The tumour microenvironment influences antigen specific T cell transmigration

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Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy, May 2011

Contents

Abstract

T cell infiltration into tumours is essential for tumour antigen recognition and tumour cell elimination. The aim of this study was to develop a better understanding of T cell infiltration into tumours, focusing on two opposing arms of an immune response, anti-tumour CD8 T cells and Regulatory T cells (Tregs). Activated CD4 T helper cells are also of importance but could not be studied due to the time constraints of the project. The effect of T cell signalling at the immunological synapse following interactions between T cells and APCs presenting cognate antigen have been well studied [1]. The endothelium is neither a stereotypical APC nor simply a passive filter barrier for non-cognate infiltrating T cells. The endothelium can actively influence the development of an inflammatory response depending on the functional state of both the endothelium and interacting T cells (resting versus recently activated T cells) and the type of interactions (cognate versus non-cognate). The hypothesis was that recognition of antigens presented in the context of major histocompatibility complex (MHC) molecules by endothelium aids T cell transmigration and hence infiltration into tissues, including into tumours.

In this study, the data highlights that high avidity TRP-2 specific CD8 T cell transmigration across murine lung endothelium requires recognition of TRP-2 peptide presented by the endothelium, aiding recruitment of antigen-specific T cells into tissues in the absence of endothelial cell killing. In order for antigen specific T cells to migrate into the tumour, the tumour endothelium therefore needs to present tumour antigens.

In addition to CD8 T cells, high numbers of Tregs have been found within tumours but the key mediators for this recruitment remain uncertain. The data shows a novel mechanism for

Treg transmigration where cognate antigen-specific recognition of self-peptides was required for transmigration with preferential transmigration of Tregs across syngeneic rather than allogeneic endothelium. Upregulation of major histocompatibility complex (MHC) class II and adhesion molecules, by IFN-γ and TNF-α, together with a gradient of the tumour-associated chemokine CXCL12 were also pre-requisites for efficient Treg transmigration. Previous studies have shown that high CXCL12 expression can induce fugetaxis of tumour cells leading to efficient metastatic spread and a poor prognosis. These results would suggest that low levels of CXCL12 and recognition of self peptides presented by self MHC on endothelial cells allows efficient migration of Tregs whereas higher levels of CXCL12 may promote tumour metastases and lead to fugetaxis of the Tregs leading to an even worse prognosis.

In conclusion recognition of cognate antigen presented by endothelium enhances antigen-specific transmigration of CD8 and Regulatory T cells. This study therefore reports a novel mechanism for T cell subset infiltration into tumours where high avidity CD8 T cells require recognition of cognate tumour antigen presented on tumour endothelium in the context of MHC class I and conversely regulatory T cell infiltration into tumours depends on the repertoire of self-peptides presented on tumour endothelium in the context of MHC class II. Alteration of antigen presentation or MHC expression on tumour endothelium therefore represents a mechanism whereby T cell infiltration can be altered to re-direct anti-tumour immune responses.

Abbreviations

APC	Antigen presenting cell
ALCAM	Activated leukocyte cell adhesion molecule
Bad	Bcl-2-associated death promoter
BcL-2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
CDR	Complementarity determining regions
CEA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CTL	Cytolytic T lymphocyte
CTLA-4	Cytolytic T lymphocytes antigen 4
DAB	3, 3'-Diaminobenzidine tetra hydrochloride
DC	Dendritic cell
DISC	Death-induced silencing complex
DNA	Deoxyribonucleic acid
DNAPK	DNA-dependent protein kinase
DPX	Distyrene, plasticiser and xylene
DSS	Disease specific survival
ECs	Endotheilial cells
EDTA	Ethylenediaminetetraacetic acid
EmGFP	Emerald green fluorescent protein
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FCS	Fetal calf serum
FDA	Food and Drug Administration
FFPE	Formalin fixed paraffin embedded
FOXP3	Forkhead box P3
FR4	Folate receptor 4
GEF	Guanine nucleotide exchange factor
GITR	Glucocorticoid inducible TNF receptor
GlyCAM	Glycosylation-dependent cell adhesion molecule-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor,
GPCR	G protein coupled receptor
GSK3B	Glycogen synthase kinase 3 beta
HA	Hyaluronate
HA	Hemagglutinin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HepB	Hepatitis B
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

HPV	Human papilloma virus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
hTERT	Human telomerase
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Inter-Cellular Adhesion Molecule 1
IDO	Indolamine -2,3-dioygenase
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
Ii	Invariant chain
IL-	Interleukin
IP	Intraperitoneal injection
ITAMs	Immunoreceptor tyrosine-based activation motifs
JAM	Junctional adhesion molecule
LAG-3	Lymphocyte-activation gene 3
LB	Lysogeny broth
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MadCAM-1	Mucosal addressin cell adhesion molecule
MAP kinase	Mitogen-activated protein kinases
MDSC	Myeloid derived suppressor cells
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MLECs	Murine lung endothelial cells
MM	Malignant mesothelioma
MMP	Matrix metalloprotease
MPEM	Malignant peritoneal mesothelioma
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NBCS	New born calf serum
NFAT	Nuclear factor of activated T-cells
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NO	Nitric oxide
NSS	Normal swine serum
nTregs	Natural Regulatory T cells
NY-ESO-1	New York oesophageal squamous-cell carcinoma 1
OVA	Ovalbumin
PAP	Prostatic acid phosphatase
PARP	Poly (ADP-ribose) polymerase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed Death 1
PECAM	Platelet-endothelial cell adhesion molecule-1
PI3K	Phosphatidylinositol 3-kinase

PKB	Protein kinase B
PSGL-1	P-selectin-binding glycoprotein-1
PTGS	Post-transcriptional gene silencing
RANK	Receptor activator of NFκB
RNAi	RNA interference
RORγ	RAR-related orphan receptor gamma
SDF-1	Stromal cell-derived factor-1
SLC	Secondary lymphoid tissue chemokine
SOC	Super optimal broth
STAT-1	Signal transducer and activator of transcription-7
TAA	Tumour associated antigen
TAP	Transporter associated with antigen processing
TBS	Tris buffered saline
TCR	T cell recepter
Tfh cells	Follicular B helper T cells
TGF-β	Transforming growth factor beta
Th cell	T helper cell
TIL	Tumour infiltrating lymphocyte
TK	Thymidine kinase
TLR	Toll-like receptor
TMA	Tissue microarray
TNF-α	Tumor necrosis factor alpha
Tr1	Inducible Regulatory T cells
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	Regulatory T cells
TRP-2	Tyrosine-related protein 2
UTR	Untranslated region
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLP	Virus like particle
WT1	Wilms tumour gene 1

Acknowledgements

Firstly, and most importantly, I would like to say a huge thank you to my supervisors Prof. Lindy Durrant and Dr Judith Ramage for their continued support and guidance throughout my PhD.

I will be forever grateful to Scancell Ltd for their help and support in many aspects of my project. A particular thanks goes to Dr Victoria Pudney for her assistance in almost every aspect of my *in-vivo* work and Dr Rachel Metheringham for opening my eyes to the world of molecular biology.

A special thanks also goes to Dr Ian Spendlove and Rob Moss for their help with the *in vivo* studies.

I would also like to take the opportunity to say a big thank you to everyone within the department, without mentioning you all by name, at some point I have sought help from each and every one of you. Thanks for making my PhD an enjoyable experience.

Finally, I'd like to thank the soon to be Dr Philip Noble, without whom my PhD (and life) would never have been the same, for his continued support and advice during this period in my life.

Chapter 1: Introduction

Immunotherapy is defined as the treatment of disease by inducing, enhancing, or suppressing an immune response. The aim of cancer immunotherapy is to reject tumours by stimulating the patient's immune system to attack the malignant tumour cells that are responsible for the disease. The identification of tumour antigens, has allowed the development of antigen-specific vaccines and a number of these are to self antigens. However, the immune system is inherently programmed to regulate immune response to self-antigens in order to prevent autoimmunity. The effectiveness of anti-tumour vaccines is also limited by the evasion strategies evolved by tumours. The ultimate goal of a cancer vaccine is to stimulate the immune system and generate effector T cells that localise and kill tumours. This thesis investigates the mechanisms of immune migration into tumours and signals that alter the tumour environment.

1.1 Immunity – The lymphoid system

The adaptive immune response allows immunological memory to develop towards a particular antigen resulting in a more rapid response on second exposure. The development of effective cancer vaccines relies on the formation of this immunological memory against tumour antigens. Lymphocytes are divided into T and B cells, which bear antigen specific receptors on their surface, however this introduction will focus on T lymphocytes.

1.1.1 T lymphocytes

T lymphocytes are derived from the bone marrow and migrate to the thymus for maturation. T cells express a membrane receptor for antigen known as the T cell receptor (TCR). Unlike

B cells, T cells are unable to recognise native antigen and instead recognise short peptide sequences presented by the MHC on the surface of an antigen presenting cells (APC) forming an immunological synapse [1]. The TCR molecule exists as a heterodimer and the majority of T cells possess alpha (α) and beta (β) chains, although gamma (γ) and delta (δ) forms also exist. Each chain of a TCR contains a variable and constant region where the variable domains are responsible for recognition of peptide via three complementarity determining regions (CDRs). Associated with the TCR on the cell surface are co-receptors such as CD3 which activate intracellular signalling pathways upon TCR binding to cognate peptide presented via MHC molecules (peptide:MHC complexes) [2]. There are two main types of T cells which are dependent on the surface expression of further co-receptors CD4 and CD8 which also contribute to the MHC restriction of the T cell [3]. Helper T cells are denoted by a CD4+CD8-T cell phenotype and recognise only antigen presented in association with MHC class II. Conversely, CD4-CD8+ T cells recognise only antigen presented in association with MHC class I and are known as cytotoxic T cells (CTLs) [4]. Due to the different antigen processing pathways leading to antigen presentation in association with MHC class I and II, CD4+ and CD8+ T cells primarily respond to exogenous and endogenous antigens respectively. This difference in antigen specificity contributes to the diverse effector functions of these two sub types of T cells.

1.1.2 The Major Histocompatibility Complex

The major histocompatibility complex contains genes essential for the normal function and diversity of the immune response where MHC molecules are cell surface glycoproteins that present protein fragments (peptides) on the cell surface. The MHC complex can therefore influence immune recognition during infection and is involved in other biological processes such as mating preferences, kin recognition and pregnancy [5,6].

The MHC complex in man is called the human leukocyte antigen (HLA) system, HLA class I antigens are denoted as HLA-A, B and C with minor antigens E, F and G also present [7]. Major HLA class II genes include HLA-DP, DQ and DR. Within mice the MHC complex is called H-2 and also contains MHC class I and II molecules where class I contains two major types, H-2K and D and the class II contains H-2A and E formed by one alpha and one beta chain to make one class II molecule [8]. The murine class II MHC complex is also referred to as the I region or Ia antigens. Homozygous mouse strains are specific for one haplotype such that the haplotype for C57Bl/6 mice is $H2^b$ and for Balb/c it is $H2^d$. With the advance of transgenic mice it is now possible to generate murine models expressing human MHC molecules to aid research into organ transplantations, infectious disease, autoimmunity and cancer [9].

1.1.3 T cell education and differentiation

Burnet first proposed the idea of tolerance to self, when he suggested that autoreactive T cells are generated randomly and deleted during development of the immune system [10]. T cell education occurs within the thymus and ensures that developing T cells encounter antigens presented on self MHC molecules. This process determines the available repertoire of T cells subsequently present in the periphery as during thymic selection few of T cells that enter the thymus actually leave. Thymic selection can be divided into two main components: positive and negative selection where around 95% of the developing thymocytes are eliminated generating central tolerance of T cells to autoantigens in the periphery [11]. Positive selection results in T cells with a functional TCR [12], followed by negative selection to ensure that the TCR does not strongly recognise self antigens [13]. During development in the thymus the recognition of self peptide by CD8+CD4+ pre T cell thymocytes occurs by interaction with thymic stromal cells [14] and thymic education is determined by the affinity of this self

peptide recognition. The high rate of elimination of developing T cells is due to most thymocytes (80−90%) either failing to express a functional TCR or synthesising TCRs with little affinity for self peptide:MHC complexes which therefore die by 'neglect' due to lack of signalling to maintain cellular homeostasis [15].

A model of activation thresholds has been proposed for the generation of T cells in the thymus that depends on a threshold for T cell activation [16]. The strength (affinity) of the interaction between a TCR and MHC on an APC determines a T cells fate. If the affinity of the interaction is too low and fails to reach the threshold of activation the T cell is eliminated (positive selection). Alternatively, if the affinity is too high and therefore over the threshold of activation, the T cell is eliminated by negative selection. Natural T regulatory cells (Tregs) in the thymus are generated as a result of an interaction with self antigen that is between thresholds for positive selection and clonal deletion, insufficient to reach the threshold for thymic deletion but is insufficient for full activation of the thymocytes [17]. In this way natural Tregs form a suppressive T cell population able to regulate peripheral immune responses and prevent autoimmuity [18]. Within the periphery, just as in the thymus, activation thresholds determine T cell fate where activation over a threshold leads to an effector T cell phenotype whereas if activation falls under a threshold this results in incomplete activation and may lead to regulatory functions [19]. Defective T cell education could lead to hyperproliferation of T cells causing problems such as severe immune deficiency and autoimmune diseases dependent of T cell subsets affected.

1.1.4 T cell activation

Mature but antigen inexperienced (naive) T cells leave primary lymphoid organs and circulate between the blood and secondary lymphoid tissues [20]. T cells interact with antigen displayed on APCs within T cell dependent areas of these secondary lymphoid tissues.

Bretscher and Cohn suggested that naive precursor T cells require the generation of two signals for full activation [21]; signal 1 is generated after the interaction of the T cell receptor with its ligand, (MHC:peptide), whereas signal 2 is generated via an interaction between costimulatory molecules on the antigen-presenting cell and counter receptors on the T cell [22]. More recently a third component has been included forming signal 3 which is generated via the presence of cytokines such as IL-12. Signal 3, although not essential for T cell activation and proliferation where high antigen stimulus is present, has been shown to be necessary for full development of T cell effector function [23]. For CD8 T cells the third signal is provided by IL-12 which is necessary for proliferation and development of cytolytic function following stimulation with antigen and B7 cosimulation.

Stimulation of T cells via TCR interaction with cognate peptide presented in the context of MHC class I results in activation of the Src family kinase Lck is leading to the phosphorylation of ITAMs (immune-receptor tyrosine-based activation motifs) on the subunits (CD3-ε,δ,γ and ζ) of the TCR [24]. This interaction results in activation of the tyrosine kinase ZAP-70 and subsequently activation of the Ras signalling pathway allowing cell proliferation, cytokine production and cytotoxicity [25]. The classical secondary co-stimulatory signal is formed by binding of CD28 on a T cell to CD80 and CD86 (B7 molecules) on activated dendritic cells (DCs). CD28 signalling initiates activation of signalling cascades, phosphorylation of a tyrosine residues and activation of PI3K [26]. The PI3K-dependent signalling pathway regulates functions such as cell cycle progression, apoptosis, cellular metabolism, and IL-2 transcription (**Figure 1.1**). The absence of CD28

engagement causes T cells to become anergic [27]. The formation of an 'immunological synapse' between a T cell and an APC also includes adhesion molecule interactions, LFA-1 (lymphocyte function-associated antigen-1) and CD2 on T cells which bind to ICAM-1 (intercellular adhesion molecule type-1) and LFA-3 on APCs [28]. These same co-stimulatory cell adhesion molecules are also involved with T cell interaction with endothelial cells (ECs) and hence T cell trans-endothelial migration.

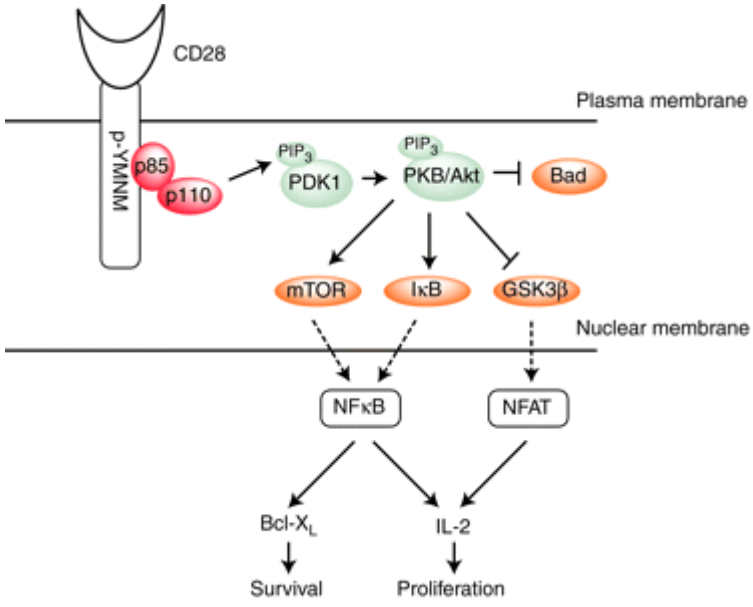

FIGURE 1.1 PI3K *dependent signal pathway following engagement of CD28 with CD80 or CD86* [26] . Tyrosine phosphorylation of the YMNM motif via Src family kinases initiates the binding of the p85 subunit of PI3K. PI3K activity leads to the production of D-3 lipids, which recruit proteins via their pleckstrin homology domain (PH), including PDK1 and PKB/Akt. Once PKB is phosphorylated by PDK1, PKB phosphorylates its downstream targets including mTOR, IκB, GSK3β and Bad. Active mTOR and IκB result in increased NF-κB transcriptional activity whereas the phosphorylation of Bad and GSK3β results in increased survival and NFAT transcriptional regulation, respectively. The activation of NF-κB and NFAT (indicated by the dotted lines) induces the transcription of both Bcl-X_L, a prosurvival factor, and IL-2, an important T-cell cytokine required for proliferation as well as other genes.

Negative regulators of T cell activation exist such as CTLA-4 [29], which also binds to B7 molecules on antigen presenting cells, and PD-1 also a member of the extended CD28/CTLA-4 family of T cell regulators [30]. CTLA-4 and PD-1 regulate T cell activation

by causing inhibition of protein kinase B (PKB/AKT) phosphorylation, however PD-1 signalling inhibits Akt phosphorylation by preventing CD28-mediated activation of phosphatidylinositol 3-kinase (PI3K) [31]. PKB/AKT phosphorylates the pro-apoptotic protein BAD [32], a pro-apoptotic member of the BcL-2 protein family, promoting apoptosis through heterodimerisation with anti-apoptotic proteins such as BcL-2 and BcL-XL [33].

1.2 CD4 and CD8 T lymphocyte subpopulations

In a simple view, MHC class I molecules present peptides from intracellular source proteins to CD8 T cells, whereas MHC class II molecules present antigenic peptides from exogenous and membrane proteins to CD4 T cells.

1.2.1 Antigen processing within CD4 T cells.

Class II molecules display exogenous peptides from proteins phago/pinocytosed and degraded in lysosomes and late endosomes. Crosstalk can occur between these two pathways and peptide presentation alters considerably upon induction of cell component degradation (autophagy). The presentation of peptides from intracellular and lysosomal source proteins has been shown to be strongly increased on MHC class II in contrast with peptides from membrane and secreted proteins [34]. MHC class II proteins contain α and β chains and they present antigen fragments to T helper cells by binding to the CD4 receptor [35]. Within T helper cells MHC class II α and β chains assemble in the endoplasmic reticulum (ER) and, together with a glycoprotein called the invariant chain (Ii), form nonameric $(\alpha\beta\text{-Ii})_3$ complexes [36]. Nonamers are then targeted to compartments of the endocytic pathway called MIICs (MHC class II compartments) where peptide loading occurs [37]. Following proteolysis Ii forms peptides called CLIP which occupy the peptide-binding groove of class II

αβ dimers and until replacement with tightly bound antigenic peptides derived from internalised antigens or endogenous proteins [38].

1.2.2 Helper CD4+ T cell subsets

Helper (Th) CD4+ T cells help both the adaptive and innate immune response by the release of different cytokine profiles designated as Th1 or Th2 type CD4 T cell subsets. The differentiation of Th1/Th2 cells is influenced by many factors such as the type and strength of co-stimulation, the dose of antigen and the cytokine milieu within the immediate microenvironment [39]. DCs influence the differentiation of Th1/Th2 effector CD4 T cells by the cytokines they produce, following CD40 or TLR ligation IL-12p70 is produced, which induces IFN-γ production and leads to a Th1 phenotype [40]. Stimulation in the absence of IL-12p70 leads to a Th2 phenotype, characterised by down regulation of Th1 responses and the production of cytokines such as IL-12p40, IL-6, or IL-10 [41]. Th2 polarisation, while occurring in the absence of IL-12, can occur in the presence of cytokines such as IL-4 [42].

In addition to different cytokine expression patterns, Th1 differentiation from naive cells is driven by TCR signalling and subsequent phosphorylation of STAT1 which induces the expression of T-bet leading to cytokine production and responsiveness to IL-12 [43]. Th1 responses producing IL-12 and IFN-γ are pro-inflammatory and essential for cell-mediated (T cell) immunity and activation of CTL responses. IFN-γ has many effects on immune responses, including upregulation of MHC class I leading to increased antigen expression on APCs and therefore improving recognition and cytotoxicity of CTLs [44]. Th2 differentiation from naive cells is also driven by STAT6 phosphorylation and leads to the induction of the transcription factor GATA3 activating production of cytokines IL-4, IL-5 and IL-13 and simultaneously down regulating STAT4 and IL-2 receptor [45]. Th2 responses help to drive humoral (antibody) responses through secretion of cytokines such as IL-4, IL-5 and IL-6 [46].

More recently further subsets such as Th17 have been defined. Th17 cells differentiate from naive cells via TGF-β and IL-6 and although this process does not require IL-23 it is essential for Th17 expansion and maintenance [47]. The orphan nuclear receptor RORγt is the key transcription factor that causes the differentiation of this effector Th17 cell lineage and is required for their expression in response to IL-6 and TGF-β enabling release of IL-17 [48]. IL-23 is also produced following activation of TLR2 and TLR4 on APCs and induces phosphorylation of STAT3 leading to further upregulation of the IL-23 receptor enabling Th17 cells to become less responsive to IL-12 and more responsive to IL-23 [49]. IL-17 produced by Th17 cells includes six IL-17 family members (IL-17A-F) which play an active role in inflammatory diseases, autoimmune diseases, and cancer by coordinating local tissue inflammation mainly via the induced release of pro-inflammatory cytokines [50].

1.2.3 Regulatory T cells

Tregs are a collective name given to cells that have the capacity to suppress T cell proliferation *in vitro* and prevent immune pathology *in vivo*. Initially named suppressor T cells [51], they are a small subset (5-10%) of the overall CD4+ T cell population which regulate immune responses by maintaining a balance between effector and regulatory T cells [52]. Tregs form a part of the control mechanisms that ensure activated immune cells do not cause a threat to homeostasis, restraining inappropriate immune responses by creating an immunosuppressive threshold. The existence of suppressor T cells in mouse models was first suggested by evidence of CD4+ T cells functioning as suppressor cells in an anti-tumour response [53], however there remained scepticism surrounding their existence for another decade as the cells could not be convincingly characterised. Evidence of the existence of these suppressor T cells began to emerge as it was demonstrated that antigen-specific suppression could be adoptively transferred by injection of T cells [54]. In 1995 high

expression of the IL-2 receptor α chain CD25 in mouse models was recognised as a phenotypic marker for CD4+ regulatory T cells [55]. Sakaguchi *et al* demonstrated that elimination of CD4+CD25+ T cells in normal naïve mice resulted in the spontaneous development of tumour-specific effector cells and elicited potent immune responses to syngeneic tumours *in vivo* [56]. In mice, FOXP3 was found to be a control gene for Treg cell development and subsequent studies confirmed FOXP3 as a specific Treg marker in humans also [57,58]. However, within humans, both CD25 and FOXP3 expression are induced following T cell activation causing difficulties in identification and isolation of Tregs [59]. On average approximately 30% of CD4 T cells express CD25 with only 1-2% with the highest CD25 expression showing suppressive activity. CD127 (IL-7 receptor α-chain), although used as a marker of conventional CD4 differentiation, has also been used as a useful alternative to CD25 for purification of human Tregs where FOXP3 expression and suppressive ability are enriched in CD4+ T cells that express low levels of CD127 [60]. Tregs have been recognised as not only having a role in maintaining self-tolerance [61] but also in having a regulatory role in the whole immune response, against infectious agents, transplantation antigens and they can also favour tumour progression by suppressing anti-tumour immune responses [62].

1.2.3.1 Regulatory T cell subsets

Uncertainty remains about the lineages, differentiation factors, antigen specificity and mechanisms of action of Tregs as cytokine expression patterns and suppressive modes of action of subsets are not mutually exclusive and often overlap. Tregs derived from thymic selection are specific for self peptides and have the ability to control autoimmunity [63] whereas Tregs that develop within the periphery will also encounter non-self or

environmental peptides and have the ability to help prevent tissue pathology during an immune response [64].

Several distinct regulatory T cell populations have been suggested (**Table 1.1**) where Tregs play a critical role in the generation and maintenance of tolerance in addition to clonal deletion and anergy. In humans, three main subsets of regulatory T cells have been described; thymus originating CD4+CD25+ natural Tregs and inducible Tr1s and Th3 cells.

Treg subset	Suggested origin	Suppressive Mechanism	Reference
CD4+ T-cell subset			
CD4+CD25+Foxp3+ (nTreg)	Thymus (Mouse and Humans)	In-vitro: cell-cell contact In-vivo: multiple modes of action	[55] [65]
CD4+CD25+Foxp3- (Tr1)	*In-vitro* (Human)	IL-10?	[66]
CD4+IL-10+Foxp3- (Tr1)	Periphery (Mouse)	IL-10	[67]
CD4+TGF-β+ (Th3)	Periphery (Mouse)	TGF-β	[68]
CD8+ T cell subset			
CD8+CD25+	Thymus (Human)	TGF-β and CTLA-4	[69]
CD8+CD28-	Periphery (Human)	Targeting ILT3 and ILT4 on DCs (Immunoglobulin-like transcript)	[70]
CD8+CD62L+CD122+	Not determined (Mouse)	Not determined	[71]
CD8+IL-10+	Periphery (Human)	IL-10	[72]

Table 1.1 ***Regulatory T cell populations.***

1.2.3.2 Natural T regulatory cells

Classically natural Tregs are thought to actively suppress the proliferation of CD4 Th cells through contact-dependent inhibition of IL-2 production [73]. CD4+CD25+ Treg suppressive

function is dependent on activation via their TCR [74] while their inhibitory effect is independent of the antigen specificity of the target T cell population. Studies with human CD4+CD25+ T cell clones confirmed that they did not produce IL-10 but that they do produce TGB-β, which was been implicated in their suppressive function [75]. Recent studies have shown that human thymically derived CD4+FOXP3+ T cells follow lineage differentiation [76] and can be distinguished using CD45RA/RO expression. CD45RA+FOXP3low CD4 T cells are designated as naive/resting Tregs with potent suppressive functions and are present in peripheral blood and prevalent in cord blood [77]. These naive Tregs proliferate after *in vitro* TCR stimulation and are highly resistant to apoptosis. Tregs with an activated phenotype CD45RA-FOXP3hi also show a suppressive phenotype and are terminally differentiated effector Tregs hyporesponsive to apoptotic stimuli. The proportions of resting or activated Tregs changes with age as natural resting Treg cells are constantly activated by endogenous and exogenous self antigens resulting in a predominantly activated Treg population within adults.

As the majority of natural Tregs are found phenotypically in an activated or antigen-primed state, it is difficult to phenotypically distinguish nTregs from other activated effector or memory T cells. FOXP3 (a forkhead family transcriptional regulator) is a key intracellular marker of natural Tregs and essential for their development and function [65]. Under non-inflamed conditions CTLA-4 (cytolytic T lymphocytes antigen 4) and GITR (a glucocorticoid inducible TNF receptor family) are also markers for natural Tregs, however these are also upregulated on effector T cells following TCR activation [78]. CTLA-4 is crucial for the suppressive function of FOXP3+ Tregs in mice both *in vitro* and *in vivo* [79]. CTLA-4 is only expressed at high levels by activated CD45RO+FOXP3hi Tregs and can inhibit T effector cell activation via regulation of CD80 and CD86 expression by dendritic cells. Another CD4+

related molecule, LAG-3, that binds MHC class II has been shown to be selectively upregulated on natural Tregs [80]. The identification of the constitutive expression of folate receptor 4 (FR4), a subtype of the receptor for the vitamin folic acid, by nTregs in high amounts has enabled a distinction to be seen between nTregs and naïve or activated T cells [81]. Treating mice with an anti-FR4 monoclonal antibody can reduce the numbers of Tregs, provoking tumour immunity in tumour-bearing animals whereas similar treatment in healthy mice caused autoimmune disease. Combinations of high or low expression of FR4 and CD25 can distinguish four functionally different CD4+ T cell subpopulations, natural Tregs (FR4hiCD25+), effector T cells (FR4loCD25+), memory-like T cells (FR4hiCD25-) and naïve T cells (FR4loCD25-).

1.2.3.3 Inducible T regulatory cells

Besides naturally occurring CD4+CD25+ Tregs, other Tregs include Tr1 cells secreting large amounts of IL-10 [82] and Th3 cells secreting high levels of TGF-β, IL-4 and IL-10 [68]. FOXP3 is not expressed by mouse Tr1s and is expressed at lower levels on human Tr1s than nTregs [83]. These inducible suppressor cells are derived from conventional CD4+CD25- T cells in the periphery by TCR activation without the need for simultaneous co-stimulation [84]. Within humans IL-10 producing Tr1 cells can be induced via stimulation of resting CD4+ T cells with anti-CD3 and anti-CD46 antibodies [85]. Following activation by antigen-specificity, Tr1 cells suppressive activity can be enhanced by bystander effects of IL-10 release [67]. Tr1 cells are characterised by high levels of IL-10, IL-5 and TGF-β and low levels of IFN-γ and IL-2 and no IL-4 expression. Tr1s also express activation markers, including CD40L, CD69, CD28, CTLA-4 and human leukocyte antigen-DR (HLA-DR) and can be induced by immunosuppressive drugs [83] and soluble protein and peptide antigens

[86]. Under chronic stimulation Tr1s can also be induced from fully differentiated Th1 and Th2 cells secreting IL-10 (detected after 4 hours and reaching a maximum concentration after 12-24 hours) without any other cytokines [87]. IL-15 supports Tr1 cell proliferation even in the absence of TCR activation and in combination with IL-2, significantly enhances the expansion of Tr1 cell clones [88]. The addition of IL-15 into Tr1 cell culture enhances the production of IFN-γ without a change in phenotype [89]. IL-10 and TGF-β expressing Tr1s can also modulate antigen presenting cells and T cells via not only cytokines but also via perforin-dependent cytotoxicity as seen in CD4+ T cells, CD14+ monocytes and DCs [90].

Inducible Th3 cells were first identified as murine TGF-β producing CD4+ Tregs and are specific for antigens not associated with any danger signals, not presented in the thymus such as food antigens, self antigens such as insulin or altered self peptides. Th3 cells suppress bystander CD4+ proliferation by IL-10 and TGF-β production [91]. Interestingly, natural Tregs also have the ability to directly suppress T cell development, leading to the induction of IL-10 secreting Tr1 cells from co-cultured conventional CD4+ T cells [92].

1.2.4 CD4 T cell plasticity

The previously described effector CD4 T cell subsets were related to the cytokines that they produce and the transcription factors expressed. In contrast to the expression of CD4 or CD8, transcriptional regulation in peripheral T cells driving gene expression profiles such as those encoding chemokine receptors and cytokines is much more variable [93] (**Figure 1.2**).

IL-12 and IFN-γ are important for Th1 cell differentiation, IL-4 drives Th2 cell differentiation and TGF-β and IL-6 together induce Th17 cell differentiation. RORγt is the transcription factor required for Th17 cell differentiation and is required for IL-17 induction. The finding that the Th1 and Th2 cytokines IFN-γ and IL-4 both inhibit induction of IL-17

further validates Th17 cells as a separate phenotype [94]. In addition to Th17 cells, TGF-β is also essential for differentiation of inducible Tregs and mice that lack TGF-β have been shown to lack FOXP3+ Tregs and Th17 cells [95].

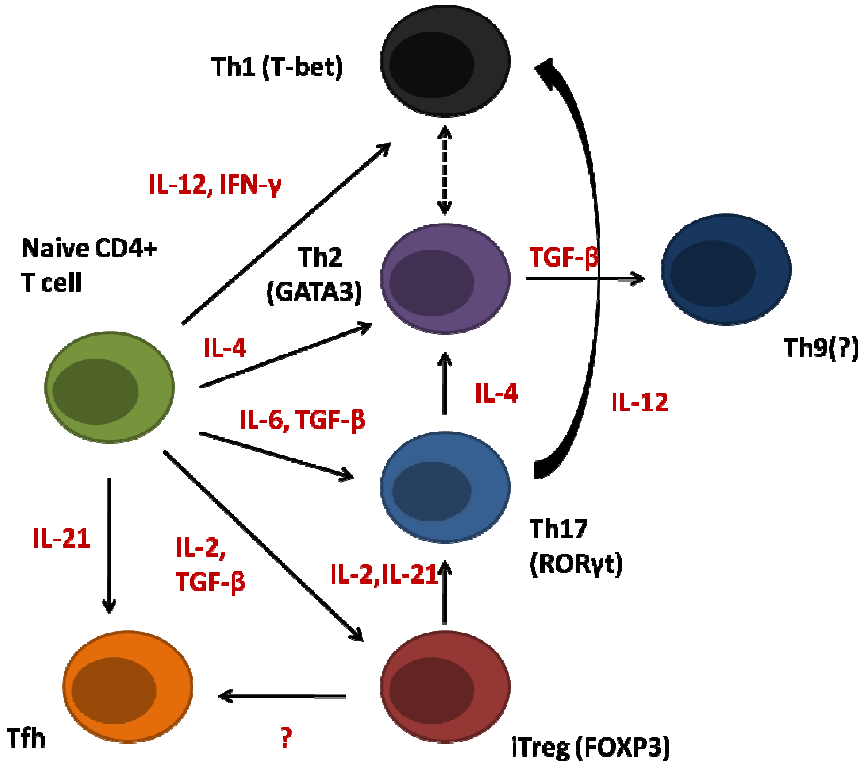

Figure 1.2 CD4+ T cell plasticity is determined by the cytokine milieus [96].
Upon encountering foreign antigens presented by antigen-presenting cells,naive CD4+ T cells can differentiate into Th1, Th2, Th17, iTreg, and Tfh cells. Conversion of naive CD4+ T cells to various recently accepted phenotypes occurs in the presence of distinct cytokine expression profiles, each regulated by separate transcription factors.

Th1 and Th2 cells are thought to display relatively stable phenotypes, inducible Tregs and Th17 cells have the ability to change to other T helper cell phenotypes under certain cytokine conditions. Inducible Tregs can become IL-17 producing cells upon stimulation of IL-6 and IL-21. Treg cells can change to T follicular helper (fh) cells requiring B cells and CD40-CD40L interaction. Re-differentiation of Tregs into effector T helper cells has been reported, maintaining FOXP3 expression while also producing IFN-γ and T-bet indicating that suppressive function is not a state of terminal differentiation [97]. Th17 cells may also

convert into IFN-γ producing Th1 cells or IL-4-producing Th2 cells when stimulated by IL-12 or IL-4, respectively. Evidence also suggests that Th2 cells can switch to IL-9 producing cells in response to TGF-β, controversy remains as to whether Th9 cells are a distinct lineage [98]. The local environment, cytokines and cell-cell contact appear to direct the fate of CD4 T cell subsets.

1.2.5 CD8 T cells

CD8+ T cells mediate the direct killing of infected host cells expressing endogenous peptides derived from viral or bacterial antigens. MHC class I molecules, are expressed on almost all somatic nucleated cells, contain an α chain & β2-microglobulin and encode heterodimeric peptide-binding proteins, as well as antigen-processing molecules [35].

Classically for endogenously synthesised proteins, the "direct" antigen processing pathway occurs within the cytoplasm. Cytosolic proteases, mainly the multi-catalytic proteasome, fragment polypeptide substrates into smaller antigenic intermediates, which are then imported into the ER by the transporter associated with antigen processing (TAP) [99]. In the ER, together with the nascent MHC I molecules, intermediate peptides undergo final trimming by proteases [100]. Stable peptides bound to MHC I molecules then leave the ER to enter the Golgi and finally reach the cell surface to be presented to circulating CD8 T cells. Professional APCs have the ability to carry out cross-presentation where exogenous antigens are presented on MHC class I molecules allowing CD8 CTLs to respond to exogenous antigens [101].

Classical cross-presentation depends on two components of the MHC class I pathway, the proteasome complex and the 'transporter associated with antigen presentation' (TAP) [102]. This proteasome dependent pathway involves the transfer of antigen from the phagosome into the cytosol [103] where the antigens are transported by the TAP complex into the ER.

Antigenic peptides are then bound by MHC class I molecules and transported to the cell surface. The phagosome-cytosol pathway is inhibited by mutations in TAP and inhibitors of the proteasome. Recently an important finding was that a protein-translocation channel called the Sec61 complex is embedded in the ER membrane and imports newly synthesised proteins into the ER and exports proteins from it, targeting the proteins for degradation by the proteasome [104]. The alternative or proteasome independent pathway for cross presentation is also called the exclusive vacuolar pathway. The proteasome-independent pathway has been shown to be used by DCs to cross-present OVA peptide (Ovalbumin) suggesting that both processing and MHC I loading could occur in the phagosome [105].The study showed peptides cross-presented by the TAP independent pathway are produced by proteolysis on the phagosome where Cathepsin S is the key protease. The nature of the antigen impacts which of these pathways can contribute to cross-presentation *in vivo* perhaps dependent on whether a particular epitope can be generated by endosomal proteases. Once activated T cells release the perforin, causing pores to form within the cell membrane, allowing granzymes to enter the cell and subsequent activation of the caspase cascade, consisting of cysteine proteases which result in cell apoptosis.

Apoptosis can also be initiated upon engagement of TCR where CTL activation causes expression of the surface protein Fas ligand which can bind to Fas molecules expressed on the target cell [106]. Fas-Fas ligand interactions are important for the disposal of non-essential T lymphocytes during their development. Engagement of Fas with FasL allows for recruitment of the death-induced silencing complex (DISC). The Fas-associated death domain (FADD) translocates with the DISC, allowing recruitment of procaspases 8 and 10. The effector caspases 3, 6, and 7 are then actvivated leading to cleavage of death substrates such as lamin A, lamin B1, lamin B2, PARP, and DNAPK and finally resulting in apoptosis.

CTLs can also recognise cells expressing altered self-antigens, facilitating the clearance of aberrantly transformed cells that could potentially be pathogenic, such as tumour cells.

1.2.5.1 CD8+ regulatory T cells

Regulatory T cells have been found to also originate from the CD8+ lineage where the CD8 T cells express CD122 and suppress the proliferation and IFN-γ production of both CD8 and CD4 target cells [71]. CD122 is the IL- 2 receptor B-chain and CD8+CD122+ Tregs have been shown to interact with activated T cells forming active regulatory cells that produce IL-10 and suppress the target cells [107].

Plasmacytoid DCs (pDC) from human ovarian tumours have been found to induce CD8 T cells expressing IL-10 which inhibit tumour associated antigen (TAA)-specific T-cell immunity *in vitro* [108]. These tumour-associated pDC induced suppressive T cells with the phenotype CD8+CD45RO+CCR7+ [109]. Suppressive CD8 Tregs potentially function in cancer patients to prevent T effector cell tumour cell killing as DC maturation state can be influenced by the tumour environment.

1.2.6 T cell memory subset differentiation

Naive T cells within secondary lymphoid organs are primed by activated APCs to become effector cells and to generate long-lived memory cells [110,111]. Stimulation with antigen for a prolonged period of at least 7 days [112], and the presence of T helper cells are required for memory cell formation [113,114,115]. Priming of T cells induces a differentiation program that involves clonal expansion and induction of effector functions where cell surface receptors expression influences migration and homing. CD45 is also known as leukocyte common antigen, a transmembrane protein tyrosine phosphatase involved in TCR signalling

[116]. The CD45 family includes three exons of the primary transcripts including CD45RA, RB and RC isoforms. CD45RA denotes naive T cells and CD45RO is located on activated and memory T cells which lack RA, RB and RC exons.

The differentiation pathway for memory subset formation is unclear, classically a linear model was suggested whereby naive T cells differentiate into effector cells and finally into a memory phenotype (**Figure 1.3**). Alternatively a divergent pathway may exist where T cells become activated without an effector function and give rise to memory cells either directly from one intermediate precursor or independently directly from an activated cell [117].

Memory T cells are divided into two main populations dependent on their location, central memory T cells (T_{CM}) are found residing in secondary lymphoid organs and are able to proliferate whereas effector memory T cells (T_{EM}) are found within the periphery with limited proliferation capacity. Sallusto *et al* first identified differences within human memory T cells via CCR7 expression [110]. CCR7 is required for homing to secondary lymphoid organs toward a CCL22 gradient and CCR7 expression denotes central memory T cells whereas CCR7- expression denotes effector memory T cells which are able to leave secondary lymphoid tissues to circulate within the periphery.

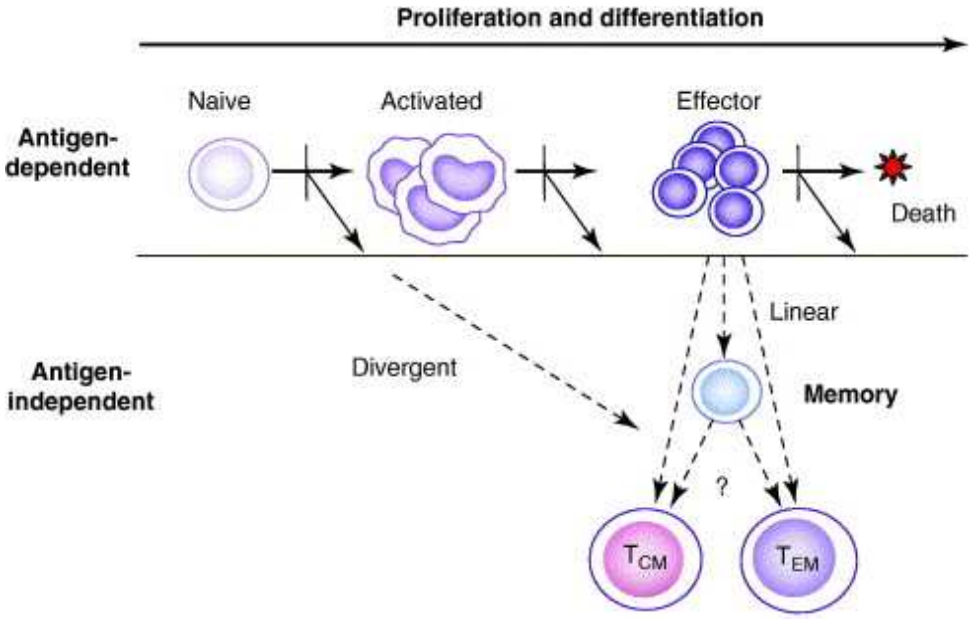

FIGURE 1.3 Potential routes of memory T-cell generation [117]. The linear pathway suggests memory T cells develop sequentially from effector cells however evidence exists for a divergent pathway where activated T cells lacking effector function can also give rise to memory cells. The arrows represent molecular and biochemical triggers for memory generation which can potentially occur before formation of an activated phenotype, after activation but prior to effector function or later prior to death. Potentially T_{CM} and T_{EM} can arise from one intermediate precursor or independently directly from one effector cell.

Both subsets produce IFN-γ but central memory T cells can also produce IL-2 enabling the proliferative capacity compared to effector memory T cells whereas the enhanced cytotoxicity of the effector memory subset can be attributed to increased perforin and granzyme B production [118]. During a secondary response to antigen within the periphery both antigen dependent and independent mechanisms can re-activate T cells as cytokines such as IL-12, IL-18 and IFN-γ via Signal 3 [119,120].

While the existence of T_{EM} and T_{CM} subsets has been proven [110], discrepancy remains in how to identify each subset, whether this should be via gene expression profiles, activation markers or cytokine release. CD44 is a receptor for hyaluronic acid and the most commonly used marker for distinguishing effector and memory CD8 and CD4 T cells from naive or antigen inexperienced cells. Once activated T cells upregulate CD44 and this expression remains high permanently [121]. For identifying murine subsets of memory T cells dual

expression of CD62L (L-selectin) and CD127 (IL-7Ra) can be used. The combined expression profile of these markers defines central memory T cells ($CD62L^{+}CD127^{+}$), effector memory T cells ($CD162L^{-}CD127^{+}$) and effector T cells ($CD62L^{-}CD127^{-}$). T cells of different memory phenotypes differ in cytokine production, proliferation capacity and cytolytic activity which is dependent on antigen interaction and time (**Figure 1.4** [122]).

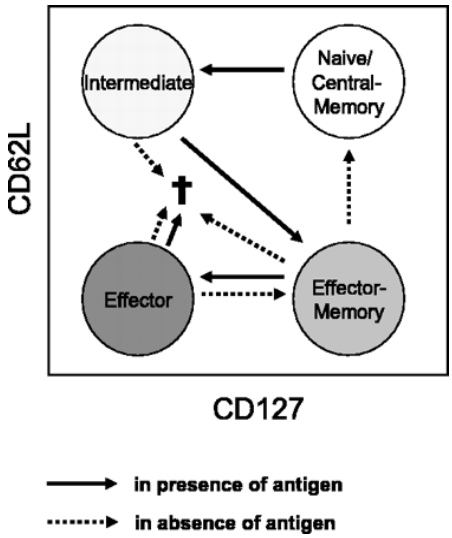

FIGURE 1.4 ***Model for in vivo differentiation phenotypes of CD8+ T cells [122]***. Naive CD8+ T cells (CD127+ CD62L+) downregulate both markers via transition of a CD127+CD62L- stage. In the presence of antigen, effector memory cells differentiate into CD127-CD62L- effector cells, which can revert to effector memory cells and eventually central memory cells in the absence of antigen.

CD62L acts as a homing receptor for leukocytes to enter secondary lymphoid tissues via interaction with GlyCAM-1, found in the high endothelial venules of the lymph nodes [123]. This CD62L expression on naive T cells therefore allows their entry into secondary lymph nodes to encounter their antigen [124]. Central memory T cells, which have encountered antigen, also express CD62L to home back to secondary lymphoid organs for re-encounter of antigen. Effector/effector memory T cells are present in the periphery ready to respond to subsequent antigen encounter, the lack of CD62L expression ensures they remain in the periphery and do not home to lymphoid organs [125]. CD127 is the IL-7 receptor α-chain and is a marker of primed $CD8^{+}$ T cells that are able to develop into long-lived memory cells

[126]. Adoptively transferred CD8 T cells with and without CD127 expression showed that CD8+CD127+ T cells survived in the absence of antigen by homeostatic proliferation and thus maintenance via CD127. CD127 down-regulation on T cells occurs during the effector phase of T cell activation whereas the formation of memory CD4 and CD8 T cells is associated with a CD127 high phenotype, where expression of CD127 determines the functional quality of antiviral T cells in both mice and humans [127].

Within humans a large number of markers can be used to distinguish memory phenotypes of CD8 T cells including CD45RA, CD27, CD62L and CCR7. CD27 is a member of the TNF receptor family and its ligand CD70 is expressed on the majority of peripheral T cells. Activation of T cells via TCR/CD3 induces high CD27 surface expression initially [128] however prolonged activation causes down-regulation. All CD4+CD45RA+CD45RO- T cells express CD27, whereas small numbers of the CD4+CD45RA-CD45RO+ subset are CD27-. In contrast, within the CD8+ population CD27 is present on both the CD45RA+ and CD45RA- subpopulations. Stimulation with CD3 monoclonal antibodies causes CD27 expression on both CD4+ and CD8+ subpopulations. CD27 has a functionally distinct role from CD28 and combining CD27 and CD28 interactions leads to an additive costimulatory effect [129]. Combining CD27 and CD45 expression patterns on T cells can distinguish between differentiation states [130]. Within CD8 T cells CD45RA+CD27+ cells are thought of as naive T cells, the loss of CD27 (CD45RA+CD27-) is associated with effector function on cells having undergone antigenic stimulation [131]. Loss of CD27 appears irreversible which supports a maturation pathway of naive to memory to effector phenotypes as central memory T cells show CD27 expression (CD45RA-CD27+). Loss of CD45RA (CD45RA-CD27-) represents a change to an effector memory function.

CCR7 is important for T cell infiltration into secondary lymphoid organs where its ligand secondary lymphoid-tissue chemokine (CCL21) is expressed in the high endothelial venules of lymph nodes and Peyer's patches and in the T cell areas of spleen and lymph nodes [132]. As previously mentioned, human naive and memory T cells can be identified by expression of the CD45RA or CD45RO isoforms. Sallusto *et al* [133] showed that double staining for CD45RA and CCR7 shows three subsets of CD4+ cells: one naive CD45RA+CCR7+ and two memory subsets, CD45RA-CCR7+ and CD45RA-CCR7-. Within CD8+ T cells, a CD45RA+CCR7- phenotype also exists.

Therefore memory subset expression of CD4 and CD8 T cells can be distinguished using the same markers; however their relative frequencies differ within peripheral blood and under immunological stress such as autoimmunity and cancer. A study by Maldonado *et al*. examined the central and effector memory phenotypes of T cell subsets in response to rheumatoid arthritis (RA) [134]. Within a cohort of 8 healthy patients the percentage of naive (CD45RA+CD62L+) was 46% and only 18% among the CD4 and CD8 populations respectively. The percentage of CD45RA-CD62L+ central memory CD4 and CD8 T cells was 37% and 6.8% respectively. The effector memory phenotype of CD45RA-CD62L- showed the least variation with 17% of CD4 and 24% of CD8 T cells showing this phenotype. Interestingly, the percentage of effector cells (CD45RA+CD62L-) T cells within the CD4 population was only 1.2% compared to 52% of the CD8 population. The study by Maldonado *et al* showed that within patients with RA T cell maturation tended towards a CD45RA–CD62L+ naïve phenotype.

