chemokine receptor, corrected for non-specific staining using concentration matched isotype controls																		
Grade	Tumour type	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CCR10	CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	CXCR6	CXCR7	XCR1	CX3CR1
2	Clear cell	9.3%	3.0%	12.1%	0.7%	28.8%	9.4%	4.8%	2.4%	-0.1%	9.7%		1.5%	6.0%	44.4%	4.1%	14.5%	-0.5%	4.3%	2.0%
2	clear cell	7.3%	8.7%	5.3%	2.0%	46.1%	11.3%	6.0%	-0.8%	12.8%	0.1%	21.7%	14.2%	0.7%	18.2%	6.8%	9.5%	9.8%	-0.2%	4.9%
2	Clear Cell	5.3%	0.7%	10.5%	3.0%	44.2%	7.6%	13.9%	6.5%	-0.3%	4.6%	4.3%	-0.3%	44.3%	21.8%	1.3%	14.1%	0.1%	3.2%	3.3%
2	Clear cell	2.5%	-2.1%	4.2%	2.4%	52.7%	9.2%	-0.5%	-0.7%	1.7%	-3.1%	1.5%	1.1%	28.2%	44.4%	-1.2%	28.5%	0.7%	-0.7%	7.0%
2	Clear cell					26.63								44.2			33.07			
2	Clear cell					39.87								63.68			66.45			
3	Chromophobe					66.21								21.98			28.192			
3	Clear cell	9.6%	4.6%	5.4%	-0.8%	35.3%	7.1%	3.9%	-5.2%	13.5%	9.6%	7.6%	3.3%	11.5%	36.2%	12.4%	35.8%	12.3%	14.3%	7.1%
3	Clear cell			25.8%	3.5%	81.0%	19.3%	10.1%						55.8%	58.8%		23.4%			
3	Clear cell			3.5%	11.9%	56.8%	19.0%	5.2%	1.9%					36.9%	45.2%		21.4%			12.1%
3	Clear cell					6427.0%								6529.0%			2314.0%			
3	Clear cell					4340.0%								4508.0%			4005.0%			
3	nd	2.3%	0.9%	3.1%	1.3%	27.6%	3.5%	1.7%	-0.7%	0.5%	-1.0%	-0.4%	-0.7%	4.6%	19.6%	0.8%	-1.0%	-0.7%	-0.8%	8.6%
4	Clear cell			13.7%	5.2%	68.5%	27.6%	11.9%						34.4%	63.2%		46.3%			
4	Papillary					28.72								12.37			27.53			
4	translocation	0.0572	0.0267	0.0116	0.0139	0.4408	0.0764	0.0436	-0.0083	-0.0066	-0.0189	0.0194	-0.0141	0.03	0.03	0.02	0.159	-0.0051	-0.0097	0.0359

Sample characteristics		Median fluorescence intensity of chemokine receptor expression, corrected for non-specific staining using concentration matched isotype controls																		
Grade		CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CCR10	CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	CXCR6	CXCR7	XCR1	CX3CR1
2	Clear cell	157	0	30	108	1920	0	0	0	4	544		172	469	1195	0	3	59	217	155
2	clear cell	114	133	133	104	987	185	142	0	255	0	379	207	56	371	121	218	249	0	212
2	Clear Cell	110	76	50	86	1529	275	69	0	0	0	55	0	906	510	24	277	15	9	43
2	Clear cell	65	0	200	76	1962	183	0	0	98	0	150	105	727	1519	0	639	96	0	275
2	Clear cell					591								1717			321			
2	Clear cell					3491								4640			611			

Sample characteristics		Median fluorescence intensity of chemokine receptor expression, corrected for non-specific staining using concentration matched isotype controls																		
3	Chromophobe					4275								1919			419			
3	Clear cell	647	143	81	447	2346	319	17	0	599	422	245	0	1762	2647	642	2567	399	607	109
3	Clear cell			278	19	1809	85	39						838	799		80			
3	Clear cell			11	32	847	30	10	8					139	327		48			77
3	Clear cell					487								1143			63			
3	Clear cell					167								174			77			
3	nd	0		25	43	1881	10	9	0	15	0	0	0	115	397	0	0	0	0	87
4	Clear cell			183	98	2371	458	120						622	1428		992			
4	Papillary					1260								1425			340			
4	translocation	198.54	126.8	0	86.08	2131.03	256.78	2.92	0	0	0	0	0	759.17	622.41	47.97	371.16	0	0	51.85

Cells are shaded according to the proportion of chemokine receptor positive cells or degree of staining as determined by median fluorescence intensity. White shading indicates the lowest levels of staining, red, the highest.

Table 2. Effector cytokine production by T cells from RCC TIL samples as measured by intracellular cytokine staining after 5 hours stimulation with PMA and ionomycin, and clinical characteristics

Sample characteristics		Mean percentage of T cells producing effector cytokines, corrected for non-specific staining using concentration matched isotype controls			
Grade	ratio % Tregs in TIL: % Tregs in PBMC	All cytokine production	IFNγ production	IL-2 production	TNF-α production
2	2.08	14.96	2.38	3.94	38.57
2	ND	12.05	5.12	1.09	29.94
2	1.05	56.92	64.73	33.84	72.21
2	43.13	1.00	0.33	1.15	1.53
2	2.35	7.28	ND	4.42	11.75
2	0.67	33.97	35.74	25.25	40.94
2	0.73	32.19	31.06	5.52	60.01
2	1.19	38.19	55.52	15.17	49.58
3	5.49	2.46	1.57	1.54	4.28
3	1.12	2.71	3.14	1.44	3.57
3	3.16	26.03	34.81	10.52	32.76
3	1.65	6.55	8.99	0.95	9.70

Sample characteristics		Mean percentage of T cells producing effector cytokines, corrected for non-specific staining using concentration matched isotype controls			
4	1.80	44.75	33.35	32.19	68.73
4	0.96	22.38	22.85	15.89	28.41
4	1.57	51.07	58.26	25.71	69.23
4	3.30	12.78	13.73	4.53	20.08
4	1.40	14.41	12.42	9.23	21.58
4	4.40	23.40	22.46	15.39	32.35

ND, no data. Cells are shaded according to the proportion of cytokine-producing T cells. White shading indicates the lowest levels of staining, red, the highest.