Another study by Kuss *et al*. [135] looked at the numbers of effector CD8+CD45RO-CD27- T cells in patients with squamous cell carcinoma of the head and neck. The study showed a significant increase in the proportion of CD27- cells among CD8+CD45RO- T cells in the

patient and hence and increase in effector CD8 T cells. These studies demonstrate that the plasticity of the differentiation state of T cell memory subsets is highly dependent on the immunological stress within the organism.

1.3 Endothelial cells

In order for T cells to target an infection within tissues they need to migrate from the blood through the endothelium and into the tissue. Vasodilation and vasoconstriction are controlled directly by the endothelium as is smooth muscle cell proliferation and migration [136]. Vasodilation is achieved by the release of nitric oxide (NO), prostacyclin and bradykinin [137]. Prostacyclin acts synergistically with NO to inhibit platelet aggregation while Bradykinin stimulates production of tissue plasminogen activator (t-PA), forming a role in fibrinolysis. Angiotensin II controls vasoconstriction and stimulates production of endothelin which together with angiotensin II allows smooth muscle cell grow [138].

The endothelium acts as a selective barrier for leukocyte migration into tissue requiring many tightly regulated steps involving cell adhesion molecule/ligand and chemokines/receptor interactions. Early phase immune responses occur within 30-90 minutes do not involve selectin upregulation but are augmented by histamine which causes vasodilation and changes in vascular permeability. Late phase responses of around 3-4 hours following antigen challenge involve infiltration of neutrophils, eosinophils and mononuclear cells, with granulocytes 6-8 hours. 24-48 hours following antigen challenge mononuclear cells are the remaining cells infiltrating tissues.

1.3.1 Classical transmigration

The recruitment of leukocytes from blood vessels into underlying tissues is mediated by a cascade of events including initial leukocyte tethering and rolling, firm adhesion and transmigration through the endothelial cell lining. Cell adhesion molecules expressed by the endothelium control each step in the leukocyte extravasation cascade, including selectins, integrins and chemokine receptor expression. Initially selectins mediate low affinity binding of endothelial E- and P-selectin (CD62-E and CD62-P) to leukocyte L-selectin (CD62-L), P-selectin ligand (CD24) and PSGL-1 (P-selectin-binding glycoprotein-1), to allow leukocytes to roll along the endothelial surface [139]. Addressins such as PNAd (peripheral lymph node addressin), CD34, GlyCAM and MAdCAM-1 (mucosal addressin cell adhesion molecule), VAP-1 (vascular adhesion protein-1), VCAM-1 (vascular cell adhesion molecule-1) and HA/CD44 (hyaluronate) expressed by ECs can also help mediate leukocyte attachment and tethering by ligation with leukocyte LFA-1 (leukocyte function associated-1 cell adhesion molecule), α4β7 and α4β1 integrins.

Activation of ECs by cytokines or chemokines in the surrounding environment increases ligand expression on ECs and corresponding expression of leukocyte integrins, strengthening cell adhesion [140]. The expression pattern of integrins is dependent on the surrounding cytokine/chemokine milieu however the main cell adhesion molecules involved in the firm adhesion of leukocytes are members of the immunoglobulin gene superfamily: ICAM-1 and -2 (intercellular adhesion molecule-1 and -2), VCAM-1, MAdCAM-1 and PECAM-1 (platelet-endothelial cell adhesion molecule-1) binding leukocyte LFA-1, α4β7 and α4β1 integrins. Once firmly attached to the endothelium diapedesis of leukocytes across the vessel wall occurs mainly via inter-endothelial junctions by PECAM-1 [141], ICAM-2, CD99, JAM-A/B/C (junctional adhesion molecule) and VE-cadherin (vascular endothelial) [142].

PECAM and JAM-A, B and C are members of the immunoglobulin gene superfamily and form homophilic interactions between ECs. Under inflammatory conditions such as IFN-γ or TNF-α within the micro-environment, these molecules are re-arranged within the cell and JAM-A be induced to redistribute to the endothelial apical surface, whilst reducing the expression of PECAM-1. Specifically IFN-γ allows redistribution of PECAM-1 to the apical surface indicating a role in diapedesis.

1.3.2 MHC expression by endothelial cells

The constitutive expression of MHC class I and upregulation of MHC class II antigens has been shown by both human and murine ECs [143]. Human EC strongly express MHC class I and class II molecules *in vivo*, MHC class I expression is reduced and class II expression is lost *in vitro* [144,145]. Cutaneous microvascular ECs which express high levels of MHC class I and II *in vivo*, perhaps due to low levels of circulating IFN- γ, can be restored *in vitro* via addition of IFN-γ. Whereas IFN-γ impacts both MHC class I and II expression on human umbilical vein endothelial cells, IFN-α/β has been shown to enhance MHC class I antigen expression with no effect on MHC class II [146]. The function of MHC class I and II expression by dermal microvascular human ECs is thought to be to present antigen to circulating memory T cells. Following contact with T cells, ECs become activated allowing increased infiltration of memory and effector T cells via antigen independent transmigration [147].

Lidington et. al. [148] carried out a comparison of primary ECs and endothelial cell lines for expression of various cell adhesion molecules and MHC expression. Human adult ECs from aorta, coronary artery, pulmonary artery, umbilical artery were all shown to upregulate MHC class II in response to IFN-γ. However, human umbilical vein ECs, (HUVEC), were negative for MHC class II but constitutively expressed MHC class I, Fas, CD40 and LFA-3. When

treated with IFN-γ HUVEC upregulated ICAM-1, MHC class I, Fas, CD40 and LFA-3 and induced VCAM-1 and MHC class II expression. The dermal microvascular endothelial cell line (HMEC-1) was also studied and showed similar results to the HUVEC primary cultures. HUVEC do not express CD80 and, although able to induce cytokine production, are unable to differentiate naïve CD4 T cells into Th1 effector cells [149].

Murine lung ECs constitutively express low levels of CD80 molecules and MHC class II expression can be induced with culture with IFN-γ [150]. Research investigating the level of endothelial MHC class I molecule expression in diabetes-prone mice on CD8 T cell migration focused on MHC class I expression in a variety of different tissues and mouse strains [151]. The study showed tissue-specific EC heterogeneity in the expression of MHC molecules with ECs from aorta, lung, heart and thymus from non-obese diabetic mice compared for MHC class II (I-A) and MHC class I (H2-Kd). NOD ECs did not express MHC class II whereas MHC class I was expressed on all tissues with higher expression on heart ECs and the lowest expression on thymic ECs. In addition MHC class I molecule expression was found to differ between NOD (H2-Kd) and Balb/c (H2-Kd) mouse strains with higher H-2K molecule expression in NOD mice than Balb/c mice. In summary this study showed that EC co-stimulation properties *in vitro* differed depending upon the species, the maturity of the T cell, and the vascular bed of origin.

1.3.3 Antigen specific transmigration

In order for T cells to transmigrate endothelium into tissue, in addition to conventional adhesion molecule and chemokine receptor/ligand interactions [152], it has been proposed that cognate antigen presentation by ECs to T cells through peptide:MHC and TCR interaction is also an important determinant for transmigration and the development of chronic inflammation and autoimmunity [147,153]. Indeed, the level of major MHC class I

expression on the endothelium in non-obese diabetic mice has been shown to influence islet auto-reactive CD8 T cell adhesion and migration [151] and antigen-specific T cells can be attracted and retained in tissues via antigen present within the tissue [154].

An *in vivo* model of HY-specific CD8 T cell transmigration into antigenic and non-antigenic tissue showed an accumulation of the male-specific HY antigen-specific CD8 T cells in the peritoneal cavity of IFN-γ treated male mice and not untreated male or IFN-γ treated female mice [155]. The data suggests that up regulation of H2 molecules by IFN-γ in peritoneal mesothelium, 72 hours prior to additions of T cells, led to recruitment of PKH26 labelled HY specific T cells in male and not in female mice. Intravital microscopy of the peritoneal membrane indicated that cognate recognition enhances diapedesis into tissue without affecting rolling and adhesion. Marelli-Berg hypothesised that due to the small molecular size of MHC:peptide complexes would mean that TCR triggering would only occur when the two cells were in close proximity, i.e. once rolling and adhesion had already occurred and therefore antigen recognition does not affect adhesion.

1.4 Chemokines

Chemokines are secreted proteins involved in immunoregulatory and inflammatory processes which play a major role in leukocyte trafficking via their interaction with a group of seven transmembrane G protein-coupled receptors (GPCR) [156]. Over 50 chemokines have been identified forming a family of heparin-binding cytokines with over 20 receptors, identified by the position of cysteine residues (C, CC, CXC and CX3C) near the N termini of each protein. Chemokine involvement in leukocyte trafficking has been studied under homeostatic conditions and during an inflammatory responses. Chemokines have also been identified

within tumour micro-environments and more recently implicated in organ-selective cancer metastasis.

Chemokine receptor and ligand binding causes phosphorylation of C-terminal serine/threonine residues that dissociate heterotrimeric G-proteins into α and βγ subunits. This inhibits adenylyl cyclase activity, increases the generation of inositol trisphosphate, intracellular calcium release and the activation of phosphatidyl inositol 3 kinase (PI3K)/Akt cascade and Ras/MAP kinase signalling [157]. During non-inflammatory conditions peripheral T cell homeostatic trafficking to lymphoid organs is mediated predominantly by CCL19 and CCL21. CCL19 is a small cytokine belonging to the CC chemokine family that is also known as EBI1 ligand chemokine (ELC) and macrophage inflammatory protein-3-beta (MIP-3-beta). CCL21 is also known as secondary lymphoid-tissue chemokine (SLC) and is expressed in the high endothelial venules of lymph nodes and Peyer's patches, in the T cell areas of spleen and in the lymphatic endothelium of multiple organs [132]. CCL19 is mainly expressed in the thymus and lymph nodes [158,159], with lower levels in the trachea and colon and even lower levels in stomach, small intestine, lung, kidney and spleen. CCL19 and CCL21, bind to the chemokine receptor CCR7 attracting certain cells of the immune system [159], including DCs, B cells and CCR7+ naive and central memory T Cells.

1.4.1 Chemokine expression in cancer

The tumour microenvironment, which is composed of immune cells, tumour cells, stromal cells and the extracellular matrix, is under constant change during the neoplastic process, to allow proliferation, survival and migration of tumour cells. Tumours survive and are able to spread as they can hijiack signalling pathways used by immune cells favouring tumour immune tolerance and resulting in escape from anti-tumour immunity.

Chemokine expression in tumours can be investigated from two perspectives, the first being chemokine expression within the tumour itself and consequential migration of leukocytes bearing the corresponding receptors. In this situation tumour regression can be correlated with the type and frequency of leukocyte infiltration. Alternatively, the tumour cells may also express chemokine receptors allowing migration to sites of metastasis where the corresponding chemokine is present. *In vivo* a combination of chemokine/receptor expression within tumours will affect immune recognition, tumour progression and ultimately patient prognosis.

Tumour associated chemokines are not produced in isolation and chemokine expression profiles will exist for different types of cancer. The CXCR4/CXCL12 axis is a key factor in tumour metastasis of many cancer types where cancer cells show CXCR4 expression and corresponding expression of CXCL12 is seen at sites of tumour metastasis [160,161,162]. CXCL12 binds to G-protein-coupled CXCR4 [163].

The homeostatic chemokine receptor CCR7 has been shown to be expressed in tumours such as breast and oesophageal squamous cancer, where CCR7 expression increases tumour metastasis towards the ligand CCL21 [164,165]. In breast cancer patients the chemokine CXCL13 and its receptor CXCR5 have been shown to be over-expressed within the tumour and in the peripheral blood where expression is associated with metastasis [166]. CCR4 and CCR3 expression in adult T cell leukaemia/lymphoma and corresponding ligand expression, CCL17 and CCL11 respectively, is associated with reduced survival [167,168]. Curiel *et al* [169] first showed that Tregs expressing CCR4 infiltrate ovarian tumours mediated by CCL22/17 expression.

Chemokines can affect tumours in four ways; firstly by altering the extent and type of leukocyte infiltration, secondly by promoting tumour angiogenesis, thirdly by controlling tumour cell metastasis and finally by affecting tumour cell proliferation [170].

1.4.2 Chemokines and leukocyte infiltration into tumours

Curiel *et al* [169] first showed that Tregs expressing CCR4 preferentially infiltrate ovarian tumours in response to CCL22. CXC chemokines, CXCL9, CXCL10, and CXCL11 are induced by IFN-γ and are typical chemoattractants of NK cells and the expression of CXCR3 on NK cell allows infiltration into tumours expressing CXCL10 [171]. In contrast, to infiltration of Tregs, overexpression of these chemokines leads to increased cytotoxic responses and long-term anti-tumour immunity.

CCL2 monocyte chemotactic protein-1 (MCP-1) contributes to the progression of colorectal cancer by influencing the number and distribution of tumour associated macrophages (TAMs) [172] and the same chemokine leads to NK cell infiltration into neuroblastomas [173]. Tumour infiltration by macrophages is associated with neoangiogenesis and reduced survival, whereas the effect of DC infiltration is less clear. DC infiltration is associated with a positive clinical outcome in association with their ability to present tumour antigens and induce specific anti-tumour T cell responses. However, studies have reported that patients with a variety of cancers have impaired DCs [174]. Within non-small cell lung cancer infiltrating DCs were kept in an immature state and migrated in response to CCL21 [175]. Moreover, recent studies have suggested that, instead of initiating immune responses against tumours, DC in the tumour microenvironment may have the ability to suppress the responding T cells and induce tolerance [176].

CXCL16 had been recently discovered and its receptor CXCR6 is detected on both CD4 and CD8 T cells, and NK cells. A significant correlation between CXCL16 expression by tumour cells and the infiltration of T cells has shown a better prognosis in colorectal cancer [177]. Within murine models of melanoma and ovarian cancer indicate that the effect of CXCL12 on the tumour immune response is dose dependent [178]. Low levels of CXCL12 expression allows infiltration T cells into tumours reducing tumour growth, whereas high levels of

CXCL12 have been shown to be a biomarker for clinical progression in ovarian cancer, that is correlated both the HPV (human papilloma virus) infection and the accumulation of $FOXP3^+$ T cells within ovarian tumours [179]. Therefore, patient outcome is dependent on which subsets of inflammatory cells infiltrate into tumours, subsequently dependent on the expression of particular chemokines, either positively or negatively responding to the tumour [180].

1.4.3 Chemokines and tumour angiogenesis

Angiogenesis is the formation of new capllaries from existing blood vessels and enables tumour cell growth and survival. Angiogenesis is a highly regulated process involving chemokines such as CXCL1, -2, -3, -5, -6, -7, and -8 which directly cause chemoattraction of ECs and can stimulate angiogenesis *in vivo* [181]. Chemokines can also be anti-angiogenic such as CXCL9 and CXCL10 [182]. The difference in the angiogenic capacity of chemokines has been attributed to the amino acids (Glu–Leu–Arg/ELR) immediately amino-terminal to the CXC motif (ELR+) which are angiogenic and that ELR− CXC chemokines are angiostatic [183].

Tumour stroma forms an important part of the tumour microenvironment and stromal fibroblasts within breast tumours have been shown to produce CXCL12 leading to vascularisation by chemoattraction of ECs [184,185]. Hypoxia has also been shown to induce CXCL12 expression within primary human ovarian tumour cells by Hypoxia-inducible factor (HIF)-1 expression [186]. Tumour angiogenesis promoted by hypoxia, results in the expression of angiogenic factors, such as vascular endothelial growth factor (VEGF), IL-8, and basic fibroblast growth factor (bFGF). Monocytes are continually recruited into tumours and are able to differentiate into tumour associated marcophages (TAMs). Tumour infiltrating

TAMs are found to localise in hypoxic areas in response to CCL5 and CCL2 and are thought to contribute to tumour angiogenesis via secretion of CXCL8 and VEGF [187].

1.4.4 Chemokines and cancer metastasis

Cancer metastasis allows tumours to migrate and re-colonise within organs or sites that support the growth of cancer cells. Stephen Paget's 1889 proposal was that metastasis depended on interactions between selected cancer cells (the 'seeds') and specific organ microenvironments (the 'soil'). Whereas Ewing theorised that the patterns of blood flow from the primary tumour can predict the first metastasised organs [188]. More recently it is thought that cancer cells migrate to specific organ sites as a result of complex signalling between the tumour cells and the cells at the organ site.

Tumour cells use the same chemokine mediated mechanisms to those that regulate leukocyte trafficking during the process of metastasis [189]. The CXCR4/CXCL12 axis has been shown to be involved in all stages of tumour growth and progression and its role in metastasis is no exception. CXCR4 and CCR7 are highly expressed in human breast cancer cells and can induce metastasis by enabling tumour cells to migrate via actin polymerisation and pseudopodia formation to organs that produce CXCL12 and CCL21 such as lymph nodes, bone marrow and lungs [164]. As well as tumour cells migrating to organs as sites of metastasis, cancer cells also traffic to lymph nodes under the control of the same lymph node homing chemokines as lymphocytes. The expression of CCR7 in gastric cancer cells has revealed its critical role in the metastasis to lymphoid organs of gastric cancer cells in response to CCL21 [190].

1.5 Immune surveillance

The immune surveillance theory was first proposed by Burnet and Thomas who used experimental evidence from tumour transplantation models to prove that tumours could be repressed by the immune system [191]. Immune surveillance implies that spontaneous tumour development can be prevented by the immune system by the elimination of neoplastic cells early in their development. Evidence was provided using murine models of cancer, formed using methylcholanthrene to initiate spontaneous tumours, whereby tumour formation and growth could be measured following depletion of immune cells such as NK and T cells [192]. Immunogenic tumours express a range of stress related molecules which act like Toll like receptors (TLRs) and alert the immune system to the danger [193,194].

More recently the immune response to tumours has been termed 'cancer immunoediting' and can be separated into three 3 phases: (i) elimination, or cancer immune surveillance; in the elimination phase, innate and adaptive immunity work together at an early stage to destroy developing tumours. When fully effective the immune response during the elimination stage can ensure the host remains free of cancer. (ii) equilibrium, a phase of tumour dormancy where tumour cells growth and anti-tumour immunity form an equilibrium that halts any further tumour expansion; equilibrium is a function of adaptive immunity only as T cells and inflammatory cytokines are required to create immunologic equilibrium [195], whereas innate cells such as NK cells have been shown to be redundant in this process [192]. During the equilibrium phase immune-editing of the tumour can occur due to the constant battle by anti-tumour T cell responses to control tumour cell growth and (iii) escape, where tumour cells have a reduced immunogenicity or have highly immunosuppressive mechanisms to prevent any successful anti-tumour immune responses leading to the appearance of progressively growing tumours [196]. Due to the role of T cells within the elimination and equilibrium phases of immunoediting, increased infiltration of adaptive immune cells, in

particular T cells, into tumours correlates with improved patient survival [197] and CD3+ T cell infiltration within tumour associated lymphoid nodules has shown increased survival in rectal cancer [198]. A study investigating tumour infiltrating lymphocytes in oral squamous cell carcinomas found that the intensity of TILs was highest in high grade tumours and these TILs consisted of FOXP3+ Tregs [199].

1.5.1 Regulatory T cells and human tumours

Treg cells mediate peripheral tolerance by suppressing self-antigen-reactive T cells and have been shown to play a role in tumour immunity where tumour rejection has been shown following depletion of CD25+ cells [200]. In addition, adoptive transfer of CD4+ T cells depleted of Tregs increased anti-tumour immune responses [201]. Higher numbers of Treg cells have been found in peripheral blood of a variety of cancers including breast cancer [202], colorectal cancer [203], oesophageal cancer [204], gastric cancer [205], lymphoma [206], melanoma [203], ovarian cancer [207] and pancreatic cancer [208,209].

Peripheral Treg cells show potent suppressive activity *in vitro* and a high frequency of Treg cells would dampen tumour antigen-specific immunity in patients with cancer. In ovarian cancer, within tumour microenvironments Tregs expressed FOXP3 and inhibited TAA-specific CD8+ T cell cytotoxicity. Removal of CD4+CD25+ Tregs but not effector cells using cyclophosphamide was shown to activate latent high-avidity TAA-specific CD8 T cells that had been suppressed by Tregs [210]. Accumulation of Treg cells in the tumour can predict reduced patient survival [207]. Studies characterising changes in intra-tumoural Tregs within normal breast and pancreatic tissue compared to benign neoplasms found no differences but a significant increase in CD4+CD25+ cells in malignant breast and pancreatic tumours [211].

1.5.2 Source of regulatory T cells in the tumour

Regulatory T cells have been proven to be present within the tumour microenvironment due to; trafficking, differentiation, expansion and conversion. Treg cells differentiate in the thymus, tumour-associated Treg cells may traffic to tumours from the thymus, bone marrow, lymph nodes and peripheral blood under the influence of tumour microenvironmental chemokines. Treg cells differentiate in the thymus, tumour-associated Treg cells may traffic to tumours from the thymus, bone marrow, lymph nodes and peripheral blood under the influence of tumour microenvironmental chemokines. [212]. The resulting immature or partially differentiated DCs express TGF-β and promote Treg cell proliferation and conversion from CD4+CD25- T cells. IL-10 also present supports the differentiation of CD4+IL-10+TGF-β+ Tregs and induces Tr1 cells. Therefore tumour microenvironments may contain thymus-derived natural Tregs, expanded and converted natural Tregs and locally differentiated and expanded Tr1s.

1.5.3 Tumour-associated antigen specificity

Tumour reactive Tregs express markers such as CD25, GITR and FOXP3 typically associated with CD4+CD25+ naturally occurring Tregs. Tregs are specific for self-antigens and so may be specific for at least one subset of TAA. Human melanoma infiltrating Treg cells have been cloned and the target antigen was found to be LAGE-1, a cancer- and testis-specific antigen [213]. The fact that in this study LAGE-1 preferentially stimulated Tregs rather than effector CD4 T helper cells indicates that the type of antigen, expression level and dose of an antigen determines whether CD4 T cells are deleted or express a helper or regulatory phenotype [214].

Evidence exists for both Treg suppression of TAA-specific T cell priming and effector function. In ovarian cancer large numbers of Tregs in the tumour mass but few in tumour-draining LNs, indicate that Tregs predominantly inhibit extranodal effector functions at this stage. Tregs may be present in the draining LNs before clinical manifestation and may block TAA-specific T-cell priming during the priming process. Recent studies demonstrated that a large proportion of naturally occurring CD4+CD25+ Tregs recognise MHC class II bound peptides more efficiently than CD25- T cells [215].

Therapeutic applications for targeting Tregs include depletion or attenuation of natural Tregs using monoclonal antibodies has improved anti-tumour immune responses by targeting Treg associated markers such as; CD25, CTLA-4, GITR, TLR4, OX40 and FR4. By targeting these Treg associated markers the aim is to enhance anti-tumour responses via depleting Tregs and increasing the ratio of effector CD8 and CD4 T cells within the tumour micro-environment able to carry out anti-tumour cell targeting or by blocking the suppressive capacity of Tregs within the tumour. Depletion of Tregs from cancer patients has been carried out using Ontak (Denileukin diftitox), a fusion protein comprising IL-2 and diphtheria toxin [216]. Within the study a group of patients who had received Ontak was also vaccinated with DCs transduced with tumour RNA and showed enhanced T cell responses to tumour antigens compared to those receiving vaccine alone. This indicates that depleting Tregs alone is not sufficient to induce/recover anti-tumour responses but that depletion in combination with a secondary immunisation to prime tumour antigen-specific T cell responses, which would otherwise have been suppressed by the presence of Tregs, enhances anti-tumour T cell immunity in these patients.

1.5.4 Myeloid suppressor cells

Myeloid cells that can also develop into suppressor cells called myeloid derived suppressor cells (MDSC) which bring about antigen-specific T cell tolerance by various methods but one mechanism is the increased metabolism of L-Arg [217] and these cells can be inhibited in mice with inhibitors of Phosphodiesterase-5 [218]. Many human tumours produce the immunosuppressive enzymes indolamine -2, 3-dioxygenase (IDO) which prevents the activation of T cells [219]. Stereoisomers of 1-methyl-tryptophan can inhibit these enzymes and may have a role to play in treatment of patients with cancer [220].

1.5.5 Tumour induced immunosuppressive environment

Tumour cells can also develop resistance to immune cell-mediated death by becoming insensitive to apoptotic signals. Down-regulation of the death receptor, Fas, has been reported in a number of human tumours. Engagement of Fas by its ligand, FasL, normally induces cellular apoptosis. Missense mutations resulting in disrupted Fas signalling have been reported in myeloma [221], non-Hodgkin's lymphoma [222] and melanoma [223]. In contrast, a variety of tumours have been reported to express FasL, which induces apoptosis in Fas susceptible target cells, such as activated T cells. This has been proposed as a mechanism by which tumours may induce activation induced cell death in infiltrating T cells [224]. Tumour cells have also been reported to block CD8-mediated cytotoxicity through the over-expression of a serine protease inhibitor that inactivates granzyme B [225]. In a last attempt to prevent T cell recognition tumour can lose expression of MHC class I. However, as complete loss of MHC class I can be recognised by NK cells, most tumours selectively down regulate only a single MHC allele that is the target for the cancer induced T cell response [226,227]. Vaccine stimulation of naïve T cells to give a new response to an allele which has

not been lost, can effectively overcome this block.

Although the tumour environment is capable of blocking anti-tumour immunity, in many tumours the presence of immune cells is still a strong prognostic factor [228,229,230,231] suggesting that it could be externally manipulated to provide an effective therapeutic approach. Perhaps potent anti-tumour targeting requires stimulation of new high avidity CD4 and CD8 T cell responses in combination with depletion of Tregs or neutralisation of immunosuppressive cytokines/high dose chemokines.

1.5.6 Migration of T cells in tumours

In order for T cells to target tumours they need to migrate from the blood through the endothelium and into the cancer. The alteration of the vasculature within tumour cells to form new vessels was proposed by Folkman in 1971 [232] where tumour cells can mediate tumour angiogenesis by secreting soluble factors that enhance endothelial cell proliferation, migration and tube formation and via direct interactions with endothelial cells. In comparison to conventional endothelium tumour endothelium is structurally and functionally abnormal, forming highly disorganised and leaky structures [233,234]. The flow of blood through the tumour capillaries is slow and even reverse direction as vessel diameter is thinner than normal vessels [235]. A comparison of gene expression patterns of endothelial cells derived from blood vessels of normal and malignant colorectal tissues of almost 200 transcripts showed that around 50% were differentially expressed, half of which were specifically increased in tumour-associated endothelium [236]. Genes including collagens 4A1, 4A2 and 1A1, SPARC, THY1 and MMP9 have been shown to be overexpressed on a variety of tumour endothelium whereas TEM7, is overexpressed on ECs of all tumour types [237].

1.6 Cancer Vaccines

In order to overcome the suppressive environment of tumours cancer vaccines need to stimulate T cell responses. Cancer vaccines come in two types: therapeutic, which are aimed at treating an existing cancer, and prophylactic, which are designed to prevent disease.

1.6.1 Prophylactic cancer vaccines

Preventative prophylactic vaccines aim to prevent cancer by targeting oncogenic infectious agents such as viruses which ultimately lead to tumour formation. Gardasil and Cervarix are both prophylactic vaccines against the human papilloma virus (HPV) [238]. Gardasil (Merck), also known as Silgard, is specific for several HPV subtypes, subtypes 16 and 18 are highly associated with cancer formation in around 70% of cervical [239] and 26% of head and neck cancers [240]. Cervarix (GSK) is also designed to prevent infection from HPV types 16 and 18, but in addition also strains 45 and 31. Cervarix contains an HPV type 16 L1 protein, HPV type 18 L1 protein [241] and also an adjuvant AS04;3-O-desacyl-4'-cmonophosphoryl lipid A (MPL) adsorbed on aluminium hydroxide, hydrated (Al(OH)3) [242]. Currently in the UK all girls aged 12 to 13 are able to receive Cervarix.

In addition to HPV vaccines, the FDA has approved a vaccine for hepatitis B (HBV), Engerix B (GSK) as HBV is associated with the initiation of hepatocellular carcinomas (HCC) in both adults and children [243]. Engerix B is active against all subtypes of HBV and contains Hepatitis B surface antigen produced by recombinant DNA technology in yeast [244]. Developing vaccines against oncogenic viruses has the advantage of eliciting strong immune responses however the latency or time taken between viral infection and tumour formation can impact on the success of prophylactic immunotherapy.

1.6.2 Therapeutic vaccines

Therapeutic vaccines aiming to stimulate potent anti-tumour T cell responses have to overcome the immunosuppressive tumour environment and for this reason are often used in combination with other cancer treatments such as surgery, to remove primary tumours, radiation and chemotherapy. Therapeutic cancer immunotherapy relies on tumour cells expressing surface antigens for recognition and initiation of anti-tumour immune responses, ideally absent on healthy cells. The molecular identification of antigens recognised on human tumour cells by T cells has aided the development of T cell based cancer immunotherapy [245] and vaccine classification is based on the genetic mechanisms leading to their expression [246]. Most tumour antigens elicit CD8 T cells responses however CD4 T cell help and efficient DC activation is also required for effective anti-tumour immune responses [247].

Oncofetal antigens are only expressed in fetal tissues and in cancerous somatic cells and to date the most widely studied is CEA in colorectal carcinoma [248], whereas over expressed accumulated antigens such as HER-2/neu are expressed by both normal and neoplastic tissue and can be associated with many types of carcinomas. T cells recognizing HER-2/neu peptides were initially derived from tumour-infiltrating lymphocytes (TILs) of ovarian carcinomas [249]. PRAME is overexpressed in almost all melanomas and in many other tumours such as lung carcinomas [250], Telomerase is a universal tumour antigen due to both its expression by the vast majority of tumours and its inherent functional involvement in oncogenic transformation [251] and WT1, (Wilms tumour gene 1) a transcription factor encoded by a gene that is over expressed in leukaemia [252]. Cancer testis antigens are expressed only by cancer cells and adult reproductive tissues such as testis and placenta, examples include the gene families MAGE [253], SAGE [254], and BAGE [255]. MAGE

expression in many tumours types has been examined and the triggering event seems to be demethylation of their promoter, which have a high CpG content [256]. Lineage-restricted antigens are expressed largely by a single cancer histotype and are also referred to as differentiation antigens where they identify tissue differentiation. Within melanoma, specific genes include tyrosinase [257], Melan-A/MART-1 [258], Pmel17/gp100 [259], TRP-1or TRP-2 [260,261,262]. Antigens resulting from mutation are formed when antigenic peptides are encoded by genes that show somatic mutations in tumour cells. Several point mutations present in antigenic peptides have an oncogenic function such as in CDK4 [263], K-ras [264], p53 [265] and MART-2 in melanomas [266]. Mutated tumour antigens show tumour specificity and resistance to immunoselection when the mutated gene products are essential for oncogenic processes. However, studies have indicated that T cell tolerance can develop rapidly towards tumour-specific antigens even when they are mutated [267].

1.6.3 Therapeutic vaccine strategies

Therapeutic cancer vaccines aim to target existing tumours by either vaccinating non-specifically against a known tumour antigen or by enhancing a patient's own anti-tumour immune response using patient-specific cells cultured *ex vivo*.

Table 1.2 highlights other therapeutic cancer vaccines currently in late-phase development in the European Union or United States.

Name (company)	Indication (Phase)	Description	Class of vaccine
Abagovomab (Menarini)	Ovarian cancer (II–III)	A murine IgG1 anti-idiotype monoclonal antibody that mimics the structure of a specific epitope on the ovarian cancer tumour-associated antigen MUC16	Antigen specific
Allovectin-7 (Vical)	Metastatic melanoma (III)	A DNA plasmid–lipid complex encoding MHC1 antigen	Antigen specific
Belagenpumatucel-L (NovaRx)	Non-small-cell lung cancer (III)	Allogeneic non-small-cell lung cancer cells transfected with a plasmid containing a TGF β2 antisense transgene	Polyvalent
BLP-25 (Merck Serono)	Non-small-cell lung cancer (III)	A liposome-encapsulated peptide derived from the MUC1 antigen	Antigen specific
BiovaxID (Biovest/Accentia)	Non-Hodgkin's lymphoma (III)	An anti-idiotype patient-specific protein	Antigen specific
GSK1572932A (GlaxoSmithKline)	Human melanoma antigen A3-positive non-small-cell lung cancer (III)	Human melanoma antigen A3	Antigen specific
MDX-1379 (Medarex/Bristol–Myers Squibb)	Melanoma (III)	gp100 melanoma peptides	Antigen specific
M-Vax(AVAX Technologies)	Metastatic melanoma (III)	Autologous melanoma cells that have been irradiated and then modified with the hapten dinitrophenyl	Polyvalent
Oncophage(Antigenics)	Renal cell carcinoma (Pre-registration)	Autologous heat shock proteins	Polyvalent
PR1 leukaemia peptide vaccine (The Vaccine Company)	Acute myeloid leukaemia (III)	A 9-amino-acid HLA-A2-restricted peptide derived from proteinase 3	Antigen specific
Sipuleucel-T (Dendreon)*	Prostate cancer	Prostatic acid phosphatase-loaded autologous antigen-presenting cells	Dendritic cell-mediated
TroVax (Oxford Biomedica)	Renal cell carcinoma (III)	A recombinant modified *Vaccinia ankara* viral vector encoding the 5T4 oncofoetal trophoblast glycoprotein	Antigen specific
gp100, glycoprotein 100; HLA-A2, human leukocyte antigen A2; IgG1, immunoglobulin G1; MHC1, major histocompatibility complex 1; MUC16, mucin 16 (also known as CA125); TGF β2, transforming growth factor β2.			

Table 1.2 ***Therapeutic cancer vaccines in development in the European Union or United States*** [268]. * Sipuleucel-T has now been approved by the U.S. FDA on April 29, 2010 to treat asymptomatic or minimally symptomatic metastatic hormone refractory prostate cancer.

1.6.4 Dendritic cell vaccines

Targeting established tumours which have an established suppressive tumour micro-environment greatly reduces any anti-tumour efficacy, in addition to which cancer patients may already be immunocompromised. Successful tumour elimination requires efficient

priming of T cells by fully functional dendritic cells. Provenge (Sipuleucel-T) is designed to stimulate T cell immunity to prostatic acid phosphatase (PAP) [269], an antigen expressed in prostate cancers [270]. Specifically, Sipuleucel-T is composed of autologous PBMCs cultured with a fusion protein (PA2024), which consists of PAP linked to GM-CSF [271]. PA2024 has been shown to provide efficient loading and processing of antigen by APCs and upregulation of costimulatory molecules [272].

To generate mature DCs capable of priming efficient T cell responses DCs can be harvested from tumour bearing patients and stimulated *ex vivo* to be adoptively transferred back into the same patient with the aim of enhancing patient-specific anti-tumour immunity [273]. Within melanoma patients a phase III trial failed to show any survival advantage for patients immunised with peptide pulsed DC [274]. The failure could be due to ineffective T cell priming of T cells once the DCs re-encountered the tumour immunosuppressive environment. However by altering the way DCs are stimulated effective responses can be seen in breast cancer patients [275] where these DCs had additional stimulation with IFNγ, TNFα and CD40 ligand enhancing cytokine release. One huge disadvantage of generating *ex vivo* stimulated patient specific DCs is the cost and time required to generate sufficient numbers and quality of cells required for adoptive transfer and subsequent clinical responses [276].

1.6.5 Whole cell vaccines

Whole cell vaccines are aimed at presenting a wide variety of tumour rejection antigens present on cancer cells to the immune system. Immunising with autologous whole cell mixtures derived from patient tumour samples removes the need to identify and isolate these mutant gene products for each individual patient. Most of these vaccines involved engineering the tumour cells to secrete pro-inflammatory cytokines prior to immunisation, in order to improve immunogenicity. A phase III trial of an allogeneic melanoma cell lysate

vaccine with IFN-α reported there to be no survival advantages of the vaccine compared with treatment with IFN-α alone [277]. Belagenpumatucel-L is a whole cell vaccine containing allogeneic non-small-cell lung cancer cells transfected with a plasmid containing a TGFβ2 antisense transgene [278]. Using an antisense gene to inhibit TGFβ2 results have linked inhibition of cellular TGFβ2 expression with increased immunogenicity of gene-modified cancer cells [279]. AVAX Technologies are developing a whole cell vaccine for the treatment of melanoma, M-Vax is being developed using irradiated and modified melanoma cells [280]. The advantage of whole cell vaccines is the removal of the need to identify specific tumour antigens however a huge selection of antigens would be present on tumour cells and could skew T cells responses against immuno-dominant epitopes from housekeeping genes stimulating or inducing Tregs further reducing any generation of anti-tumour immunity [281].

1.6.6 Peptide vaccines

In direct contrast to whole cell vaccines peptide vaccines aim to generate specific T cells responses against known tumour antigens either in the context of MHC class I or class II molecules. The advantage in this approach is in focusing the anti-tumour response on one or a select few particular antigens with the aim of removing immune competition from competing epitopes. In this way the effectiveness of generating specific anti-tumour T cells responses can be monitored by identification of these antigen-specific T cells within cancer patients.

PR1 leukaemia peptide vaccine (The Vaccine Company) is a 9 amino-acid human leukocyte antigen (HLA)-A2 restricted peptide derived from proteinase 3 which is a serine proteinase overexpressed in leukemic cells and is involved in angiogenesis and metastasis [282]. A trial in patients with persistent myeloid leukaemia showed PR1-specific T cell responses in 35 of 44 (57%) of patients and improved overall clinical response and a trend towards longer event-free survival. GV1001 is a peptide vaccine targeting telomerase, representing a 16 amino acid

hTERT sequence binding both HLA class I and II epitopes, therefore ecapable of stimulating both CD8 and CD4 T cell responses. A phase I/II trial has demonstrated GV1001-specific T-cell responses in more than 50% of pancreatic and pulmonary cancer patients, without toxicity [283].

The advantage of vaccines based on peptide response epitopes is that they are ideal for large scale production. However one problem is their short serum half life as they become rapidly proteolytically degraded. A major failure of peptide vaccines is that although generating expansions of T cells against immunising peptide, in some cases less than 5% of treated patients showed clinical responses [284]. T cells are highly responsive to peptide concentration and therefore the correct dose is essential to generate high avidity T cell responses, too high a concentration would lead to T cell death and low avidity T cell production unable to elicit tumour elimination.

1.6.7 Protein vaccines

Protein vaccines are some way between individual peptide vaccines and whole tumour cells vaccines. GSK1572932A (GlaxoSmithKline) is an antigen-specific protein vaccine used as adjuvant therapy in patients with resectable MAGE-A3 (melanoma-associated antigen 3) positive Non-Small Cell Lung Cancer (NSCLC) [285]. MAGE-A3 is a tumour associated antigen overexpressed in melanoma, head and neck cancer, and bladder cancer in addition to NSCLC. The advantage of using whole proteins as opposed to selective peptides is that multiple epitopes can be presented to both CD4 and CD8 T cells, however as mentioned previously this can be disadvantageous when immunodominant epitopes stimulate both Tregs and Th1 cells [286].

1.6.8 DNA vaccines

DNA vaccines aim to target tumours by injecting genetically engineered DNA to produce an anti-tumour response. Immunisation with naked DNA offers an alternative approach to directly target antigens to the endogenous MHC class I processing pathway without the need for a viral vector in order to stimulate CD8 T cell responses. This strategy has been shown to efficiently generate CTL responses to encoded antigens and can provide protective immunity in a range of models of infectious disease [287]. Plasmid DNA vaccines are easy to synthesise and administer compared to other vaccine strategies and there have been no reported adverse toxicities associated with transfection of cells *in vivo* [288] as they are safer than using live attenuated vaccines which can cause pathogenic infection *in vivo*. Repeated DNA vaccines do not elicit anti-DNA antibody production [289]. Bacterial DNA plasmids contain CpG motifs which act as adjuvants by enhancing the immunogenicity of DNA vaccines. The ability to introduce antigen to the host immune system eliciting both CD4 and CD8 cytotoxic T cells separates DNA vaccines from peptide vaccines [290].

DNA vaccines that are currently being tested for HIV-1, Ebola, Severe Acute Respiratory Syndrome did not show sufficient integration into host cellular DNA [291]. A phase I/II trial, conducted with a DNA vaccine encoding human prostatic acid phosphatase (PAP) in combination with GM-CSF, in prostate cancer patients resulted in 14% of patients developing PAP-specific IFN-γ secreting CD8 T cells and 41% of patients developed PAP-specific CD4 and/or CD8 T cell proliferation, confirming initiation of immune responses [292]. A DNA vaccine currently in phase III trials for melanoma patients is Allovectin-7® which containins the DNA sequences encoding HLA-B7 and ß2 microglobulin, which form the MHC class I complex. The aim is to cause recognition of the tumour at the local site as the vaccine is injected directly into tumour lesions [293]. The vaccine has three proposed mechanisms of

action; firstly, in HLA-B7 negative patients, an allogeneic response would be expected against the foreign MHC class I antigen. Secondly, in all patients, ß2 microglobulin may recover normal class I antigen presentation increasing tumour antigen presentation. Thirdly, in any patient intralesional injection of the pDNA/lipid complex may promote innate pro-inflammatory responses.

1.6.8 Inhibition of checkpoint proteins

In a recent phase three trial, Ipilimumab, a human IgG1κ monoclonal antibody which is a checkpoint protein inhibitor blocking CTLA-4, was administered with or without a glycoprotein 100 (gp100) peptide vaccine and compared to gp100 alone in 676 patients with previously treated metastatic melanoma [294]. Ipilimumab is the first agent ever proven to improve survival in advanced melanoma has now been approved by the FDA for second line therapy in adult patients. Tremelimumab is another human anti-CTLA-4 antibody that has been used in phase I and II trials with encouraging results. Out of 39 patients with advanced melanoma, two patients had a complete response and two had a partial response. The mechanism whereby Tremelimumab was able to elicit anti-tumour responses was shown to be due to suppression of Treg activity while also increasing the effector and memory CD4 and CD8 T cells populations [295]. This effect was also seen in a murine model whereby CTLA-4 blockade was seen to alter the balance of Tregs to effector cells favouring anti-tumour responses [296]. However Tremelimumab was not as successful as Ipilumimab as a phase III trial evaluating the effect of as a first-line treatment compared to conventional treatment strategies on patients with advanced melanoma showed no significant difference in overall survival due to three deaths in the Tremelimumab treated patients resulting in the study being abandoned [297].

1.7 Generating an effective cancer vaccine

An ideal cancer vaccine should induce a strong immune response against the antigen expressed only on malignant cells. The failure of antigen specific cancer vaccines is not in generating antigen-specific T cells as these can be shown to be present, but that these CD8 T cells are of low avidity and not capable of carrying out effective anti-tumour cytotoxicity. Low avidity T cells may also fail to extravasate into tumours or be retained once they have migrated [298].

1.7.1 T cell avidity

T cell affinity represents the interaction between a TCR and MHC:peptide on APCs. T cells show a range of affinities which affect the T cell specificity and activity [299]. The term affinity refers to the strength of an interaction between a single receptor and its ligand. Whereas avidity is the sum of the strengths of interaction occurring between two cells and is determined by affinity, the number of synapses formed, the length of time the synapses are engaged, cytokines such as IL-12 and costimulation [300]. The interaction between a T cell and a target cell can be measured in terms of its functional avidity which is determined by the peptide concentration at which a T cells elicits effector functions and measured by the amount of peptide needed to elicit a half maximal response. Subsequently T cells with low avidity respond only to high concentrations of peptide. The concentration of an *in vitro* stimulating antigen has been shown to influence the expansion of high or low avidity T cells [301]. Studies in TCR transgenic mice have shown that any cell can divide to produce further cells with different avidities suggesting that avidity can be modulated at the level of a single cell [302]. During T cell activation the reorganisation of membrane microdomains (lipid rafts) results in the formation of raft-associated molecules at the site of TCR engagement [303]. At the immunological synapse the activation of T cells is dependent on lipid

raft integrity resulting from the ability of these microdomains to properly localise and concentrate signalling molecules in the correct orientation [304].

Antigen specific CD8 T cells stimulated with various concentrations of peptide can produce CD8 T cells with varying avidities where expansion with low-dose peptide (resulting in high avidity T cells) enables lysis of target cells sensitised with more than 100 times less peptide than CD8 T cells generated with high-dose peptide (resulting in low avidity T cells) [305]. The high avidity T cells, recognising low does peptide, when adoptively transferred into severe combined immunodeficient mice were up to 1000 times more effective at viral clearance than the low avidity T cells however both T cell lines lysed virus-infected targets *in vitro*. Therefore, low dose peptide selects high avidity T cells whereas high dose stimulates low avidity responses or even cell death at too high a concentration.

1.7.2 T cell avidity in anti-tumour response

Differences in T cell avidity correlate with the *in vivo* anti-tumour efficacy with higher avidity T cells leading to better anti-tumour responses [306], [307]. The influence of T cell avidity on tumour recognition *in vivo* was assessed using a murine melanoma model by Zeh *et al* [306]. High avidity T cells specific for tumour antigens TRP-2 or p15E were generated by stimulating T cells with very low amounts of antigenic peptide. Adoptive therapy using T cells of high and low avidity demonstrated that the high avidity T cells eliminated lung metastases from B16 melanoma. Dutoit *et al* used CD8 T cells clones derived from T cells isolated from melanoma patients and showed that an avidity of over 10^{-9}M, (the antigen concentration at which a half maximal response is achieved), was required for tumour recognition and these CD8 T cell clones from spontaneously regressing cancer patients had avidities of around 10^{-12}M [308].

Scancell Holdings Plc developed the ImmunoBody®, a modified DNA vaccine encoding an antibody molecule. Complementarity determining regions (CDRs) of the antibody have been modified and replaced with unique restriction sites. The ImmunoBody® expression system contains cytotoxic CD8 and helper CD4 epitopes within a human IgG1 or mouse IgG2a framework, removing the need for adjuvants and stimulating effective T cell responses [309]. Vaccination using the ImmunoBody® has shown anti-tumour immunity as a result of the generation of high-frequency and high-avidity T cell responses [307] where no significant correlation was found between avidity and frequency suggesting they are independent responses. The melanoma antigen tyrosinase related protein 2 (TRP2) was engineered into the ImmunoBody® expression system compared to DCs pulsed with HepB/TRP-2 linked peptide. The study found that the avidity of TRP-2 specific T cell responses following DNA immunisation was up to 10 times higher than with peptide-pulsed DCs. The avidity of T cell responses generated by a TRP2/HepB human IgG1 Immunobody DNA vaccine are an average of $5x10^{-10}$M, significantly higher than that of the same peptide immunisation [307], and able to induce tumour lysis *in vivo*.

1.8 Summary

To summarise, effective cancer vaccines need to stimulate high avidity T cells that can get into tumours. The next generation of cancer vaccines need to achieve three essential criteria: firstly generating T cells with highly avid recognition of tumour antigens *in vivo*. Secondly these high avidity T cells must be able to infiltrate tumours where trafficking to the tumour is dependent on chemokine profiles and potentially antigen recognition required for extravasation from the blood. Thirdly, the T cells must be activated sufficiently within the tumour, overcoming the suppressive mechanisms at the tumour site to carry out effector mechanisms causing tumour destruction. The presence of regulatory T cells within the tumours microenvironment highlights the importance of active suppressor mechanisms arising both from the tumour and from the immune system itself that can inhibit anti-tumour immune reactions *in vivo*. Work from CTLA-4 inhibition would suggest that altering the balance of T effector cells to T regulatory cells can result in clinical benefit. This thesis therefore addresses the migration of CD8 and Treg cells into tumours and the chemokine effect within the tumour environment in order to further our understanding of how to shift the balance to an effective CD8 T cell response.

1.8.1 Hypothesis

Due to the growing body of evidence that antigen recognition influences the transmigration of T cells the hypothesis for this thesis was that recognition of cognate antigen presented on tumour endothelium influences the transmigration and subsequent infiltration of CD8 and regulatory T cells into tumours.

1.8.2 Project aims

In order to further analyse factors affecting T cell infiltration into tumours four aims were addressed. The aims of the project were:

1) To analyse the importance, in CD8 T cell transmigration, of recognition of peptide: MHC on ECs by tumour associated antigen TRP-2 specific CD8 T cells.

2) To analyse the effect of CD8 infiltration into colorectal tumours on the survival and clinicopathological characteristics of patients.

3) To analyse the importance of cognate antigen recognition, chemokines and inflammation on natural regulatory T cell transmigration.

4) To identify the effect of CXCL12 and CXCR4 within cancer patients on survival and correlation with clinicopathological characteristics.

Chapter 2: Materials and Methods

2.1 Mice

All animal work was carried out under a Home Office approved project license. C57BL/6 mice were purchased from Charles River UK Ltd and were housed in the animal facility at Nottingham Trent University. HHD II transgenic mice (Institut Pasteur) were used as an HLA-A*0201 transgenic murine model. These mice have a HLA-A*0201/D_b transgene consisting of the α1 and α2 domains of the HLA-A*0201 linked to the α3 transmembrane and cytoplasmic domain of H-$2D_{b,}$ as well as the human β2-microglobulin gene. The mice are on a C57Bl/6 x SJL, H-$2D_{b-/-}$ $\beta2m_{-/-}$ double knockout background. DR4 transgenic mice are a transgenic strain containing a hybrid MHC class II molecule (the peptide binding domains of human HLA-DR4 and the membrane proximal domains of mouse I-E). All mice were bred in house at the animal facility in Nottingham Trent University and were aged between 6 and 12 weeks.

2.1.1 Peptide

The synthetic TRP-2 peptide used for T cell culture and endothelial pulsing was SVYDFFVWL (Department of Biomedical Sciences, Nottingham University, UK) and reconstituted in PBS at a stock concentration of 20mg/ml.

2.1.2 Murine cell culture

All cell culture was carried out in a class II safety tissue culture hood using aseptic techniques and all reagents were purchased from Sigma (Dorset, UK) unless otherwise stated. Murine splenocytes were cultured in RPMI 1640 media was supplemented with 10% NBCS

(newborn calf serum) 2mM L-glutamine, 100U/ml Penicillin /Streptomycin, 10mM Hepes buffer, 5mM Non essential amino acids, 5mM Sodium Pyruvate and 5 x 10^{-5}M 2-mercaptoethanol. Murine lung endothelial cells (primary cells) were cultured in Hams-F12 media, 10% NBCS, 2mM L-glutamine, 100U/ml Penicillin /Streptomycin, 10mM Hepes buffer, 5mM Non essential amino acids, 5mM Sodium Pyruvate, 5 x 10^{-5}M 2-mercaptoethanol, 100µg/ml murine EGF (Peprotech, London,UK) and 10µg/ml murine bFGF (Peprotech). The B16 melanoma cell line and RMAS TAP-2 deficient murine tumour cell line (ATCC) were cultured in RMPI 1640 and 10% NBCS.

2.1.3 Flow cytometric analysis

For flow cytometric analysis sample cells were placed at $1x10^6$ cells/ml and 100µl used for each samples. Each sample was resuspended in 50ul of staining solution containing indicated antibodies at described concentrations. For surface staining samples were mixed and incubated on ice for 1 hour, washed x2 with PBS and resuspended in 400ul PBS for analysis. For intracellular staining, following initial surface staining, samples were resuspended in 500µl FACs permeabilisation buffer (eBioscience 00-5123-43) for 45 minutes on ice. Samples were washed x2 with PBS, resuspended in 50ul intracellular staining solution and placed on ice for an hour. Samples washed x2 with PBS and resuspended in 400ul PBS for analysis on FACS-calibur flow cytometer using WinMDI 2.8 software.

2.2 Murine lung endothelial cell isolation and culture

To investigate the effect of antigen recognition on T cell transmigration across endothelium an *in vitro* murine model was developed using murine lung endothelial cells (MLECs) as a model endothelium and isolated murine T cells for transmigration.

2.2.1 Isolation of murine lung endothelial cells

Murine microvascular endothelial cells (MLECs) were isolated from DR4/C57Bl/HHDII murine lung tissue by collagenase digestion as shown previously [310]. The lung tissue was diced into 1mm^2 pieces and placed in a solution of 200U/ml collagenase type II (Invitrogen, Paisley
UK) in Hanks Balanced Salts Solution (Invitrogen). Lung tissue was incubated in collagenase for 1 hour at 37^{o}C, shaking vigorously every 20 minutes. MLECs were cultured in 0.2% gelatin-coated T75 flasks at 37°C with 5% CO_2. At confluence MLECs were detached from the culture flasks using 0.1% trypsin in 0.2% EDTA and passaged 1:3. For use in functional assays MLECs were used between passage 4 and 10, activated by culture with a combination of 100ng/ml of IFN-γ (Peprotech), 100ng/ml TNF-α (SantaCruz, Heidelberg, Germany) and 50ng/ml VEGF (SantaCruz) for 24 hours to induce/reduce the expression of cell adhesion molecules.

2.2.2 Characterisation of murine lung microvascular endothelial cells.

Murine lung endothelial cells were cultured on the surface of transwell inserts to form a monolayer. **Figure 2.1A** highlights the 3μm pores of the PET transwell insert prior to addition of ECs whereas the insert covered with a C57Bl MLEC monolayer is shown in **Figure 2.1B.** CD31or PECAM-1 (platelet endothelial cell adhesion molecule 1) expression has been shown to be a marker of endothelial lineage [311].

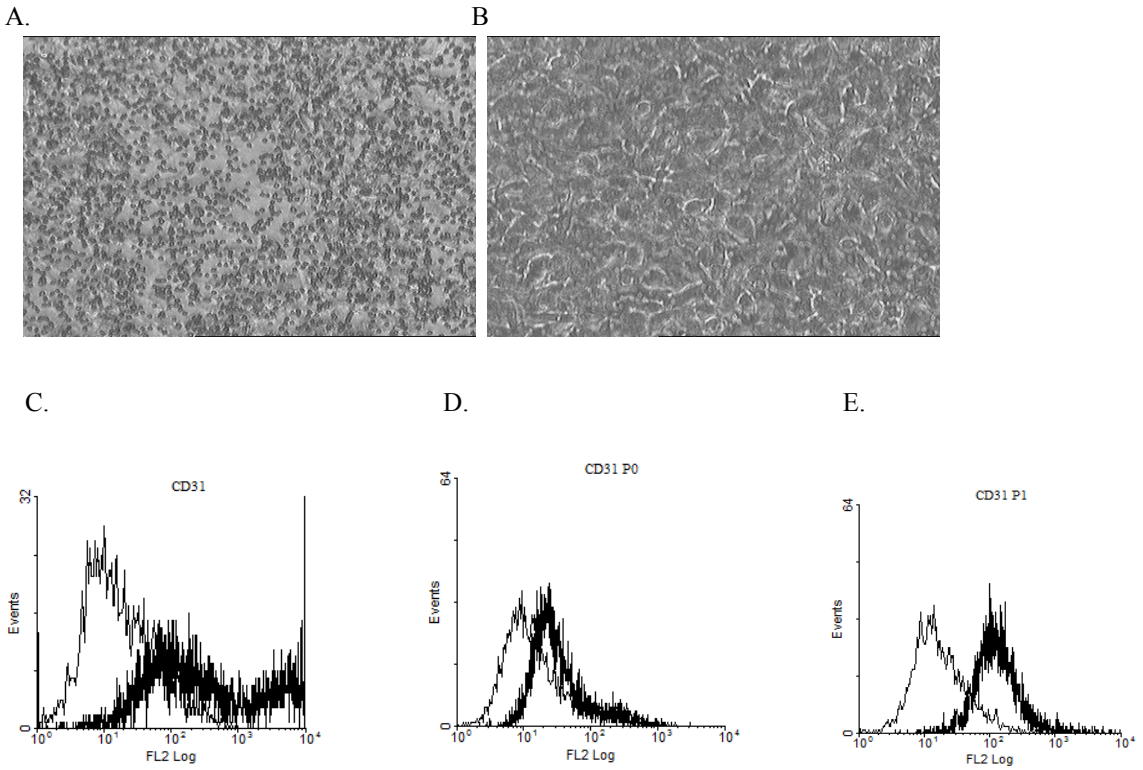

FIGURE 2.1. ***Characterisation of murine lung microvascular endothelial cells.***
Murine lung endothelial cell monolayer formation, **A** 3um pores of a 24 well PET transwell insert. **B** MLEC monolayer formation across the insert following coating with 2% gelatin and growth overnight at 37°C, 5%CO2. **C** Indirect immunofluorescence staining with anti-CD31mabs (Thick black lines) and flow cytometric analysis of murine lung cells following degradation for 1 hour with collagenase type II, **D** The same MLEC cells following adherence to 0.2% gelatin coated T25 flask and **E** following passage 1. Isotyped matched immunoglobulin was used as a negative control (thin line).

To ensure that the isolated endothelial cells from murine lungs were indeed derived from an endothelial cell lineage the cells were stained by indirect immunofluorescence and analysed by flow cytometry. Samples were analysed with anti-murine CD31-PE (eBioscience, Hatfield, UK 12-0311-81) at 1:50 dilution. Endothelial activation was assessed using anti-murine antibodies; ICAM-1-FITC (eBioscience, Hatfield, UK 11-0541), VCAM-1-FITC (eBioscience 11-1061), ALCAM-PE (eBioscience 12-1661), MHC Class 1-FITC (eBioscience 11-5999), MHC Class II-PE (eBioscience 12-5322) and CXCR4-APC (eBioscience 17-9991) at 1:50 dilutions. CD31 expression of DR4 murine lung cells following degradation in collagenase type II is shown in **Figure 2.1C**. Collagenase

degradation causes dissociation of the native collagen that holds the lung tissue together allowing retrieval of endothelial cells. CD31 expression increases following preferential adherence of endothelial cells to 0.2% gelatin coated T25 tissue culture flasks **Figure 2.1D**. Finally the same cells at passage 1 following subsequent culture resulting in increased numbers of cells expressing CD31 to 80-90% and therefore a purer population of endothelial cells **Figure 2.1E**.

2.2.4 Isolation of murine CD4 and CD8 T cells

Murine CD4, CD8 and CD4CD25FOXP3 T cell subsets were isolated using the Miltenyi MACs separation system for use in transmigration assays.

Splenocytes were obtained from murine spleens, adhered to tissue culture flasks prior to use in transmigration assays to partially enrich for T cells by monocyte adherence and depletion. For isolation of CD8 and CD4 T cells murine CD4+ and CD8+ T Cell Isolation Kits (Miltenyi 130-095-248 and 130-095-236) were used. Splenocytes were labelled with Biotin-Antibody Cocktail (Miltenyi, Surrey, UK), for CD4 T cell isolation, containing biotin-conjugated monoclonal antibodies against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, anti-MHC class II, and Ter-119. For isolation of CD8 T cells the antibody cocktail contained anti-CD4 to replace CD8a. T cell populations were obtained by negative selection by passage down MS MACS columns (Miltenyi). Purity of the obtained CD8 and CD4 population was assessed using Flow Cytometry.

2.2.5 Isolation of CD4+CD25+ T cells

Splenocytes were obtained from murine spleens, adhered to tissue culture flasks prior to use in transmigration assays to partially enrich for T cells by monocyte adherence and depletion, cultured in RPMI 1640 medium supplemented with 10% FCS, 2mM glutamine (Sigma), HEPES buffer (Sigma), 2-Mercaptoethanol and 100U/ml penicillin and 50μg/ml streptomycin (Sigma) at 37°C with 5% CO2. For isolation of CD4+CD25+ T cells from splenocytes a murine CD4+CD25+Regulatory T Cell Isolation Kit (Miltenyi 130-091-041) was used. Splenocytes were indirectly magnetically labelled to remove non-CD4+ T cells with Biotin-Antibody Cocktail and Anti-Biotin MicroBeads (Miltenyi). This was followed by positive selection of CD25+ cells by labelling with anti-CD25-PE antibody. Magnetic separation using LD and MS columns, for negative and positive selection was carried out respectively. The purity of the obtained CD4+CD25+ T cells was assessed using indirect immunofluorescence staining and Flow Cytometric analysis.

2.3 Murine immunisation

To generate anti-tumour specific T responses mice were immunised via gene gun with gold bullets coated with plasmid DNA.

2.3.1 Plasmids

The ImmunoBody^{TM} DNA plasmid used in immunisations was a kind gift from Scancell Ltd. The ImmunoBody^{TM} construct contains CDRs within the single heavy and light chain vectors that had been replaced with unique restriction sites enabling rapid insertion of epitope sequences [309]. To generate the human IgG1 TRP2 constructs, oligonucleotides encoding

the TRP2 epitope SVYDFFVWL [312] has been incorporated into the CDRH2 or in direct replacement of CDRH3.

2.3.2 Preparation of Gold bullets

100 µl 0.05M spermidine (Sigma) was added to 8.3mg 1µm gold particles (Biorad) and sonicated. 18 µg DNA was added and the mixture sonicated again. 100 µl 1M $CaCl_2$ was added dropwise to the mixture whilst sonicating then tubes were left to stand for 10 minutes at room temperature. Gold was spun at 350g in a microfuge. Pellet was washed 3 times by resuspending in 1ml dry ethanol and spinning at 350g. Finally, pellet was resuspended in 1ml of 0.025mg/ml PVP (Biorad, Hertfordshire, UK). Gold was loaded into Tefzel tubing that had previously been pre-dried under nitrogen for 10 minutes, whilst sonicating. Tubing was loaded onto the prep-station (Biorad) and left to stand at room temperature for 30 minutes. Ethanol was gently aspirated using a syringe, leaving the gold sediment undisturbed. Tubing was turned 180° and held for 5 seconds then set to spin on setting 1. After 30 seconds, nitrogen was switched on to level 4 and tubing was spun for a further 5 minutes. Bullets were cut using the supplied guillotine (Biorad) and stored at 4°C

2.3.3 Immunisation schedule

Mice were immunised 3 times at weekly intervals with DNA coated onto gold particles, prepared as previously described. Each mouse received 1 µg DNA/immunisation into the shaved abdomen. Hair was removed from the abdomen by shaving before delivery of the gold bullets by the Helios Gene Gun (Bio-Rad).

2.4 Generation of a TRP-2 specific cell line

Splenocytes obtained from immunised mice were used to generate TRP-2 specific T cell lines for subsequent use in *in vitro* transmigration assays across murine lung endothelium and *in vivo* infiltration into tumours.

2.4.1 ELISPOT

The Enzyme Linked Immunosorbent Spot (ELISPOT) assay was used to quantify the number of cells responding to TRP-2 peptide by assessment of IFN-γ secretion by CD8 T cells within the splenocyte population of immunised mice. The ELISPOT kit for mouse IFN- γ (AP system), MABTECH (cat # 3321-2A) was used to verify the presence of antigen-specific CD8 T cells for CTL generation.

96 well Multiscreen Immunopore plates with Immobilon-P membrane (Millipore) were washed once with 15 µl/well 30% ethanol to pre-activate the membrane, then 4 times with sterile PBS. Coating antibody (AN18 anti-IFN-γ) was diluted 1:100 in sterile PBS and 50 µl added into each well. Plates were incubated overnight at 4°C. The following day, coating antibody was aspirated and plates were washed 4 times with sterile PBS. Plates were blocked with 200 µl per well of complete RPMI 1640 10% NBCS 5 x 10^{-5} M 2-mercaptoethanol (2-Me) for at least 20 minutes at room temperature. Media was aspirated from the plate then immediately, 100 µl per well serial dilutions of peptide, prepared in supplemented RPMI 1640 10% FCS 5 x 10^{-5} M 2-Mercaptoethanol, were added to the plate in triplicate for each mouse. 100 µl per well PMA (Sigma) (10ng/ml)/Ionomycin (Sigma) (1000ng/ml) was added as a positive control and 100 µl per well complete RPMI with 2-Me was added as a negative control. 5 x 10^{5} splenocytes from immunised mice were added to each well; in 100 µl to take

the total volume per well up to 200 µl. Plates were wrapped in tin foil and incubated for 48 hours at 37°C 5% CO_2.

Plates were washed 4 times with 0.5% FCS in PBS and 100 µl per well of biotinylated anti-murine IFN-γ detection antibody was added to each well, diluted 1:1000 in PBS. Plates were incubated for 2 hours at room temperature. Detection antibody was removed from the plates and plates were washed 4 times with PBS. Streptavidin-AP (AP-conjugate substrate system, BioRad (cat # 170-6432) was diluted 1:1000 in 0.5% NBCS in PBS and 100 µl added to each well. Plates were incubated for one hour at room temperature. Plates were again washed 4 times with PBS then a further twice with distilled H_2O. Substrate solution was prepared according to the manufacturer's instruction and 100 µl was added to each well. Plates were incubated in the dark for 45 minutes then were rinsed with distilled H_2O then allowed to dry at room temperature overnight. The following day, the number of spots was quantified automatically using an automated ImmunoSpot® plate reader from Cellular Technologies Ltd.

2.4.1.1 Calculation of funtional avidity

The functional avidity of the response is calculated as the peptide concentration at which a half maximal response is achieved either in ug/ml of by Molarity (M). Molarity is calculated by: Concentration (ug/ml)x1000ml/L/ Molecular Mass = uM

For a nonomer peptide such as TRP-2 a concentration of 1ug/ml equates to a Molarity close to $1x10^{-6}$M.

2.4.2 LPS Blasts

Splenocytes were harvested from syngeneic naïve mice and washed once with complete RPMI 10% NBCS. Cells were counted and put into culture at 1 x 10^6/ml in complete RPMI media, 10% NBCS with 5 x 10-^{5}M 2-Me, 25 µg/ml LPS (Sigma), 7 µg/ml dextran sulphate (Sigma) and incubated at 37°C, 5% CO_2. 48 hours later, LPS blast cells were irradiated at 3000 rads and washed twice with RPMI 10% NBCS. Cells were counted with trypan blue exclusion, resuspended at 2 x 10^7/ml and pulsed for 1 hour with 10 ng/ml TRP-2 peptide at 37°C. At the end of the incubation period, cells were diluted to 1 x 10^6/ml ready for use.

2.4.3 Culture of cytotoxic T lymphocytes (CTLs)

Seven days following the final immunisation, splenocytes (5x10^6/ml) were co-cultured at 37^{o}C with syngeneic, irradiated (3000 rads), peptide-pulsed LPS blasts (0.5 to1x10^6 cells/ml). LPS blasts were obtained as previously described. Prior to use 2x10^7/ml LPS blasts were labelled with 10 ng/mL synthetic peptide for 1 h, washed once with complete media and cultured at 5x10^6/ml. The $CD8^+$ T-cell lines, specific for the TRP-2 peptide epitope SVYDFFVWL presented by H2-K^b, was maintained in culture by re-stimulation every week with LPS blasts from C57Bl mice presenting TRP-2 peptide (10ng/ml) and recombinant interleukin-2 (rIL-2) (20 U/mL). Cultures were assayed for cytotoxic activity on day 6 in a ^{51}Cr-release assay.

2.4.4 Phenotyping of CTL lines

Phenotypic analysis of CTL lines was carried out using four colour flow cytometry where 2x10^5 cells were used for each condition in 50ul PBS. Samples were analysed with anti-

murine CD62L-FITC (eBioscience 11-0621), anti-murine CD8-PeCy5 (eBioscience 15-0083) and anti-murine CD127-PeCy7 (eBioscience 25-1271) at 1:25 dilution. In addition, anti murine TRP-2 specific pentamer PE labelled (ProImmune, Oxford, UK) was added at a 1:10 dilution.

2.4.5 Chromium release assay

Target cells were passaged 24 hours before the assay. C57Bl MLECs were pre-treated with 100ng/ml human IFN- γ (Peprotech) to upregulate MHC class I expression for 24 hours. Target cells were suspended at 2 x 10^6/ml in serum free medium containing increasing concentrations of TRP-2 peptide and incubated with 1.85mBq 51[Cr] Sodium Chromate (Amersham) for 1 hour at 37°C 5% CO_2. Cells were washed 3 times with serum free medium. Cell lines of central memory, effector or effector memory CTLs were washed once and viable cells counted with trypan blue exclusion. Cells were resuspended at 2.5 x 10^6 per ml in supplemented RPMI 1640 10% NBCS. Effector cells were plated out, 50 µl per well, in 96 well round-bottomed plates. Serial dilutions were performed to obtain the necessary effector:target ratios. Supplemented RPMI 1640 10% NBCS media was added to make the final volume in each well up to 175 µl per well. After washing with serum free medium, viable target cells were counted with trypan blue exclusion and resuspended at 1 x 10^5 cells/ml in supplemented RMPI 1640 10% NBCS. 25 µl target cells were added to each well of effector cells. Effector cells were incubated with target cells at 37°C 5% CO_2 for 4 hours. To assess the level of spontaneous release of Chromium, each target cell line was also incubated alone in 200 µl of media. To assess maximum release, each target cell line was incubated alone in 200 µl media with 25 µl Triton X (Sigma). After 4 hours, 50 µl supernatant from each well was transferred to a Lumaplate (Packard) plate. Plates were left to

dry at room temperature. The following day plates were read on a Topcount Microplate Scintillation Counter (Packard). The percentage specific lysis was determined using the following formula: Specific lysis (%) = ((Experimental release – Spontaneous release)/(Maximum release – Spontaneous release)) x 100

2.5 *In vitro* transmigration assays

A murine model of T cell transmigration was developed to investigate the effect of antigen-recognition of peptide presented by endothelial cells in the context of MHC molecules on T cell transmigration.

Transmigration assays were carried in Costar Transwell tissue culture inserts (6.5mm diameter) with 3.0μm pore size and Polyester membranes (**Figure 2.1A**.). Inserts were coated with 2% gelatin for 1 hour at 37°C and seeded with 1-2x10^5 ECs overnight to form a monolayer. Where used, monolayers were cultured with 100ng/ml IFN-γ, 100ng/ml TNF-α and 50ng/ml VEG for 24 hours prior to transmigration.

2.5.1 CD8 T cell transmigration assay

Purified murine CD8 T cells (0.5-2x10^5) in 200μl RPMI 1640 with 10% NBCS were added to the upper chamber, 500μl media per well was added to the lower chamber and left for 24 hours to migrate through the monolayer. MLEC monolayers of C57Bl (syngeneic) and HHDII (allogeneic) were used (**Figure 2.2**). C57Bl ECs were untreated (control) or treated with 0.5ug/ml anti-H-2K/Db (Abcam ab64523) for 1 hour prior to transmigration. ECs were pulsed with 10ng/ml TRP-2 peptide for 4 hours prior to addition of splenocytes for

transmigration. For chemotaxis experiments the media within the lower chamber was supplemented with 100ng/ml of either CXCL12 or CCL16 (Peprotech). Those cells having migrated were determined by counting cells present in the lower well media, expressed as a percentage of total cells added and T cell subset expression determined using FACS analysis after 24 hours.

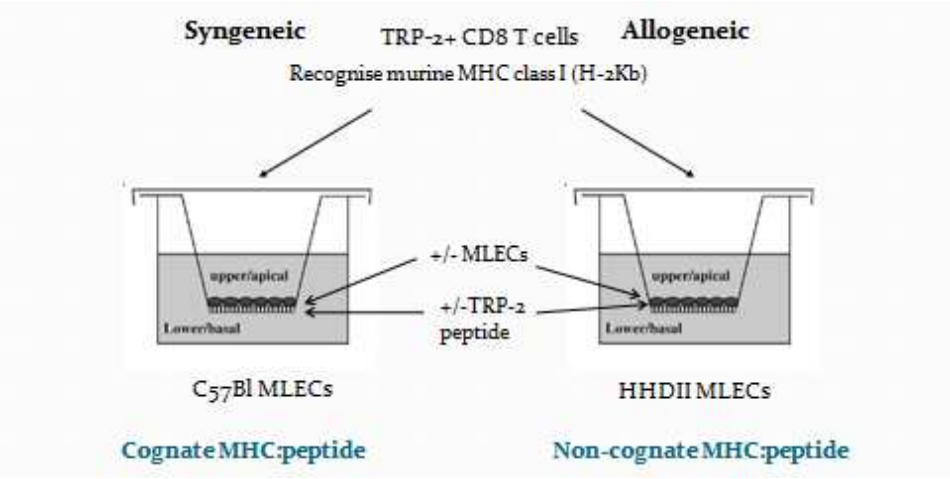

FIGURE 2.2. **In-vitro *CD8 T cell transmigration assay***
Transmigration of CD8 T cells containing a population of TRP-2 specific CD8 T cells from C57Bl mice across murine lung endothelial cell (MLEC) monolayers derived from C57Bl and HHDII mice to form models of syngeneic and allogeneic transmigration respectively.

2.5.2 Regulatory T cell transmigration assay

Murine splenocytes or purified murine CD4 T cells ($0.5\text{-}2x10^5$) in 200µl RPMI 1640 with 10% NBCS were added to the upper chamber, 500µl media per well was added to the lower chamber and left for 24 hours to migrate through the monolayer (**Figure 2.3**). For chemotaxis experiments the media within the lower chamber was supplemented with 100ng/ml of either CXCL12 or CCL22 (Peprotech). Where used ECs were treated with 0.5ug/ml anti-HLA-DR (Biolegend) for 1 hour prior to transmigration. Those cells having migrated were determined by counting cells present in the lower well media, expressed as a percentage of total cells added and T cell subset expression determined using flow cytometric analysis after 24 hours.

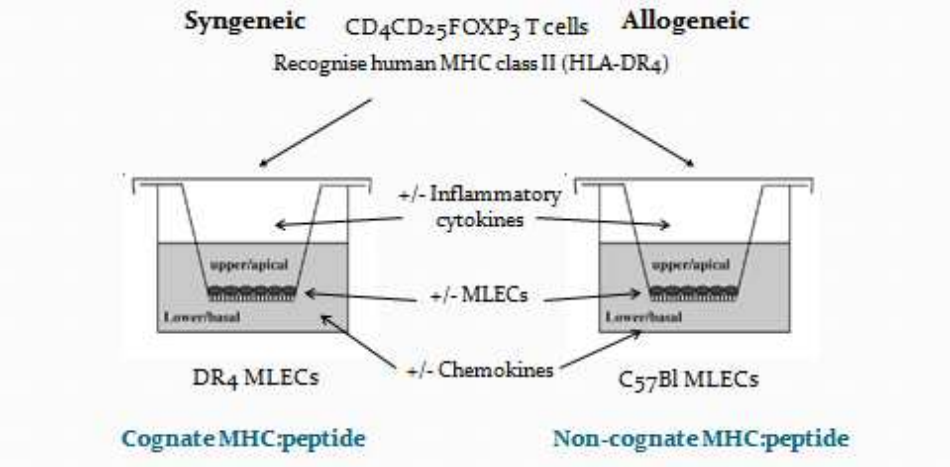

FIGURE 2.3. **In-vitro *Regulatory T cell transmigration assay***
Transmigration of $1x10^5$ isolated CD4CD25+ Tregs from DR4 mice across murine lung endothelial cell monolayers derived from DR4 and C57Bl mice to form models of syngeneic and allogeneic transmigration respectively.

2.5.3 Analysis of Treg transmigration

Single-cell suspensions of cells recovered from the lower chambers of transwells were incubated with all of the following mouse anti-murine monoclonal antibodies for Treg transmigration assays: anti-CD4-FITC, anti-CD25-PE and anti-FOXP3-PE-Cy5, (all 1:25 Insight Biotechnology) for 60 minutes on ice. Recordings were made from at least 100,000 cells on a FACS-calibur flow cytometer using WinMDI 2.8 software.

An example of flow cytometric analysis is shown in **Figure 2.4** for splenoctes derived from a DR4 mouse. Within the whole *ex vivo* splenocyte population single staining with anti-CD4-FITC shows 21% of the cells are CD4 positive **(2.4A).** Gating on this CD4 T cell population and analysing double with anti-murine CD25-PeC and FOXP3-PeCy7 (**2.4B**) highlights only 1% of the whole CD4 population are CD4+CD25+FOXP3+ regulatory T cells.

In this way percentages of total CD4+ and CD4+CD25+FOXP+ T cells within transmigrated populations can be assessed by flow cytometric analysis and actual cell counts before and after transmigration were used to determine the percentage of each cell subset transmigrating under experimental conditions.

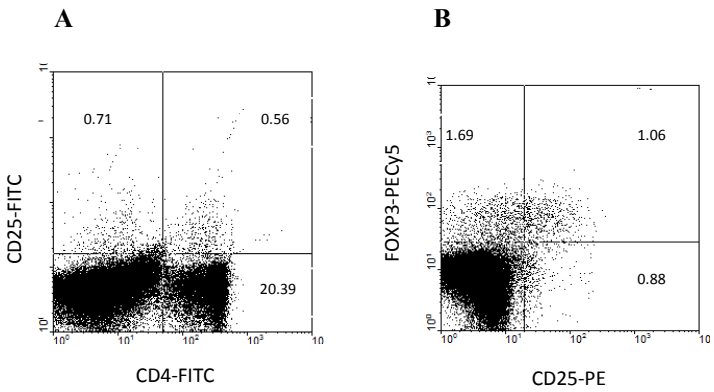

FIGURE 2.4 CD4CD25FOXP3 T cell expression by flow cytometric analysis.
Splenocytes were derived from a DR4 mouse and analysis by flow cytometry where the cells were detected by 3 colour flow cytometry for CD4CD25FOXP3 T cells using anti-murine CD4-FITC, CD25-Pe and intracellular FOXP3-PeCy7. Scattergrams of FACS analysis for CD4CD25 of the whole splenocyte population is shown in **A** and **B** represents CD25FOXP3 expression gated on the CD4 T cell population.

2.5.4 Analysis of TRP-2 specific CD8 T cell transmigration

Single-cell suspensions of cells recovered from the lower chambers of transwells were incubated with all of the following mouse anti-murine monoclonal antibodies: anti-murine CD62L-FITC (eBioscience 11-0621), anti-murine CD8-PeCy5 (eBioscience 15-0083) and anti-murine CD127-PeCy7 (eBioscience 25-1271) at 1:25 dilution. In addition, anti-murine TRP-2 specific pentamer PE labelled (ProImmune) was added at a 1:10 dilution for 60 minutes on ice. Recordings were made from at least 100,000 cells on a FACS-calibur flow cytometer using WinMDI 2.8 software.

An example of flow cytometric analysis is shown in **Figure 2.5** for splenocytes derived from a C57Bl mouse immunised with TRP-2 Immunobody^{TM}. Within the whole *ex vivo* splenocyte population single staining with anti-CD8-PeCy5 shows 16% of the cells are CD8 positive **(2.5A).** Double staining of the same population with anti-murine CD8-PeCy5 and TRP-2 specific pentamer (PE) (**2.5B**) highlights only 0.22% of the whole splenocyte population are CD8+TRP-2+ T cells. Following CD8 T cells isolation the percentage of CD8 T cells increases to around 94% (**2.5C**) with 2.16% of the population being CD8+TRP-2+ T cells.

Figure 2.5D represents a scattergram for a CTL line stimulated with 10ng/ml TRP-2 and rested for 14 days where an effector memory phenotype was identified by CD62L-CD127+ expression. Within the effector memory population 22% were CD8+TRP-2+ T cells.

In this way percentages of total CD8+ and CD8+TRP-2+ T cells within transmigrated populations can be assessed by flow cytometric analysis and actual cell counts before and after transmigration were used to determine the percentage of each cell subset transmigrating under experimental conditions.

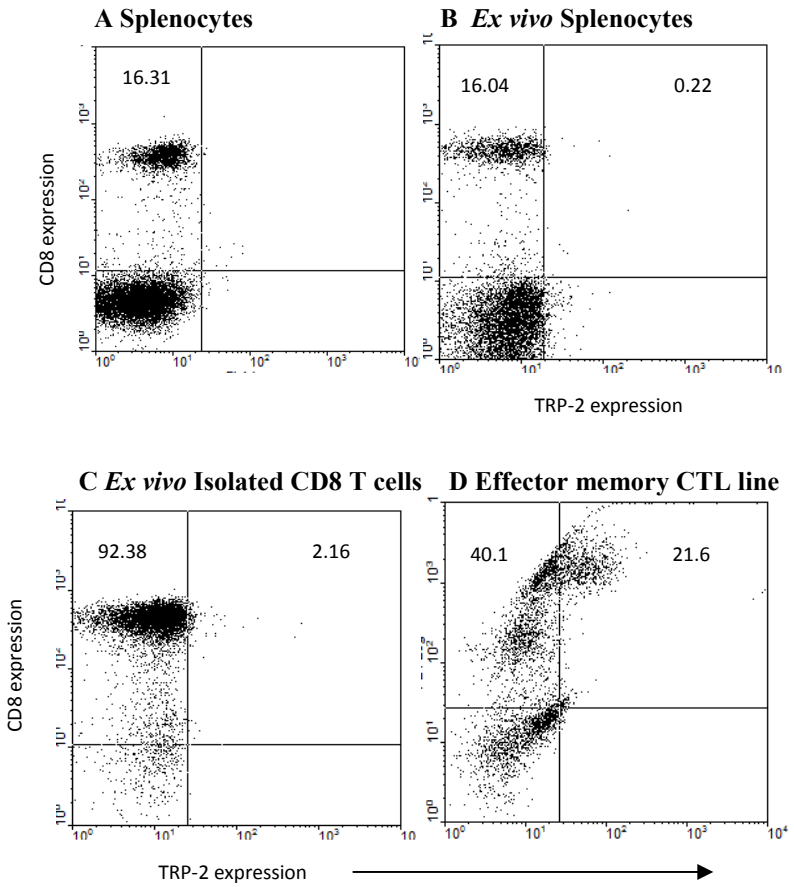

FIGURE 2.5 TRP-2 specific CD8 T cell expression by flow cytometric analysis.
Splenocytes were derived from a C57Bl mouse immunised 3 times with TRP-2 Immunobody^{TM} construct via gene gun. Scattergrams of FACS analysis for CD8+ and CD8+TRP-2+ expression within the *ex vivo* splenocyte population is shown in **A** and **B** respectively. CD8+TRP-2+ expression within an *ex vivo* isolated CD8 T cell population and 14 day cultured CTL line is shown in **C** and **D** respectively.

2.6 Synthesis of TRP-2 knockout B16 lines

To investigate the effect of antigen recognition on the *in vivo* transmigration of TRP-2 specific CTL lines into B16 tumours a TRP-2 knock-out murine B16 melanoma cell line was produced to compare infiltration of T cells against TRP-2+ B16F10 wild type tumours.

The BLOCK-iT^{TM} Inducible Pol II miR RNAi Expression Vector Kit was used (Invitrogen). RNA interference (RNAi) describes the phenomenon by which short, homologous RNA duplexes induce potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA (mRNA), and is functionally similar to the processes of post-transcriptional gene silencing (PTGS).

TRP-2 or Mus musculus dopachrome tautomerase (Dct), mRNA sequence is shown below **Figure 2.6**).

Base Sequence

```
   1 gaactgagtt caaggcaatt aaggtcaagg gctagggaga gaaggaggag gcttagaaac
  61 agcagcataa taagcagtat ggctggagca ctctgtaaat taactcaatt agacagagcc
 121 tgatttaaca aggaagactg gcgagaagcg ctcccctcat taaacctgat gttagaggag
 181 cttcggatga aattaaatca gtgttagttg tttgagtcac ataaaattgc atgtgcgtgt
 241 acacatgtgc acacgtgtag gctctgtgat ttaggtggga attttgagag gagaggaaag
 301 ggctagaact aaacccaaag aaaaggaaag aagagaagag gaaaggaaag aaaaaagaaa
 361 aggcaatttg agtgagtaaa ggttccagaa ctcaggagtg gaagacaagg agtaaagtca
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 661 caaacagaca ccagaccctg gagtggccct tatatccttc gaaaccagga tgaccgtgag
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1081 aaggccattg atttctctca ccaagggcct gcctttgtca cgtggcacag gtaccatctg
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1561 gtcatgaacc ttcataactt ggctcactcc ttcctgaatg ggaccaatgc cttgccacac
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1921 ttggggttgc tggcttttct tcaatacaga aggcttcgca aaggctatgc gcccttaatg
1981 gagacaggtc tcagcagcaa gagatacacg gaggaagcct agcatgctcc tacctggcct
2041 gacctgggta gtaactaatt acaccgtcgc tcatcttgag acaggtggaa ctcttcagcg
2101 tgtgctcttt agtagtgatg atgatgatgc cttagcaatg acaattatct ctagttgctg
2161 ctttgcttat tgtacacaga caaaatgctt gggtcattca ccacggtcaa agtaaggtgt
2221 ggctagttat atgtgacctt tgattaaaag tccttatatt gaaaaaaaaa aaaaaaaaa
```

FIGURE 2.6 TRP-2 or Mus musculus dopachrome tautomerase (Dct), mRNA sequence, NCBI Reference Sequence: NM_010024.3

2.6.1 DNA plasmid generation

The BLOCK-iT^{TM} RNAi Designer (Invitrogen) was used to generate miR RNAi oligonucleotides (oligos), the selected oligos started at 1360: CTGTGTAATGGAACCTATGAA, shown in bold in **Figure 2.6**.

Forward Primer 5' to 3' NM_010024_1360

TGCTGTTCATAGGTTCCATTACACAGGTTTTGGCCACTGACTGACCTGTGTAAGAACCTATGAA
CAAGTATCCAAGGTAATGTGTCCAAAACCGGTGACTGACTGGACACATTCTTGGATACTTGCCT

Reverse Primer 3' to 5' NM_010024_1360

Two complementary single-stranded DNA oligonucleotides, encoding the TRP-2 miRNA were annealed to generate double stranded (ds) oligos. The ds oligos were cloned into the linearised pcDNA™6.2-GW/EmGFP-miR vector and transformed into One Shot® TOP10 chemically competent *E. coli* and select for spectinomycin-resistant transformants. The pcDNA™6.2-GW/EmGFP-miR vector that allows the expression of the engineered TRP-2 pre-miRNA under the control of the strong, Pol II human CMV (cytomegalovirus) promoter and Herpes Simplex virus (HSV) thymidine kinase (TK) polyadenylation signal. The coding sequence of EmGFP (Emerald Green Fluorescent Protein) is incorporated into the vector such that the pre-miRNA insertion site is in the 3' untranslated (3'UTR) region of the fluorescent protein mRNA (**Figure 2.7**).

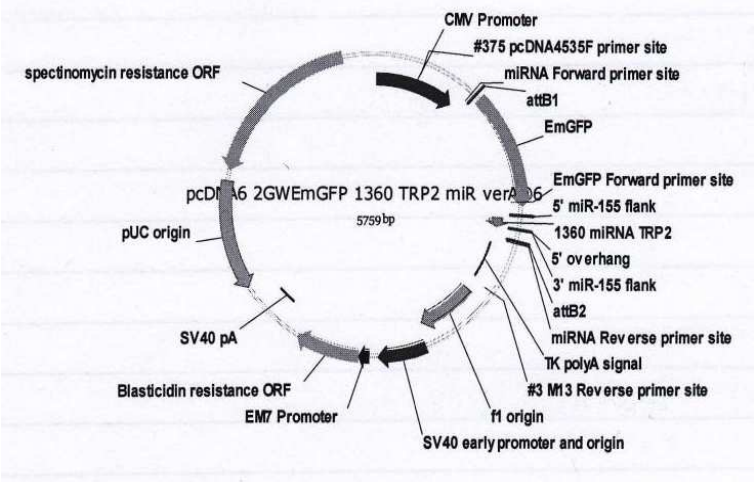


FIGURE 2.7 pcDNA6.2GWEmGFP 1360 TRP-2 miR plasmid.

2.6.1.1 Generation of double stranded oligonucleotides

To anneal single-stranded (ss) oligonucleotides to generate the double stranded (ds) oligonucleotides the lyophilised single-stranded oligonucleotides were used at 200µM each in DNase/RNase free sdH_2O.

Annealing reaction:

Top strand DNA oligo (200 μM)	5ul
Bottom strand DNA oligo (200 μM)	5ul
10X Oligo Annealing Buffer	2ul
DNase/RNase-Free Water	8ul
Total volume	20ul

The reaction was incubated at 95°C for 5 minutes, removed from the heat block and set on the laboratory bench at room temperature for 30 minutes to allow the single-stranded oligos to anneal. The samples were mixed gently in a microcentrifuge and 1μl of the 50μM annealing mixture was added to 99μl H_2O (500nM). Subsequently 1μl of this 500nM oligo mix was then added to 5μl 10x oligo annealing buffer and 44μl DNase free H_2O to make a final concentration of 10nM ds oligo stock.

2.6.1.2 Ligation

Ligation reaction:	5x Ligation buffer	4ul
	pcDNA6.2GW/GMGFPmiRlinearised	2ul
	miR 1360 oligo stock (10nM)	2ul
	DNase/RNase free H_2O	8ul
	T4 DNA ligase	1ul

The ligation buffer was mixed by pipetting and incubated at room temperature for 1 hour and then placed on ice.

2.6.1.3 Transformation of E.coli

The ligation reaction was transformed into One Shot® TOP10 Chemically Competent E. coli. 2μl of the ligation reaction was added to 50μl of Shot® TOP10 chemically competent E. coli and mixed gently. It was incubated on ice for 30 minutes, heat shocked for 30 seconds at 42°C and transferred back to ice. 250μl of room temperature S.O.C. (super optimal broth) medium (Sigma) was added and incubated at 37°C for 1 hour. SOC medium contains 50 and

150µl from each transformation were spread onto pre-warmed LB agar plates containing 50µg/ml spectinomycin and incubated overnight at 37°C.

Plates were assessed for colony growth and 10 individual colonies were selected, picked with a sterile pipette tip and placed into universals containing 1.5mls of LB containing 50 µg/ml spectinomycin. Samples were then placed at 37°C overnight.

Plasmid DNA purification performed using QIAprep Miniprep kits (QIAGEN, Sussex, UK) on the 10 clones for sequencing positive transformants to confirm the sequence of the ds oligo insert and 15µl of each were sent to Lark Technologies for sequence analysis.

2.6.2 Transfection of B16 cells

DNA plasmid was purified using QIAprep Maxiprep kit and spectrophotometric DNA quantification performed. B16 cells were then transfected with the plasmid using lipofectamine 2000 reagent (Invitrogen) and cells selected for Blasticidin resistance. 4µg of DNA plasmid was added to 250µl of OPIMEM media while 10ul of Lipofectamine 2000 reagent was added to a further 250µl of OPIMEM media. They were mixed gently and incubated at room temperature for 5 minutes. DNA and Lipofectamine were added together and incubated at room temperature for 20 minutes. B16 cells, were grown overnight at $1.5x10^5$ cells/ml in 6 well tissue culture plates (Costar, Sigma). The media was replaced with 2mls of fresh media and DNA/Lipofectamine complex was added dropwise and mixed gently prior to incubation for 24 hours at 37^oC.

2.6.3 Analysis of TRP-2 knockdown

Each well was trypsinised and cells replated into 24 well tissue culture plates in fresh complete medium containing 10μg/ml Blasticidin. Medium was replaced with fresh medium containing Blasticidin every 3-4 days until Blasticidin- resistant colonies were identified (generally 10-14 days after selection). Blasticidin-resistant colonies were analysed for knockdown of TRP-2 expression by flow cytometric analysis of GFP expression (EmGFP excitation 487nm). Intracellular TRP-2 expression was analysed using rabbit anti-murine TRP-2 antibody (Abcam, Cambridge, UK, ab74073) and a goat anti-rabbit-PeCy7 secondary antibody (SantaCruz). Colonies were selected that showed no/minimal TRP-2 expression with the presence of GFP.

2.7 *In vivo* TRP-2 specific transmigration assay

To analyse the effect of antigen-recognition on CD8 T transmigration into tumours an *in vivo* model of transmigration was formed using B16 melanoma as a murine tumour model and TRP-2 specific CD8 T cells of an effector memory phenotype.

2.7.1 Tumour growth

The B16 tumour cell lines were grown in T75 tissue culture flasks in RPMI 1640 plus 10% NBCS at 37^{o}C 5% CO_2 until confluent. B16 cells harvested by trypsinisation and resuspended at 2x10^5 cells/ml in PBS. C57Bl mice were injected subcutaneously with 2x10^4 B16 WT cells on the right flank and 2x10^4 B16 TRP-2 knockout cells on the left flank. Tumour growth was analysed every 2 days and used for analysis of transmigration when they reached 500mm^3.

2.7.2 Labelling and immunisation of CTL line

The CTL line stimulated with LPS blasts and 10ng/ml TRP-2 peptide was rested for 2 weeks, harvested and cells placed at $1x10^6$ cells/ml in PBS. 1nM CFSE (diluted from 5nM stock in PBS) was added and the cell solution incubated at 37^oC for 15 minutes. Staining was quenched with the addition of ice-cold media for 5 minutes. CTLs were resuspended in PBS at $2x10^7$ cells/ml and 500ul was injected by intraperitoneal injection into each tumour bearing mouse.

2.7.3 Analysis of *in vivo* T cell infiltration

T cell infiltration was analysed within TRP-2 positive and negative B16 tumours from the same mouse. Tumours, spleens and peripheral blood samples were taken and analysed by flow cytometry for CFSE expression to identify adoptively transferred cells. Once identified, these samples were analysed with anti-murine CD8-PeCy5 (eBioscience 15-0083) at 1:25 dilution and anti murine TRP-2 specific pentamer PE labelled (ProImmune, Oxford, UK) at 1:10 dilution.

2.8 Immunohistochemical staining

Immunohistochemistry (IHC) exploits the specific interaction between an antibody and its antigen to estimate expression of that antigen on a section of tissue. This study used formalin fixed paraffin embedded (FFPE) tissue for both an ovarian and a colorectal TMA. All tissues used the same technique with slight alterations depending on type of tissue and antigen.

2.8.1 Colorectal patient cohort

The study population cohort encompasses a consecutive series of 462 archived primary invasive colorectal cancer specimens obtained from patients undergoing elective surgical resection of a histologically proven primary colorectal cancer at Nottingham University Hospitals, Nottingham, UK.

The samples were collected between January 1994 and December 2000 from the established institutional tumour bank and were identified from the hospital archives. No cases were excluded unless the relevant clinico-pathological material/data were unavailable. The average follow-up period was 42 months (range 1-116 months) to ensure a sufficient duration of follow-up to allow meaningful assessment of the prognostic value of the markers examined. Follow-up was calculated from the date of resection of the primary tumour, and all surviving cases were censored for data analysis on December 2003. Patient and tumour characteristics for the cohort have been previously published [313]. All patients underwent surgical resection; patients with lymph node positive disease routinely received adjuvant chemotherapy with 5-flurouracil and folinic acid.

All tumours received following resection in the histopathology lab were incised, fixed immediately in formaldehyde and processed through to embedding in paraffin wax, ensuring optimal tissue fixation and preservation for histological examination. Tissue microarrays were built as described previously [314].

2.8.2 Colorectal specimen characteristics

For each tumour, 5μm section slides stained with haematoxylin-eosin were first used to locate representative areas of viable tumour tissue. 0.6 mm needle core-biopsies from the corresponding areas on the paraffin-embedded tumour blocks were then placed at pre-specified coordinates in recipient paraffin array blocks using a manual tissue-arrayer (Beecher Instruments, Sun Prarie, WI). Array blocks were constructed with between 80–150 cores in each, with analysis of a single core from each case. Fresh 5μm sections were obtained from each TMA block and placed on coated glass slides to allow the immunohistochemical procedures to be performed, preserving maximum tissue antigenicity.

2.8.3 Ovarian patient cohort

A total of 360 patients with ovarian cancer were entered in this study, and these consisted of patients undergoing a laparotomy for primary ovarian cancer. Information on cancer size, stage, presence or absence of residual disease after surgery, histologic type and grade, age at diagnosis, and type of adjuvant treatment was collected for all patients. From this original population, histologic material was available for analysis in 339 cases. The paraffin-embedded tissue blocks from these patients dated back from January 1, 1984 until December 31, 1997. Disease-specific survival was calculated from the operation date until November 31, 2005 when any remaining survivors were censored. The database was audited to ensure validity; there were no major discrepancies with >97% of data available.

2.8.4 Ovarian patient characteristics

During the study period, patients with high-grade stage I and stage II to IV disease received chemotherapy. The specific chemotherapy varied but reflected the best current practice; most recently, this treatment was platinum based. Sixty-two patients participated in the International Collaborative Group for Ovarian Neoplasia trials I to IV during which the allocated chemotherapy was randomized. Although the study spans a 14 year period, there was no significant change in the survival of patients treated in the earlier or latter part of the study. This is in line with the unaltered survival of ovarian cancer patients over the last 30 years [315].

2.8.5 Immunohistochemical analysis of CD8

Immunohistochemical analysis of CD8 expression was performed using a routine streptavidin-biotin peroxidase method. Tissue array sections were first deparaffinised with xylene, rehydrated through graded alcohol and immersed in methanol containing 0.3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity. In order to retrieve antigenicity, sections were immersed in 500 mls of pH 9.0 EDTA buffer and heated for 10 min in an 800 W microwave at high power, followed by 10 min at low power. Endogenous avidin/biotin binding was blocked using an avidin/biotin blocking kit (Vector Labs, USA). In order to block non-specific binding of the primary antibody all sections were then treated with 100 µl of 1/5 normal swine serum (NSS) in TBS for 15 min.

CD8 expression was determined using 100 µl of a rabbit polyclonal antibody (ab4055, AbCam), recognising a synthetic peptide comprising the 13 C-terminal amino acids of the cytoplasmic domain of the CD8 alpha chain, at an optimal concentration of 1:200 in TBS for

1 hour at room temperature. Positive control tissue comprised whole sections of colorectal cancer tissue. The primary antibody was omitted from the negative control, which was left incubating in TBS.

After washing with TBS all sections were incubated with 100 µl of biotinylated goat anti-mouse/rabbit immunoglobulin (Dako Ltd, Ely, UK) diluted 1:100 in NSS, for 30 min. Sections were washed again in TBS and next incubated with 100 µl of pre-formed streptavidin-biotin/horseradish peroxidase (HRP) complex (Dako Ltd, Ely, UK) for 60 min at room temperature. Subsequently, visualisation of CD8 expression was achieved using 3, 3'-Diaminobenzidine tetra hydrochloride (DAB, Dako Ltd, Ely, UK). Finally, sections were lightly counterstained with haematoxylin (Dako Ltd, Ely, UK), dehydrated in alcohol, cleared in xylene (Genta Medica, York, UK) and mounted with distyrene, plasticiser and xylene (DPX – BDH, Poole, UK).

2.8.6 Immunohistochemical analysis of CXCL12 and CXCR4

Immunohistochemical analysis of active CXCL12 and CXCR4 expression was performed using a routine streptavidin-biotin peroxidase method. Tissue array sections were first deparaffinised with xylene, rehydrated through graded alcohol and immersed in methanol containing 0.3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity. In order to retrieve antigenicity, sections were immersed in 500 mls of pH 9.0 EDTA buffer and heated for 10 min in an 800 W microwave at high power, followed by 10 min at low power. Endogenous avidin/biotin binding was blocked using an avidin/biotin blocking kit (Vector Labs, USA). In order to block non-specific binding of the primary antibody all sections were then treated with 100 µl of 1/5 normal swine serum (NSS) in TBS for 15 min.

Test sections were incubated with 100 µl of mouse polyclonal antibody, CXCL12/SDF-1 (MAB350) recognising human CXCL12 (R&D Systems) which was found to show optimal staining at a dilution of 1/50 in TBS for 1 hour at room temperature. CXCR4 expression was determined using a mouse monoclonal antibody (MAB172, R&D Systems) recognising human CXCR4 at an optimal concentration of 1:600 in TBS for 1 hours at room temperature. Positive control tissue comprised whole sections of colorectal cancer tissue. The primary antibody was omitted from the negative control, which was left incubating in TBS.

After washing with TBS, all sections were incubated with 100 µl of biotinylated goat anti-mouse/rabbit immunoglobulin (Dako Ltd, Ely, UK) diluted 1:100 in NSS, for 30 min. Sections were washed again in TBS and next incubated with 100 µl of pre-formed streptavidin-biotin/horseradish peroxidase (HRP) complex (Dako Ltd, Ely, UK) for 60 min at room temperature. Subsequently, visualisation of CXCL12 or CXCR4 expression was achieved using 3, 3'-Diaminobenzidine tetra hydrochloride (DAB, Dako Ltd, Ely, UK). Finally, sections were lightly counterstained with haematoxylin (Dako Ltd, Ely, UK), dehydrated in alcohol, cleared in xylene (Genta Medica, York, UK) and mounted with distyrene, plasticiser and xylene (DPX – BDH, Poole, UK).

2.8.7 Evaluation of staining

The tumour cores were first imaged using a NanoZoomer (Hamamatsu, NJ, USA). The cores were then assessed at X20 magnification by two observers (AP and RS) in the Nanozoomer Digital Pathology viewer (Hamamatsu), both with experience in the analysis of TMAs. Both observers were blinded to the clinical and pathological parameters of the case. In the few cases (<5%) where there was a discrepancy between the classification of cores a review of those cores was performed and a consensus reached. Tumours were classified by H scores

where tumours were assessed for high, moderate, low and negative CXCL12 and CXCR4 expression. For the CD8 staining, a different approach was used due to whole cells present. Using the NanoZoomer Slide Viewer, the area of the tumour was measured and number of positive cells in each area counted, giving a value of positive cells per mm^2.

Following scoring, H score data needed appropriate grouping to facilitate statistical analysis. This was most commonly achieved using a binary cut off of either high or low expression using the program X-Tile (Yale University, CT, USA) was used to determine low/high expression groups.

2.9 Statistical analysis

Statistical analysis of the study data was performed using the SPSS package (version 16 for Windows, SPSS Inc., Chicago, IL). Pearson χ^2 chi squared tests were used to determine the significance of associations between categorical variables. Disease-specific survival calculations included all patients whose death related to cancer. In contrast, patients whose deaths resulted from non-cancer related causes were censored at the time of death. Kaplan-Meier curves were used to assess factors which influenced survival. The statistical significance of differences in disease-specific survival between groups with differing expression was estimated using the log-rank test. The Cox proportional-hazards model was used for multivariate analysis in order to determine the relative risk and independent significance of individual factors. In all cases p-values < 0.05 were considered as statistically significant.

For statistical analysis of T cell transmigration P values were calculated by using two-sided Student's t test; $P<0.05$ was considered to be statistically significant.

Chapter 3: TRP-2 antigen-dependent CD8 T cell transmigration

3.1 Background

Cytotoxic CD8 T cells can recognise tumour antigens and have the potential to eliminate tumour cells *in vivo* [316]. The endothelium is a gateway for T cell transmigration into tumours and it is essential to understand the endothelial barrier and the immunological switches that promote transmigration if an effective immunotherapy is to be generated.

T cell trafficking from blood to tissues is a multi-step process including leukocyte tethering and rolling, firm adhesion and transmigration through the endothelial cell lining. Several studies have shown that recognition of MHC: peptide complexes may also provide a vital signal for extravasation [153,317,318,319]. Marelli-Berg *et al* proposed a 'shop window model' whereby endothelial cells acquire antigens from the surrounding tissue and present them in the context of MHC molecules to circulating T cells within the blood. Cognate peptide:MHC recognition allows entry into tissues [318]. However, it remains unclear how antigen can be acquired from tissues by endothelial cells and presented on MHC. One mechanism that has recently been described is transfer of peptides via gap junctions from the underlying tissue to the endothelial cells and then presented on MHC [320]. This would allow specific enrichment of the relevant T cells for the tissue. Unlike a viral infection the tumour microenvironment may prevent the endothelium displaying the complete architecture of an inflamed tissue, perhaps making the role of MHC peptide more vital. As only small amounts of peptide are transferred to endothelial cells this may mean that only high avidity T cells recognising low levels of cognate MHC: peptide will get sufficient signal to extravasate. As

only high avidity T cells clear viral infections and eradicate tumours [305,321,322], the selective enrichment by the endothelium would be a distinct advantage.

In this study an *in vitro* model is used for transmigration of high avidity antigen-specific CD8 T cells across murine lung endothelium. The CD8 T cells used to transmigrate were specific for a TRP-$2_{(180\text{-}188)}$ peptide epitope SVYDFFVWL presented by H2-K^b. TRP-2 is a melanogenic enzyme (dopachrome tautomerase, *dct*) that is expressed by both melanocytes and melanomas and has been identified as a melanoma rejection antigen [262,312]. TRP-$2_{(180\text{-}188)}$ is an epitope identified as a shared HLA-A*0201 epitope recognised by both human and murine CTLs [323,324]. Murine lung endothelial cells (MLECs) are easy to isolate in substantial numbers and were therefore used as a source of endothelial cells. As the lungs have been shown to be the primary site of metastasis in melanoma patients [325,326] lung endothelium would be an essential barrier for tumour antigen specific T cells to reach metastases making lung endothelium a biologically relevant model.

TRP-2 specific CD8 T cells were derived from mice immunised with a DNA vaccine platform called the ImmunoBody™ [309]. The ImmunoBody™ epitope expression system represents a plasmid DNA vector encoding an IgG1 human monoclonal antibody in which the complementarity determining regions (CDRs) are replaced with T cell epitopes. Upon immunisation these T cell epitopes are efficiently processed and presented to break tolerance and stimulate high avidity CTL responses capable of anti-tumour activity, prevention of metastases and inhibition of already established tumours [306,307].

By using a novel murine model of TRP-2 specific CD8 transmigration the aim of this study was to investigate whether cognate MHC/peptide recognition was required for efficient endothelial transmigration. A static transmigration model allows the effect of transmigration to be assessed independently of adhesion as the requirement for rolling and tethering is

removed. Splenocytes derived from immunised C57Bl mice were used to transmigrate across lung endothelium derived from C57Bl wildtype mice and HHDII (human HLA-A*0201) transgenic mice forming models of syngeneic and allogeneic transmigration respectively. HHDII transgenic mice (Institut Pasteur) were used as they were on the same genetic background as C57Bl mice but mis-matched for MHC class I as they have human HLA-A*0201 class I and no mouse MHC class I molecules. *In vivo* studies in C57Bl mice transplanted with mouse tumours expressing TRP-2 or in which TRP-2 had been knocked out confirmed the antigen specificity of transmigration.

The hypothesis is that high avidity T cells may selectively transmigrate across endothelium due to cognate MHC:peptide recognition (H-2Kb/TRP-2) confirming that antigen specific transmigration is important in CD8 T cell transmigration. Thus we sought to investigate whether cross-presentation of specific antigens by the endothelium within tumour environmental conditions is one of the requirements for the homing of CTL into tumours.

3.2 Results

3.2.1 Generation of high avidity CD8 T cells recognising TRP-2 peptide.

The effect of cognate peptide:MHC recognition of TRP-2 presented by the endothelium on the migration of antigen specific CD8+ T cells was investigated using a murine model of transendothelial T cell migration (**Figure 2.2**).

Previous research has shown that immunisation with the TRP-2 Immunobody construct results in high frequency high avidity T cells [305]. CD8 T cells were derived and pooled from three C57Bl mice immunised with the TRP-2 ImmunoBody™ construct and the presence of TRP-2 specific CD8 T cells was measured in an ELISPOT assay by IFN-γ release (**Figure 3.1A)** with titrating concentrations of TRP-2 peptide. These results confirm that the Immunobody generates TRP-2 antigen specific T cells of high avidity and high frequency responses were seen. Functional avidity of the response was calculated as the peptide concentration at which a half maximal response is achieved. In this case, for pooled splenocytes the maximal response is 700 spots/10^6 splenocytes and the peptide concentration required to achieve half of this response (350 spots) is 1ng/ml TRP-2 peptide or $1x10^{-9}$M. Therefore the avidity was shown to be 1ng/ml ($1x10^{-9}$M) of peptide.

The splenocyte population derived and pooled from 3 mice immunised with TRP-2 Immunobody™ construct was analysed using four colour FACs with; anti-murine CD62L-FITC, anti-murine CD8-PeCy5 and anti-murine CD127-PeCy7, anti-murine TRP-2 specific pentamer PE labelled Flow cytometry demonstrated that 15% of the initial population were CD8+ T cells, following CD8 T cell isolation using MACS separation the purity was around 95% (**Figure 2.5**). Pentamer staining confirmed that 2.2% of the isolated CD8 T cells were

TRP-2 specific and that the majority of these cells were CD62L+CD127+ central memory T cells (**Figure 3.1C**). However, this central memory population would not be a true representative of CD8 T cells within peripheral blood as central memory, effector and effector memory cells are all found in blood. Therefore, the proportion of memory/effector subsets present within murine peripheral blood relative to the spleen was determined. **Figure 3.1B** shows that Flow cytometric analysis of CD8 T cells derived from the spleen of a naive C57Bl mice show a predominantly central memory phenotype (70%) whereas CD8 T cells within the peripheral blood from the same mouse show 50% effector phenotype (CD62L-CD127-), 20% central memory and 20% effector memory (CD62L-CD127+).

In order to generate TRP-2 specific CD8 T cells of central memory, effector memory and effector phenotypes, CTL lines were derived from the splenocytes of three immunised mice, pooled and maintained in culture in the presence of 10ng/ml TRP-2 peptide and IL-2 (20U/ml). **Figure 3.1C** shows the phenotype of one representative cultured CD8+TRP-2 specific CTL line up to 14 days post-stimulation. The data shows that after the initial stimulation, the phenotype switches from a predominantly central memory (CD62L+CD127+) to an intermediate phenotype (CD62L+CD127-). Histograms for one representative CTL line are shown for days 7 to 14 (**Figure 3.1D).** After 7 days the line was predominantly effector CD8 T cells with 85% CD62L-CD127- CD8 T cells (**3.1Di**). From 7-14 days the percentage of effector cells decreased as effector memory cells increased (CD62L-CD127+) resulting in 70% of the line being effector memory TRP-2 specific CD8 T cells and 30% effector at 14 days (**3.1Diii**).

For subsequent experiments *ex vivo* isolated CD8 T cells, of a predominant central memory phenotype, were referred to as central memory CD8 T cells and functional avidity was assessed via ELISPOT assays. CD8 T cells cultured with peptide for 10-14 days were

referred to as effector memory CD8 T cells as determined by flow cytometric analysis prior to use in each assay. Effector memory CD8 T cells are determined by a switch from an effector phenotype (CD62L-CD127-) to a predominant (CD62L-CD127+) phenotype by upregulation of CD127, this occurred between 10-14 days post-stimulation (**Figure 3.1C&D**). The percentage of CD8+TRP-2+ T cells within each central memory and effector memory population was evaluated prior to each assay by flow cytometry.

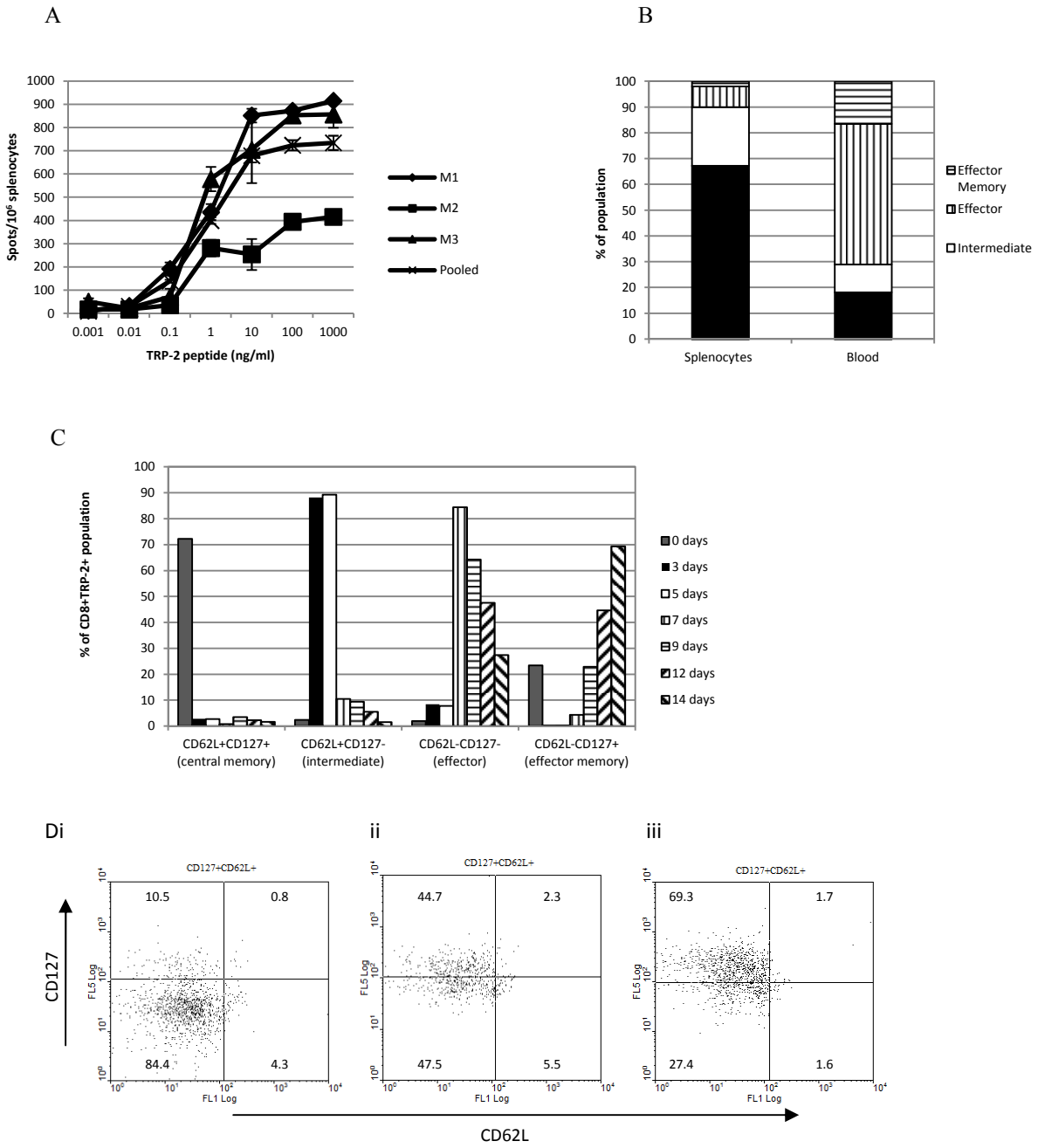


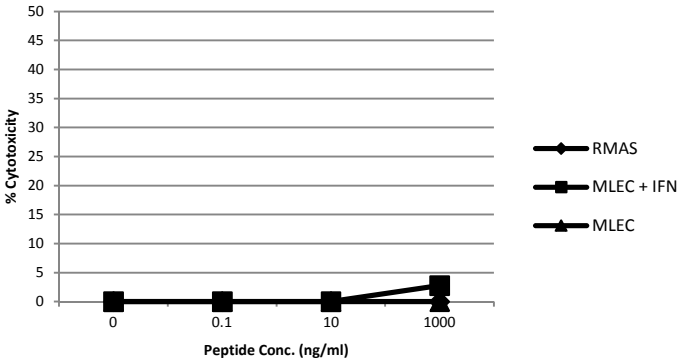
FIGURE 3.1 ***Generation of high avidity CD8 T cells recognising TRP-2 peptide.***
A Murine IFN-γ Elispot assay showing responses to TRP-2 peptide following vaccination of three C57Bl mice with Immunobody^{TM} DNA construct incorporating TRP-2 (DCIB15) via gene gun weekly for 3 consecutive weeks. Data in **A** shows the number of spots developed per 10^6 splenocytes at increasing peptide concentrations from 1pg/ml ($1x10^{-12}$M) to 1ug/ml ($1x10^{-6}$M). The functional avidity of the response is calculated as the peptide concentration at which a half maximal response is achieved. **B** Subset phenotypes of CD8 T cells from the spleen and peripheral blood of a naive C57Bl mouse determined by flow cytometry using; anti-murine CD62L-FITC, anti-murine CD8-PeCy5 and anti-murine CD127-PeCy7 at 1:25 dilution. In addition, anti-murine TRP-2 specific pentamer PE labelled (ProImmune) was added at a 1:10 dilution for 60 minutes on ice. Recordings were made from at least 100,000 cells on a FACS-calibur flow cytometer using WinMDI 2.8 software. **C** Splenocytes derived from C57Bl mice immunised 3 times with TRP-2 Immunobody^{TM} construct via gene gun. The $CD8^+$ T-cell line, specific for the TRP-2 peptide epitope SVYDFFVWL presented by H2-K^b, was maintained in culture by restimulation every week with LPS blasts from C57Bl mice presenting TRP-2 peptide (10ng/ml) and recombinant interleukin-2 (rIL-2) (20 U/mL). The data represents memory subset expression over time for one CTL line where each CTL line generated is assessed in the same way for effector memory phenotype generation (n>6). **D** Shows histograms of CD62L and CD127 expression on gated CD8+TRP-2+ T cells at 7 (**i**), 12 (**ii**) and 14 days (**iii**) post stimulation for one representative line.

3.2.2 CTL mediated lysis of target cells via memory subsets of CD8 T cells.

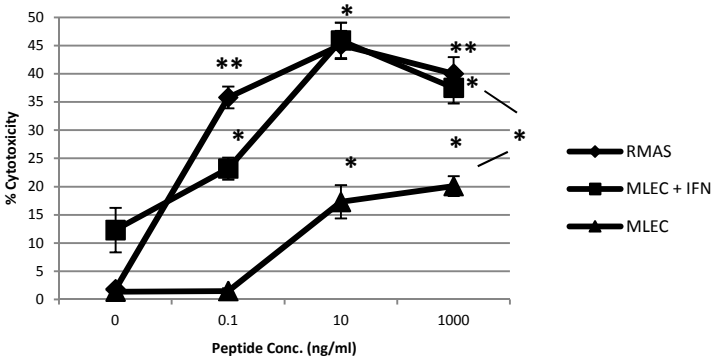
As the CD8 T cells specific for TRP-2 were to be used in transmigration assays with endothelial monolayers pulsed with TRP-2 peptide it was vital to determine that these cells would not kill endothelial cells as this would alter the integrity of endothelial monolayers. **Figure 3.2** shows data for a CTL killing assays carried out on separate occasions using CD8 T cells isolated from splenocytes derived and pooled from three mice immunised with TRP-2 Immunobody^{TM} and used directly ex vivo (**A**), of a CD62L-CD127- (effector) phenotype following 6 days culture (**B**) and of a CD62L-CD127+ (effector memory) phenotype following 14 days (**C**) with 10ng/ml TRP-2 peptide pulsed blasts. These represent central memory, effector and effector memory CD8 T cells respectively. The TAP-deficient RMAS cell line was used as a control. **Figure 3.2A** and **3.2C** demonstrate that the central and effector memory CD8 T cells did not kill endothelial cells. However, after one week of culture, splenocytes that are predominately of an effector phenotype (**Figure 3.2B)** killed resting endothelial cells. Seven days of culture of splenocytes is normal experimental conditions which are used to generate a standard 4 hour 51Cr release killing [327]. Maximum 20% killing was achieved at a 50:1 effector:target ratio which is increased to around 40% following endothelial cell activation with IFN-γ and TNF-α ($p=0.011$). This is similar to the level of killing of peptide pulsed RMAS cells. Cytotoxic killing of peptide pulsed RMAS and cytokine treated endothelium by effector cells was achieved at 0.1ng/ml peptide pulsing whereas for resting endothelial cells 10ng/ml of peptide was required ($p<0.05$). Maximum killing by effector cells of resting and activated endothelial cells was achieved at a concentration of 10ng/ml TRP-2 peptide ($p<0.05$). These results show that endothelial cells can be killed by effector CD8 T cells at low levels even in the absence of inflammation. These results suggest that effector CD8 T cells can kill endothelial cells

whereas central memory and effector memory T cells recognising antigen presented by endothelial cells do not. This raises the question as to why effector CD8 T cell populations within peripheral blood do not cause extensive endothelial damage. However, to avoid destruction of the endothelial monolayer by effector T cells, for our transmigration studies we concentrated on studying central and effector memory cell migration. As shown is **Figure 3.1B** these cells comprise 50% of the peripheral blood population.

A Central memory



B Effector



C Effector memory

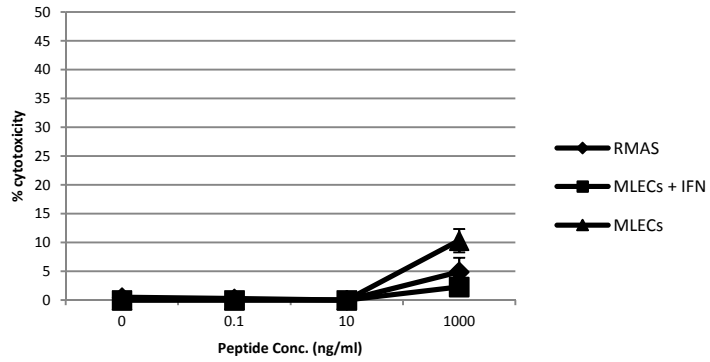


FIGURE 3.2 ***CTL mediated lysis of target cells via memory subsets of CD8 T cells.***
Splenocytes derived from three C57Bl mouse immunised 3 times with TRP-2 Immunobody^{TM} construct via gene gun were pooled and either used directly **A** *ex vivo* (central memory) or **B** cultured for 6 days (effector) or **C** 14 days (effector memory) with LPS blasts pulsed with 10ng/ml TRP-2 peptide. Therefore the assay was carried out on separate occasions and the targets used were C57Bl endothelial cells with (Square) and without stimulation (Triangle) with IFN-γ (100ng/ml) and TNF-α (100ng/ml) for 24 hours and the endogenous antigen-processing deficient RMAS cell line (Diamond). Target cells pulsed with peptide concentrations from 0-1000ng/ml TRP-2 peptide and 1.85mBq 51[Cr] Sodium Chromate for 1 hour and incubated for 4 hours with effector cells. The percentage cytotoxicity was calculated at a 50:1 effector:target ratio as the actual cell counts-spontaneous lysis/maximum lysis x 100. P values were calculated by using two-sided Student's t test were carried out for each target cell type at varying peptide concentrations compared to the control; $P<0.05$ was considered to be statistically significant where * represents $p<0.05$ and ** represents $p<0.001$ for n=3.

3.2.3 Cognate recognition of ECs enhances transendothelial migration of TRP-2-specific CD8+ T cells *in vitro*.

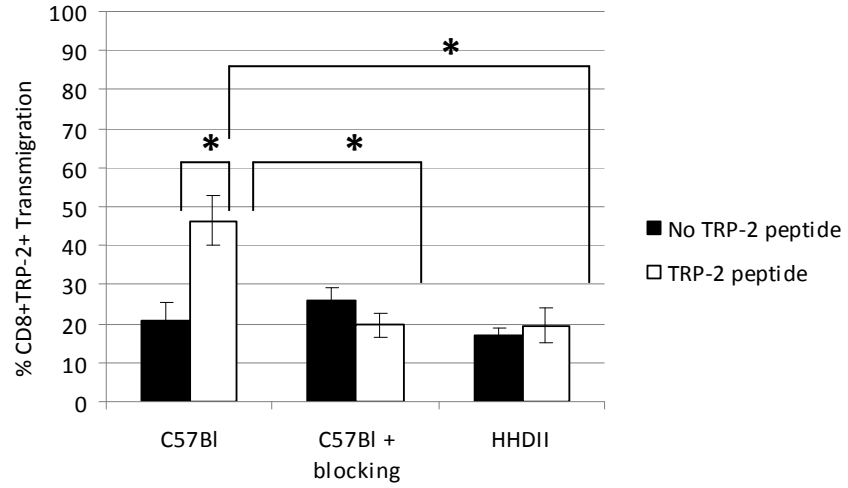
TRP-2 antigen-specific CD8 T cells, of central (CD62L+CD127+) and effector memory (CD62L-CD127+) phenotypes were transmigrated across C57Bl and HHDII murine lung endothelial cells to form syngeneic (cognate MHC) and allogeneic (non-cognate MHC class I) models of transmigration. For the central memory transmigration assay CD8 T cells were isolated from TRP-2 immunised C57Bl mice and the purity for this experiment was around 80% CD8+ T cells for all mice. For effector memory transmigration, data is an average of three separate experiments. Pre and post transmigrated cells were stained for CD8+TRP-2 pentamer positive T cells. During analysis by flow cytometry the cells were gated on leukocyte populations and double stained using anti-CD8-PECy5 and anti-TRP-2 pentamer-PE labelled antibodies. MLEC monolayers of C57Bl (syngeneic) and HHDII (allogeneic) were used. C57Bl ECs were untreated (control) or treated with anti-H-2K/Db for 1 hour prior to transmigration to block murine MHC class I and prove the involvement of peptide/MHC on transmigration. All ECs were pulsed with 10ng/ml TRP-2 peptide for 4 hours prior to addition of splenocytes for transmigration.

Table 3.1 shows how the percentage transmigration is calculated from the actual numbers of CD8+TRP-2+ T cells transmigrating under each condition. The percentage transmigration of TRP-2 antigen-specific CD8 T cells was calculated from the absolute numbers of all cells having transmigrated and the relative percentages of CD8+TRP-2+ T cells within each migrated population relative to cell numbers in the initial population prior to transmigration. Within the isolated population of Mouse 1, 2.2% of cells (8640 of 300,000 cells) were CD8+TRP-2+, following transmigration across untreated syngeneic endothelium these cells made up 2.4% of the migrated population (2187 of 90,000 cells). This equates to 25%

transmigration (2187/8640*100) of the CD8+TRP-2+ cells (**Table 3.1**). Whereas following transmigration across TRP-2 peptide pulsed syngeneic, 5800 TRP-2+ CD8 T cells from an initial population of 8640 transmigrated endothelium which equates to 67% transmigration of the CD8+TRP-2+ cells**.**

Figure 3.3Ai shows the percentage transmigration of central memory CD8+TRP-2+ T cells across syngeneic resting endothelium was 20±4.9%, in the presence of TRP-2 peptide (10ng/ml) this was significantly increased to 46±6.4% (p=0.039) suggesting antigen specific transmigration. In contrast, only low level transmigration (17±1.8%) was seen across allogeneic HHDII endothelium and this was not increased with peptide pulsing of the allogeneic MHC. To confirm that the migration in response to peptide on syngeneic MHC was due to recognition of MHC:peptide CD8+TRP-2+ T cells where migrated across C57Bl MLECs pulsed with peptide in the presence or absence of MHC class I blocking antibody. The percentage of transmigrating TRP-2 positive CD8 T cells was reduced from 46±6.4% to 20±3% (p=0.024) in the presence of blocking antibody. In contrast, as expected for total CD8 T cell transmigration (**Figure 3.3Aii**) around 40% of CD8 T cells transmigrated across endothelium with and without TRP-2 peptide in the presence or absence of MHC class I blocking showing non antigen-specific transmigration. **Figure 3.3B** shows the percentage transmigration of CD8+TRP-2+ T cells of an effector memory phenotype under the same experimental conditions. Initially, 36±4% of the effector memory CD8 T cells specific for TRP-2 migrated through unpulsed syngeneic endothelium. This number increased significantly to 49±3.9% (p=0.04) in the presence of TRP-2 peptide and this increase was blocked with MHC blocking antibody down to 28±9.6% ($p<0.05$). There was no peptide specific migration through allogeneic endothelium. This data indicates that recognition of TRP-2 peptide on the endothelium enhances transmigration of both central and effector memory TRP-2 antigen-specific CD8 T cells.

A Central Memory (i)



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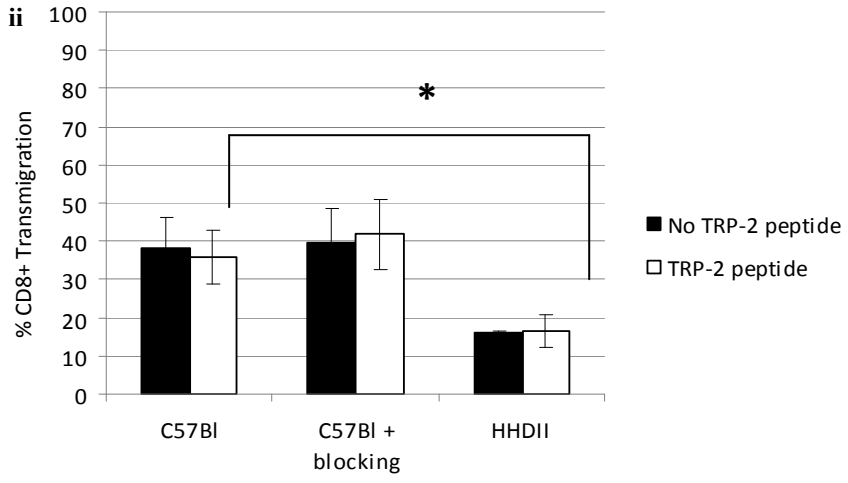


B Effector Memory

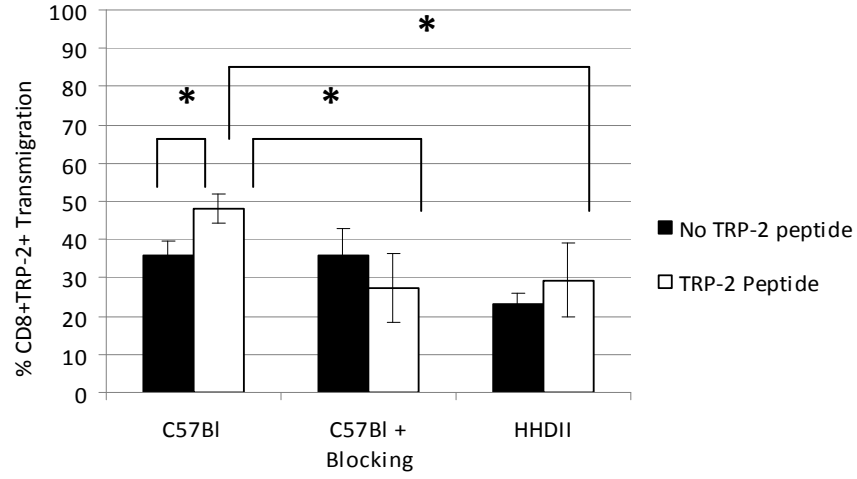


FIGURE 3.3 ***Abrogation of TRP-2 antigen-specific transmigration following blocking with MHC Class I (H-2K/Db) antibody***

Splenocytes were derived from C57Bl mice immunised 3 times with TRP-2 Immunobody^{TM} construct via gene gun. 50,000 isolated CD8 T cells were added to each well, 6 wells for each condition. **A** Transendothelial antigen-specific central memory CD8 T cell migration and **B** antigen-specific effector memory CD8 T cell migration through MLEC monolayers. For central memory transmigration the assay was carried out for 3 mice on one day whereas for effector memory transmigration the data is the average of three separate experiments. Following transmigration under each condition the cells were surface stained for CD8+TRP-2+ (**Ai&B**) and CD8+ (**Aii**) positive T cells and compared to the initial population. MLEC monolayers of C57Bl (syngeneic) and HHDII (allogeneic) were used. C57Bl ECs were untreated (control) or treated with 0.5ug/ml anti-H-2K/Db for 1 hour prior to transmigration. ECs were pulsed with 10ng/ml TRP-2 peptide for 4 hours prior to addition of splenocytes for transmigration. Black lines indicate the comparison of transmigration across C57Bl TRP-2 pulsed endothelium to peptide pulsed MHC blocked C57Bl and HHDII ECs separately. P values were calculated by using two-sided Student's t test; $P<0.05$ was considered to be statistically significant.

Endothelial monolayer used in transmigration assay	Mouse	Initial number of cells added to transwells	Number of CD8+TRP-2+ T cells in initial population	Total migrated cell number	Number of CD8+TRP-2+ T cells in migrated population	% Transmigration of CD8+TRP-2+ T cells
Untreated EC	1	300000	8640 (2.16%)	90000	2187 (2.43%)	25.31 (2187/8640*100)
Untreated EC	2	300000	8040 (2.01%)	65000	2054 (3.16%)	25.54
Untreated EC	3	300000	7960 (1.99%)	45000	859 (1.91%)	10.79
TRP-2 peptide pulsed EC	1	300000	8640 (2.16)	165000	5800 (2.9%)	67.12
TRP-2 peptide pulsed EC	2	300000	8040 (2.01)	170000	3340 (1.67%)	41.54
TRP-2 peptide pulsed EC	3	300000	7960 (1.99)	150000	3940 (1.97%)	49.49

Table 3.1 ***Properties of antigen specific transmigration of central memory TRP-2 specific CD8 T cells***.
Transmigration data is shown for CD8 T cells isolated from three C57Bl mice (M1, M2 and M3) and migrated across untreated and TRP-2 (10ng/ml) peptide pulsed C57Bl endothelium. Following transmigration cells from the 6 replicates were pooled for analysis. 50,000 isolated CD8 T cells were added to each well giving an initial number of 300,000 cells. The number of CD8+TRP-2+ T cells in the initial population is calculated from the % of TRP-2+ CD8 T cells determined by flow cytometry. Total migrated cell numbers are the total cells within the lower well following transmigration. The number of CD8+TRP-2+ T cells in the migrated population is calculated from the % of TRP-2+ CD8 T cells following transmigration determined by flow cytometry. The percentage transmigration is calculated from the cell numbers of TRP-2 specific CD8 T cells having migrated out of the total TRP-2 specific CD8 T cells within the initial population in each well.

3.2.4 Antigen-specific transmigration is dependent on peptide concentration.

The previous experiments demonstrated that CD8+TRP-2+ T cells are preferentially recruited across MHC matched, peptide pulsed endothelial cells. To determine if this was affected by peptide concentration a gradient of TRP-2 peptide pulsed endothelium was established. The hypothesis was that high avidity CTL lines recognising cognate antigen on the endothelium would need low peptide concentrations to migrate. **Fig**ure **3.4A** shows the avidity of a CTL line of an effector memory phenotype, having been stimulated with 0.1ng/ml TRP-2 peptide and rested for 14 days was 1ng/ml ($1x10^{-9}$M). The data in **Figure 3.4B** confirms that transmigration of effector memory antigen-specific CD8 T cells, recognising the corresponding antigen, was highest at low peptide concentration (0.1ng/ml ($p=0.029$)), with no further enhancement of transmigration at higher levels of peptide. The CD8 T cell line in this experiment was cultured in 0.1ng/ml TRP-2 peptide and the data shown in **Figure 3.4B** demonstrated 2% transmigration across C57Bl endothelium pulsed with 10ng/ml TRP-2 peptide. Whereas when CD8 T cells were cultured in the higher concentration of 10ng/ml TRP-2 peptide as in **Figure 3.3B** almost 50% transmigration was demonstrated. Therefore culturing of CTL lines in with LPS blasts pulsed with 0.1ng/ml in **Figure 3.4B**, as compared to 10ng/ml in **Figure 3.3B**, shows the same antigen-specific effect on transmigration but appears to decrease the overall maximal transmigration. Additional transmigration assays of effector memory CD8 T cell lines cultured with varying peptide concentrations have indicated differences in antigen specific transmigration (data not shown) however further validation is required. Potentially CTL lines cultured in lower peptide concentrations require recognition of less cognate peptide on the endothelium to reach a maximal response.

A B

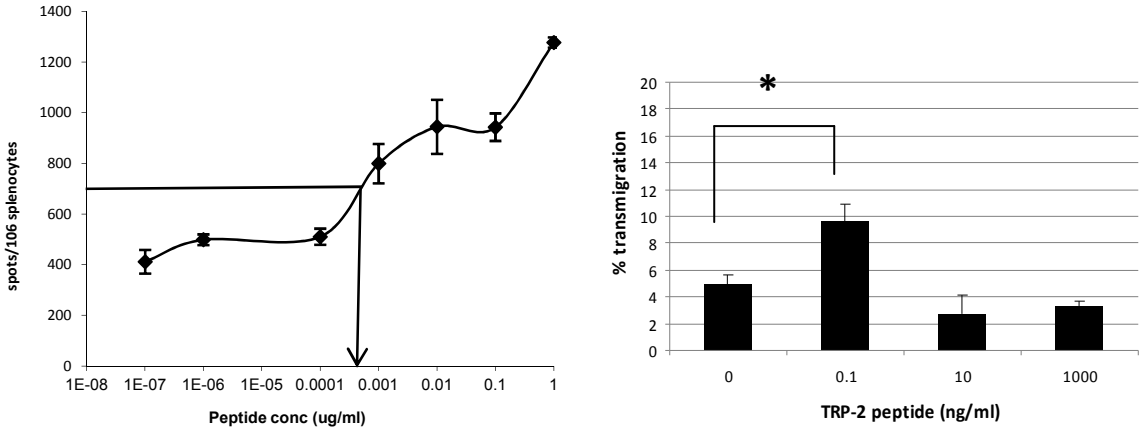
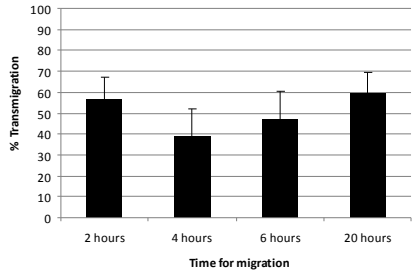


FIGURE 3.4. ***Antigen-specific transmigration is dependent on peptide concentration.***
Splenocytes were derived from three C57Bl mice immunised 3 times with TRP-2 Immunobody^{TM} construct via gene gun and was maintained in culture by restimulation every 2 weeks with splenocytes from C57Bl mice pulsed with 0.1ng/ml TRP-2 peptide and recombinant interleukin-2 (rIL-2) (20 U/mL). **A** Murine IFN-γ Elispot assay prior to culture of the CTL line showing the number of spots developed per 10^6 splenocytes at increasing peptide concentrations from 1pg/ml to 1ug/ml ($1x10^{-12}$M to $1x10^{-6}$M). The functional avidity of the response is calculated as the peptide concentration at which a half maximal response is achieved. **B** Transmigration of the CD8 T cell line having been rested for 14 days, across C57Bl endothelium (6 replicates for each condition, in triplicate) pulsed with increasing concentrations of TRP-2 peptide for 4 hours. Results are given as the percentage transmigration of TRP-2 specific CD8 T cells across 4 P values were calculated by using Student's t test; $P<0.05$ was considered to be statistically significant.

3.2.5 Antigen-dependent transmigration occurs more rapidly than antigen-independent transmigration of CD8 T cells.

Antigen-recognition by CD8 T cells enhances transendothelial migration where cognate peptide is presented on the endothelium in the context of MHC Class I. However, migration of non-antigen specific CD8 T cells is still seen in this assay. To evaluate whether the antigen-specific transmigration follows different time kinetics than conventional antigen-independent transmigration, CD8+TRP-2+ T cells migration was followed over time compared to CD8+ T cells. **Figure 3.5** shows the percentage transmigration of effector memory (CD62L-CD127+) TRP-2 antigen specific CD8 T cells at 2, 4, 6 and 20 hours. The data indicates no significant difference in the percentage (50±11%) transmigration of CD8+TRP-2+ T cells along the time course, with maximum antigen-specific transmigration occurring at 2hr (**Figure 3.5A**). However, TRP-2 pentamer positive CD8 T cells constituted only 5% of the CD8 line population. **Figure 3.5B** shows the percentage transmigration of all CD8 T cells and shows significantly increased transmigration at 20 hours than at 2 hours (28±1.2% compared to 15±3.1%; p=0.038). This is in contrast to the antigen-specific transmigration. Although the level of transmigration of the antigen-specific CD8 T cells did not increase over time when the proportion of antigen-specific cells within each migrated population was examined these made up a significantly higher percentage of the population at 2 hours, 14±0.3% compared to 7±1% (p=0.026) at increasing timed intervals (**Figure 3.5C**). In conclusion the data demonstrated that antigen-dependent transmigration occurred more rapidly than antigen-independent transmigration.

A



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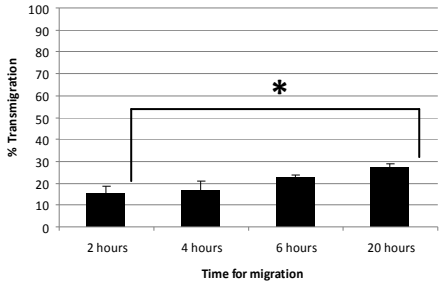

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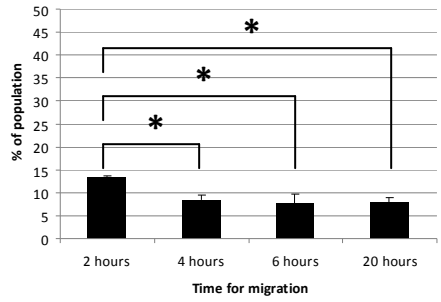


FIGURE 3.5 ***Antigen-dependent transmigration occurs more rapidly than antigen-independent transmigration of CD8 T cells.***
CD8 T cells were derived and from a C57Bl mouse immunised 3 times with TRP-2 Immunobody^{TM} construct via gene gun, stimulated with LPS blasts pulsed with 10ng/ml TRP-2 peptide and rested for 14 days. 50,000 isolated CD8 T cells were added to each well, 6 wells for each condition **A** shows results for transendothelial antigen-specific CD8+TRP-2+ T cell migration, of an effector memory phenotype, through MLEC monolayers at timed intervals for one CTL line (6 replicates for each condition in triplicate). Following transmigration under each condition the cells were surface stained for CD8TRP-2 positive T cells and compared to the initial population. ECs were pulsed with 0.1ng/ml TRP-2 peptide for 4 hours prior to addition of CTLs for transmigration. **B** represents the percentage transmigration of the whole CD8 CTL line at times intervals and **C** represents the proportion of antigen-specific cells within each migrated population at each time interval. Black lines in **C** indicate that the transmigration at 2 hours was compared to transmigration at 4, 6 and 20 hours separately. P values were calculated by using two-sided Student's t test; $P<0.05$ was considered to be statistically significant.

3.2.6 Antigen-dependent transmigration of central memory CD8 T cells occurs more slowly than transmigration of antigen-dependent effector memory CD8 T cells.

Following the observation that antigen-dependent transmigration of effector memory CD8 T cells occurs more rapidly than antigen-independent transmigration it was of interest to see whether similar kinetics also applied to central memory transmigration or whether these cells required longer times. In order to test the hypothesis that antigen-specific effector memory CD8 T cells transmigrate more rapidly than central memory cells, *Ex vivo* transmigration of CD8 T cells isolated from immunised mice was assessed at timed intervals of 2, 4, 6 and 20 hours. Indeed as hypothesised, transmigration of central memory CD8+TRP-2+ T cells increased from 2 hours (8±0.9%) with a peak at 20 hours (22±2.5%, p=0.002) (**Figure 3.6A**). The proportion of antigen-specific cells within each migrated population was examined and increased significantly from 40±2.5% of the population at 2 hours to 53±3.1% at 20 hours (**Figure 3.6B** p=0.036). This is direct contrast to **Figure 3.5C** where the proportion of effector memory CD8+TRP-2+ T cells is highest at 2 hours. These results suggest that central memory cells may require time following MHC encounter to allow them to transmigrate.

Four colour flow cytometry of the migrated population under each condition was carried out and the proportion of each memory subset within the antigen-specific CD8+TRP-2+ population is shown for each condition in **Figure 3.6C**. Within the initial population of CD8+TRP-2+ T cells, 92% were of a central memory (CD62L+CD127+) phenotype, 7% were effector memory (CD62L-CD127+) and no cells showed an effector phenotype. After 2 hours the CD8+TRP-2+ T cells within the transmigrated population were 82% central memory, 4% effector memory and 11% were an intermediate phenotype (CD62L+CD127-). Absolute cell numbers are shown in **Table 3.2** and highlight that the increased numbers of cells with an intermediate phenotype following 2 hours transmigration have converted from

another memory subset as approximately 400 cells were present in the initial population whereas 700 and 750 cells were present after 2 and 4 hours respectively, suggesting that memory phenotypes can differentiate following transmigration. Interestingly, at the 2 hour time-point effector cells (CD62L-CD127-) are present in the transmigrated population (2% or 150 cells) but are not detected in the initial population or at later time-points and therefore they must have differentiated from another subset. At 20 hours the transmigrated population of CD8+TRP-2+ T cells contained 84% central memory, 13% intermediate and 3% effector memory phenotypes, where cells with an intermediate phenotype were 7 times higher than in the initial population (2800 compared to 400). The data here suggests that transmigration of memory subsets of antigen-specific CD8 T cells occurs with different time kinetics, where those with an effector memory phenotype appear to transmigrate more rapidly than central memory phenotypes. In addition the memory phenotypes of antigen-specific CD8 T cells change following transmigration perhaps due to antigen-recognition and subsequent T cell differentiation.

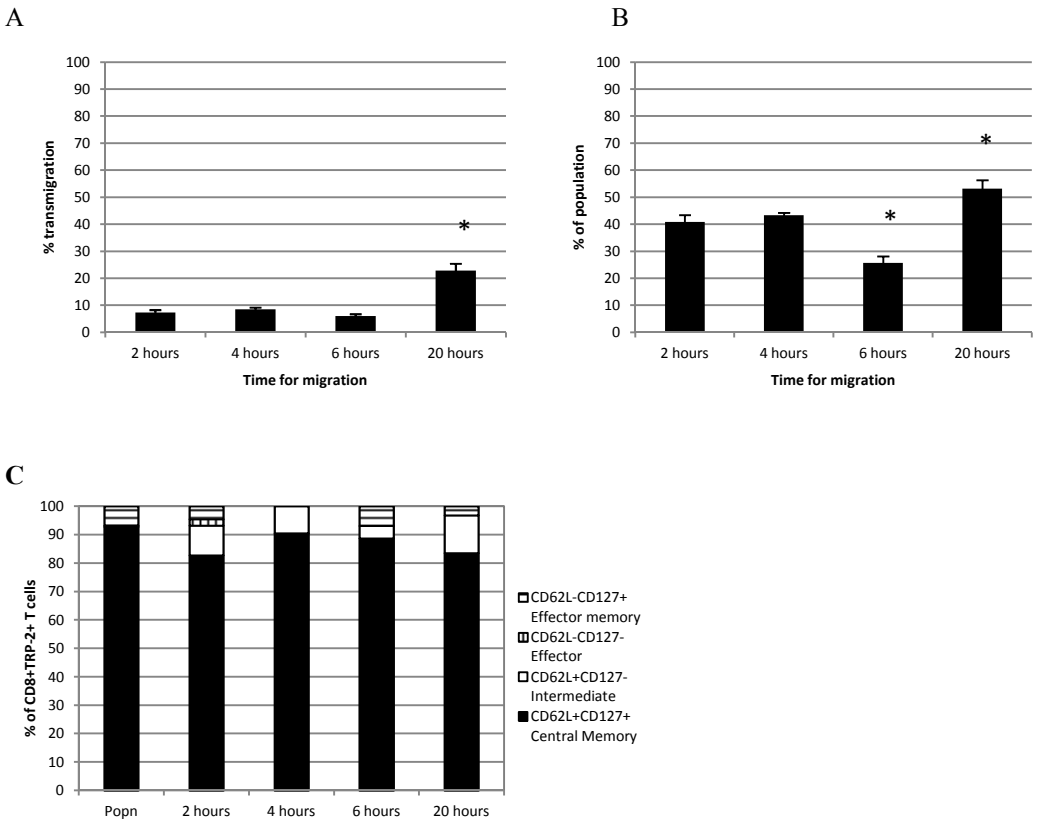


FIGURE 3.6 ***Antigen-dependent transmigration of central memory CD8 T cells occurs more slowly than antigen-dependent transmigration of effector memory CD8 T cells.***
A Transendothelial antigen-specific CD8+TRP-2+ T cell migration, of *ex vivo* central memory phenotype, through MLEC monolayers at timed intervals. Following transmigration under each condition the cells were surface stained for CD8TRP-2 positive T cells and compared to the initial population. ECs were pulsed with 0.1ng/ml TRP-2 peptide for 4 hours prior to addition of T cells for transmigration. 100,000 isolated CD8 T cells were added to each well, 2 wells for each condition, repeated 3 times. **B** The proportion of antigen-specific cells within each migrated population at each time interval. P values are relative to transmigration at 2 hours and calculated by using two-sided Student's t test; $P<0.05$ was considered to be statistically significant. **C** The proportion of memory subset phenotypes within CD8+TRP-2+ T cells under each condition.

CD8+TRP-2+ T cells	Population	2 hours	4 hours	6 hours	20 hours
CD62L+CD127+ Central Memory	86953	5651	7172	4954	17868
CD62L+CD127- Intermediate	384	722	749	242	2839
CD62L-CD127- Effector	0	151	0	0	0
CD62L-CD127+ Effector memory	6331	303	0	383	655

Table 3.2 ***Memory phenotypes of CD8+TRP-2+ T cells expressed as absolute numbers present.***
Transendothelial antigen-specific CD8+TRP-2+ T cell migration, of a central memory phenotype, through MLEC monolayers at timed intervals. Following transmigration under each condition the cells were surface stained for CD8TRP-2 positive T cells and compared to the initial population. ECs were pulsed with 0.1ng/ml TRP-2 peptide for 4 hours prior to addition of T cells for transmigration. 100,000 isolated CD8 T cells were added to each well, 2 wells for each condition, repeated 3 times. The absolute numbers of memory subset phenotypes within CD8+TRP-2+ T cells under each condition are shown where 'Population' refers to those CD8+TRP-2+ cells of each phenotype in the upper chamber prior to transmigration.

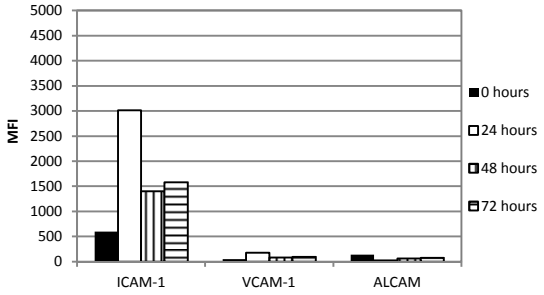
3.2.7 Activation by inflammatory cytokines increases cell adhesion molecule and MHC expression by murine lung endothelial cells but does not alter antigen specific transmigration.

During an immune response inflammation causes endothelial activation and upregulation of cell adhesion molecules (CAMs) on the endothelial surface enhancing leukocyte infiltration into inflamed tissues. In order to assess the effect of inflammation on TRP-2 specific transmigration the effect of inflammatory cytokines was first assessed on murine lung endothelial cells. The hypothesis was that inflammatory cytokines increasing cell adhesion molecule and MHC expression on the endothelium would enhance CD8 T cell transmigration. MLECs were assessed for ICAM, VCAM and ALCAM and MHC expression following culture with the inflammatory cytokines IFN-γ and TNF-α at 100ng/ml. C57Bl derived MLECs showed a constitutive low level of ICAM-1 (MFI=600) expression (**Figure 3.7A**) which peaked at 24 hr following stimulation with IFN-γ and TNF-α (MFI=3000) and then decreases at 48 hr and 72 hr, however remaining higher than the basal level. VCAM-1 expression was not seen on resting endothelium but was induced following 24 hr stimulation. However, expression was at a much lower intensity than ICAM-1. In contrast, ALCAM expression was present on resting endothelium and diminished on activated endothelium. CAM expression for HHDII and DR4 derived MLECS, similar to that of C57BL MLECs, is shown in **Figure 3.7B** and **C** respectively. The basal level of ICAM-1 expression for DR4 mice was similar to C57Bl (MFI=400) and lower for HHDII (MFI=30), however both increased following stimulation to 2000 and 75 respectively. The MHC class I expression for MLECs is shown in **Figure 3.7D** and highlights that MHC class I expression was present on resting endothelial cells of all types at a low level (MFI of around 200-300) which was

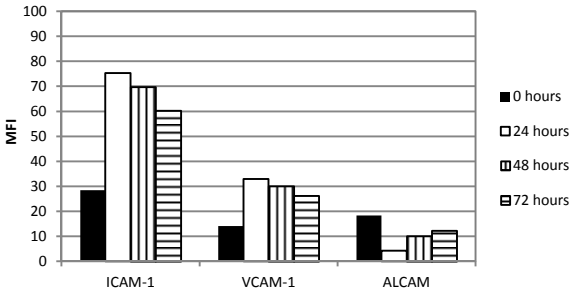
greatly increased to around 4000 with 24 hr stimulation with IFN-γ and TNF-α for C57Bl and DR4 mice and 400 for HHDII MLECs.

In order to achieve tethering and rolling T cells must interact with non-specific adhesion molecules such as selectins and integrins on the surface of the endothelium; however their role in transmigration is less clear. Upregulation of cell adhesion molecules and MHC molecules occurs in response to endothelial cell activation by inflammatory cytokines as shown in **Figure 3.7.** However activation of endothelium with the inflammatory cytokines IFN-γ and TNF-α had no effect on antigen-specific CD8 T cell transmigration (data not shown).

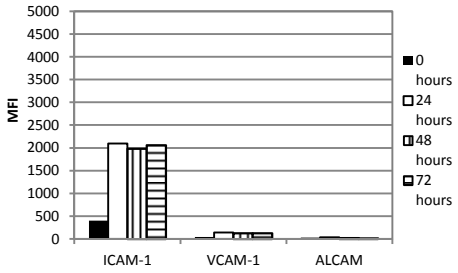
A C57Bl



B HHDII



C DR4



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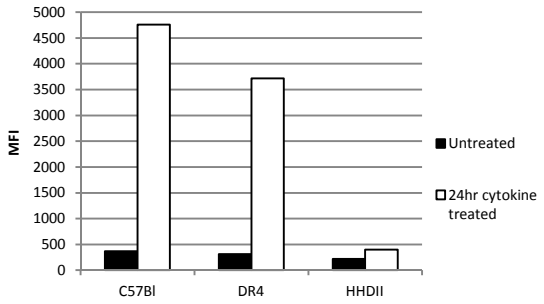

FIGURE 3.7. ***Inflammatory cytokines upregulate cell adhesion molecule and MHC expression on murine lung endothelial cells.*** Murine lung endothelial cells were seeded in 12 well tissue culture plates, 150,000 cells per well overnight. 100ng/ml of murine IFN-γ and TNF-α were added to half of the wells for 24, 48 and 72 hours. MLECs were trypsinised and analysed for ICAM-1, VCAM-1, ALCAM, MHC class I and II expression by FACS analysis. **A, B** and **C** show CAM expression for C57Bl, HHDII and DR4 MLECs respectively over time expressed as MFI (mean fluorescent intensity). **D** shows MHC Class I expression for MLEC following 24 hours incubation with IFN-γ and TNF-α.

3.2.8 Antigen-specific transmigration is enhanced by the addition of chemokine gradients.

Within the tumour microenvironment chemokine gradients determine both T cell infiltration/fugetaxis and tumour cell metastasis. As TRP-2 specific CD8 T cells of an effector memory phenotype undergo antigen-specific transmigration across untreated endothelium, the effect of two tumour-associated chemokines, CXCL12 and CXCL16 were also assessed. The chemokine CXCL16 has been associated with T cell infiltration in colorectal cancer [177] and CXCL12 with FOXP3+ cell infiltration in cervical cancer [179].

The tumour associated chemokine CXCL12 and CXCL16 were used at 100ng/ml to create chemokines gradients and transmigration of effector memory TRP-2 specific CD8 T cells was measured under each condition. Importantly, the results (**Figure 3.8**) show transmigration of TRP-2 specific CD8 T cells is doubled following pulsing of the endothelium with TRP-2 peptide under all conditions ($p<0.05$). In addition, both CXCL12 and CXCL16 increased antigen-specific transmigration but not antigen-independent transmigration (no TRP-2 peptide present on the endothelium). The level of antigen-independent transmigration across unpulsed endothelium remained at 27±6.7% transmigration with both CXCL12 and CXCL16. Peptide pulsing the endothelium caused the percentage transmigration to increase from 20±4.4% to 47±3.9% across unactivated endothelium and from 27±6.7% to 57±0.3% with CXCL12 ($p=0.03$) and 67±1.5% with CXCL16 ($p=0.01$). The ratio of transmigration for peptide/ no peptide for each condition was 2.3±0.2 highlighting that antigen-specific transmigration of TRP-2 specific CD8 T cells occurs independently of chemokine gradients. The ratio between antigen-specific and non-specific transmigration remains the same while the background level for both can be

enhanced by the chemokines CXCL12 and CXCL16, suggesting that the additive effect of chemokines occurs independently of TCR ligation.

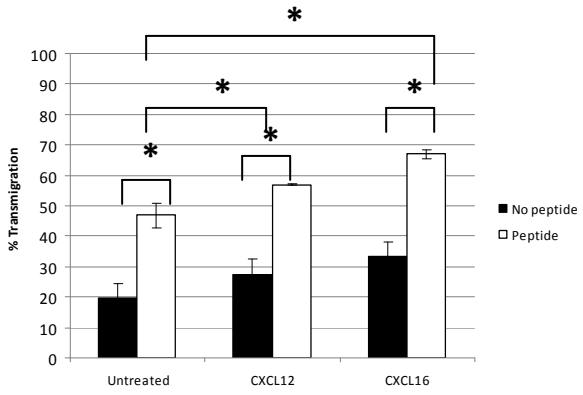

FIGURE 3.8 Antigen-specific transmigration is enhanced by the addition of chemokine gradients.
Data for transendothelial migration of antigen-specific effector memory TRP-2 specific CD8 T cell migration across C57Bl MLEC monolayers. Effector memory TRP-2+ CD8 T cells were generated by stimulation of splenocytes derived from TRP-2 immunised mice, stimulated twice at weekly intervals with 10ng/ml TRP-2 LPS blasts and rested for 14 days. 100,000 cells were added to each well, 4 wells for each condition. Transmigration is shown across non-pulsed endothelium (Black bars) and across 10ng/ml TRP-2 peptide pulsed endothelium (White bars). Following transmigration under each condition the cells were surface stained for CD8+TRP-2 positive T cells and compared to the initial population. Chemokines were added into the lower chamber of transwells at 100ng/ml. Black lines indicate transmigration across TRP-2 pulsed untreated ECs compared to the inclusion of CXCL12 and CXCL12 gradients separately. Student's t test; $P<0.05$ (n=3) was considered to be statistically significant.

3.2.9 Increased antigen-specific transmigration of TRP-2 specific CD8 T cells across tumour conditioned endothelium.

In vitro pulsing of endothelium with the melanoma specific TRP-2 peptide has shown to increase transmigration of TRP-2 specific CD8 T cells. As this is an artificial system and the question remains at to whether melanoma cells are able to transfer TRP-2 peptide to be presented by endothelial cells. In order to test whether endothelial cells in contact with tumour cells can acquire peptide that can be recognised by antigen specific CD8 cells, transmigration assays of TRP-2 specific CD8 cells was tested across endothelium conditioned with the murine TRP-2 expressing melanoma cell lines B16F10 (**Figure 3.9A**). C57Bl derived lung endothelial cells were grown on transwell inserts with and without the addition of TRP-2 peptide. For B16 conditioning, C57Bl endothelium was grown over the top of a monolayer of B16 cells which represents transmigration from the vasculature across the endothelium and into the tumour ((B16+EC)**Aii**). In addition, a monolayer of B16 cells was grown on the top of the C57Bl endothelium representing transmigration from the tumour, across the endothelium into the vasculature ((EC+B16)**Ai**). The percentage transmigration of CD8 and CD8+TRP-2+ T cells across each condition was assessed by Flow cytometry and is shown in **Figure 3.9B** and **C** respectively. Interestingly, the percentage transmigration of antigen non-specific CD8 T cells is reduced across all conditions where the endothelium has been in contact with B16 ($p<0.05$). For antigen-specific transmigration of CD8+TRP-2+ T cells, as expected the transmigration is increased across peptide pulsed endothelium from 32±6% to 73±10.8% ($p=0.008$) and is also significantly increased across C57Bl endothelium with an underlying layer of B16 cells from 32±6% to 51±5% ($p=0.04$).

This data is consistent with the hypothesis that endothelium can uptake antigen from tumour cells and present it to T cells however further investigation is required to prove this hypothesis. Here the layer of endothelium on the top represents the TRP-2 specific T cells transmigrating across vasculature endothelium and into the tumour. Where the B16 monolayer was reversed and on top of the endothelium antigen-specific transmigration was significantly reduced ($p=0.017$).

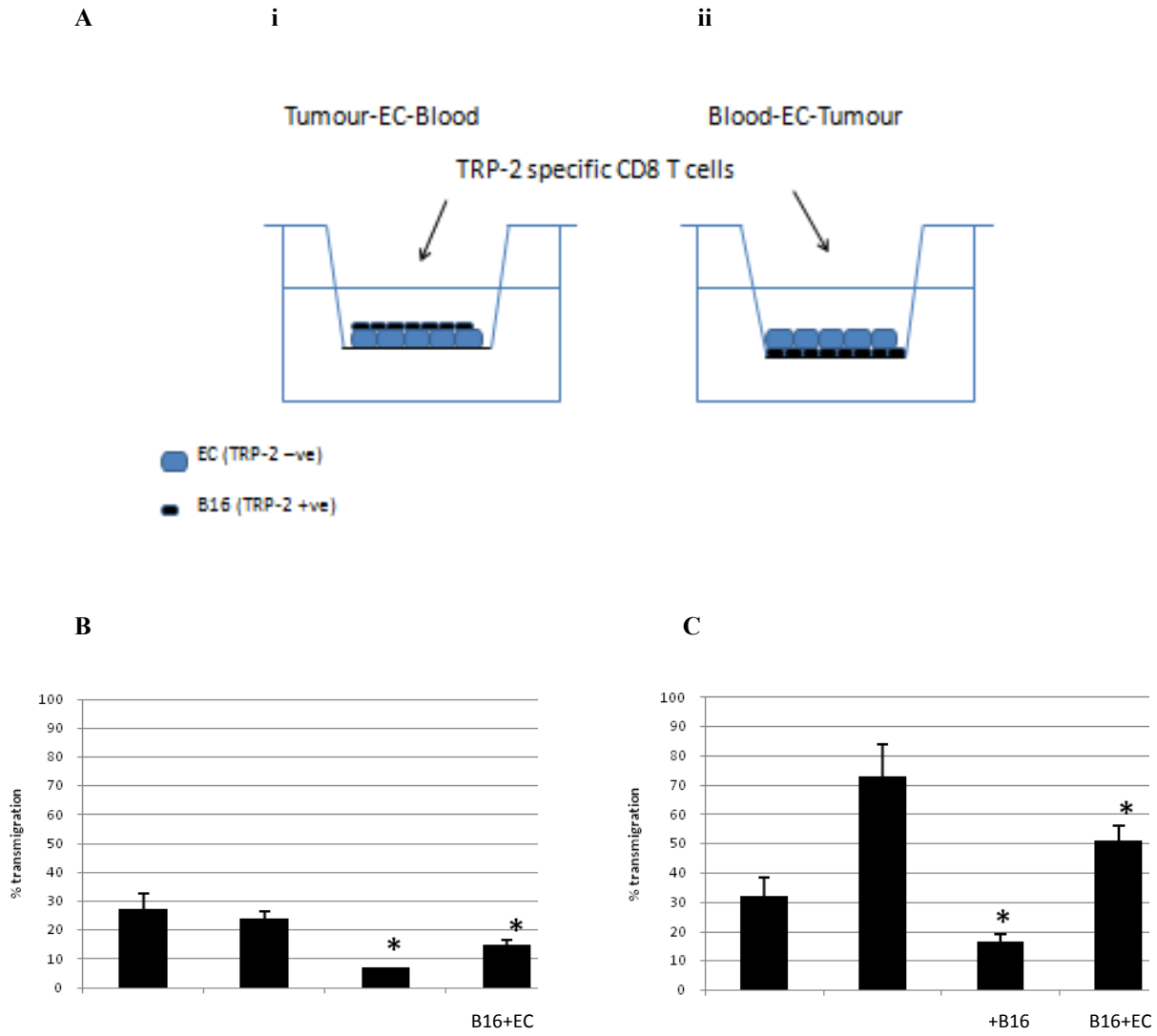

FIGURE 3.9. Increased antigen-specific transmigration of TRP-2 specific CD8 T cells across tumour conditioned endothelium.

A Diagram to depict the transmigration of CD8 T cell across B16F10 melanoma cell monolayers grown over MLECs ((EC+B16)**Ai)** and conversely across MLECs grown over B16F10 melanoma cell monolayers ((B16+EC)**Aii)**. **B** Transendothelial CD8+ T cell migration, of an effector memory phenotype, through MLEC monolayers under various B16 conditions. Following transmigration under each condition the cells were surface stained for CD8TRP-2 positive T cells and compared to the initial population. ECs were pulsed with 10ng/ml TRP-2 peptide for 4 hours prior to addition of CD8 T cells for transmigration. CD8 T cells were derived and from a C57Bl mouse immunised 3 times with TRP-2 Immunobody^{TM} construct via gene gun, stimulated with LPS blasts pulsed with 10ng/ml TRP-2 peptide and rested for 14 days to achieve a CD62L-CD127+ phenotype. 30,000 isolated CD8 T cells were added to each well, 2 wells for each condition, repeated 3 times. **C** The % transmigration of antigen-specific CD8+TRP-2+ T cells within each migrated population. P values are relative to transmigration across untreated ECs and calculated by using two-sided Student's t test; $P<0.05$ was considered to be statistically significant.

3.2.10 Detection of tumour infiltrating TRP-2 specific CD8 T cells.

In vitro transmigration assays presented here have shown that recognition of cognate antigen on endothelium in the context of MHC enhances antigen-specific transmigration of TRP-2 specific CD8 T cells. In order to test whether antigen-specific transmigration also enhances CD8 T cell infiltration into tumours an *in vivo* model of transmigration was required. The murine melanoma cell line B16, expresses the melanoma rejection antigen TRP-2 and data here would suggest a hypothesis that TRP-2 specific CD8 T cells are able to infiltrate B16 tumours via tumour vasculature where they recognise TRP-2 peptide presented on the tumour endothelium. In order to test this hypothesis firstly the presence of TRP-2 specific CD8 T cells needed to be assessed within B16 tumours. A TRP-2 specific CTL line was generated from the immunisation of three mice, pooled and cultured twice with 10ng/ml TRP-2 peptide, and rested for 14 days (effector memory). The CTL line contained 20% CD8+TRP-2+ T cells and was labelled with 1nm CFSE, resulting in 95% of the cells showing fluorescence (**Figure 3.10A**). $1x10^7$ labelled CTLs were injected I.P. into three mice bearing B16 tumours and migration allowed to occur for 72 hours. Subsequently the tumours were resected and assessed for infiltration of CFSE labelled TRP-2 specific CD8 T cells by flow cytometry. The percentage distribution of the injected CFSE labelled CD8+TRP-2+ T cells are shown for three tumours of varying sizes in **Figure 3.10B**. The data indicates that approximately 50% of the adoptively transferred cells were recovered by resection of the tumour, spleen and peripheral blood sample. The tumours varied in size from $440mm^2$ (M1), $600mm^2$ (M2) to $1280mm^2$ (M3) where the smallest tumour showed the lowest infiltration of CD8+TRP-2+ T cells (0.8%) but the largest amount present in the spleen (42%). The largest tumour did not show the highest level of CD8+TRP-2+ T cells infiltration, 2% compared to 8% within the medium sized tumour. The mouse with the largest tumour showed a larger proportion of

antigen-specific CD8 T cells within the blood, 19% compared to 8% and 3 % in the medium and small tumours respectively.

CFSE labelling of adoptively transferred effector memory CD8 T cells into B16 tumour bearing mice enabled the amount of proliferation of CD8+TRP-2+ CFSE labelled T cells to be measured within the tumours, spleen and blood to be measured (**Figure 3.10C)**. The largest amount of proliferation was seen for cells collected from murine spleens where 83±2.3% of adoptively transferred CD8+TRP-2+ T cells proliferated compared to a significantly lower level of 67±7.3% within the blood ($p=0.04$). The amount of proliferation having occurred within the tumour was highly variable across the three mice with an average of 65±16.8%. **Figure 3.10D** shows the histograms for immunofluorescence of the CFSE labelled CD8+TRP-2+ T cells isolated from the three tumour bearing mice shown in **3.10B**. The high variability in proliferation appears to correlate with tumour CD8+TRP-2+ infiltration as the highest amount of proliferation was 94% for M2 which had the highest percent of infiltration of CFSE labelled CD8+TRP-2+ T cells, 10% of the total input. The lowest amount of proliferation was seen for M3, 36%, which was the largest tumour at 1280mm^3.

In conclusion, adoptively transferred TRP-2 specific CD8 T cells, of a predominantly effector memory phenotype, can be detected within the spleens, peripheral blood and tumours of mice. The amount of infiltration appears to depend on the tumour size and in addition the presence of antigen-specific CD8 T cell presence in the blood positively correlates with tumour size.

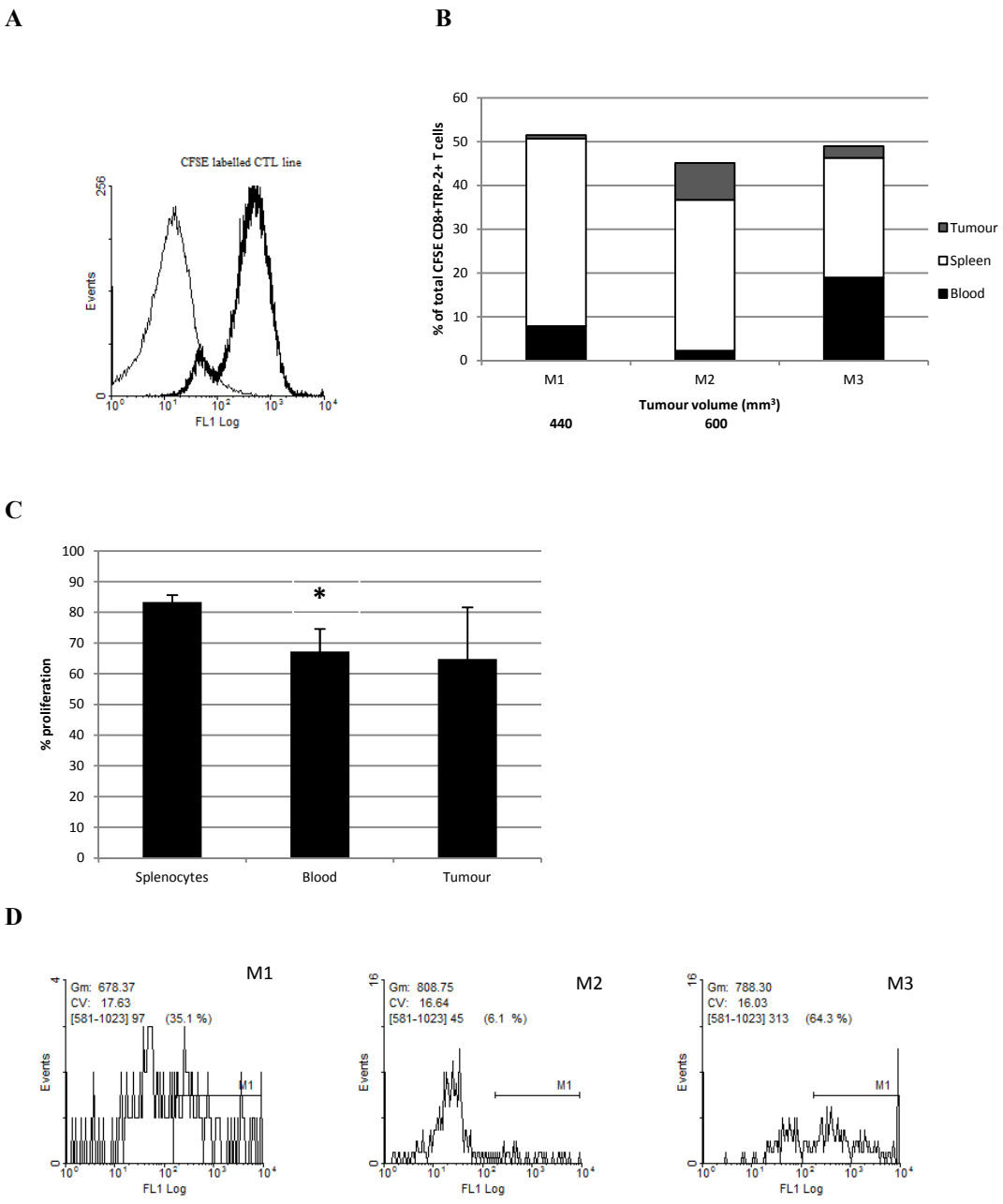

FIGURE 3.10 ***Detection of tumour infiltrating TRP-2 specific CD8 T cells***

A CTL line was generated from CD8 T cells derived from a C57Bl mouse immunised 3 times with TRP-2 Immunobody^{TM} construct via gene gun, stimulated with LPS blasts pulsed with 10ng/ml TRP-2 peptide and rested for 14 days to achieve a CD62L-CD127+ phenotype. **A** Immunofluorescence of the CTL line stained with 1nM CFSE (Thick black line), unlabelled cells used as a negative control (thin line). **B** The distribution of CFSE labelled CD8+TRP-2+ T cells having been I.P. injected into three mice. $2x10^4$ B16 cells were injected S.C. into the right flank of each C57Bl mouse, after 14 days $1x10^7$ CFSE labelled CTLs were I.P. injected into each C57Bl mouse and allowed to migrate for 72 hours before resection of tumours, spleens and a blood sample taken. The distribution of labelled cells assessed by 3 colour flow cytometry detecting CFSE labelled cells also positive for CD8 and pentamer (TRP-2). **C** The percentage of CFSE labelled CD8+TRP-2+ T cells having proliferated, collected from the spleen, blood and tumours of three C57Bl mice. Students T tests: where $p<0.05$ (n=3) considered significant. **D** Immunofluorescence of CFSE labelled CD8+TRP-2+ T cells isolated from three B16 tumours.

3.2.11 The effect of tumour antigen expression on infiltration of antigen-specific CD8 T cells into tumours.

A model of transmigration was developed to test whether antigen-specific transmigration of TRP-2 specific CD8 T cells occurs *in vivo*. The murine melanoma cell line B16, expresses the melanoma rejection antigen TRP-2. A TRP-2 knockdown B16 cell line was produced using siRNA and the intracellular TRP-2 expression by control B16 and transfected B16 cells are shown in **Figure 3.11A.** The mean fluorescent intensity is 43.07 for control B16 cells, reduced to a MFI of 20.04 following transfection and equating to approximately 90% of the transfected B16 cells showing knocked-down TRP-2 expression.

It was hypothesised that TRP-2 specific CD8 T cells would be able to infiltrate TRP-2+ B16 tumours via antigen-specific transmigration across the tumour vasculature where they recognise TRP-2 peptide presented on the tumour endothelium, but would have lower levels of infiltration into the B16 tumour with TRP-2 knockout.

To screen for antigen specific transmigration *in vivo*, C57Bl mice were injected with B16 TRP-2+ and B16 TRP-2- in contra-lateral flanks of three mice and when tumours reached around 500mm^3 1x10^7 CFSE labelled CD8+TRP-2+ cells where adoptively transferred.. 1x10^7 CFSE labelled CTLs were injected I.P. into each mouse for a period of 72 hours, all tumours were excised and labelled cells enumerated by flow cytometry. As hypothesised higher numbers of TRP-2 specific CD8 T cells were found in TRP-2 positive tumours 40,000 compared to 30,000 for Mouse 1 or 4.5% and 3.5 % of the I.P. injected CFSE labelled CD8+TRP-2+ T cells (**Figure 3.11B**). For Mouse 2 25,000 compared to 15,000 were detected in B16 TRP-2+ and B16 TRP-2 knock-out tumours respectively equating to 3% and 1.7% of I.P. injected cells (**3.11C**). As analysis was only possible on two mice a paired

Student's T test on the data was not possible however there was a difference between TRP-2 specific CD8 T cell infiltration into B16 and B16 TRP-2 deficient tumours with a trend towards an increase in infiltration into TRP-2+ tumours. The percentage of antigen non-specific CD8 T cell transmigration was very low in both TRP-2 positive and negative tumours (**D**) at less than 0.5% of the injected CD8 T cells.

Initial *in vivo* transmigration data supports the hypothesis as increased transmigration of TRP-2 specific CD8 T cells was seen into TRP-2+ B16 tumours. However as this was only possible for two samples further validation of this assay is required.

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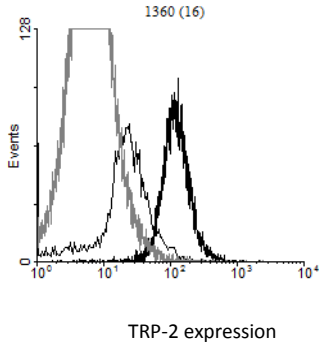

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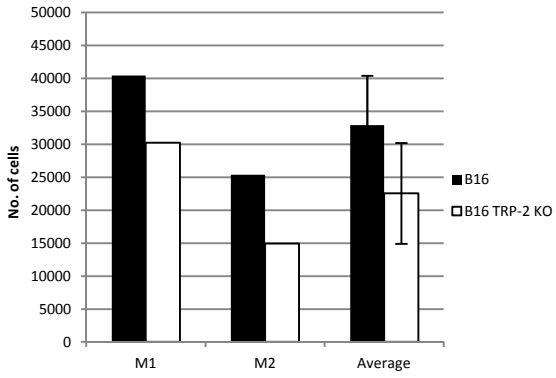

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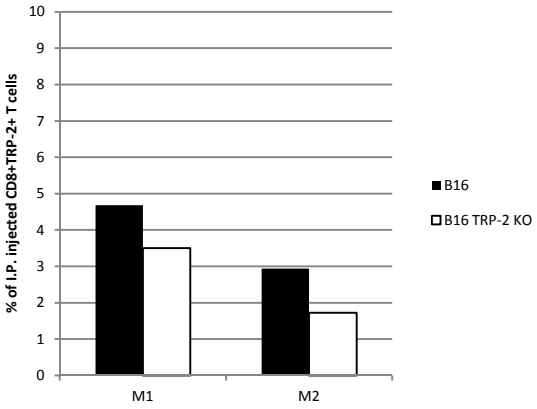

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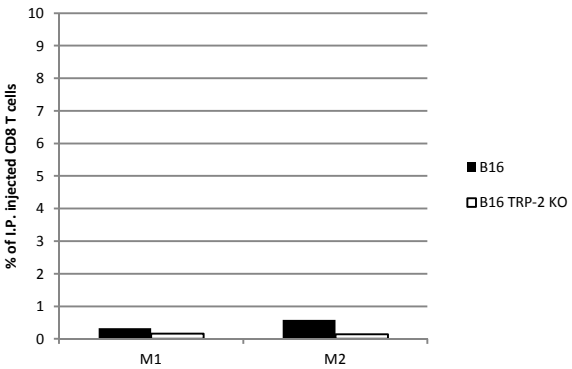

FIGURE 3.11 ***Infiltration of TRP-2 specific CD8 T cells into B16 melanoma tumours.***
A Indirect intracellular immunofluorescent staining of transfected B16 cells with anti-TRP-2 antibody (thin black line) and untransfected (control) B16 cells (thick black line). Isotyped matched immunoglobulin was used as a negative control (thick grey line).Infiltration of CFSE labelled TRP-2+CD8+ T cell transmigration was assessed by flow cytometry into control B16 tumours (Black bars) and TRP-2 knockout B16 cell line 1360(16) (White bars). The actual numbers of TRP-2+CD8+ CFSE labelled T cells are shown for two mice in **B** and the percentage of TRP-2+CD8+ CFSE and TRP-2-CD8+ CFSE labelled T relative to those injected (I.P.) are shown in **C** and **D** respectively. N=2.

3.3 Discussion

In order for T cells to enter a tumour they must first transmigrate from the blood into the tumour tissue via interaction with the tumour endothelium. Uniquely, our results showed that cognate peptide: MHC recognition, enhanced tumour specific CD8 T cell migration. Antigen specific CD8 cells migration was enhanced on peptide pulsed, syngeneic endothelial cells but not in the presence of an MHC class I blocking monoclonal antibody, in the absence of peptide or on peptide pulsed allogeneic MHC. Furthermore, antigen specific transmigration was enhanced across endothelium in the presence of tumour cells expressing the cognate antigen, whereas, antigen non-specific migration was inhibited. These *in vitro* results translated to *in vivo* studies with enhanced accumulation and proliferation of antigen specific CD8 cells within tumour expressing cognate antigen but not in tumours in the same mice that lacked antigen.

Previous research by Marelli-Berg et. al. [319] has shown that memory CD4 T cell transmigration across endothelium could increase the recruitment of specific T cells into the tissue by cognate recognition of tissue peptide presented by MHC [153,328]. Marelli-Berg hypothesised that the endothelium acts as a shop window [318] where tissue derived antigen is picked up by endothelial cells (acting as antigen presenting cells), processed and presented by MHC molecules. Together with chemokine stimulation migration of antigen-specific T cells is favoured into the tissue over non-specific T cells. Subsequent research by Savinov et. al. [317] supported this theory and showed antigen recognition to be important for CD8, as well as CD4 transmigration. Savinov used pancreas-homing TCR-transgenic insulin-specific CD8+ T cells and showed that insulin production and MHC class I expression were necessary for trafficking of these CD8 T cells into the pancreas. Interestingly Savinov et. al. also showed CD8 T cells demonstrated adhesion to endothelial cells under shear stress in the

presence of specific peptide recognition and trafficking *in vivo* was chemokine dependent but occurred in the absence of inflammation. Our data showed antigen-specific transmigration in the absence of both chemokine and inflammation in this static model however chemokines but not inflammation increased antigen specific migration in this model. This does not preclude the fact that inflammation may still be required *in vivo* to allow rolling and adherence of lymphocytes. Indeed, an *in vivo* model of HY-specific CD8 T cell transmigration into antigenic and non-antigenic tissue [155] showed upregulation of H2 molecules by IFN-γ in peritoneal mesothelium led to recruitment of HY specific T cells in male and not in female mice. Intravital microscopy indicated that cognate recognition enhances diapedesis into tissue without affecting rolling and adhesion. This led Marelli-Berg to hypothesise that due to the small molecular size of MHC: peptide complexes would mean that TCR triggering would only occur when the two cells were in close proximity, i.e. once rolling and adhesion had already occurred and therefore antigen recognition does not affect adhesion.

Interestingly, our data would suggest that T cell immune surveillance occurs more rapidly than antigen independent transmigration. When cognate antigen is present within the tissue, it is taken up and presented by the endothelium, allowing rapid antigen-dependent transmigration of specific CD8 T cells into the tissue. Antigen-independent (non-cognate) transmigration occurs much more slowly in this static model where recognition of endothelium causes functional changes in T cells such as upregulation of adhesion molecule receptors such as CD11a, activation markers (CD69) and costimulation (CD86) receptors allowing transmigration to occur [329]. Whether this is physiologically relevant in a flow or *in-vivo* model remains to be determined, although we did see very low numbers of antigen non-specific CD8 T cells migrating in a B16 *in vivo* model. Potentially TCR signalling during

antigen-specific transmigration enhances the speed of transmigration due to additional activation of T cells via signalling pathways such as the PI3K pathway (phosphoinositide 3-kinase) (PI3Ks) which control various cell functions, including motility and trafficking [330]. Antigen-specific transmigration of TRP-2 specific CD8 T cells of an effector memory phenotype occurs much more rapidly than for those of a central memory indicating that the functional state of the T cell not only alters trafficking patterns, such as central memory T cells trafficking to lymphoid organs, but that the speed of transmigration is also affected between subsets. An explanation for this could be the expression of costimulatory molecules such as CD28, where CD28 mediated signals enhance T cell transmigration via integrin clustering and integrin mediated migration [331]. Central memory populations which express CD28 [332] may require time to fully activate these signalling effects whereas effector memory populations show predominantly low/negative CD28 expression are independent of CD28 mediated effects and transmigrate more rapidly.

The guanine nucleotide exchange factor (GEF) Vav-1 plays a role in coupling TCR and CD28 to signalling pathways and regulates T cell activation and migration. T cell receptor and CD28 induced Vav-1 activity has been shown to be required for the accumulation of primed T cells into antigenic tissue [331]. The contribution of TCR and CD28 induced Vav-1 activity has been assessed on the trafficking and localisation of primed HY-specific CD4 T cells. Defects in the migration of Vav-1-/- T cells *in vitro* were not seen under flow and in the presence of chemokines leading to normal recruitment *in vivo*. In contrast, Vav-1 -/- T cell retention into antigen rich tissue was impaired, showing T cells inability to engage in sustained TCR and CD28 mediated interactions with tissue resident APCs. In our *in vivo* model we showed proliferation of antigen specific CD8 T cells within small tumours when compared to blood or spleen implying that cognate peptide: MHC recognition either by the

endothelium or tumour resident APCs gave them an additional boost. However, in larger tumours, proliferation appeared to be reduced implying a more immunosuppressive tumour environment. Perhaps due to an increased immunosuppressive environment created by infiltration of other cell types such as regulatory T cells and secretion of immunosuppressive cytokines such as TGF-β produced by tumour cells and immune cells that can polarise the immune response [333].

Here, cognate peptide: MHC recognition enhanced both antigen specific effector and central memory T cells. We were unable to show effector CD8 T cell migration as these cells efficiently killed peptide pulsed endothelium. Other studies have shown that endothelial cells naturally processing antigens are poor targets for CTLs as they present 50-5000-fold less endogenously processed antigenic proteins compared with any other target cell analysed [334]. HLA-A2-specific CTL clones killed endothelial cells less efficiently due to the lower peptide presentation or suggesting that endothelial cells present a different antigen repertoire compared with other cell types. Further investigation into antigen-dependent transmigration of CD8 T cells of an effector phenotype is needed to find whether effector CTLs, primed to kill target cells expressing TRP-2 peptide, are able to kill endothelium *in vivo.*

In contradiction to results presented here, Marelli-Berg [335] found that CD8 migration across peptide pulsed endothelium decreased under both resting and activated endothelium, initiating a stop signal to CD8 T cells. One explanation for the contrast in migratory ability of antigen-specific T cells could be the dose of peptide used to pulse the endothelium, which was 100nM (100ng/ml for 16 hours). High doses of peptide reduced CD8 transmigration, potentially by over-activation and subsequent killing of high avidity CD8 T and therefore use

of low doses of peptide in the range of 0.1-10ng for 4 hours are optimal for high avidity transmigration.

CD8 T cells vary in antigen specificity and in addition CD8 T cells specific for the same antigen can vary in avidity where different peptide concentrations are sufficient for activation of effector function [301,305]. Higher avidity CD8 T cells show effector functions when in contact with lower peptide concentrations and therefore would be at an advantage during recognition of peptide on ECs at low concentrations due to the inefficiency of exogenous peptide loading of ECs [336]. Recognition of too much peptide leads to too much signal one without costimulation and kills high avidity T cells [337,338].This represents an efficient mechanism which would allow those higher avidity antigen specific CD8 T cells, recognising low doses of cognate peptide presented by endothelium, to enter tissue rapidly to mount an immune response.

Endothelial cells have been characterised as antigen-presenting cells with the ability to cross-present antigenic peptides to both CD4 and CD8 T cells. However controversy still remains as to how endothelial cells acquire tumour antigens. Studies have suggested that endothelial cells derive and cross-present antigenic peptides from phagocytosed dead tumour cells [339]. Another study has suggested melanoma-derived exosomes allow acquisition of melanoma antigen by ECs [340]. However, more recently melanoma-derived ECs have been shown to acquire melanoma antigen following cytosolic exchange of tumour antigens from melanoma cells directly via gap junctions allowing the endothelium to cross-present peptides from the tumour microenvironment and be eliminated via anti-melanoma CD8 T cells [320]. In this study Benlalam et. al showed the importance of the interaction between tumour cells and ECs in the formation of gap junctions and that the classical antigen cross-presentation pathways

were not involved. Here, co-culture of ECs with B16 cells prior to transmigration significantly enhanced the transmigration of TRP-2 specific effector memory CD8 T cells while reducing the overall CD8 T cell transmigration. This provided evidence to support the idea of gap junctions between tumour cell and ECs as the T cells came into contact with the apical side of the endothelium while the B16 tumour cells were underneath. This suggested that TRP-2 peptide passed from the B16 cells to ECs via gap junctions and was then presented on MHC class I molecules. Potentially, the reduced transmigration is due to transmigration being in the direction from the tumour into the vasculature where tumour associated conditions prevent T cell migration. To further prove that this increase in transmigration across B16 conditioned endothelium is due to TRP-2 peptide uptake via gap junctions, an inhibitor of gap junction formation (18GA) could be used in addition to mis-matched HHDII endothelium. The gap junction inhibitor 18GA, which prevents the formation of channels could be used to fully determine whether the uptake of TRP-2 by the endothelium was indeed due to transfer within GAP junctions between ECs and B16 cells. The implications of this mechanism support the theory that *in vivo* within TRP-2 expressing B16 melanomas, TRP-2 peptide could be obtained by the tumour endothelium and presented to TRP-2 specific CD8 T cells within the tumour vasculature. An *in vivo* adoptive transfer experiment was designed to test this theory by adoptively transferring TRP-2 specific effector memory CD8 T cells into mice bearing TRP-2+ and TRP-2- B16 tumours and T cell infiltration measured. Antigen-specific transmigration in response to a naturally occurring tumour antigen showed increased numbers of TRP-2 positive CD8 T cells were found in TRP-2 positive tumours. However this pilot data is representative for two mice and requires further validation by the group.

Antigen specific infiltration of adoptively transferred CD8 T cells has been shown previously using in vivo imaging [341]. The study used CT44 hemagglutinin+ (HA+) and CT26 (HA-) tumours implanted onto each footpad of Thy1.1 Balb/c mice and after 7 days when the tumour had reached around 25mm^2 ^{111}In oxine labelled HA specific CD8 T cells were injected I.V. and followed over 24 hours. Using SPECT-CT (single photon emission computed tomography) to analyse transmigration, the imaging showed that after 2 hours the labelled cells had accumulated within the lung, moving down into the kidneys, spleen and liver and after 24 hours had preferentially accumulated in the HA positive CT44 tumour. Between 7 and 14 days the CTLs controlled the progression of the HA+ CT44 tumours. This study proved that HA+ effector CTLs preferentially accumulated within HA+ tumours and due to the effector phenotype caused regression in these tumours. The effector phenotype of the HA specific CD8 T cells within this study means that these are capable of killing tumour endothelium presenting HA antigen and therefore it is impossible to say whether the increased transmigration is due to damaged endothelium or enhanced antigen specific migration. Within our study the CD8 T cells were of an effector memory phenotype and unable to kill endothelium pulsed with TRP-2 peptide. Therefore, the increased TRP-2 specific CD8 T cell infiltration into TRP-2 positive tumours is likely to be due to TRP-2 expression on the tumour endothelium leading to increased transmigration.

In conclusion the data here has shown MHC recognition to be important in tumour antigen specific CD8 T cell transmigration. In order for tumour antigen specific CD8 T cells to infiltrate tumours they must first transmigrate across the tumour endothelium. This mechanism suggests that tumour endothelium presenting tumour cell derived antigen peptides specifically selects for high avidity potent T cells. This has implications for T cell vaccines as adoptively transferred high avidity CD8 T cells specific for certain tumour antigens would

require these antigens to be presented on the tumour endothelium in order to infiltrate tumours and exert anti-tumour responses.

Chapter 4: CD8 T cell infiltration into colorectal tumours

4.1 Background

Within cancer patients tumour-infiltrating T lymphocytes (TILs) are considered to play important roles in anti-cancer immune mechanisms of the tumour bearing host. Increased survival times have been correlated with increased T lymphocyte infiltration into melanomas and ovarian tumours [228,231]. Animal models have proved that infiltration of tumours by tumour-reactive T lymphocytes is essential for efficient tumour regression [342]. CD8 T cells are cytotoxic T lymphocytes that recognise particular tumour-associated antigens presented on MHC class I molecules at the cancer cell surface and possess the ability to destroy cancer cells directly. Results in the previous chapter demonstrated that antigen-recognition of tumour antigens presented on endothelium increases antigen-specific CD8 T cell infiltration.

In human cancers, infiltration of cytotoxic CD8 T cells has indeed been shown to enhance survival in those cancer patients with high numbers of CD8 T cells per tumour area in esophageal carcinoma, colorectal cancer and renal cell carcinoma [343,344,345]. An early study in 1998 by Naito et.al showed a significant correlation between the degree of CD8 T cell infiltration within tumour cell nests and Dukes' staging, where both factors significantly impacted upon a patients overall survival.

CD4 T cells recognise peptides presented on MHC class II molecules expressed primarily on antigen presenting cells. Although most tumour cells do not express MHC class II molecules, CD4 T cells can have an effect on anti-tumour responses in the absence of CD8 T cells by either directly killing tumour cells or secreting cytokines, such as interferon-gamma (IFN-γ) [346], or by activation and recruitment of effector cells such as macrophages and eosinophils

[247]. However, another role of CD4 T cells in the immune response to cancer is to prime CD8 cells and maintain their proliferation. In addition to CD8 T cell infiltration, CD4 infiltration is a favourable prognostic factor in non-small-cell lung carcinoma [347]. However the role of immune system in the pathogenesis and progression of tumours is a subject of controversy and tumour-infiltrating CD4 T lymphocytes have been associated with lymph node metastasis and the spread of neoplasia to lymph nodes in patients with early breast cancer [348]. Perhaps due to the wide variety of T helper cell subsets encompassed under CD4 staining within tumours such as Th1, Th17 and regulatory T cells having opposing effects. A recent study of immune regulated genes associated with CD4+ helper T cell subset expression within colorectal cancer found that patients with high expression of the Th17 cluster had a poor prognosis whereas patients with high expression of the Th1 cluster had prolonged disease-free survival and none of the Th2 clusters predicted survival [349].

The purpose of the present study was to evaluate the infiltration of T cell subsets in order to determine the individual or synergistic role of TILs in colorectal cancer. The expression of CD8 within colorectal tumours was investigated using a tumour microarray consisting of 464 colorectal cancer samples and a detailed database of clinicopathological variables. T cell infiltration and subsequent immune responses at the primary tumour site could be used to stratify patients for immunotherapy.

4.2 Results

4.2.1 Clinical and pathological data of colorectal cancer patients

The clinic-pathological features of the 462 cases included in the present study are shown in **Table 4.1**.

Variable	Categories	Frequency of total cohort (%) n=462	Frequency of CD8 stained cohort (%) n=353
Gender	Male	266 (58)	207(59)
	Female	199 (42)	146(41)
Age (years)	Median	72	72
	Range	58-89	57-93
Status	Alive	278(60)	220(62)
	Dead	184(40)	133(38)
Tumour Grade	Well differentiated	29 (6)	22(6)
	Moderately differentiated	353 (77)	270(77)
	Poorly differentiated	71 (15)	53(15)
	Unknown	8 (2)	8(2)
Tumour Site	Colon	238 (52)	181(51)
	Rectum	181 (39)	137(39)
	Unknown	43 (9)	35(10)
TNM Stage	0 (T_{is})	3 (1)	2(1)
	1	69 (15)	59(17)
	2	174 (28)	131(37)
	3	155 (33)	120(34)
	4	54 (12)	34(10)
	Unknown	7 (2)	7(2)
Extramural Vascular Invasion	Negative	224 (48)	169(48)
	Positive	128 (28)	94(27)
	Unkown	110 (24)	90(26)
Histological Type	Adenocarcinoma	392 (85)	301(85)
	Mucinous carcinoma	51 (11)	39(11)
	Columnar carcinoma	4 (1)	4(1)
	Signet ring carcinoma	7 (1)	3(1)
	Unknown	8 (2)	2(0)

Table 4.1. Clinicopathological variables for the patient cohort (n = 462) and cores stained for tumour CD8 (n=353).

Patients had a median follow-up of 37 months (range 0 to 116) and there were slightly more male than female patients (58% and 42% respectively). Fifty two per cent of the tumours were of colonic origin and 39% of rectal origin, and in 9% of cases, the site was not recorded. Well-differentiated tumours comprised 6% of the series, while 77% showed moderate

differentiation, and 15% were poorly differentiated. Examination of the standard clinicopathological features identified the expected associations between disease-specific survival (DSS) and TNM stage (log-rank = 211.37, $p < 0.0001$), DSS with extramural vascular invasion (log-rank = 44.30, $p < 0.0001$) but not with differentiation (logrank=5.75, p=0.12).

4.2.2 CD8 T cell infiltration of colorectal tumours

Analysis of CD8 T cell infiltration was possible in 353 of the total 462 cores (76%) with the remainder not available on the cut slide, being lost during antigen retrieval or not demonstrating viable tumour cells in the core. CD8 staining was seen in the tumours and within the tumour-associated stroma, only tumour-infiltrating CD8 T cells were scored (**Figure 4.1**). Of the 353 samples, 114 (32%) were negative for CD8 infiltrating T cells within the tumours (**Figure 4.1C**.). Cut –points to divide both tumours into low and high expression of tumour infiltrating CD8 T cells were determined using the X-tile program (Yale University, CT, USA). This provided low/high groups of cells/mm^2 of 0-20 (low) and 20-3000 (high) for tumour CD8 expression (**Figure 4.1D**) representing 60% and 40% respectively.

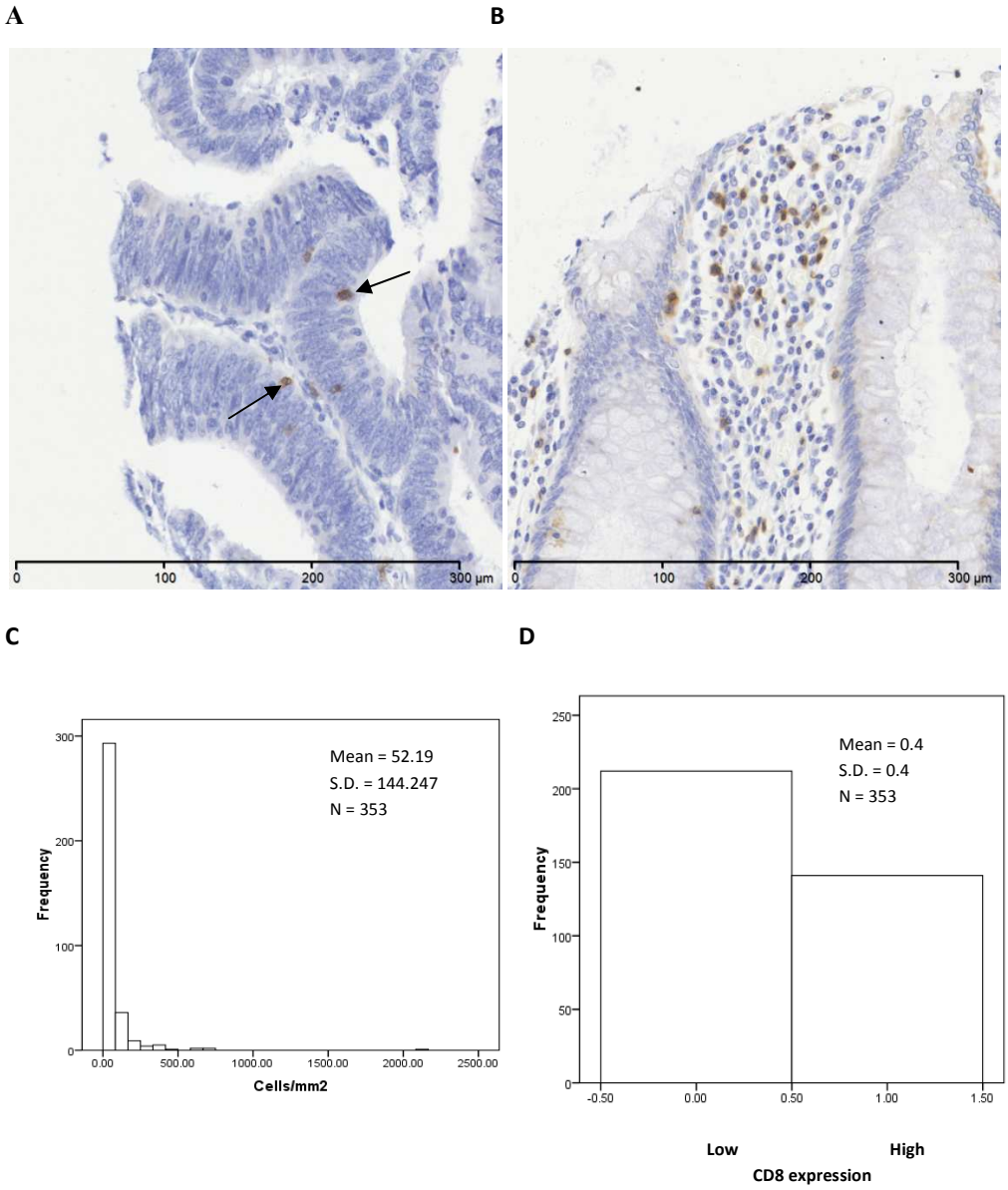

Figure 4.1 ***CD8 expression within colorectal tissue***
Photomicrographs of colorectal tissue microarray cores immunohistochemically stained for CD8 within the tumour **A** and stroma **B**. Magnification: x100. **C** Frequency of CD8 expression in cells/mm^2. **D** Low/high groups of cells/mm^2 0-20 (low) and 20-3000 (high) for tumour infiltrating CD8 T cells within the 353 sample cohort of colorectal tumours.

4.2.3 Influence of lymphocyte infiltration of tumours on survival of colorectal cancer patients

Kaplan-Meier plots were used to analyse the relationship between low and high expression of CD8 positive cells within tumours and disease-specific survival. **Figure 4.2** demonstrates that patients with a higher number of CD8 T cells per unit area within tumours expression have significantly increased mean survival (p=0.012; 83 months) than those with a lower CD8 infiltration (67 months).

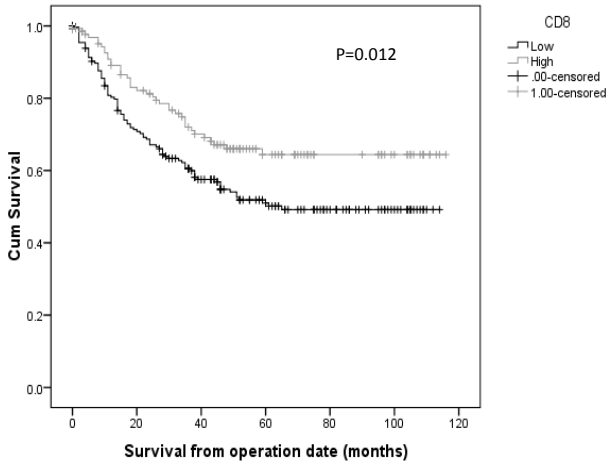

FIGURE 4.2 Kaplan-Meier plot analysis of low/high groups of cells/mm^2 0-20 (low-black) and 20-3000 (high-grey) for tumour infiltrating CD8 T cells within the 353 sample cohort of colorectal tumours against patient survival. Cum Survival = cumulative survival. The statistical significance of differences in disease-specific survival between groups with differing expression was calculated using the log-rank test where $p < 0.05$ was considered as statistically significant.

4.2.4 Relationship between CD8 expression and standard clinicopathological variables.

The relationship between CD8, expression within colorectal tumours and standard clinicopathological variables was measured using the Pearson χ^2 test. The current cohort of colorectal tumour samples have previously been analysed for CD3, STAT-1 and MHC Class I expression [350]. Similarly the presence of macrophages or NK cells was enumerated using the CD68 or CD16 markers [350] and the expression of BCL2 and p53 have also previously been reported in colorectal cohort [351]. Here, expression of CD8 was significantly associated with extramural vascular invasion ($p=0.017$) Duke's stage ($p=0.045$) and CD3 infiltration ($p<0.001$), (**Table 4.2**). Interestingly CD8 expression was highly significantly associated with cytoplasmic Stat-1 expression ($p<0.000$) but not nuclear Stat-1 expression ($p=0.322$). When analysed against expression of MHC class I and MHC class II expression by the tumour cells, CD8 T cell infiltration was associated with MHC class II expression ($p=0.02$) on the tumour but not MHC class I ($p=0.488$).

Variables	CD8 T cells
Gender	0.88
Tumour Site	0.131
Tumour Type	0.973
Tumour Grade	0.220
Duke's Stage	0.045
T Stage	0.138
Distant Metastases	0.099
Extramural vascular invasion	**0.017**
CD3 T cells	**0.0004**
MHC class I	0.488
MHC class II	**0.02**
Stat-1 (nuclear)	0.322
Stat-1 (cytoplasmic)	**0.0001**

Table 4.2.Univariate analysis of CD8 expression in correlation with standard clinicopathological variables using the X^2 test. Values <0.05 are accepted to be significant.

4.2.5 Multivariate analysis of CD8 expression with standard clinicopathological variables.

In order to determine the relative influence of CD8 infiltration into tumours on patient and tumour variables known to affect prognosis, a multivariate analysis was performed using the Cox proportional hazards model. The variables included were those that have been shown to be significantly related to disease specific survival (DSS) on univariate analysis (extramural vascular invasion and TNM stage). In this model, extramural vascular invasion ($p<0.004$) and TNM stage ($p<0.001$) were seen to retain independent prognostic significance (**Table 4.3**.). Expression of CD8 T cells within tumours was not seen to be an independent prognostic marker (CI 95% 0.949-2.231, $p=0.086$).

	Exp(B)	95.0% CI for Exp(B)		P-VALUE
		Lower	Upper	
TNM stage				
1	1			0.001
2	1.537	0.661	3.575	
3	2.634	1.154	6.010	
4	19.725	8.015	48.544	
Vascular invasion				
absent	1			0.004
present	2.419	1.599	3.66	
CD8 T cells				
Low	1			0.086
High	0.687	0.488	1.054	

Table 4.3. The Cox proportional-hazards model was used for multivariate analysis in order to determine the relative risk and independent significance of individual factors including CD8 expression. In all cases p-values < 0.05 were considered as statistically significant.

4.3 Discussion

Within human tumours T cell infiltration has been shown to predict patient survival [343,345,352] potentially as an indication that an anti-tumour immune response is underway. Galon *et al*. [353] used large cohorts of human colorectal cancers to characterise tumour-infiltrating immune cells by gene expression profiling and in situ immunohistochemical staining. The study showed immunological data for the immune cell type, density, and location within the tumour samples to be a better predictor of patient survival than the histopathological methods currently used to stage colorectal cancer. Here CD8, T cell infiltration was analysed within 353 colorectal tumour samples.

The current cohort of colorectal tumour samples have previously been analysed for CD3, STAT-1 and MHC Class I expression [350]. Following staining of the cohort for CD3 expression, of the 355 available tumours, 121 patients (34%) showed low intra-tumoural T cells (ITTC) designated as 15cells/mm^2 or less. Of these, 53 had no T cell infiltration. The presence of a high level of intra-tumoural T cells (ITTC) correlated with improved survival compared with a low level of ITTC, with a mean difference in survival of 16.3 months ($p=0.006$). Within this current study high numbers of CD8 T cells per unit area of tumour, described as those tumours having more than 20 CD8 positive cells per mm^2 of tumour tissue, was shown to predict a better prognosis and increased survival time. This is also in agreement with previous studies looking at CD8 T cell infiltration within colorectal tumours [344]. In addition, as would be expected, CD8 infiltration correlated with CD3 infiltration ($p<0.001$) and interestingly also correlated with cytoplasmic Stat-1 expression ($p<0.001$). Stat-1 expression has been shown to be important for the efficient expansion of CD8 T cells by promoting their survival [354] and in addition Stat-1 expression predicts an IFN-γ mediated

immune response, which would enhance MHC expression, including MHC class II, and possible extravasation correlating with CD8.

A study by Naito et. al. [344] analysed 131 resected colorectal tumours and separated CD8 T cell infiltration into 4 groups; 0, 1-19, 20-50 and >50 cell/mm^2 and found an inverse correlation between CD8 T cell infiltration and Dukes' staging. One difference between the study by Naito and the present study was that the authors found 43% of the tumours to have no CD8 infiltration whereas the present study found 32%. Perhaps, a larger cohort of tumour samples shows that a higher percentage of tumours have CD8 T cell infiltration.

More recently T cell infiltration within tumours has been assessed via ratios of T cell subsets, this is not surprising as CD4 T cells can act to help activate CD8 T cell and FOXP3 expressing Tregs can work to have opposing effects. Therefore, the relevant ratios of these cells within tumours may show a more accurate representation of the immune response occurring in patient tumour samples. Within ovarian cancer [352] intraepithelial CD8 T cells and a high CD8: Treg ratio showed a favourable prognosis, where Treg staining was denoted by CD25+FOXP3+ stained cells. Sato *et al* also showed that CD4 infiltration alone did not predict survival whereas a high CD8+: CD4+ ratio showed improved survival than high CD8+ alone. Diederichsen *et al* studied the prognostic value of the CD4+:CD8+ ratio of tumour infiltrating lymphocytes in colorectal cancer and HLA-DR expression on tumour cells using flow cytometry [355]. Patients with low CD4+/CD8+ ratios had significantly higher survival, p=0.046, independent of the Dukes stage and age. Analysis of CD4 and FOXP3 expression within the tumour samples in this study would allow the CD8: CD4 and CD8: FOXP3 ratios to be assessed.

In conclusion the presence of intra-tumoural CD8 cells was associated with improved survival in colorectal cancer. Activation/memory states and antigen specificity of the infiltrating cells and MHC expression by tumour endothelial cells would be useful to understand the role of these infiltrating T cell subsets within colorectal cancer.

Chapter 5: Antigen-dependent Regulatory T cell transmigration

5.1 Background

Natural Regulatory T cells constitutively express the interleukin-2 (IL-2) receptor α chain (CD25) and the transcription factor forkhead box P3 (FOXP3). Natural Tregs (CD4+CD25+FOXP3+) are found phenotypically in an activated or antigen-primed state, selected in the thymus by self-MHC recognition in an affinity range where they appear to have either escaped thymic deletion or be positively selected as part of an 'anti-self' repertoire [356]. Natural Tregs are essential for maintaining peripheral tolerance to self and non-self antigens, including tumour-associated antigens and have been detected in high numbers in a variety of cancer-bearing patients [169]. Potentially these Tregs result in dampening of the anti-tumour immune response by suppressing effector T cell targets, either in a contact-dependent manner by competing directly for stimulatory ligands on APCs, by scavenging IL-2, or using bystander effects such as secreting suppressive cytokines IL-10 and TGF-β.

The endothelium acts as a selective barrier for leukocyte migration into tissue requiring recognition of non-specific adhesion molecules, chemokine gradients and possible cognate MHC peptide. Inflammatory cytokines such as IFN-γ and TNF-α have been shown to upregulate cell adhesion molecules on the endothelial surface such as ICAM-1 [357] and VCAM-1 [358] and also upregulate MHC class II expression by the endothelium [143]. ECs have been shown to present endogenous antigen to activated T cells as they possess antigen processing machinery including proteasome subunits, TAP proteins and both MHC class I and II [359]. In this context normal housekeeping proteins could be presented on MHC class

II and be recognised by natural Tregs conditioned in the thymus. This would allow recruitment of Tregs to survey normal tissues or to regulate immune responses within tissues. High numbers of Tregs have been found within tumours but the key mediators for this recruitment remain uncertain. Curiel et. al. [169] first showed that Tregs preferentially move to and accumulate within ovarian tumours and ascites, mediated by CCL22/17. Similar results have recently been shown in gastric cancer [360]. A study by Shimizu et. al. [361] implicated another chemokine, CXCL12, in Treg transmigration. CXCL12 was examined by immunostaining tissue specimens from malignant pleural mesothelioma (MPM) and malignant peritoneal mesothelioma (MPEM) and was found to be expressed in the cytoplasm of all malignant mesothelioma (MM) patients but not in the control groups. CXCR4 mRNA was expressed by CD3+FOXP3+CD25+ T cells located adjacent to the border of CXCL12 expressing epithelial MPM. This research suggested that CXCL12 contributed to tumour-related inflammation by inducing accumulation of CXCR4-expressing cells with Treg markers around MM.

Selective accumulation of T cells, specifically Tregs, in tumour tissue could be due to an increased ability to transmigrate endothelium in response to tumour-associated chemokines and/or antigen recognition. It is proposed that T cells recognising highly expressed, high affinity epitopes in the thymus are either deleted or differentiated into natural Tregs. The hypothesis is that these high affinity self epitopes will be preferentially transferred from tissues to endothelial cells by the "shop window" model [317]. The model suggests that tissue derived self epitopes would be taken up by the surrounding endothelium and presented in the context of MHC class II molecules to circulating CD4 T cells within the blood. Recognition of these self-epitopes would allow preferential recruitment of natural Tregs to prevent autoimmunity.

The hypothesis is that syngeneic transmigration of MHC matched Tregs across ECs from DR4 mice would allow the endothelium to present cognate high affinity self peptide in the context of the correct MHC. Whereas, allogeneic transmigration of DR4 Tregs across C57Bl endothelium would lead to mis-matched MHC class II, unable to present self-peptides to Tregs. This avoids the need to clone and identify the peptide recognised by Tregs. It is therefore anticipated if cognate MHC:peptide is required for Treg migration there should be enhanced migration across cells expressing syngeneic MHC when compared to allogeneic MHC.

5.2 Results

5.2.1 Natural Regulatory T cell transmigration is not affected by resting syngeneic or allogeneic endothelium.

The hypothesis was that regulatory T cells undergo antigen-specific transmigration following recognition of self-peptides presented in the context of MHC class II molecules on the endothelium. To test this hypothesis splenocytes were derived from naive DR4 mice, which contain human MHC class II molecules in replacement of murine MHC class II and were migrated within a transwell system across DR4 or C57Bl MLECs forming models of syngeneic and allogeneic transmigration respectively (**Figure 2.3**).

The transmigrated cells within each population were stained for CD4+CD25+FOXP3+ expressing cells by flow cytometry and the percentage transmigration calculated. **Figure 5.1A(i-iii)** represents flow cytometry data for the whole splenocyte population of one naive DR4 mouse where around 20% of the cells are CD4+, by gating on the CD4+ T cells (**5.1A(iv))** the percentage of CD4CD25FOXP3 T cells within the CD4 population was found to be only 1%. Following transmigration, gating on either the whole CD4 T cell population or Tregs there was no difference in transmigration of either CD4 or CD4CD25FOXP3 T cells across syngeneic when compared to allogeneic endothelium (**Figure 5.1B**). Therefore, under conditions of no inflammation no antigen-specific transmigration of Tregs was found. This may have been due to low levels of MHC class II expressed on resting endothelial cells. **Figure 5.1C** shows that MHC class II expression was not present on resting C57Bl and DR4 MLECS but was induced by endothelial activation. The amount of MHC class II expression

by DR4 endothelium is lower than that of the C57Bl as the DR4 mice are a transgenic strain containing a hybrid MHC class II molecule (the peptide binding domains of human HLA-DR4 and the membrane proximal domains of mouse I-E). Despite a low basal level the MHC class II expression on DR4 endothelium it increased 4 fold following a 24 hour stimulation with cytokines IFN-γ and TNF-α. Similar results presented in chapter 3 also showed that 24 hour stimulation with inflammatory cytokines was sufficient time to up-regulate cell adhesion molecule and MHC class I expression on the endothelium.

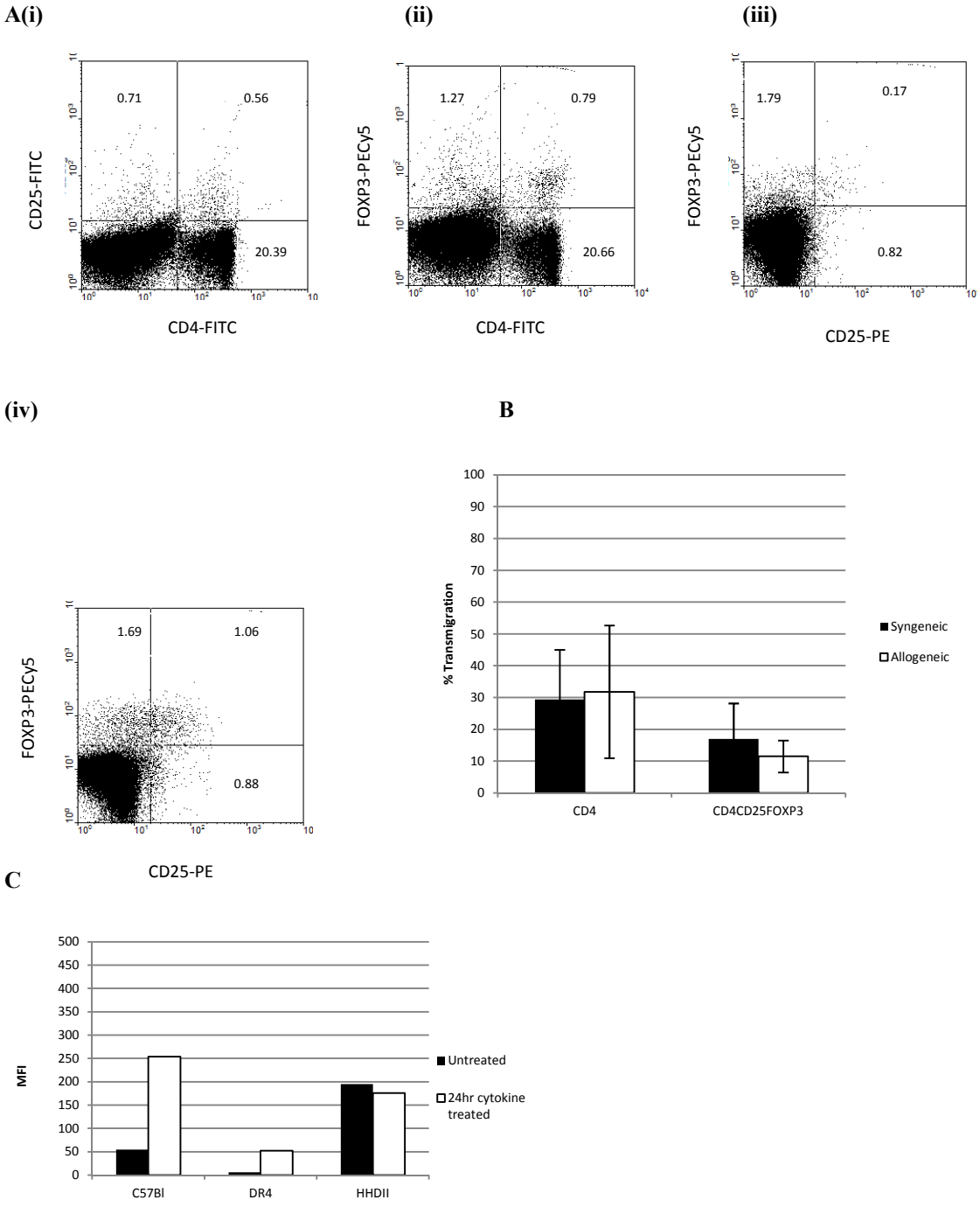

FIGURE 5.1 ***Regulatory T cell transmigration is not affected by syngeneic or allogeneic endothelium.*** Transendothelial CD4CD25FOXP3 T cell migration through MLEC monolayers. Analysis of splenocytes derived from a DR4 mouse is shown in **A** where the cells were detected by 3 colour flow cytometry for CD4CD25FOXP3 T cells. **A(iv)** represents CD25FOXP3 expression gated in CD4 T cells. Following transmigration under each condition the cells were surface stained for CD4 and CD25 and intracellular stained for FOXP3 and compared to the initial splenocyte population. MLEC monolayers were derived from DR4 (syngeneic) or C57Bl (allogeneic) mice. 200,000 cells from DR4 mice were added per well with 6 wells per condition. Each experiment was carried out 4 times and the data represents pooled results for all 4. **B** represents the percentage transmigration of CD4CD25FOXP3 T cells transmigrating under each condition across either syngeneic endothelium (black bar) or allogeneic endothelium (white bars). **C** shows MHC Class II expression for MLECs following 24 hours incubation with IFN-γ and TNF-α

5.2.2 Natural Regulatory T cells show higher levels of transmigration across activated syngeneic endothelium in the presence of CXCL12.

To answer the question of whether additional signals are required for regulatory T cell transmigration, splenocytes from unimmunised mice containing natural Tregs (CD4CD25FOXP3) were migrated across syngeneic and allogeneic MLECS (**Figure 5.2**) under ten experimental conditions (**Table 5.1**.) This included migration towards tumour associated chemokines such as CCL22 and CXCL12, across endothelium activated with inflammatory cytokines IFN-γ and TNF-α and/or pre-treated with the pro-angiogenic factor VEGF.

	Conditions
1.	Control
2.	IFN-γ (100ng/ml)
3.	IFN-γ and TNF-α (100ng/ml)
4.	CXCL12 (100ng/ml)
5.	CCL22 (100ng/ml)
6.	IFN-γ, TNF-α and CXCL12
7.	IFNγ, TNF-α and CCL22
8.	VEGF (50ng/ml)
9.	VEGF, IFN-γ and TNF-α
10.	VEGF, IFN-γ, TNF-α and CXCL12

Table 5.1 Isolated splenocytes from DR4 and C57Bl mice were allowed to transmigrate under the 10 conditions shown. Control endothelium corresponds to untreated endothelium, IFN-γ, TNF-α and VEGF were used to treat endothelial monolayers for 24 hours. The chemokines CXCL12 and CCL22 were present in the media of the lower chamber of the transwell during transmigration.

If the initial hypothesis is correct and Tregs do require recognition of self-peptides in the context of MHC class II molecules to transmigrate an increased level of transmigration would be expected across syngeneic endothelium. Endothelial cells require activation via cytokines to upregulate MHC class II expression (**Figure 5.1C**). When comparing

CD4+CD25+FOXP3+ T cell migration for each syngeneic condition in comparison to untreated (control) endothelium the number of Tregs was measured by indirect immunofluorescence and flow cytometry on the splenocyte population before and after transmigration. A significantly increased transmigration was seen across endothelium treated with IFN-γ, TNF-α with a CXCL12 gradient (p=0.02) (**Figure 5.2A)**. In contrast, there was no significant increase in transmigration of Tregs across any of these conditions on its own or in response to CCL22 or VEGF either on their own or in combination with inflammatory cytokines. Specific migration of Tregs in response to IFN-γ, TNF-α and a CXCL12 gradient was not seen across allogeneic endothelium indicating there was a difference between migration across syngeneic and allogeneic MLECs. The number of Tregs transmigrating syngeneic endothelium in response to CXCL12, IFN-γ and TNF-α was 8000 compared to only 3000 across allogeneic. **Figure 5.2B** shows the percentage of CD4CD25FOXP3 T cells that transmigrate across syngeneic endothelium in combination with CXCL12 and inflammatory cytokines was 45±12%, whereas only 17±5% transmigrated across allogeneic endothelium under the same conditions (p=0.029). **Figure 5.2C** shows the percentage transmigration of the whole CD4 population, where no significant difference in transmigration was seen across any condition. This provides possible evidence to support the theory that peptide recognition is required for Treg migration.

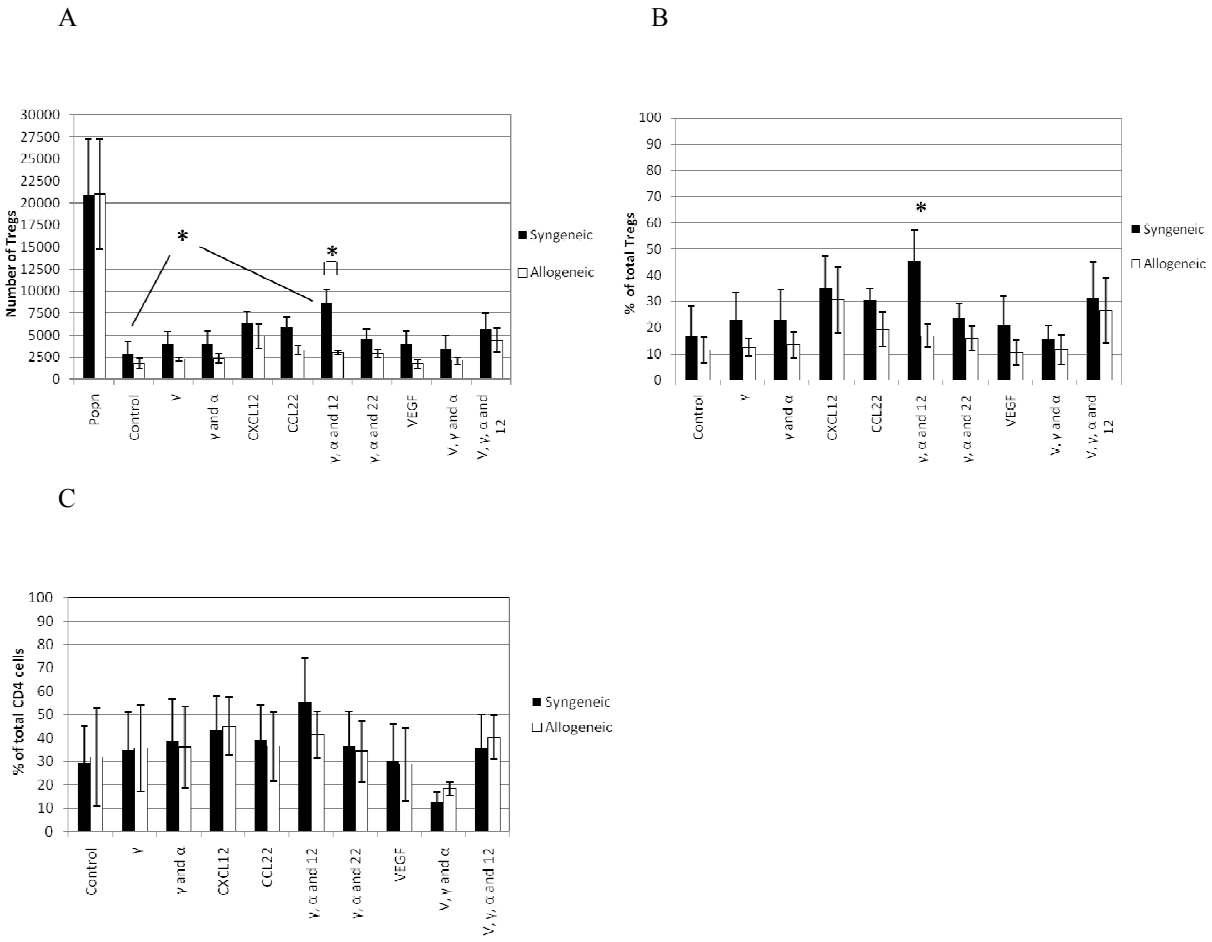

FIGURE 5.2 ***Natural Regulatory T cells show higher levels of transmigration across syngeneic endothelium.*** Transendothelial CD4CD25FOXP3 T cell migration through MLEC monolayers. MLEC monolayers, derived from DR4 mice, were treated with one of the 10 conditions, cytokines added for 24 hours each. 200,000 cells were added per well with 6 wells per condition, 100ng/ml of each chemokine was added in the lower chamber of each well. Following transmigration under each condition the cells were surface stained for CD4 and CD25 and intracellular stained for FOXP3 and compared to the initial splenocyte population.. **A** represents the actual numbers of CD4CD25FOXP3 T cells transmigrating under each condition across either syngeneic endothelium (black bar) or allogeneic endothelium (white bars). **B** represents the percentage transmigration calculated from the absolute numbers of splenocytes having transmigrated and the percentage of CD4CD25FOXP3 T cells within each of these populations from the FACS data. **C** represents the percentage transmigration of the whole CD4 T cell transmigration under each condition. Transmigration of splenocytes shown as means and 95% confidence intervals of absolute numbers of transmigrated cells for each condition. Student's T tests were carried out for each condition relative to the control and in this case the significance shown is for syngeneic versus allogeneic where ***** represents $p<0.05$ and ****** $p<0.001$. Each experiment was carried out 4 times (in triplicate) and the data represents pooled results for all 4.

5.2.3 Recognition of self antigen presented by MHC on syngeneic endothelium increases migration of natural Regulatory T cells.

The hypothesis once again states that MHC: peptide recognition is required for Treg transmigration and therefore blocking of MHC class II molecules on the endothelium would be expected to decrease Treg transmigration across endothelium. Naive splenocytes containing Tregs were migrated across syngeneic and allogeneic MLECS in the presence of CXCL12, inflammatory cytokines and the presence or absence of MHC class II anti-HLA-DR blocking antibody. Absolute numbers of Tregs transmigrating under each condition as an average of 3 experiments (each in triplicate) where Tregs were derived from 3 donors are shown in **Figure 5.3**. The number of Tregs transmigrating syngeneic endothelium in response to CXCL12, IFN-γ and TNF-α was 1400±190 compared to only 500±70 ($p<0.05$) across allogeneic and significantly lower ($p<0.05$; 800±45 cells) following MHC class II blocking. This data verifies that, under conditions of inflammation and CXCL12, Tregs preferentially migrate across syngeneic rather than allogeneic MLECS where cognate antigen-recognition can only occur in the syngeneic model and this antigen-specific transmigration can be blocked by an anti-MHC class II antibody.

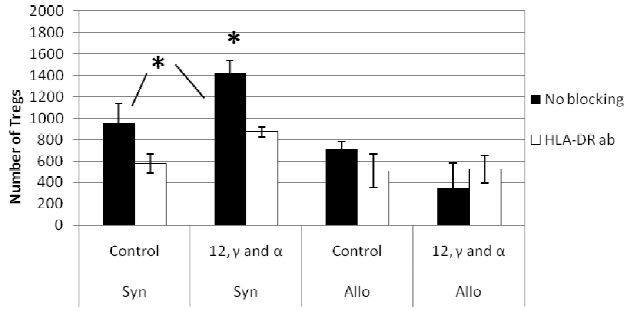

FIGURE 5.3 ***Recognition of self antigen presented by MHC on syngeneic endothelium increases migration of natural Regulatory T cells.***
Transendothelial CD4CD25FOXP3 T cell migration through MLEC monolayers. CD4CD25FOXP3 T cell transmigration across either DR4 (Syn) or C57Bl (Allo) murine lung endothelium, untreated or conditioned with 100ng/ml IFN-γ and TNF-α for 24 hours. In addition the endothelium was treated with 0.5ug/ml anti-HLA-DR (white bars) for 1 hour prior to transmigration or untreated (black bars). Splenocytes derived from DR4 mice were placed at 200,000 cells per well with 6 wells per condition, in triplicate for each experiment. The absolute numbers of splenocytes having transmigrated and the percentage of CD4CD25FOXP3 T cells within each of these populations from Flow cytometric analysis was used to calculate absolute numbers of Tregs transmigrating. Numbers of Tregs is shown as means and 95% confidence intervals of absolute numbers of transmigrated cells for each condition. Student's T tests were carried out for each condition relative to the control and in this case the significance shown is for syngeneic versus allogeneic where ***** represents $p<0.05$ and ****** $p<0.001$. The experiment was repeated three times, each with a separate donor in triplicate (n=3).

5.2.4 CXCR4 expression on Regulatory T cells is intracellular until interaction with CXCL12.

T cell transmigration towards CXCL12 is known to be dependent on CXCR4 expression on the T cells. Data here has shown that Tregs preferentially transmigrate across syngeneic endothelium towards CXCL12 and therefore CXCR4 expression was assessed on Tregs by flow cytometry. **Figure 5.4** shows surface staining of CXCR4 and intracellular CXCR4 for CD4 T cells (**A**) and on CD4CD25FOXP3 Tregs (**B**) prior to transmigration. Only 0.05% of the whole CD4 population express CXCR4, at the cell surface whereas, 98% express intracellular CXCR4. 10.5% of the Treg population express CXCR4, at the cell surface whereas, 43.5% express intracellular CXCR4. This data indicates that chemokine receptor expression, particularly in the case of CXCR4, may be intracellular until interaction with corresponding chemokines. In addition it also shows that Tregs do not show preferential expression of CXCR4 over conventional CD4 T cells. It would be interesting to assess CXCR4 expression following transmigration across syngeneic and allogeneic endothelium towards a CXCL12 gradient to identify the effect of CXCL12 ligation and transmigration on CXCR4 expression.

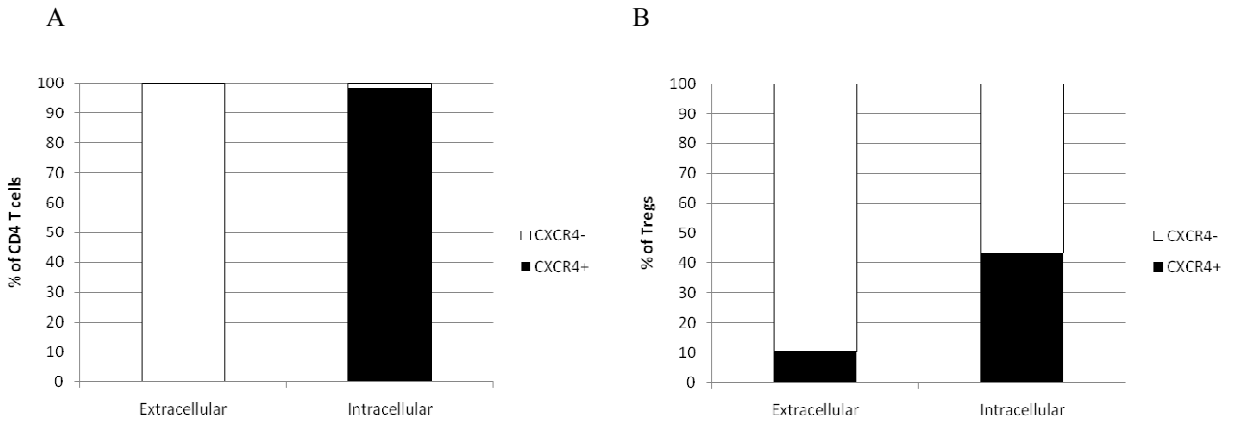

FIGURE 5.4 ***CXCR4 expression on Regulatory T cells.***
Surface expression of tumour associated chemokines was assessed by flow cytometry. To facilitate visualisation splenocytes from DR4 mice were surface stained for 30 minutes with anti-murine antibodies for CD4-FITC, CD25-PE, permeabilised for 1 hour and subsequently intracellular staining was carried out with anti-murine FOXP3-PECy7 and CXCR4-Alexa647 for 30 minutes. **A** and **B** represent the percentage of CD4 and CD4CD25FOXP3 T cells expressing CXCR4 following extracellular and intracellular staining respectively.

5.2.5 Regulatory T cell transendothelial migration is dependent on a CXCL12 gradient and reduced under high concentrations.

As Tregs appear to preferentially transmigrate across syngeneic endothelium in response to CXCL12, it was hypothesised that this transmigration would be concentration dependent. CD4CD25FOXP3 T cells were transmigrated along a CXCL12 gradient **Figure 5.5A**. Significantly increased transmigration of Tregs was seen across syngeneic endothelium at 100ng/ml, (10% to 50% transmigration) but not across allogeneic endothelium showing the antigen-specificity in the presence of inflammatory cytokines and CXCL12. Above 100ng/ml there was no significant increase in the transmigration of CD4CD25FOXP3 Tregs across syngeneic endothelium. **Figure 5.5B** shows the total splenocyte population following transmigration across allogeneic and syngeneic endothelium, conditioned with cytokines, in response to CXCL12 at 0, 10ng, 100ng and 1µg/ml. The data show an increase in splenocyte transmigration along the chemokine gradient, with significantly increased transmigration observed at 100ng/ml CXCL12. However there was no difference between syngeneic and allogeneic endothelium. In conclusion, both CD4+CD25-FOXP3- and CD4+CD25+FOXP3+ T cells follow a CXCL12 gradient that peaks at 100ng/ml however Tregs are the only cell type to show antigen-specific transmigration at the peak of transmigration towards CXCL12.

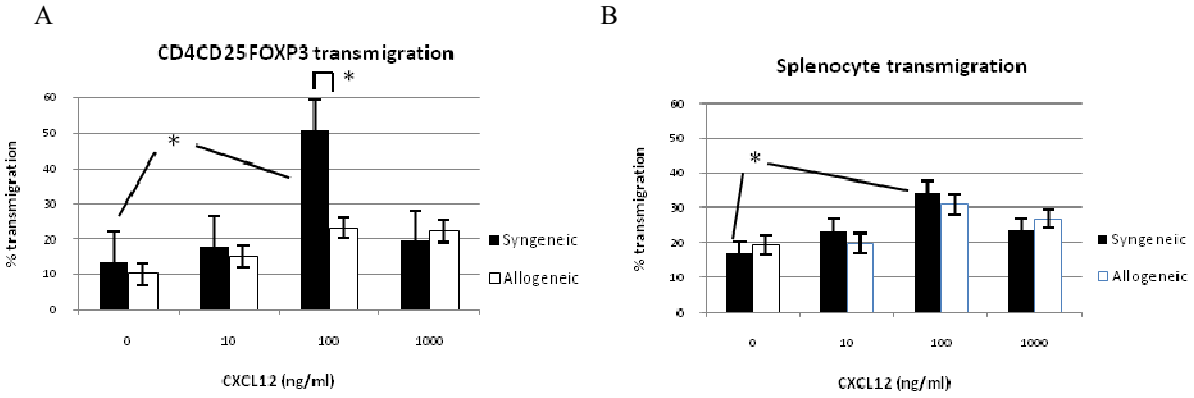

FIGURE 5.5 ***Regulatory T cell transendothelial migration is dependent on a CXCL12 gradient and reduced under high concentrations.***

Transendothelial splenocyte migration through MLEC monolayers. MLEC monolayers from DR4 (syngeneic) and C57Bl (alogeneic) were treated with 100ng/ml IFN-γ and TNF-α for 24 hours each. A range of CXCL12 concentrations were added into the lower chamber of corresponding wells from 0, 10, 100, and 1000ng/ml. 200,000 splenocyte cells derived from three DR4 mice were added per well with 6 wells per condition. **A** represents the percentage of the total splenocyte population having migrated across either syngeneic DR4 (black bars) or allogeneic C57Bl (white bars) MLECs under each condition. **B** shows the percentage transmigration of CD4CD25FOXP3 T cells under each condition across either syngeneic endothelium (black bar) or allogeneic endothelium (white bars) and number of cells calculated using cell counts of cells within the lower chamber. Transmigration of splenocytes is expressed as means and 95% confidence intervals of absolute numbers of transmigrated cells for each condition. Student's T tests were carried out where * represents $p<0.05$ and ** $p<0.001$. Data represents an average of 3 donors (n=3).

5.3 Discussion

Regulatory T cells mediate peripheral tolerance by suppressing self-antigen-reactive T cells and high numbers of Tregs have been found in peripheral blood of a variety of cancers including breast cancer [362], colorectal cancer [363], oesophageal and gastric cancer [360], lymphoma [364], ovarian cancer [169] and pancreatic cancer [208,209]. Vaccination strategies to remove the inhibitory effect of Tregs have proven successful; in particular the blockade of CTLA-4 has been shown to promote the rejection of pre-implanted B16 melanomas [365,366]. Combinational therapy blocking both PD-1 and CTLA-4 has shown up to 50% regression within B16 melanomas. Combination of CTLA-4 blockade and depletion of $CD25^+$ Treg cells results in tumour rejection where efficacy of the antitumor therapy correlated with an increased frequency of tyrosinase-related protein $2^{180–188}$–specific CTLs detected in the periphery [367]. The effect seen with combination approaches leads to an increase in effector T-cell (Teff) infiltration, resulting in highly advantageous Teff: Treg ratios with the tumour. The high CD8:Treg ratio resulted from increased CD8 infiltration and expansion, impaired conversion of CD4 T cells into Tregs resulting from CTLA-4 blockade, and reduced Treg suppression through PD-1 blockade [368]. More recently in a phase III study in over 600 metastatic melanoma patients, Ipilimumab which blocks CTLA-4, has been shown to improve overall survival in patients with previously treated metastatic melanoma [369].

Studies showing the importance of removing the suppressive effects of Tregs for the effectiveness of anti-cancer therapies meant that the trafficking of Tregs across endothelium and more importantly into tumour tissue was of interest. Having previously shown that

antigen recognition is required for CD8 T cell transmigration into tumours, potentially antigen recognition may also be required for Treg transmigration into tumours. In order to study this transmigration assays under 10 conditions combining the effects of inflammatory cytokines and tumour associated chemokines CCL22 and CXCL12 was carried out. Our data suggest that Tregs preferentially transmigrate, across murine lung endothelium, towards the tumour-associated chemokine CXCL12, in the presence of endothelial cell activation by inflammatory cytokines and preferentially across syngeneic rather than allogeneic endothelium. Furthermore, the preferential migration across syngeneic endothelium could be blocked with an MHC class II binding antibody, demonstrating that Treg transmigration across endothelium and therefore infiltration into tissue is antigen-specific and requires MHC class II expression by the endothelium.

Marelli-Berg hypothesised that the endothelium acts as a shop window [318] where endogenous self antigens and tissue derived antigen is picked up by endothelial cells (acting as antigen presenting cells), processed and presented by MHC molecules on the endothelium. Together with chemokine stimulation, migration of antigen-specific T cells is favoured into the tissue over non-specific T cells potentially selectively recruiting Tregs into tissues to prevent autoimmunity.

Beckhove et. al. [370] studied regulatory T cell infiltration in human pancreatic carcinoma and showed that tumour endothelium selectively recruited regulatory T cells (CD4+CD25+FOXP3+). The study showed increased density of blood vessels in the tumour tissue and increased expression of addressins such as CD62-E, ICAM-1/-2, MAdCAM-1, VCAM-1 and CD166. Tumour infiltrating Tregs but not tumour-infiltrating CD4+ T cells showed differential over-expression of β7 integrin, CD166L and CD62L, showing an

increased capacity of Tregs to adhere to and transmigrate selectively into tumour tissue. Our data suggests that cognate MHC: peptide recognition of endogenous antigens is a further key factor in Treg migration into tissues.

Regulatory T cells develop due to TCR interactions with cognate self-peptide: MHC class II complexes, on thymic stromal cells, in an affinity range between positive and negative thymic selection [17]. High levels of peptide expression result in deletion of TCR transgenic T cells whereas moderate expression results in partial deletion. Of the remaining cells up to 50% express CD25 and exhibit Treg functions. In addition to recognition of self peptides in the thymus, Tregs have been shown to recognise self-peptides bound to MHC class II expressed by peripheral antigen presenting cells [371]. By using TCR gene transfer, studies have shown that the CD4+CD25+ T cell TCR repertoire was as diverse as the CD4+CD25- TCR repertoire, exhibiting a higher frequency of TCRs with enhanced affinity against self-peptides [372]. This diverse repertoire could enable Tregs to recognise a wide variety of self-peptides presented by the endothelium and high affinity interactions would further enable selective recruitment of Tregs into tissues.

The chemokine CXCL12 is important in T cell trafficking and was first reported in human ovarian cancer in 2001 [178,373]. Within the tumour microenvironment stroma and cancer cells are the two main components that can produce CXCL12, activated tumour stromal fibroblasts produce CXCL12 [185] and contribute to tumour vascularisation by endothelial stem cell attraction. Our data showed that Tregs expressed minimal CXCR4 surface expression, with the majority being intracellular, supporting previous research that expression of the receptor is not confined to the cell surface [374]. Upon activation the amount of cell surface expressed CXCR4 on lymphocytes increases two-fold within seconds and is

completely down-regulated within the next 2 minutes. Receptor cross-linking caused by incubation of cells with anti-CXCR4 mAb was shown to trigger receptor trafficking and the receptor is rapidly internalized and recycled to the cell surface. Thus supporting our hypothesis that the CXCR4 receptor may be internal until encounter with the endothelium where the receptor is trafficked to the cell surface and allows interaction to occur with CXCL12, activating signalling cascades which allow the cell to transmigrate.

Antigen-specific Treg transmigration required endothelial activation and subsequent upregulation of MHC class II by IFN-γ and TNF-α in addition to a CXCL12 gradient. Interestingly, within ovarian cancer cell lines and primary epithelial cancer cells TNF-α stimulation is involved in the CXCL12/CXCR4 axis by increasing expression of CXCR4 cell surface expression and subsequent migration towards CXCL12 [375]. This role of TNF-α within the CXCL12/CXCR4 axis could help to explain the synergy seen with CXCL12 for antigen specific Treg transmigration.

Data here demonstrates that the level of CXCL12 can alter Treg transmigration with peak migration at 100ng/ml of CXCL12 and lower levels of transmigration seen at 1µg/ml. Previous research using CD4 T cells demonstrated that increasing the CXCL12 concentration from 100ng/ml to 1µg/ml inhibited cells responding towards a concentration gradient and initiated the movement of T cells away from CXCL12 [376]. Movement away was highest at 1 µg/ml CXCL12 for naive and memory CD8 T cells and memory CD4 T cells, whereas for naive CD4 cells, migratory activity was higher at 10µg/ml. Movement away from CXCL12 (fugetaxis) was significantly greater than chemotaxis for each subset of cells. Data presented here showed that all CD4 T cells showed concentration dependent transmigration towards a CXCL12 gradient; however Tregs were the only subset to show this occurred in an antigen-dependent manner. An *in vivo* murine tumour study investigated the

effect of engineering B16 melanomas to produce CXCL12 at high and low levels and found chemo-repellent activity of antigen-specific T cells away from melanoma expressing high concentrations of CXCL12 [377].

Results from *in vitro* transmigration assays has shown natural Tregs preferentially transmigrate across syngeneic endothelium under conditions of inflammation and towards a CXCL12 gradient. Potentially Tregs use this same antigen-specific mechanism to infiltrate tumours by recognition of self-peptides in the context of MHC class II on the tumour endothelium. In order to demonstrate this, an *in vivo* transmigration assay could be carried out to assess the contribution of peptide: MHC recognition of Treg infiltration into tumours. To achieve antigen-specific Treg transmigration B16 melanomas could be grown on C57Bl and DR4 mice to form models of syngeneic and allogeneic endothelium and isolated CD4 T cells from naive C57Bl mice fluorescently labelled with CFSE (1nm) and injected I.P into each mouse. The hypothesis would suggest that increased infiltration of natural labelled Tregs would be seen in tumours of C57Bl mice where tumours, endothelium and Tregs are matched for MHC class II.

MHC recognition is important for CD4 T cell transmigration across endothelium and increases the recruitment of specific T cells into the tissue by cognate recognition of tissue peptide presented by MHC [153,328]. We have demonstrated that cognate recognition of peptides presented by MHC class II aids recruitment of Tregs across endothelium. This enhanced T cell transmigration due to MHC/peptide recognition may be due to the TCR engagement leading to activation of signalling pathways such as Ras/Rho family of GTPases that control cell adhesion molecule activation such as integrins and cytoskeletal rearrangements allowing polarisation and motility to be enhanced [378]. Our observation that

antigen-specific Treg transmigration occurred in the presence of a CXCL12 gradient is very interesting as while TCR signals through initiation of tyrosine kinase cascades, chemokine receptors are members of the serpentine receptor family linked to heterotrimeric G proteins and potentially a link between the two pathways can occur via Protein Kinase C isoforms implicated in downstream regulation. Activation of the TCR with anti-CD3 monoclonal antibody elicits a desensitisation effect on CXCR4 activity [379].

The hypothesis was that CXCL12 is involved in antigen-specific transmigration of Tregs where it acts to further enhance the TCR signal during antigen-recognition. CXCL12 may work synergistically to promote downstream signalling cascades. In addition, the role of ZAP-70 on CXCL12 functionality has been assessed in different T-cell lines and in $CD4^+$ T cells obtained from a patient with ZAP-70 deficiency [380]. Zap-70 is the protein tyrosine kinase zeta-associated protein, a key signalling element in T-cell activation. The study by Ticchioni *et al* showed that ZAP-70 is required for CXCL12-induced transendothelial T-cell migration and for downstream signalling components including Vav1 and ERKs. Abrogation of Vav1 function blocked CXCL12-induced T-cell migration and showed that ZAP-70 plays a role in CXCL12 activation of the ERK signalling pathway. CD4 T cells from ZAP-70 deficient patients resulted in an impairment of transendothelial migration that was reversed by the transfection of ZAP-70. CXCL12 stimulates the physical association of CXCR4 and the TCR and uses the ZAP-70-binding immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR for signal transduction [381].These studies support the hypothesis that chemokine receptor responses can depend upon TCR signalling.

A study using T cells from patients with chronic lymphocytic leukemia found ZAP-70-negative samples showed significantly less migration towards CXCL12 compared to T cells from ZAP-70-positive samples and that this was not due to defective CXCR4 down-

regulation [382]. This further supports role for ZAP-70 in migration towards CXCL12 and as a potential target to alter T cell subset infiltration into tumours.

Tregs transmigrate across endothelium in a MHC class II restricted manner in the presence of inflammatory cytokines and CXCL12. Homeostatically, MHC dependent transmigration of Tregs recognising self-antigens into tissues would only occur during infection/inflammation and represents a mechanism to enhance the threshold for T cell recognition to prevent concomitant autoimmunity. Within tumour microenvironments our results would suggest that recognition of naturally presented high affinity self peptides presented by self MHC on endothelial cells would preferentially recruit Tregs. Therefore, efficient transmigration of Tregs recognising self-peptides presented on the tumour endothelium in combination with moderate levels of CXCL12 leads to a suppressive tumour environment and poor prognosis. Potentially altering the availability of self-peptides by targeting the MHC class II expression on tumour endothelium would help to prevent the infiltration of Tregs, however this may also result in reduced antigen-specific transmigration of CD4 effector T cells into tumours. However, neutralising CXCL12 within the tumour-microenvironment could have the potential to selectively inhibit CD4 and not CD8 T cell migration. CD4 effector T cells would also require MHC class II expression for antigen-specific transmigration into tumours, however being an effector population, the CD4 T cells may not depend on CXCL12 signalling via CXCR4 in the same way as Tregs. Although CXCL12 has been shown to enhance tumour metastasis it may also promote Treg recruitment providing a less immunogenic environment for the incoming tumour cells. Targeting the CXCL12/CXCR4 axis has been shown to reduce tumour metastasis [383], in addition neutralising CXCL12 within the tumour microenvironment would limit Treg antigen-specific infiltration into tumours while potentially still allowing effector CD4 and CD8 T cell infiltration.

Chapter 6: CXCL12 and CXCR4 expression within tumours

6.1 Background

Stromal derived factor-1 (SDF-1α, or CXCL12) is a 68-amino acid small (8kDa) cytokine belonging to the CXC chemokine family. CXCL12 is constitutively expressed in the bone marrow and other tissues including the skin, heart, liver, lung and brain endothelium [384]. CXCL12 is a strong chemotactic factor for T cells and monocytes [385] and in B cell lymphopoiesis and bone marrow myelopoiesis where its corresponding receptor is CXCR4. Data within the previous chapter has highlighted the role of CXCL12 in antigen-specific Treg transmigration, this chapter aims to assess the expression levels of CXCL12 and CXCR4 within colorectal and ovarian tumours relative to clinicopathological variables and overall survival of patients.

CXCL12 belongs to the intercrine family, expressed on stromal cells and vascular endothelium and induced by pro-inflammatory stimuli such as LPS, TNF and IL-1 and hypoxia [186]. CXCL12 binds to G-protein-coupled CXCR4 [163] and plays an important role in the regulation of stem/progenitor cell trafficking. This constitutive expression is responsible for trafficking and localisation of immature and maturing leukocytes to these tissues suggesting a role in immune surveillance [385]. It is also a potent costimulator of CD4 helper T cells [386]. More recently it has been shown that CXCL12 can act as an anti-inflammatory chemokines during the inflammatory process by promoting the polarisation of helper cells to become antigen specific regulatory cells [387]. CXCL12 is an efficient chemoattractant for T cells as it increases adhesion to ICAM-1 (CD54) by up-regulation of the binding activity of LFA-1 on the T cells [387]. This is in line with the observation that

CXCL12 is a potent chemotactic factor for plasmacytoid dendritic cells and promotes them to produce IL-10 [388]. This contradictory role of CXCL12 during homeostasis which promotes leukocyte extravasation and immune surveillance, whilst down regulating immune responses, during acute inflammation may be explained by concentration effects, with low doses promoting inflammation and high doses inhibiting inflammation. This is supported by the recent study showing that low doses of CXCL12 are chemotactic for T cells whereas higher doses are fugetactic or repellent [376].

The CXCL12 receptor, CXCR4, is a 352 amino acid rhodopsin-like seven transmembrane G protein-coupled receptor [389] and is a co-receptor for HIV entry [390]. CXCL12/CXCR4 axis plays an important role in the regulation of stem/progenitor cell trafficking [391]. AMD3100 is a highly selective CXCR4 chemokine receptor antagonist and has recently been approved as a drug (in combination with GM-CSF) for hematopoietic stem cell mobilisation from the bone marrow to the blood where they can be collected for autologous stem cell transplantation [392,393]. Endothelial cells can express both CXCL12 and CXCR4 and are key regulators of angiogenesis [394] and in addition CXC12 and CXCR4 are essential for life as mice deficient in either gene are unable to survive past birth [395,396].

Endothelial cells can express both CXCL12 and its receptor CXCR4 and expression in tumour has been shown to be important for tumour progression and metastasis as this chemokine/receptor expression has been found to be a key regulator of angiogenesis [394]. More recently CXCL12 expression has been shown to be expressed within the tumour microenvironment where it impacts upon patient survival. Tumour stroma is an active element of the tumour microenvironment and it has recently been shown that in breast cancer, activated stroma fibroblasts produce CXCL12 [184] leading to vascularisation by chemotaxis of endothelial progenitor cells [185]. The CXCR4/CXCL12 axis has been shown to be a key

factor in tumour metastasis of many cancer types where cancer cells show CXCR4 expression and corresponding expression of CXCL12 is seen at sites of tumour metastasis [397].

Various tumours and in particular the androgen dependent tumours such as prostate, breast and ovarian cancers also produce CXCL12 and express CXCR4 [388,398,399,400]. In these tumours CXCL12 can have pleiotropic roles in autocrine growth stimulation [373,401], a chemoattractant for tumour cells [164,402,403,404,405], contributes to tumour vascularisation by endothelial stem cell attraction [185] and can suppress tumour immunity [387,388]. Furthermore, expression of CXCR4 and CXCL12 predicts lymph node metastasis in colorectal [10] esophageal [406] and breast cancer [407]. CXCR4 has been shown to be a predictor of poor survival in Nasopharyngeal carcinoma [408], renal cell carcinoma [409], gastrointestinal tumours [161] and ovarian cancer [410]. In contrast, it has been shown that endogenous CXCL12 expression in colorectal cancer cell lines provides a barrier to metastases by increasing anoikis via activation of a Bim-mediated intrinsic apoptotic pathway [411]. Thus expression of CXCL12 could have a correlation with good or poor prognosis. Kollmar et. al. [412] used a murine model of established extrahepatic colorectal metastasis and showed that CXCL12 promotes tumour cell migration *in-vitro* and tumour growth of established extrahepatic metastasis *in vivo* due to angiogenesis-dependent induction of tumour cell proliferation and inhibition of apoptotic cell death.

While CXCR4 expression on tumour cells has shown enhanced metastasis towards CXCL12, few studies have looked at the expression of both CXCR4 and CXCL12 by tumour cells and the relationship with clinicopathological features and survival of patients. In this study a large cohort of 292 colorectal and 289 ovarian tumour samples were analysed for CXCL12 and CXCR4 expression by tumour cells. Correlations were assessed between chemokine expression patterns, patient clinicopathological features and survival.

6.2 Colorectal Cancer Results

6.2.1 Clinical and pathological data of colorectal cancer patients

The clinic-pathological features of the total 462 cases and 292 included in the present study are shown in **Table 6.1**.

Variable	Categories	Frequency of total cohort (%)	Frequency of stained cohort (%)
Gender	Male	266 (58)	169(58)
	Female	199 (42)	123(42)
Age (years)	Median	72	72
	Range	58-89	57-93
Status	Alive	278(60)	174(60)
	Dead	184(40)	118(38)
Tumour Grade	Well differentiated	29 (6)	23(8)
	Moderately differentiated	353 (77)	220(75)
	Poorly differentiated	71 (15)	43(15)
	Unknown	8 (2)	6(2)
Tumour Site	Colon	238 (52)	141(48)
	Rectum	181 (39)	123(42)
	Unknown	43 (9)	28(10)
TNM Stage	0 (T_{is})	3 (1)	2(1)
	1	69 (15)	49(16)
	2	174 (28)	101(35)
	3	155 (33)	102(35)
	4	54 (12)	33(11)
	Unknown	7 (2)	5(2)
Extramural Vascular Invasion	Negative	224 (48)	140(48)
	Positive	128 (28)	81(28)
	Unknown	110 (24)	71(24)
Histological Type	Adenocarcinoma	392 (85)	249(85)
	Mucinous carcinoma	51 (11)	32(11)
	Columnar carcinoma	4 (1)	4(1)
	Signet ring carcinoma	7 (1)	2(1)
	Unknown	8 (2)	5(2)

Table 6.1. Clinicopathological variables for the patient cohort (n = 462) and cores stained for tumour-associated CXCL12 (n=292).

The patient cohort was comprised of 226 (58%) males and 196 (42%) females. The median age at the time of surgery was 72 years, consistent with a median age at diagnosis of colorectal cancer of 70-74 years in the UK. Fifteen percent of tumours arrayed were TNM 1,

38% TNM 2, 33% TNM 3 and 12% TNM 4 comparable with recently published national figures [413]. The majority of tumours (85%) were adenocarcinomas, and were often of a moderate histological grade (77%). Patients had a median follow-up of 37 months (range 0 to 116) and there were slightly more male than female patients (58% and 42% respectively). Fifty two per cent of the tumours were of colonic origin and 39% of rectal origin, and in 9% of cases, the site was not recorded. Well-differentiated tumours comprised 6% of the series, while 77% showed moderate differentiation, and 15% were poorly differentiated. Evidence of extramural vascular invasion was documented in 76% of all tumours. Among all the clinicopathological parameters scored, only tumour stage and the presence or absence of vascular invasion had strongly significant influence on survival. This is consistent with previously published data.

At the time of censoring for data analysis 49% of patients had died from their disease, 13% had died from other causes, and 37% were alive which is comparable with a national average of approximately 45% five-year survival for colorectal cancer in the UK [413]. The median disease-specific survival was 58.2 months

6.2.2 CXCL12 expression in colorectal cancer tissue

Analysis of CXCL12 was possible in 292 of the total 462 cores (63%) with the remainder not available on the cut slide, being lost during antigen retrieval or not demonstrating viable tumour cells in the core. Due to the large loss of cores, the clinicopathological data for the 292 samples was assessed to confirm that the stained cohort represented the original cohort (Table 1). CXCL12 staining was seen in the cytoplasm of tumour cells (**Figure 6.1**). Of the 292 samples, using an H score system, 20 (7%) were negative (a score of 0) for CXCL12 in tumour cells with the majority of tumours having low (104; 35.6% (<=100)) and moderate CXCL12 103 (103: 35.3% (<=200)) expression but 65 tumours (22.3% (<=300)) showing strong expression. Cut –points to divide both tumours into low and high expression of CXCL12 were determined using the X-tile program (Yale University, CT, USA). This provided low/high groups of H Score 0-145 (low) and 145-300 (high) for tumour CXCL12 expression representing 55% and 45% respectively.

Figure .6.1

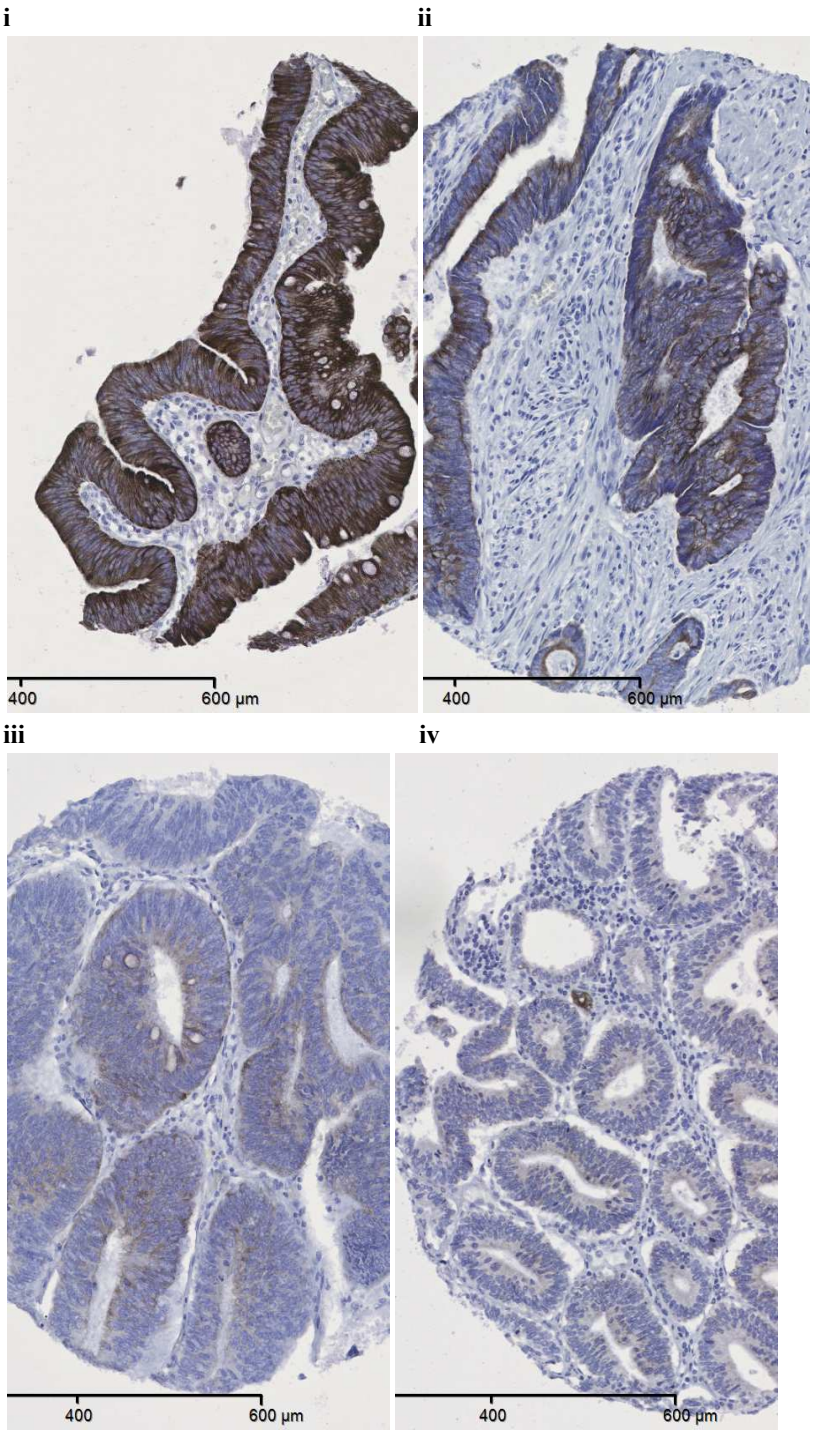

Figure 6.1 ***CXCL12 expression in colorectal tissue.***
Photomicrographs of colorectal tissue microarray cores immunohistochemically stained for CXCL12. The level of expression ranged from strong (i) to moderate (ii) to weak (iii) to negative (iv). Magnification: x100.

6.2.3 CXCR4 expression in colorectal cancer tissue

Analysis of CXCR4 was possible within the 292 samples of the total 462 cores (63%). CXCR4 staining was seen mainly in the nucleus of tumour cells, with the exception of high CXCR4 expression which was seen in the nucleus and cytoplasm (**Figure 6.2**). Of the 292 samples, using an H score system, only 6 (2%) were negative (a score of 0) for CXCR4 expression on tumour cells with the majority of tumours having low (103; 35.3% (<=100)) and moderate CXCR4 (144: 49.3% (<=200)) expression but 39 tumours (13.4% (<=300)) showing strong expression. Cut –points to divide both tumours into low and high expression of CXCR4 were determined using the X-tile program (Yale University, CT, USA). This provided low/high groups of H Score 0-70 (low) and 70-300 (high) for tumour CXCR4 expression representing 16% and 84% respectively.

Figure 6.2

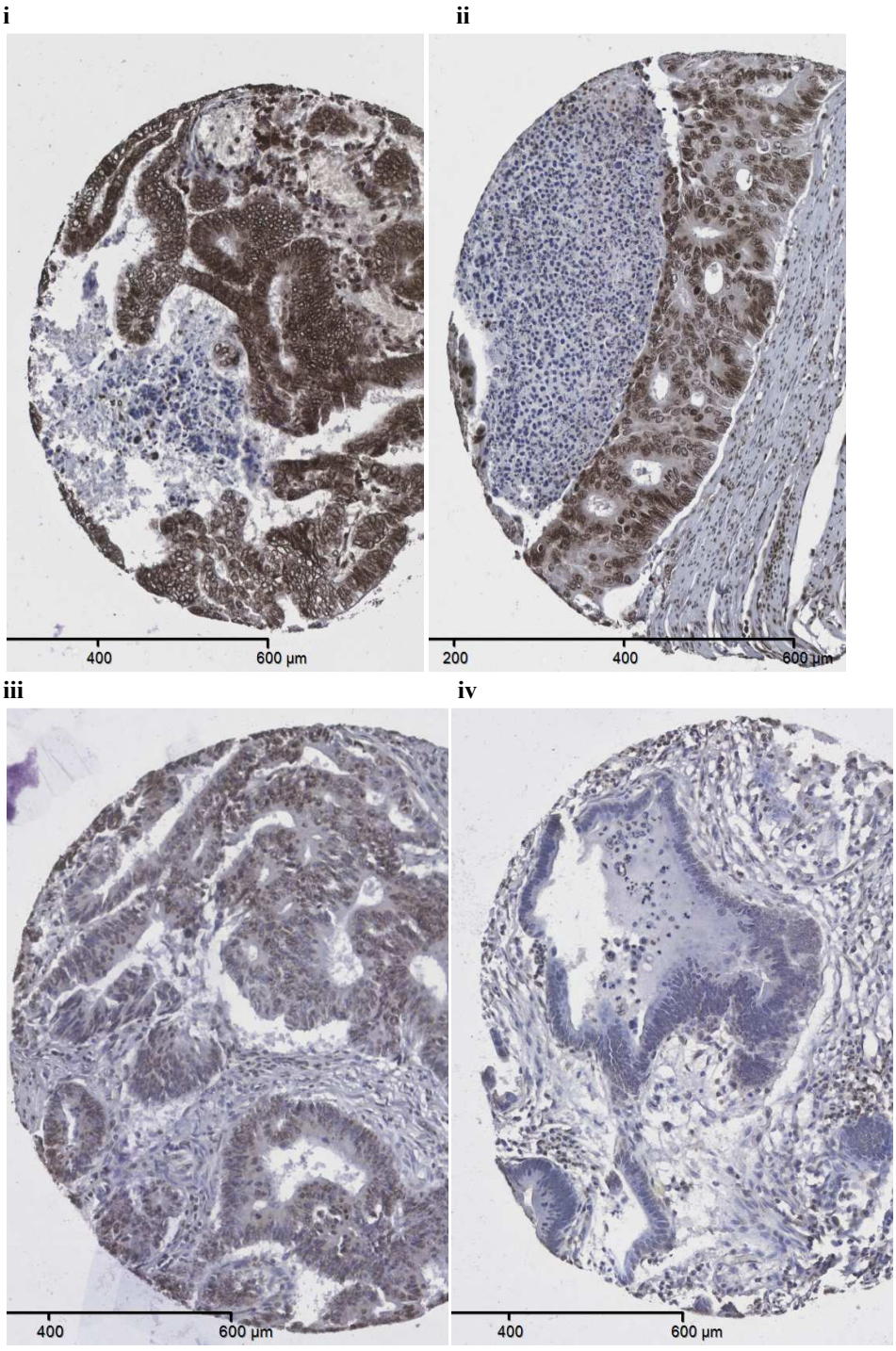

Figure 6.2 ***CXCR4 expression in colorectal tissue.***
Photomicrographs of colorectal tissue microarray cores immunohistochemically stained for CXCR4. The level of expression ranged from strong (i) to moderate (ii) to weak (iii) to negative (iv). Magnification: x100.

6.2.4 Relationship between CXCL12 and CXCR4 expression and survival

Kaplan-Meier plots were used to analyse the relationship between low and high expression of CXCL12 and CXCR4 and disease-specific survival. There is a complex relationship between CXCL12 expression and survival in colorectal cancer. Patients who express strong or no CXCL12 have a poor survival with mean survival times of 50.8 and 55.6 months respectively (**Table 6.2**). In contrast, patients whose tumours express low or moderate levels of CXCL12 have longer survival times of 81.2 and 73.4 months respectively (p=0.013; **Figure 6.3A**). Patients were regrouped into two groups using the X-tile program (Yale University, CT, USA). This provided low/high groups of H Score 0-145 (low) and 145-300 (high) for tumour CXCL12 expression representing 55% and 45% respectively. Patients with a higher level of tumour associated CXCL12 expression showed a significantly reduced mean survival (p=0.008; 59 months) than those with a lower level of CXCL12 expression (76 months; **Figure 6.3B**). Whereas if patients with either no or high expression and patients with low or moderate expression where regrouped into two groups forming 'good' and 'bad' prognosis groups, there was an even stronger association with survival (p=0.003;**Figure 6.3C**)

There was no effect on survival of H score groupings for CXCR4 (p=0.882; **Figure 6.4A and Table 6.3**). When comparing low and high levels of CXCR4 (**6.4B**) expression with disease specific survival no difference was found (p=0.274). When tumour cell expression of high and low CXCL12 was compared to high and low CXCR4 expression (**Figure 6.4C**), patients that showed low expression of CXCL12 and low CXCR4, 22 of 194 patients (11%) had the best survival (76.4 months), with patients with high CXCL12 and high CXCR4 displaying the worst survival (47.3 months), 68 of 124 patients (55%). Patients with high tumour CXCL12 and low tumour CXCR4 expression or patients with low tumour CXCL12 and high

tumour CXCR4 expression had a similar survival of 59.5 and 62.1 months. Overall there was no linear correlation between CXCL12 and CXCR4 however expression patterns showed significant effects on survival of colorectal cancer patients (p=0.046).

CXCL12 Expression	Mean survival time (months)		
	Estimate (months)	95% Confidence Interval	
		Lower Bound	Upper Bound
Negative	50.8	28.9	72.7
Low	81.2	72.1	90.4
Moderate	73.4	63.2	83.5
High	55.6	43.7	67.6
Overall	72.8	66.7	78.8

Table 6.2. Mean survival time in relation to CXCL12 expression.

CXCR4 Expression	Mean survival time (months)		
	Estimate (months)	95% Confidence Interval	
		Lower Bound	Upper Bound
Negative	55.4	35.1	75.7
Low	67.1	57.3	76.9
Moderate	67.9	59.3	76.5
High	69.6	53.4	85.8
Overall	68.4	62.4	74.4

Table 6.3. Mean survival time in relation to CXCR4 expression**.**

Figure 6.3

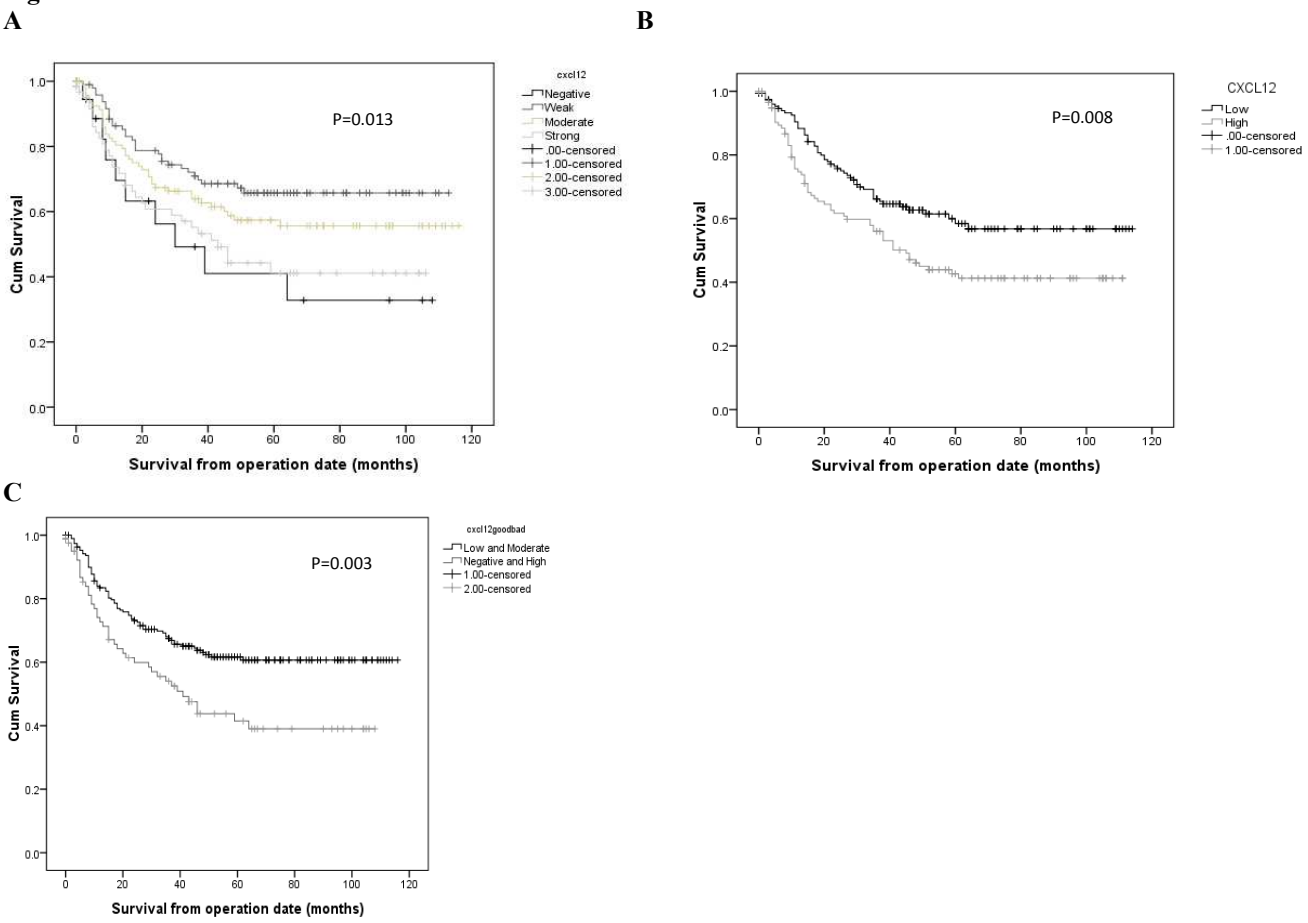

Figure 6.3. CXCL12 expression in colorectal cancer tissue: correlation with survival.
Kaplan-Meier survival graphs for CXCL12 H scores (A), high versus low CXCL12 (0-145 and 145-300) (B) and good (negative and high) versus bad (low and moderate) CXCL12 expression (C).

Figure 6.4

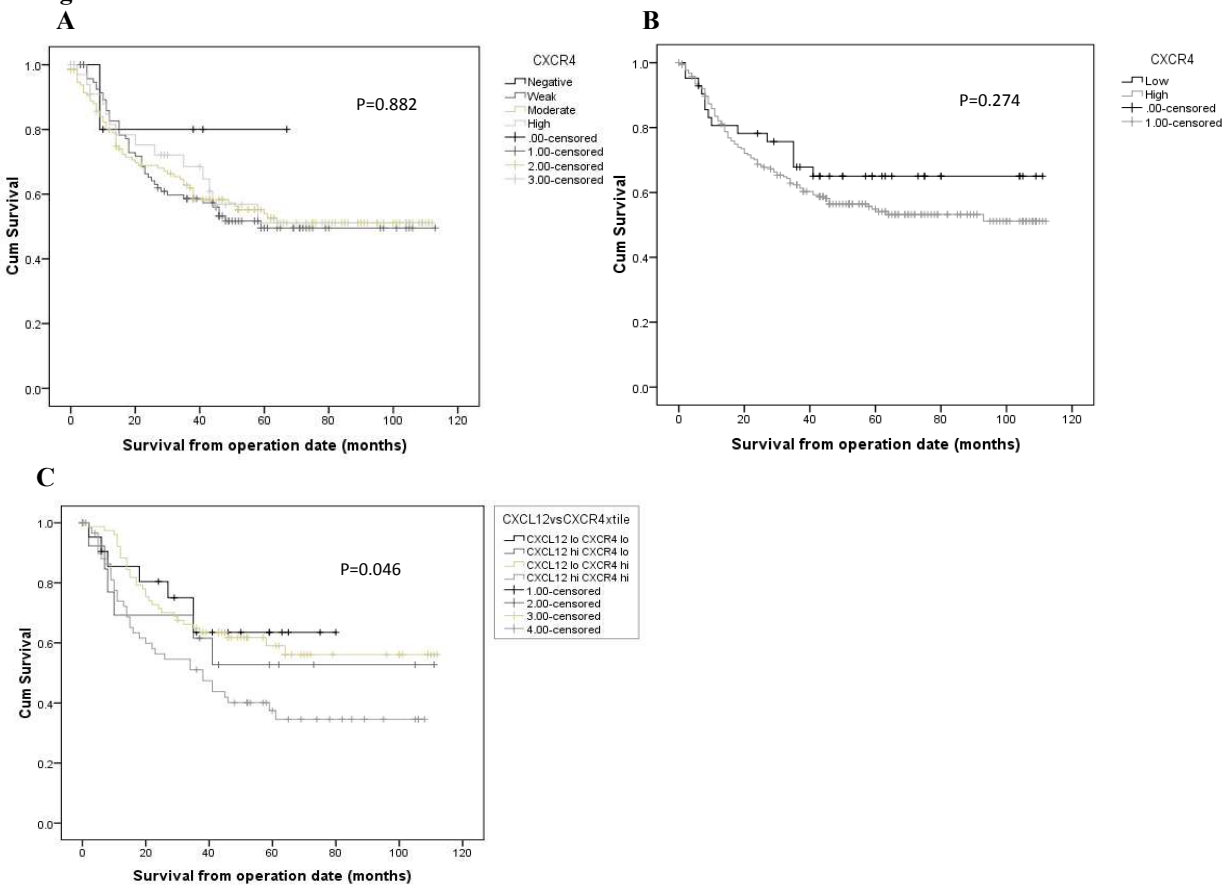

Figure 6.4. CXCR4 expression in colorectal cancer tissue: correlation with survival.
Kaplan-Meier survival graphs for CXCR4 H scores (A) and high versus low CXCR4 expression (0-70 and 70-300) (B) and high and low CXCL12 expression versus high and low CXCR4 expression (C).

6.2.5 Relationship between CXCL12 and CXCR4 expression and standard clinicopathological variables.

The relationship between CXCL12 and CXCR4 expression within colorectal tumours and standard clinicopathological variables was measured using the Pearson χ^2 test. Expression of CXCL12 was significantly associated with Duke's stage (p=0.021), TNM stage (p=0.037), nodal status (p=0.007; distant metastases (p=0.025), TRAIL expression (p=0.013), BCL2 loss (p=0.05) and VEGFa expression (p=0.045). CXCR4 was not significantly correlated with any clinicopathological variables, with the exception of VEGFc (p=0.017) (**Table 6.4**).

Variable	CXCL12	CXCR4
Histological type	0.672	0.108
Histological grade	0.129	0.359
Site	0.939	0.942
Duke's stage	0.021	0.298
TNM stage	0.037	0.415
Nodal status	0.007	0.694
Distant metastases	0.025	0.385
Extramural invasion	0.592	0.734
P53	0.551	0.316
Ki67	0.185	0.137
Bcl2	0.05	0.618
TRAIL-R2	0.013	0.527
pAKT	0.119	0.013
pERK	0.436	0.403
VEGFa	0.045	0.517
VEGFc	0.065	0.017

Table 6.4. Univariate analysis of CXCL12 and CXCR4 expression in correlation with standard clinicopathological variables using the X^2 test. Values <0.05 are accepted to be significant.

6.2.6 Multivariate analysis of CXCL12 and CXCR4 expression and standard clinicopathological variables

In order to determine the relative influence of CXCL12 and CXCR4 alongside other patient and tumour variables known to affect prognosis, a multivariate analysis was performed using the Cox proportional hazards model. The variables included were those that have been shown to be significantly related to DSS on univariate analysis (extramural vascular invasion and TNM stage). In this model, extramural vascular invasion ($p<0.001$) and TNM stage ($p=0.005$) were seen to retain independent prognostic significance (**Table 6.5**.). Expression of CXCL12 by tumour cells was also seen to be an independent prognostic marker (CI 95% 1.017-2.796, $p=0.043$). Expression of CXCR4 by tumour cells was not seen to be an independent of extramural vascular invasion and TNM, as expected (CI 95% 0.693-2.601, $p=0.383$).

	Exp(B)	95.0% CI for Exp(B)		P-VALUE
		Lower	Upper	
TNM stage				
1	1			<0.001
2	2.003	0.714	5.618	
3	3.948	1.475	10.563	
4	25.038	8.273	75.778	
Vascular invasion				
absent	1			0.005
present	2.173	1.259	3.749	
CXCL12				
No or high expression	1			0.043
Low or moderate expression	1.686	1.017	2.796	
CXCR4				
Low	1			0.383
High	1.342	0.693	2.601	

Table 6.5. Cox multivariate regression analysis of variables in relation to disease specific survival.
The Cox proportional-hazards model was used for multivariate analysis in order to determine the relative risk and independent significance of individual factors including CXCL12 and CXCR4 expression. In all cases p-values < 0.05 were considered as statistically significant.

6.3 Ovarian Cancer Results

6.3.1 Clinical and pathological data of ovarian cancer patients

CXCL12 and CXCR4 expression was analysed on colorectal cancer tissue and now assessed in a cohort of ovarian cancer samples. In the current cohort of ovarian cancer patients, the mean age at diagnosis was 61 years (range, 24-90). Using the current Surveillance, Epidemiology, and End Results (SEER) Program age categorization system, 59% of the patients were in group 3 (>60 years at diagnosis), 40% were 30 to 60 years at diagnosis, and only 2 of 357 were <30 years. Serous cystadenocarcinoma was the commonest histologic type (49%) followed by undifferentiated (15%), endometrioid (12%), mucinous cystadenocarcinoma (10%), clear cell (7%), and other types (7%). All patients were treated surgically, of which 42% had their masses optimally debulked with no macroscopic disease left. Clinicopathologic staging showed that the majority of patients (50%) were stage III followed by 27% in stage I, 11% in stage IV, and 11% in stage III. When histologic grading was applicable, almost two thirds of the tumours were poorly differentiated (grade 3). Twenty-two percent were moderately differentiated, and only 11 were deemed well differentiated. All patients' characteristics are summarised in **Table 6.6**.

Variable	Categories	Frequency of total cohort (%)	Frequency of CXCL12 stained cohort (%) n=289	Frequency of CXCR4 stained cohort (%) n=241
SEER age characteristics (n=357)	<30 yrs at diagnosis	2 (1)	2(1)	1 (1)
	30-60 yrs at diagnosis	143 (40)	112 (39)	92 (38)
	>60 yrs at diagnosis	212 (59)	172 (60)	146 (61)
Macroscopic residual disease (n=348)	Absent	143(42)	116 (42)	95 (41)
	Present	201(58)	161 (58)	136 (59)
Tumour Grade	Well differentiated (3)	225 (63)	177 (60)	152 (63)
	Moderately differentiated (2)	73 (20)	62 (22)	51 (21)
	Poorly differentiated (1)	39 (11)	34 (12)	28 (12)
	Unknown	23 (6)	16 (6)	10 (4)
Tumour FIGO Stage	I	95 (26)	77 (27)	62 (26)
	II	38 (11)	32 (11)	27 (11)
	III	175 (49)	141 (49)	118 (49)
	IV	40 (11)	30 (10)	26 (11)
	Unknown	12 (3)	9 (3)	8 (3)
Histological Type	Serous Cystoadenocarcinoma	178 (49)	148 (51)	128 (53)
	Mucinous Cystoadenocarcinoma	35 (10)	29 (10)	22 (9)
	Endometrioid	42 (12)	35 (12)	30 (12)
	Clear cell	25 (7)	20 (7)	18 (8)
	Undifferentiated	54 (15)	39 (14)	31 (13)
	Others	24 (7)	18 (7)	11 (5)
Adjuvant therapy (n=356)	No	101 (29)	78 (28)	67 (28)
	Yes	249 (71)	204 (72)	168 (72)

Table 6.6. Clinicopathological variables for the patient cohort (n = 360) and cores stained for both CXCL12 (289) and CXCR4 (n=241).

6.3.2 CXCL12 expression in ovarian cancer tissue

Analysis of CXCL12 was possible in 289 of the total 360 cores (80%) with the remainder not available on the cut slide, being lost during antigen retrieval or not demonstrating viable tumour cells in the core. Due to the loss of cores, the clinicopathological data for the 289 samples was assessed to confirm that the stained cohort represented the original cohort (**Table 6.6**). CXCL12 staining was seen in the cytoplasm tumour cells (**Figure 6.5).** Of the 289 samples, 112 (31%) were negative for CXCL12 in tumour cells with the majority of tumours having negative or low CXCL12 expression making up 67% of the cohort collectively. Cut –points to divide both tumours into low, moderate and high expression of CXCL12 were determined using the X-tile program (Yale University, CT, USA). This provided low/moderate/high groups of H Score 0-3 (low), 3-90 (moderate) and 90-300 (high) for tumour CXCL12 expression representing 39%, 41% and 20% respectively. When the X-tile program was used to divide the cohort into 2 groups: low/high groups of H Score 0-10 (low) and 10-300 (high) for tumour CXCL12 expression represented 55% and 45% respectively.

Figure 6.5

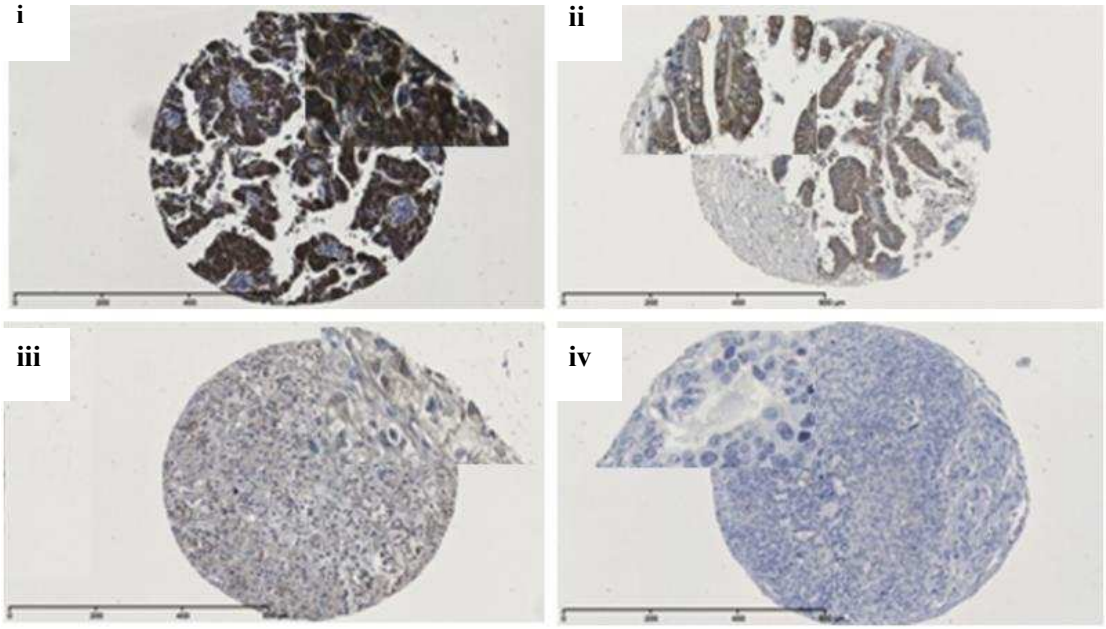

Figure 6.5 ***CXCL12 expression in ovarian tissue.***
Photomicrographs of ovarian tissue microarray cores immunohistochemically stained for CXCL12. The level of expression ranged from strong (i) to moderate (ii) to weak (iii) to negative (iv). Magnification: x20, inset x100.

6.3.3 CXCR4 expression in ovarian cancer tissue

Analysis of CXCR4 was possible in 241 of the total 360 cores (67%) with the remainder not available on the cut slide, being lost during antigen retrieval or not demonstrating viable tumour cells in the core. Due to the loss of cores, the clinicopathological data for the 241 samples was assessed to confirm that the stained cohort represented the original cohort (**Table 6.6**). CXCR4 staining was seen in the nucleus of tumour cells (**Figure 6.6**). Of the 241 samples, none were negative for CXCR4 in tumour cells with the majority of tumours having moderate CXCR4 expression making up 49% of the cohort. Cut –points to divide tumours into low, moderate and high expression of CXCL12 were determined using the X-tile program (Yale University, CT, USA). This provided low/moderate/high groups of H Score 0-60 (low), 60-115 (moderate) and 115-300 (high) for tumour CXCL12 expression representing 11%, 20% and 69% respectively. When the X-tile program was used to divide the cohort into 2 groups: low/high groups of H Score 0-105 (low) and 105-300 (high) for tumour CXCL12 expression represented 27% and 73% respectively.

Figure 6.6

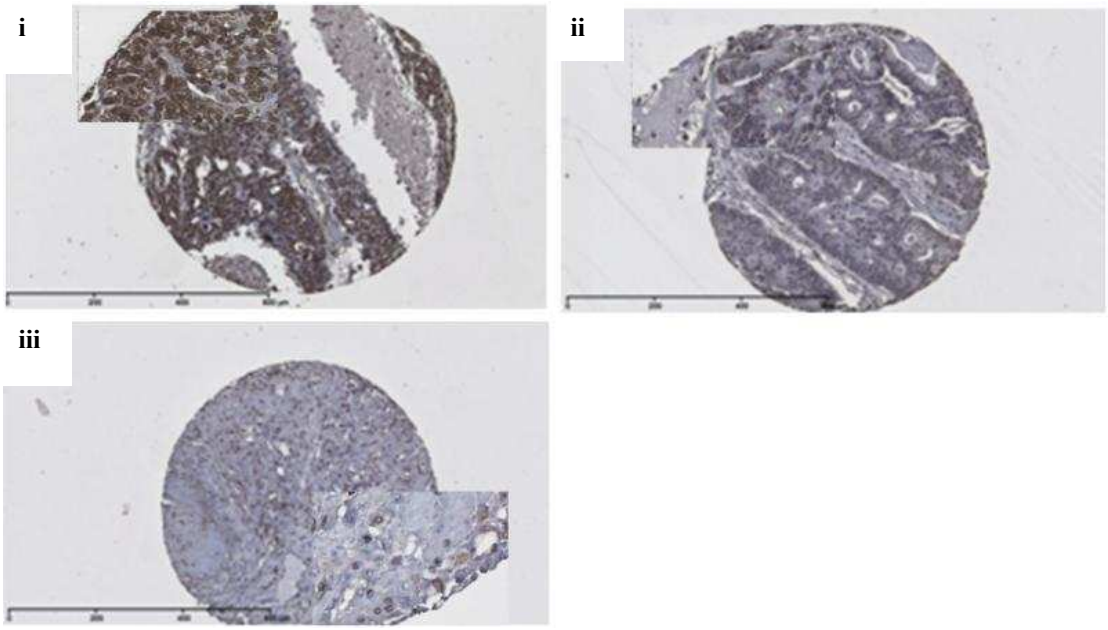

Figure 6.6 ***CXCR4 expression in ovarian tissue.***
Photomicrographs of ovarian TMA cores immunohistochemically stained for CXCR4. The level of expression ranged from strong (i) to moderate (ii) to weak (iii). Magnification: x20, inset x400.

6.3.4 CXCL12 and CXCR4 expression in ovarian cancer tissue: correlation with survival

Kaplan-Meier plots were used to analyse the relationship between expression levels of CXCL12 and CXCR4 and disease-specific survival. **Figure 6.7A** demonstrates that increasing expression of CXCL12 within tumours significantly reduces patient survival (p=0.026). Patients with low CXCL12 expression lived a mean of 75.9 months, patients with moderate expression a mean of 59.1 months and patients with high expression 24.2 months (**Table 6.7**). **Figure 6.7B** demonstrates that patients with a higher level of tumour associated CXCL12 expression have a significantly reduced mean survival (p=0.019; 53 months) than those with a lower level of CXCL12 expression (73 months).

When comparing differing levels of CXCR4 (**Figure 6.8A**) expression with disease specific survival, no difference was found (p=0.712). Patients with low CXCR4 expression lived a mean of 47.5 months, patients with moderate expression a mean of 74.6 months and patients with high expression 58.1 months (**Table 6.8**). When comparing low and high levels of CXCR4 (**Figure 6.8B**) expression with disease specific survival no difference was found (p=0.525). There was no linear correlation between CXCL12 and CXCR4 expression (p=0.409). When tumour cell expression of high and low CXCL12 was compared to high and low CXCR4 expression (**Figure 6.8C**), although no overall significant effect was seen on survival, patients that showed low expression of CXCL12 and low CXCR4, 32 of 235 patients (32%) had the best survival of 82 months, with patients with high CXCL12 and low CXCR4 displaying the worst survival, 14 of 235 patients (33%) and a survival time of only 39 months. Patients with high tumour CXCL12 and high tumour CXCR4 expression or patients with low tumour CXCL12 and high tumour CXCR4 expression had a similar survival of 66 and 60 months respectively. Overall there was no linear correlation between

CXCL12 and CXCR4 and expression patterns showed no significant effects on survival of ovarian cancer patients (p=0.173).

CXCL12 Expression	MEAN SURVIVAL TIME (MONTHS)		
	Estimate (months)	95% Confidence Interval	
		Lower Bound	Upper Bound
Low	75.9	59.1	92.6
Moderate	59.1	45.5	72.6
High	24.2	13.9	34.5
Overall	66.6	55.3	77.8

Table 6.7 Mean survival time in relation to CXCL12 H score expression.

CXCR4 Expression	MEAN SURVIVAL TIME (MONTHS)		
	Estimate (months)	95% Confidence Interval	
		Lower Bound	Upper Bound
Low	47.5	22.8	72.1
Moderate	74.6	45.6	103.7
High	58.1	46.5	69.7
Overall	65.1	53.1	77.1

Table 6.8 Mean survival time in relation to CXCR4 H score expression.

Figure 6.7.

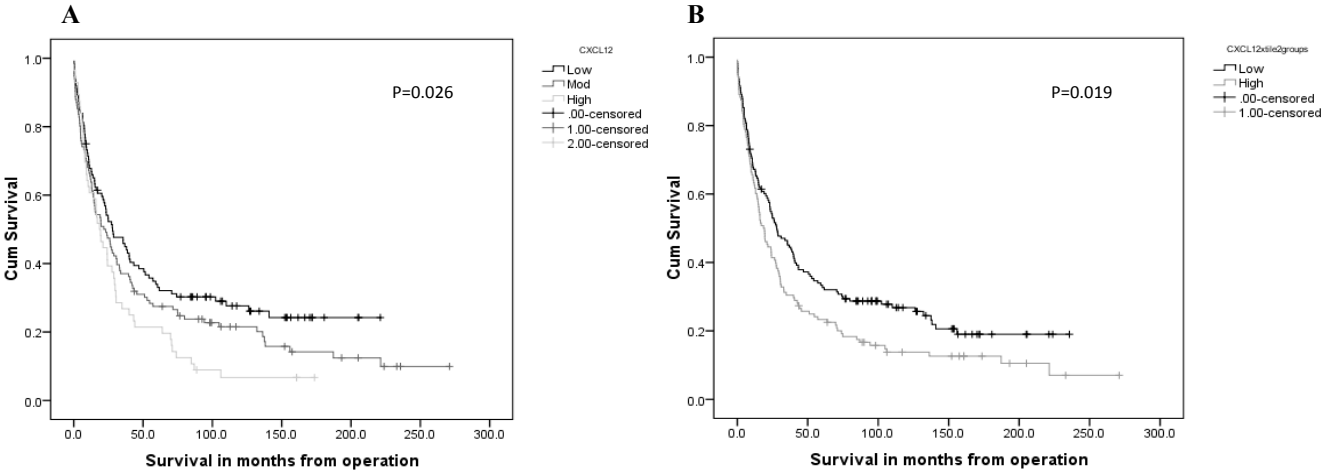

Figure 6.7 ***CXCL12 expression in ovarian cancer tissue: correlation with survival.***
Kaplan-Meier survival graphs for graded CXCL12 expression (low (0-3) moderate (3-90) and high (90-300)) (A) and low versus high CXCL12 expression (low (0-10) and high (10-300)) (B).

Figure 6.8.

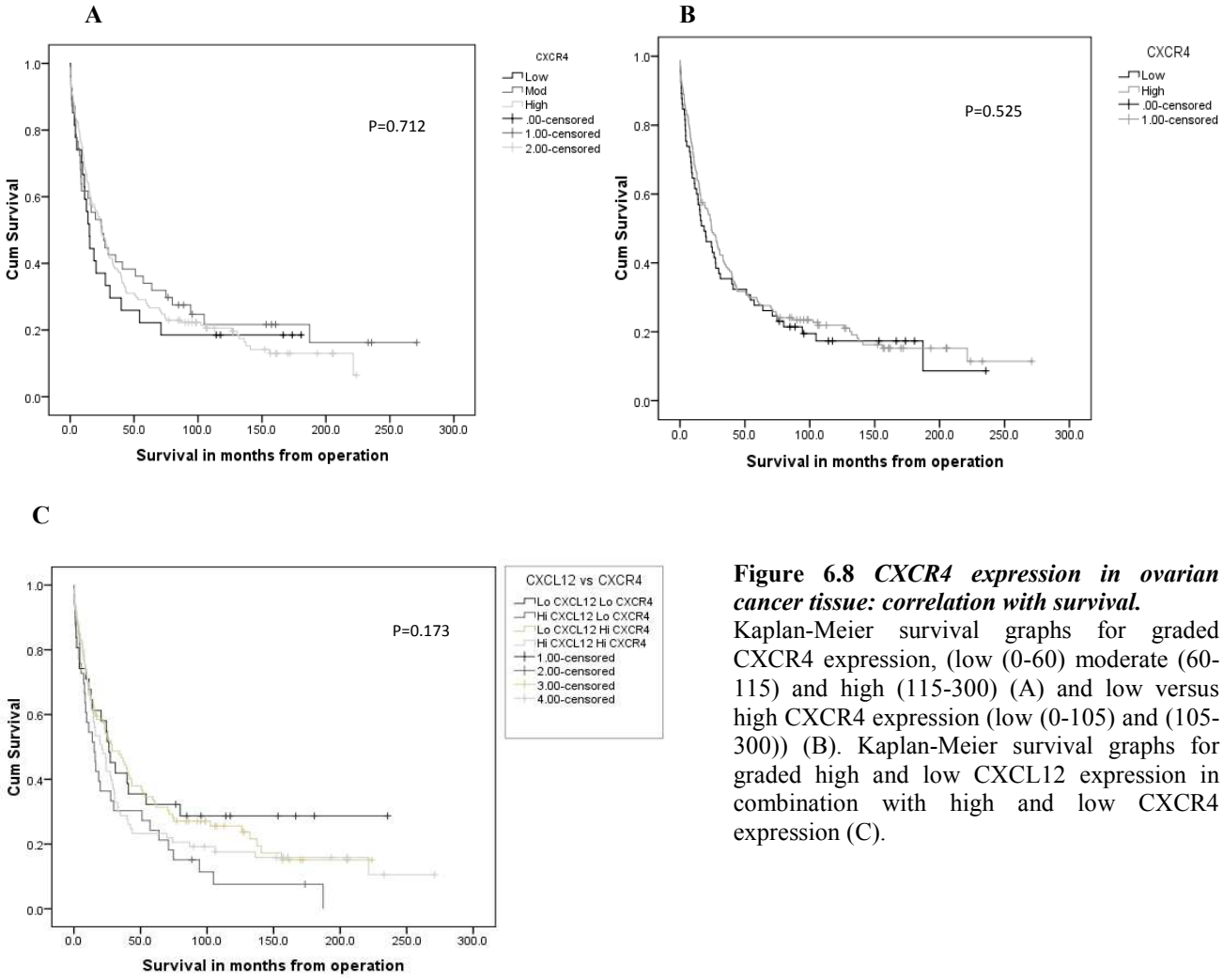

Figure 6.8 ***CXCR4 expression in ovarian cancer tissue: correlation with survival.***
Kaplan-Meier survival graphs for graded CXCR4 expression, (low (0-60) moderate (60-115) and high (115-300) (A) and low versus high CXCR4 expression (low (0-105) and (105-300)) (B). Kaplan-Meier survival graphs for graded high and low CXCL12 expression in combination with high and low CXCR4 expression (C).

6.3.5 CXCL12 and CXCR4 expression in ovarian cancer tissue: correlation with clinicopathological features.

The relationship between CXCL12 and CXCR4 expression within ovarian tumours and standard clinicopathological variables was measured using the Pearson χ^2 test. Expression of both CXCL12 and CXCR4 was not significantly correlated with any clinicopathological variables, including stage (**Table 6.9**).

Variable	X^2 test (*p* value)	
	CXCL12	CXCR4
Tumour FIGO Stage	0.184	0.603
Tumour Grade	0.865	0.239
Macroscopic Residual Disease	0.769	0.141
Adjuvant Therapy	0.144	0.704
Histological type	0.347	0.658

Table 6.9.Univariate analysis of CXCL12 and CXCR4 expression in correlation with standard clinicopathological variables using the X^2 test. Values <0.05 are accepted to be significant

6.3.6 Multivariate analysis of CXCL12 and CXCR4 expression with standard clinicopathological variables.

In order to determine the relative influence of CXCL12 and CXCR4 and other patient and tumour variables known to affect prognosis, a multivariate analysis was performed using the Cox proportional hazards model. The variables included were those that have been shown to be significantly related to DSS on univariate analysis (macroscopic residual, adjuvant therapy and FIGO stage). In this model, macroscopic residual disease (p<0.0001), adjuvant therapy (p=0.001) and FIGO stage (p<0.0001) were seen to retain independent prognostic significance (**Table 6.10**). Expression of CXCL12 by ovarian tumours was also seen to be an independent prognostic marker (p=0.016). Expression of CXCR4 by ovarian tumours was not seen to be independent of macroscopic residual, adjuvant therapy and FIGO stage, as expected (p=0.364; **Table 6.11**).

	Exp(B)	95.0% CI for Exp(B)		p-value
		Lower	Upper	
FIGO stage				
Stage 1	1			<0.001
Stage 2	2.483	1.370	4.501	
Stage 3	5.639	3.235	9.829	
Stage 4	6.047	3.168	11.543	
Macroscopic residual disease				
Absent	1			<0.001
Present	2.002	1.374	2.917	
Adjuvant therapy				
No	1			<0.001
Yes	0.493	0.331	0.735	
CXCL12				
Low	1			0.016
Moderate	1.215	0.892	1.655	
High	1.684	1.180	2.404	

Table 6.10. Cox multivariate regression model for CXCL12 expression. CI = confidence intervals.

	Exp(B)	95.0% CI for Exp(B)		P-VALUE
		Lower	Upper	
FIGO stage				
Stage 1	1			<0.001
Stage 2	2.633	1.391	4.986	
Stage 3	5.638	3.120	10.189	
Stage 4	5.698	2.878	11.281	
Macroscopic residual disease				
Absent	1			0.002
Present	1.928	1.284	2.894	
Adjuvant therapy				
No	1			<0.001
Yes	0.471	0.309	0.719	
CXCR4				
Low	1			0.364
Moderate	0.667	0.381	1.166	
High	0.796	0.505	1.257	

Table 6.11. Cox multivariate regression model for CXCR4 expression. CI = confidence intervals.

6.4 Discussion

The chemokine stromal-derived factor (CXCL12) plays complex roles in tumour pathogenesis by either promoting tumour growth, enhancing tumour angiogenesis, suppressing tumour immunity and participating in tumour metastasis via expression of CXCR4 [162,414] or by inhibiting metastases by inducing anoikis [411]. However, research to determine the source and regulation of CXCL12 expression within the tumour microenvironment is limited. Stroma and tumour cells, two main components of the tumour microenvironment, can produce CXCL12. CXCL12 regulation within breast and ovarian tumours has been attributed to estradiol which activates estrogen receptors and induces the production of CXCL12 by tumour cells [415]. Hypoxia has also been shown to induce CXCL12 expression by primary human ovarian tumour cells where Hypoxia-inducible factor (HIF)-1 is the central mediator [186] .

The CXCL12/CXCR4 axis in human cancers is now becoming an important target for the development of novel anti-cancer therapies. In this study, Tissue microarray technology was used to assess the expression of CXCL12 and CXCR4 in relation to clinic pathological characteristics and overall survival of 292 colorectal and 289 ovarian cancer patients.

6.4.1 Discussion for Colorectal data

Colorectal cancer (CRC) accounts for more than 9% of all new cancer cases [413] and is also the second most common cause of cancer-related deaths. Despite the recent advances in treating cancer, the 5-year survival rate from CRC remains at 50% and 10% for TNM stages III and IV respectively. Currently, four treatment options for CRC exist, including surgery, chemotherapy, radiotherapy and monoclonal antibody therapy.

In this study, Tissue microarray technology was used to assess the expression of CXCL12 and CXCR4 in relation to clinicopathology and overall survival of 292 colorectal cancer patients. CXCL12 and CXCR4 expression was seen in 93% and 78% of patient samples respectively. This study demonstrated in colorectal cancer that the level of CXCL12 impacted on patient survival with patients whose tumours showed no or high CXCL12 expression showing reduced disease specific survival (p=0.013). There was a 20 month survival advantage, for patients whose tumours lacked or had high CXCL12 expression, when compared to patients whose tumours expressed low or moderate levels of this chemokine.

In agreement with previous studies [160,416], expression of CXCL12 correlated with lymph node metastases, tumour stage and survival. However, in our study we show that in a multivariate analysis it was shown to confer independent prognostic value. Low or moderate expression of CXCL12 correlated with expression of Bcl2 which is consistent with its role in anoikis via the intrinsic Bcl-2 pathway [411]. It also correlated with expression of VEGFa which is consistent with its role in angiogeneisis. Hypoxia has also been shown to induce CXCL12 expression by primary human ovarian tumour cells where Hypoxia-inducible factor is the central mediator [186]. Kryczek *et al* studied CXCL12 in association with VEGF and found hypoxia simultaneously induces tumoral CXCL12 and VEGF production [186]. Hypoxia-induced signals would be an important factor for initiating and maintaining an active synergistic angiogeneic pathway mediated by CXCL12 and VEGF.

In contrast to CXCL12, the expression of CXCR4 within our study, had no significant effect on patient survival (p=0.525) or correlation with any other clinicopathological variable. This is in contrast with another studies which showed that CXCR4 expression correlated with

survival in colorectal cancer patients [410]. This may be a reflection of the differing antigen retrieval methods showing greater or less sensitivity of detection.

A study by Yoshitake *et al* within a sample of 60 colorectal cancer patients indicated that nuclear CXCR4 predicted lymph node metastasis in colorectal cancer [160]. Within this study, 78% of colorectal cancer patients showed CXCR4 positive tumours, of which 61% nuclear CXCR4 expression correlating with significantly more lymph node metastasis than those with cytomembrane expression. Another study showed CXCR4 is associated with the progression of colorectal carcinoma where high CXCR4 expression is associated with metastasis [417]. CXCR4 was positive in 39 cancer tissue specimens (73.6%) and its high expression rate (in > 50% of cells) was 45.3%. High CXCR4 expression was associated with the vascular and lymphatic vessel invasions ($P<0.01$), this is in agreement with our observation that expression of CXCR4 correlated with VEGF-C expression. Wang *et al* analysed expression of CXCR4 in 388 colorectal clinical samples and showed that CXCL12 affected the expression of CXCR4 in colon cancer cells [418]. The frequency of cytoplasmic and nuclear expression of CXCR4 in colorectal cancers was 35.6% and 36.9%, respectively. Nuclear but not cytoplasmic expression of CXCR4 was associated with advanced CRC ($p<0.001$) and lymphovascular invasion. However, as shown in data presented here, in multivariate analysis, nuclear expression of CXCR4 did not correlate with patients' outcome. Wang *et al* also showed that in an *in vitro* study CXCL12 was used to stimulate three colorectal carcinoma lines and led to enhanced the CXCR4 nuclear expression. Therefore, the explanation for the percentage of tumours expressing CXCR4 between studies could be explained by the relative levels of CXCL12 produced by the tumour. Here, with 94% of tumours expressing CXCL12 this may result in 98% of tumours expressing CXCR4. One explanation for the differences in the effect of CXCR4 on survival between the studies may

be the localisation of CXCR4 within the cell. CXCR4 expression is not confined to the cell surface and the CXCR4 receptor may be internal until activation/signalling occurs whereby the receptor becomes present at the cell surface and allows interaction to occur with CXCL12, activating signalling cascades [374]. Recent studies have shown that 5T4 is one of several proteins that are required for functional cell surface expression of CXCR4 in differentiating murine embryonic stem cells [419]. As 5T4 is also over-expressed in colorectal cancer and is a target for both vaccine and monoclonal antibody therapy it may play a similar role in this disease [420]. In our study 100% of the tumours showed nuclear staining with 69% strong nuclear and cytoplasmic staining. While in the Yoshitake *et al* study CXCR4 expression was seen either within the nucleus or within the cytoplasm of tumours the latter being associated with survival [160].

A further concern is that CXCL12 can also been shown to bind to CXCR7 [421]. Although it is unclear how this receptor signals it has been shown to heterodimerise with CXCR4 and alter both the kinetics and dynamics of CXCR4 responsiveness to CXCL12 [422]. Future studies should also look for expression of this receptor in colorectal cancer.

As the CXCR4/CXCL12 pathway has been shown to be involved in the metastasis of tumour cells, this has important implications for the survival of colorectal cancer patients where the main cause of death is attributed to metastasis of the cancer. Indeed, in breast cancer high levels of expression of CXCL12 were shown in organs representing the first destination for breast cancer metastasis [164]. However, we have demonstrated high levels of CXCL12 expression at the primary site of the tumour to be involved in poor prognosis. This raises the question whether chemokines may not just work at target organs but could also be involved in the departure of cells from the primary tumours. Indeed, high levels of CXCL12 have been shown to be fugetactic to lymphocytes [376], and a B16 melanoma engineered to produce

CXCL12 at high levels was found to be chemo-repellent to antigen specific T cells [377]. Alternatively, CXCL12 stimulates the production of metalloproteases which are important in local tumour invasion [423,424]. In contrast, complete loss of CXCL12 within tumours also conferred a poor prognosis on patients. This may reflect the protective role of CXCL12 in protecting cells from anoikis or apoptosis triggered by a loss of cellular adhesion to the underlying extracellular matrix, a vital role in tumour metastases. As CXCL12 is constitutively expressed by colon and is down regulated in adenomas perhaps this initially protects tumour cells from anoikis but ultimately the benefits of the tumour promoting aspects of CXCL12 make it an advantage to secrete it in moderate amounts.

6.4.2 Discussion for Ovarian data

Ovarian cancer is the fifth leading cause of cancer among women and the leading cause of mortality from gynaecologic cancers [425]. Human epithelial ovarian cancer constitutes approximately 90% of ovarian malignancies and is thought to arise from the ovarian surface epithelium. It can be classified into 4 major categories: serous, mucinous, endometriod and clear cell. Each subtype has different clinical, molecular and biological characteristics and may represent different diseases [426]. However, the main cause of treatment failure and death is metastases. This can be locally within the peritoneum or can be too distant sites such as liver and lung. It has been suggested that chemokines involved in lymphocyte homing and migration can also be used by tumour cells to metastasise. In a study of expression 14 chemokine receptors, only CXCR4 was expressed within ovarian cancer cell lines [427]. The only CXCR4 ligand, CXCL12, induced migration, integrin expression, proliferation, and invasion. CXCL12 was abundantly expressed within 15/18 ovarian cancer biopsies [428] and within ascites from 63 ovarian cancer patients [373].

In this study, tissue microarray technology was used to assess the expression of CXCL12 and CXCR4 in relation to clinic pathological characteristics and overall survival of 289 ovarian cancer patients. This study has the advantage of over 14 years of patient follow up. CXCL12 and CXCR4 expression was seen in 69% and 100% of patient samples respectively. This study demonstrated in ovarian cancer that the level of CXCL12 impacted on patient survival with patients whose tumours showed moderate or high CXCL12 expression showing reduced disease specific survival (p=0.026). There was a 51 month survival advantage, for patients whose tumours lacked CXCL12 expression, when compared to patients whose tumours expressed high levels of this chemokine. Furthermore, CXCL12 expression was an independent predictor of poor survival and was equally expressed by all ovarian tumour types. This data contrasts with a smaller immunohistochemical study by Jiang *et al* using 80 patients of with a shorter follow up (median 37 months). CXCL12 was detected in 40/44 (91% of patients) however in this study CXCL12 did not correlate with disease survival [410]. This contrasted with our study which analysed 289 patients with a median follow up of 167 months (range 95-335 months).

In contrast to CXCL12, the expression of CXCR4 within our study, had no significant effect on patient survival (p=0.712) or correlation with any other clinicopathological variable. This is in contrast with other studies which showed that CXCR4 expression was an independent prognostic factor for poor survival in epithelial ovarian cancer patients [410]. Both these studies used lower numbers of patients with less follow up than our study. One explanation for the differences in the effect of CXCR4 on survival between the studies may be the localisation of CXCR4 within the cell. CXCR4 expression is not confined to the cell surface and the CXCR4 receptor may be internal until activation/signalling occurs whereby the

receptor becomes present at the cell surface and allows interaction to occur with CXCL12, activating signalling cascades [374]. In our study 100% of the tumours showed nuclear staining with 69% strong nuclear and cytoplasmic staining. While in the Jiang study CXCR4 expression was only seen within the cytoplasm of 59% of patients. This may be a reflection of the differing antigen retrieval methods showing greater or less sensitivity of detection.

CXCL12 regulation within breast and ovarian tumours has been attributed to estradiol which activates oestrogen receptors and induces the production of CXCL12 by tumour cells [415]. Over 70% of ovarian and breast cancers over-express the oestrogen receptor. The binding of CXCL12 to its receptor CXCR4 is thought to induce proliferation of tumour cells. In this way CXCL12 mediates the cancer cell proliferation action of oestrogen and may be one of the reasons for the poor prognosis of patients with high CXCL12 [415].

6.4.3 Overall conclusions

CXCL12 has contradictory roles in immune responses. During homeostasis, tissues constitutively express CXCL12 promotes leukocyte extravasation and immune surveillance. However, CXCL12 is not produced within normal tissue and it is only induced by pro-inflammatory mediators such as IL-1 and TNF-α and during hypoxia [186]. Under conditions of inflammation it has been shown that CXCL12 can down regulating immune responses [429]. It can polarise helper T cells, macrophages and plasmacytoid dendritic cells to secrete the immunoregulatory cytokine IL-10 [387,388]. CXCL12 can also promote TNF-α expression which can active NK-κB and act back on tumour cells to induce cell surface CXCR4 expression. In effect, TNF-α, can amplify the CXCL12 signal [375] and therefore suggests that neutralising TNF-α, CXCL12 [429], antagonising CXCR4 [430,431,432,433] or inhibiting NK-κB [434] may be effective therapies for cancer.

In conclusion, CXCL12 expression levels within colorectal and ovarian tumours impacts on survival within patients where increasing CXCL12 expression leads to decreased disease specific survival. Expression of CXCL12 was found to be an independent prognostic indicator in a large series of patients with all stages of ovarian cancer and colorectal cancer. In addition high CXCL12 expression only occurs in a small proportion of ovarian cancers but may denote a specific group in which targeting CXCL12 expression as a therapy may be an effective target. Chemokine expression profiles are becoming more widely recognised for their role in tumour progression and the CXCL12-CXCR4 signalling pathway is becoming an attractive target for anti-tumour immunotherapy. CXCR4 has been widely accepted as an efficient target for reducing tumour metastasis and antibodies directed against CXCR4 have been reported to have effects against both HIV-1 infection and cancer cell migration [435]. A small peptide antagonist of CXCR4 has been tested on ovarian cancer cells and shown to induce cell death by mitotic catastrophe [436]. CTCE-9908 inhibited ovarian cancer cell migration to CXCL12 and caused cell death in CXCR4 positive cells. CTCE-9908 did not cause apoptosis or cellular senescence, but induced multi-nucleation, G_2-M arrest, and abnormal mitosis in ovarian cancer cells.

This study has shown that expression of CXCL12 is an independent prognostic indicator of poor survival in ovarian cancer. Furthermore, it identifies a group of ovarian cancer patients (20%) and colorectal cancer patients (22%) with high CXCL12 expression in which targeting CXCL12/CXCR4 axis may be an effective target. In addition with respect to colorectal cancer patients this study has also shown that no or high expression of CXCL12 is an independent prognostic indicator of poor survival. Due to the role of CXCL12, at high levels, in tumour cell activation, proliferation and migration and in immune cell infiltration targeting both CXCR4 and CXCL12 would potentially have synergistic effects on patient survival.

Chapter 7: Final Discussion

T cell infiltration into tumours is essential for tumour antigen recognition and tumour cell elimination. The aim of this study was to develop a better understanding of T cell infiltration into tumours, focusing on two opposing arms of an immune response, anti-tumour CD8 T cells and Regulatory T cells. Activated CD4 Th cells are also of importance but could not be studied due to the time constraints of the project. The effect of T cell signalling at the immunological synapse following interactions between T cells and APCs presenting cognate antigen have been well studied. The endothelium is neither a stereotypical APC nor simply a passive filter barrier for non-cognate infiltrating T cells. The endothelium can actively influence the development of an inflammatory response depending on the functional state of both the endothelium and interacting T cells (resting versus recently activated T cells) and the type of interactions (cognate versus non-cognate). The hypothesis was that recognition of antigens presented in the context of MHC molecules by endothelium aids T cell transmigration and hence infiltration into tissues, including into tumours.

The data here highlights the complexity of the tumour microenvironment and potential downstream effects on T cell infiltration and tumour metastasis. The presence of CD8 T cells within colorectal tumours correlated with improved patient survival. Both CD8 T cells and CD4CD25FOXP3 regulatory T cells showed enhanced transmigration across endothelium and hence entry into tissues following cognate antigen recognition. Chemokine expression profiles, in particular CXCL12, also play a role in this antigen-specific transmigration via activation of synergistic signalling pathways but in addition affect the metastatic potential of tumours via CXCR4 expression on the tumours themselves. The interplay between expression levels of CXCL12, tumour metastasis and T cell infiltration are summarised in **Figure 7.1.**

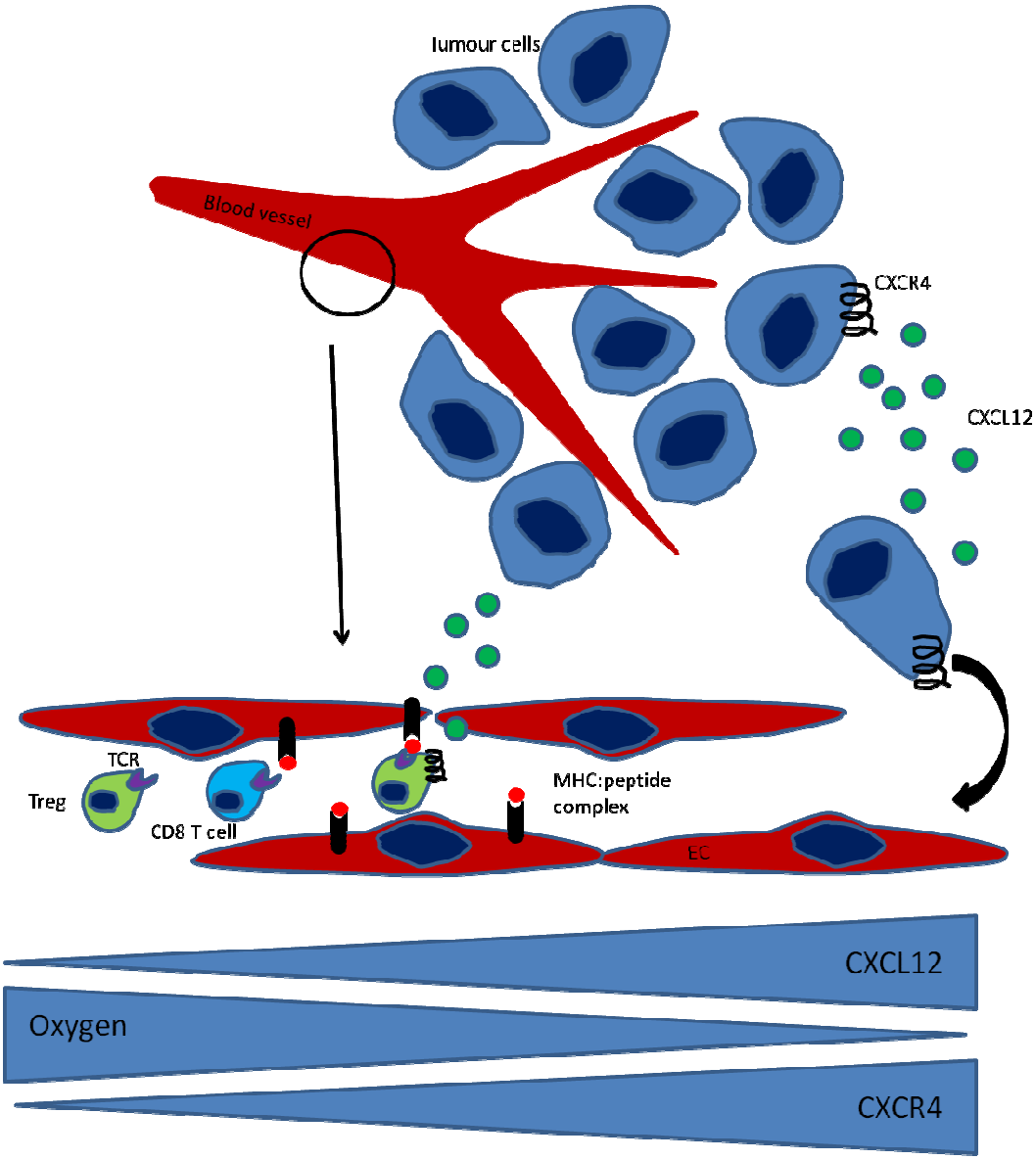

Figure 7.1 ***The importance of the CXCR4 chemokine receptor and its ligand, CXCL12, in the tumour microenvironment.***
Cognate antigen recognition on endothelium is required for T cell infiltration into tumour. Within hypoxic areas of tumours CXCL12 can be expressed by fibroblasts and tumour cells. Increasing CXCL12 expression aids antigen-specific transmigration of Tregs expressing CXCR4 and simultaneously CXCR4 expression on tumour cells increases. At high levels CXCL12 expression no longer aids T cell transmigration but stimulates tumour cell motility and invasiveness via fugetaxis. Targeted metastasis to other sites of CXCL12 expression involves CXCR4 activation on circulating tumour cells that "hijack" the CXCR4-CXCL12 axis.

CD8 infiltration into tumours, as shown here in colorectal tumours, is a good prognostic marker for survival. High avidity T cells, recognising and responding to lower amounts of cognate antigen, have superior function over low-avidity cells in CD8 CTL-mediated immunity against tumours where antigen presentation may be impaired [306]. Therefore, there is an advantage of selective/enhanced recruitment of high avidity antigen specific T cells into tumours via recognition of cognate antigen on the endothelium. These high avidity CTLs recognising low doses of tumour derived antigen on the endothelium would potentially be 'fast-tracked' into the tumour allowing secondary recognition of the same tumour antigen on the tumour cells themselves.

The present study has demonstrated that CD8 antigen-specific transmigration is important for infiltration into tumours in response to tumour antigens presented by the endothelium. One explanation for the effect of cognate recognition on transmigration is that TCR ligation induces a high affinity conformation of T cell surface integrins. TCR engagement has been shown to induce high affinity conformational changes in integrins via signalling through TCR/CD8 [437,438]. In addition, T cells transiently express CD40 ligand upon TCR engagement [439,440]. Upregulation of CD40 ligand following TCR engagement by the EC might in turn lead to the ligation of the CD40 on the EC and consequently enhance antigen-specific T-cell recruitment. CD40 ligation on endothelium leads to upregulation of cell adhesion molecules by the endothelium, production of inflammatory cytokines and overexpression of VEGF [441,442].

Transmigration of natural Tregs in response to peptides presented in the context of MHC class II molecules requires endothelial activation and subsequent upregulation of MHC class II molecules. The data here also suggests that the chemokine CXCL12, in combination with

inflammatory cytokines, was also a requirement for antigen-specific transmigration. Analysis of CXCL12 expression within colorectal and ovarian tumours has shown increasing levels of CXCL12 leads to a poor prognosis and reduced patient survival. Previous research suggests that at high levels CXCL12 expression aids tumour cell metastasis, a murine model of established extrahepatic colorectal metastasis has shown that CXCL12 promotes tumour growth of established extrahepatic metastasis *in vivo* due to angiogenesis-dependent induction of tumour cell proliferation and inhibition of apoptotic cell death [412]. Indeed, at high levels, CXCL12 exerts many effects on the tumour microenvironment. CXCL12 and CXCR4 expression plays a role in tumour cell metastasis in many tumour types. Regulatory T cell activation has been shown to up regulate CXCR4 expression and enabling them to migrate to the bone marrow in a CXCL12-dependent manner [443], suggesting that bone marrow could serve as a functional reservoir for activated Tregs where high levels may provide an immune suppressive environment to facilitate bone marrow metastasis. High levels of CXCL12 expression have been shown to cause fugetaxis of migrating cells [376] and could be used as an immune escape mechanism within tumour where increasing concentrations of CXCL12, caused by other factors as previously mentioned, can have an additional advantage to the tumour of repelling infiltrating anti-tumour T cells [377]. Due to the influence of CXCL12 on Treg transmigration, T cell infiltration into and CXCL12 expression by tumours was assessed in colorectal tumour samples. Whereas higher CD8 T cell infiltration correlated with improved patient survival, FOXP3+ cell infiltration had no significant effect and in addition the data showed no significant correlation between either CD8 or Treg (FOXP3+) T cell infiltration into tumours and CXCL12 expression by tumour cells (data not shown). No correlation between CXCL12 and CD8 T cell infiltration into colorectal tumours could be due to antigen-specific CD8 T cell transmigration occurring in the absence of chemokines. In order to further investigate the lack of correlation between

FOXP3+ T cell infiltration with CXCL12 it would be interesting to look at MHC class II expression on the tumour endothelium. Even if CXCL12 was present, without MHC class II expression no antigen-specific Treg infiltration would be expected. Another consideration, as mentioned previously, is the use of FOXP3 as a marker for regulatory T cells.

TRP-2 specific transmigration was seen for memory subsets of TRP-2 specific CD8 T cells and therefore the major issue to be resolved is the interaction between effector CTLs in the blood and recognition of cognate peptide on endothelium. Our results show that even under little/no inflammation, effector CTLs are able to kill endothelial cells, so why do we not see massive destruction to the endothelium *in vivo*. A simple explanation could lie in the markers used to denote effector populations. Here loss of memory markers such as CD62L and CD127 are used where perhaps upregulation of 'effector' markers would be more appropriate. Perforin, granzyme B, and FasL are cytotoxic molecules used by CD8 T lymphocytes and natural killer (NK) cells to induce apoptosis [444]. A study looking at these activation markers on T cells within 10 healthy patients and emphysema patients showed that within the peripheral blood of healthy non-smoking individuals the average percentage of CD8 T cells expressing granzyme B was 40% and perforin 60% [445], indicating primed and activated CD8 T cell populations do exist in the peripheral blood. Therefore, the mechanism for recognition of cognate antigen on endothelium with lack of killing *in vivo* would have to occur at the immunological synapse of the EC: T cell.

Interestingly, TCR ligation upon antigen recognition on the endothelium could enhance endothelial cell survival. Chromium release assays are carried out with target endothelial cells in suspension and therefore when in a monolayer or in contact with other parenchymal cells endothelial cells could have a survival advantage, escaping cytotoxicity. Receptor

activator of NFκB (RANK) is highly expressed in endothelial cells of normal adult arterial blood vessels, which are not actively involved in angiogenesis and vasculogenesis [446]. The ligand for RANK, RANKL molecule is important in bone metabolism. This natural and necessary surface-bound molecule found on osteoblasts serves to activate osteoclasts, which are the cells involved in bone resorption [447]. However, RANKL is secreted by arterial smooth muscle cells and can act in a paracrine manner on RANK expressed on endothelial cells and RANKL expression has also been shown on T helper cells [448] where T cell activation was reported to induce expression of the RANKL gene [449]. The RANKL protein was shown to activate anti-apoptotic kinase PI 3-kinase AKT/ through a signalling complex involving SRC kinase and tumour necrosis factor receptor-associated factor (TRAF) 6. T helper cells within peripheral blood may therefore be essential in providing RANKL survival signals to the endothelium to prevent CD8 T cell cytotoxicity. TCR signalling between T cells and ECs, while activating signalling pathways to prepare T cells for transmigration, may simultaneously inhibit apoptosis of endothelial cells.

The observation that Tregs but not CD8 T cells require additional activation via chemokines to undergo antigen-specific transmigration suggests a threshold for TCR activation is required in order to facilitate transmigration across endothelium. Further evidence for this comes from the data showing that effector memory TRP-2 specific CD8 T cells transmigrate more rapidly than the same cells of a central memory phenotype, potentially requiring additional activation prior to transmigration. Therefore, a model of antigen-specific transmigration involving thresholds for T cell transmigration can be suggested where the threshold required depends on cell type, activation state, amount of available MHC or peptide presented by the endothelium and additional activation signals such as chemokine gradients and endothelial activation via inflammatory cytokines (**Figure 7.2**).

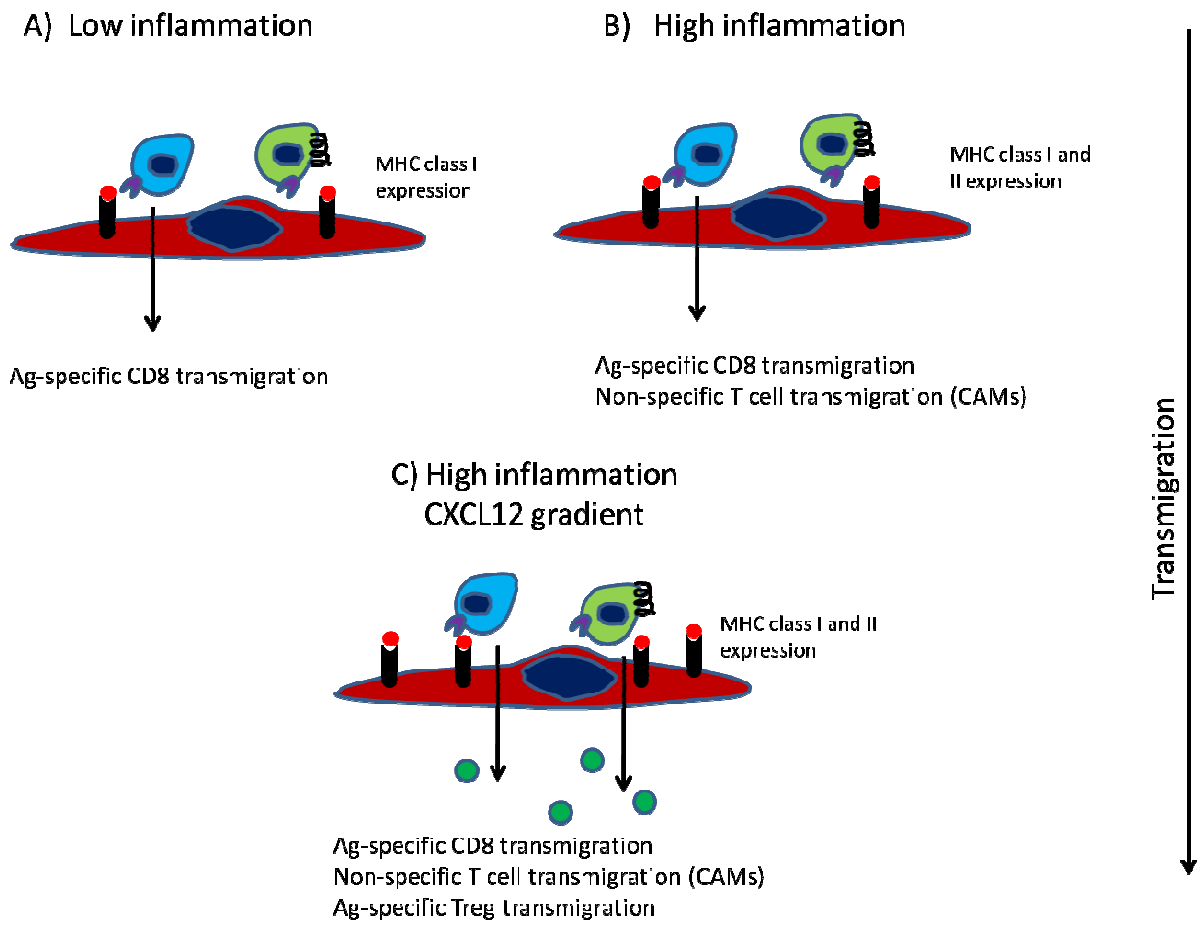

Figure 7.2 ***Antigen-specific transmigration involving thresholds for murine T cell transmigration.***
A model for transmigration of antigen specific CD8 (blue) and CD4CD25FOXP T cells (green) across endothelium in response to inflammatory activation of the endothelium and chemokine (CXCL12) gradients under conditions of low inflammation and no chemokine gradient (A), high inflammation with a CXCL12 gradient (B) and high inflammation with a CXCL12 gradient (C).

Under conditions of low inflammation (**Figure 7.2A**) the endothelium constitutively expresses MHC class I molecules and therefore supports the transmigration of antigen-specific CD8 T cell recognising cognate peptide presented by the endothelium and into tissues. Retention and further activation of these antigen-specific CD8 T cells would then be dependent on cognate antigen recognition within the tissue. Antigen-specific Treg transmigration is not possible due to no constitutive expression of MHC class II in the absence of inflammation. With increasing inflammation (**B**) endothelial activation occurs, inducing MHC class II expression and also cell adhesion molecule expression. This may allow antigen specific transmigration of effector CD4 cells but this was beyond the scope of

this thesis. Antigen-specific CD8 T cell transmigration again is possible due to MHC class I expression, however even in the presence of MHC class II expression the threshold for TCR signalling on Tregs following recognition of self-peptides is not enough to allow transmigration in the absence of a CXCL12 gradient. The increased endothelial activation would also allow non-specific T cell transmigration to occur due to increased adhesion to cell adhesion molecules. Under conditions of increased inflammation and a CXCL12 gradient (**C**) both antigen-specific CD8 and Treg transmigration occurs as the CXCL12:CXCR4 signalling on Tregs synergises with TCR recognition lowering the threshold for antigen-specific transmigration of Tregs and subsequent down-regulation of inflammation.

This model highlights that antigen-specific Treg transmigration, as mentioned previously, would occur temporally at a later stage in an immune response than CD8 T cells as endothelial activation is required for the induction of MHC class II expression. In addition, Tregs appear to require a higher threshold for activation/transmigration via additional signalling via chemokines such as CXCL12. Within tissues a higher numbers of self epitopes presented to Tregs would aid Treg infiltration over conventional CD8 and CD4 T cells. Tumoural hijacking of chemokine networks, such as CXCL12, has advantages for tumour cell proliferation, survival and metastasis but in addition could facilitate increased Treg infiltration. This could be achieved by lowering the threshold for Treg antigen-specific transmigration causing indirect enhanced tumour survival and maintenance of a high Treg: CD8 T cell ratio.

T cells continuously circulate between the blood, tissues and lymphatics and this study focused on the interaction of T cells with endothelium in order to transmigrate into tissues. CD8 T cells were able to recognise TRP-2 presented on resting endothelium in the context of

MHC class I molecules which facilitated their transmigration across the endothelium. Homeostatically this antigen specific, high avidity CD8 T cell transmigration in the absence of or under low inflammation, involving chemokine gradients, shows rapid transmigration into tissues as depicted in **Figure 7.2**. Potentially once inside the tissue these CD8 T cells aim to eliminate the cause of the infection. The activation of immune responses following CD8 T cell recognition of tumour cells could increase inflammation or activate endothelial cells directly during transmigration causing upregulation of class II molecules on the endothelium. This in turn would lead to infiltration of Tregs recognising self-antigens presented in the context of these MHC class II molecules, with the ability to dampen down immune responses.

In a tumour situation, the main role of rapid infiltration of high avidity tumour antigen specific CD8 T cells would be to kill tumour cells and help control tumour growth. Higher avidity CD8 T cells would be able to recognise lower amounts of cognate peptide presented by the endothelium, potentially requiring a lower threshold for transmigration across the endothelium and more rapidly entering tumour tissue. A model for antigen-specific transmigration of high and low avidity CD8 T cells is hypothesised in **Figure 7.3.** Under conditions of low inflammation, with lower levels of peptide: MHC on endothelial cells there will be limited antigen-specific transmigration of low avidity CD8 T cells will occur (**A**). Conversely under the same conditions, high avidity CD8 T cells requiring lower amounts of cognate peptide an increase in antigen-specific CD8 T cell infiltration across the endothelium would be expected (**B**). Under high inflammation MHC class I expression by the endothelium is enhanced, increasing the amount of peptide presented to antigen-specific CD8 T cells resulting in both high and low avidity antigen-specific transmigration (**C**).

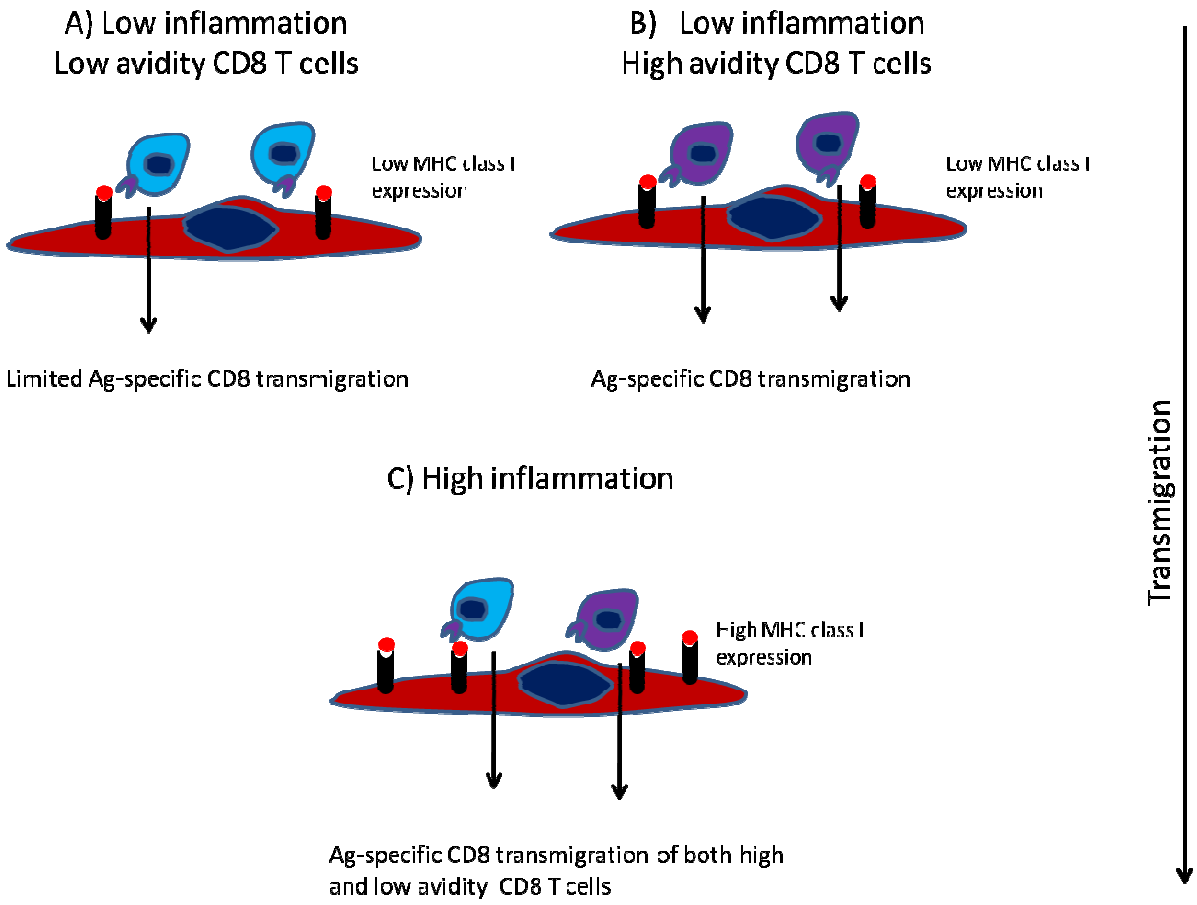

Figure 7.3 ***Antigen-specific transmigration involving thresholds for murine T cell transmigration.***
A model for transmigration of antigen specific CD8 T cells of low (blue) and high (purple) avidity across endothelium in response to inflammatory activation. Low and high avidity CD8 T cell transmigration across unactivated endothelium is shown in (A) and (B) respectively. Both high and low avidity CD8 T cell transmigration across activated endothelium is shown in (C).

ECs are genetically more stable than tumours and less prone to develop mechanisms of immune escape such as MHC class I suppression [450]. Therefore, MHC class I expression and presentation of tumour derived peptides should be relatively unaltered. IFN-γ classically is thought of as exerting anticancer effects through direct and indirect mechanisms, it can slow tumour growth by inhibiting tumour cell proliferation and by activating host immune cells, including macrophages, DCs, NK cells, and T cells [451]. However, melanoma antigen–specific $CD8^+$ lymphocytes have been shown to produce IFN-γ in a major histocompatibility complex class I–restricted fashion in the absence of tumour cell lysis [452]. Release of pro-inflammatory cytokines such as IFN-γ activating tumour endothelium

to upregulate MHC class II would aid antigen specific Th effector and subsequently Treg transmigration into the tumour tissue ultimately controlling anti-tumour responses.

Tumour infiltrating lymphocytes have shown effective recognition and elimination of tumour cells following adoptive transfer [453]. The importance of CD4 T cells during the effectors phase of the anti-tumour response has been emphasised within tumour, by studies adoptively transferring CD4 T cells [454]. Adoptive transfer of CD4 T effectors cells combined with CD8 effector cells isolated from tumour draining lymph nodes has provided synergistic therapy for mice bearing subcutaneous, intracranial, or advanced pulmonary metastases [455]. In addition to augmenting IFN-γ production by CD8 T cells, CD4 T cells infiltrated and proliferated extensively in pulmonary tumours, while also stimulating tumour antigen–specific CD8 T cells. CD8 T cells showed limited intra-tumoral proliferation in the absence of CD4 cells. This research highlights the necessity of adoptively transferred CD4 T cells in addition to CD8 T cells during the effector phase of immunotherapy.

Although CD4 T cells are vital for high avidity CD8 T cell anti tumour responses [456], tumour antigen specific CD4 T cell transmigration was not assessed in this study. Future experiments could use the same B16 melanoma model in C57Bl mice could form a model of gp100 specific CD4 T cell transmigration. The gp100 protein is expressed endogenously in both melanoma and non melanoma cells and processed for presentation of multiple epitopes by MHC class II molecules [457]. Due to the requirement for gp100 peptide presentation by MHC class II molecules, endothelial activation to upregulate MHC class II would be expected to be required for gp100 specific transmigration as required for regulatory T cell transmigration. However, as with the TRP-2 specific CD8 T cells potentially the gp100

specific T cell may not require chemokine gradients to lower the threshold for transmigration as required by Tregs.

Accurate detection of gp100 specific CD4 T cells following transmigration was not possible in this study as ProImmune's Class II Ultimers™ for detecting these gp100 specific cells do not give as accurate results as pentamers used here for TRP-2 positive CD8 T cells. Few MHC class II tetramers incorporating peptides from tumours have been developed due to the high polymorphism of class II molecules and the low binding capacity to these peptides [458]. However, with new generations of class II tetramers are becoming available making it possible to test gp100 specific CD4 T cells transmigration. New generation MHC class II tetramers specific for the tumour antigen NY-ESO-1 have been developed using molecularly defined His-tagged peptides and isolation of folded MHC/peptide monomers by affinity purification prior to tetramerization [459,460]. This technique has allowed the isolation of tetramers bound to ESO-specific CD4 T cells and will potentially advance the production of other MHC class II/peptide tetramers specific for tumour antigens.

Adoptive cell therapy with autologous tumour-infiltrating lymphocytes (TIL) has shown promising results in metastatic melanoma patients. Disadvantages of this approach are the labour-intensive TIL production limiting its widespread applicability. A phase II clinical trial using a new method for TIL production which includes unselected, minimally cultured, bulk TILs (Young-TIL) for adoptive transfer [461]. The use of Young-TIL means the treatment is not restricted to human leukocyte antigen (HLA)-A2 patients. The trial studied the efficacy and toxicity of adoptively transferred Young-TIL following lympho-depleting chemotherapy in 20 metastatic melanoma patients. Fifty percent of the patients achieved a measurable clinical response including two complete remissions and eight partial responses. The trial showed that lympho-depleting chemotherapy followed by transfer of short-term cultured TIL

mediated tumour regression in 50% of metastatic melanoma with manageable toxicity. A current ongoing phase II trial by Rosenberg *et al* uses short term cultured CD8 TILs in metastatic digestive tract cancers (NCI-10-C-0166). This process may have a major effect on cell therapy of cancer as a response was seen in 50% of the patients and perhaps this percentage can be further increased by combining adoptive transfer of tumour specific CD4 alongside CD8 T cells. Interestingly, the TMA data presented here showed 40% of colorectal tumours showed high levels of CD8 infiltrating cells which could be the high avidity antigen-specific T cells able to transmigrate across the tumour endothelium which would potentially make up the 50% of patients where TILs are able to be isolated and cultured for subsequent adoptive transfer.

More recently Xu et. al. has further modified the adoptive transfer technique to include CpG dinucleotides activation of TILs and CpG administration alongside adoptive transfer [462]. TILs from human lung cancer patients were adoptively transferred into autologous tumour bearing nude mice. The study showed CpG dinucleotides enhanced the efficacy of adoptive cell transfer by modifying Th1 polarisation and infiltration of Th17 cells. Combinational therapy combining CpGs with anti-CTLA-4 and PD-1 improved long-term survival and led to increased levels of tumour-reactive T cells and reduced numbers of Tregs at the tumour site in treatment of murine experimental bladder cancer [463]. Providing further evidence that altering the balance of T cell subsets to a higher Th1 based phenotype leads to a better prognosis within cancer patients.

Clinical data suggests that the combination of CTLA-4 blocking antibody with tumour antigen vaccination had synergistic activity and administration of ipilimumab has been shown in phase III trials to improve survival in 20-25% of metastatic melanoma patients [464]. Anti CTLA-4 therapy has been shown to overcome T cell tolerance and help sustain an active immune response against cancer cells, if used in combination with adoptive transfer of high

avidity, tumour antigen-specific T cells perhaps this 20% increased survival could be enhanced. In this way high avidity T cells selectively infiltrating tumours via antigen recognition on endothelium could maintain an active state by removal of the suppressive effects of CTLA-4 engagement. Huang *et al* [465] showed that patients with metastatic melanoma treated with the CTLA4 blocking antibody Tremelimumab had enhanced CD8 T cell infiltration into tumours in both responding and non responding patients. There were similar levels of expression of T cell activation markers (CD45RO, HLA-DR) in both groups, and no difference in markers for cell replication (Ki67) or the suppressor cell marker FOXP3. Perhaps the non responders had lost target antigen or HLA and therefore showed increased T cell infiltration but these T cells were unable to recognise tumour antigens to exert and anti-tumour response.

In addition to removing suppressive signalling targeting CTLA-4 interferes with co-inhibition, whereas targeting co-stimulatory molecules such as CD28 [466], OX40 [467] and CD137 [468] has also been shown to elicit anti-tumour responses [469]. The aim of targeting co-stimulatory molecules is to increase the numbers of tumour-specific T cells primed to elicit anti-tumour responses and to expand this population of memory cells. Other combinational strategies could include helping to prevent the immunosuppressive tumour microenvironment by targeting other tumour infiltrating immune cells such as myeloid-derived suppressor cells (MDSCs) which inhibit tumour-specific immune responses via IL-10 secretion. Targeting MDSCs may allow T cells, having infiltrated the tumour, to be effectively re-primed by activated APCs and retained in the tissue to carry out effective anti-tumour responses.

Therapeutic implications of the requirement for antigen-specific transmigration mean increasing MHC class I expression on the tumour endothelium would be an advantage. An alternative form of therapy to attempt to increase antigen-specific transmigration into

tumours would be to use targeted therapy to increase specific peptide uptake via the endothelium using vehicles such as liposomes containing the peptide of interest. Liposomes penetrate tumours spontaneously via the tumour endothelium, leading to known enhanced permeability and subsequent drug retention effect [470].

In conclusion the requirement for cognate peptide recognition on tumour endothelium shown to be important for T cell infiltration into tumours adds another factor to the requirement for an efficient T cell vaccine. Understanding the requirements for CD8 infiltration into tumour is crucial for the future development of cancer vaccines. High avidity T cells are required to infiltrate tumours where low avidity T cells would be unable to recognise sufficient cognate antigen on the tumour endothelium being unable to infiltrate into tumour tissue. The effect of T cell avidity on the ability to infiltrate and target tumours could be further investigated in the same TRP-2 positive B16 model using TRP-2 specific T cell lines of varying avidities adoptively transferred into mice bearing tumours with varying TRP-2 expression levels. The ideal experimental outcome would be to determine the expression level of tumour antigens required for efficient targeting by cognate antigen-specific CTLs. Knowing the ideal T cell avidity and antigen expression levels on tumour endothelium required for T cell infiltration and subsequent anti-tumour responses could be used to predict patient responses to therapy.

This study highlights that anti-tumour responses require high frequency, high avidity T cells that recognise cognate peptide processed by tumour cells, taken up by endothelial cells and presented in the context of MHC molecules for efficient T cell infiltration. In addition, kinetics, inflammation and chemokine gradients can influence the Teff: Treg ratio.

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