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Repolarisation of the Immune-suppressive Millieu of the Ovarian Tumour using Targeted Therapeutics



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Thesis submitted to the University of
Nottingham for the degree of
Doctorate of Philosophy

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“I have virtues that others may deem vices. Virtues which guide me, and together, have made me. I embrace pain, suffering and sacrifice and gain strength from obsession. It is true they have given me scars that I know will never fade but for that I am grateful because they show me what is within me and what is within reach of me. And through all of this I live each day without compromise, without regret and without excuse. So as I endure the misfortune that lies in my path. I chase down my vision without diversion or retreat and in the pursuit of my passions I am neither timid nor afraid. Everyone has a fair turn to be as great as he pleases and his greatness lies in the power of his thought.”

Adapted from Relentless 2010

Abstract

Ovarian cancer is a disease which is fatal in the majority of cases. The evolution of surgery and chemotherapy over the past 30 years has resulted in improvements in overall and progression-free survival. However, the rate of relapse in ovarian cancer is very high, suggesting that current treatment strategies are ineffective. Therefore, to overcome the poor prognosis of ovarian cancer, immunotherapeutic strategies have been devised such as the use of anti-CTLA-4 antibody therapy in melanoma. The principle that the immune system can affect either cancer development or clearance has been the subject of debate for over a century. Clinical results of novel immunotherapeutic approaches that aim to exploit and enhance this immunogenicity have had mixed success such as IL-2 therapy in renal cell carcinoma. It is clear that whilst many tumours possess antigenic component in their make-up, they do not stimulate durable and effective immune responses *In vivo*. This may reflect the fact that tumours develop a network of escape mechanisms to circumvent tumour-specific immunity.

Due to the ineffectual nature of current treatment options and the complexity of the tumour microenvironment a coherent stratagem needs to be composed. This thesis explores, in principle, a contemporary strategy to propagate an anti-tumour immune response within ovarian cancer by using existing drugs in combination to target three different facets of ovarian cancer immunity; Regulatory T cell (Treg) migration, poor release of the tumour-associated antigen, MUC1, and reduced cytotoxic T cell (CTL) proliferation.

The migration of Regulatory T cell (Treg) to ovarian cancer is principally mediated by the CCR4-CCL22 chemokine receptor-chemokine axis. AZ1, a specific antagonist for the chemokine receptor CCR4, which is highly

expressed on Treg, abrogated the migration of these cells to the chemokine. This compound did not alter Treg function suggesting that its activity was specifically against Treg migration.

In order to induce an adequate T cell response, sufficient antigen needs to be provided. Camptothecin, a classical topoisomerase inhibitor, demonstrated effective tumour cell death and release of the tumour-associated antigen, MUC1. The increase in tumour antigen release and decrease in tumour load was offset by significant immune toxicity. The incorporation of Camptothecin into a synthetic drug delivery system led to a decrease in immune toxicity while retaining the drug's anti-tumour activity.

Finally, in order to take advantage of tumour antigen release, it would be desirable to stimulate CTL. Imiquimod, the toll-like receptor 7 agonist, widely used in basal-cell carcinoma and melanoma was able to demonstrate a potential enhancement of an anti-tumour response in three ways. Firstly, the drug enhanced the activation and antigen uptake capacity of plasmacytoid dendritic cells. It also had a direct effect on CTL themselves whilst also reducing the suppressive effect of Treg.

This thesis illustrates, in principle, the possibility that a poly-pharmaceutical approach can be taken to target ovarian cancer. It indicates that readily available compounds, when used in the correct combination, could be key in developing effective anti-cancer therapy. Future work in this area should focus on using existing chemotherapeutic and immunotherapeutic drugs in combination to illicit enhanced anti-tumour cytotoxicity. Critically, the next step in developing this strategy is to acquire suitable *in vivo* models. This is key as there is conflicting evidence regarding the efficacy of certain drugs in mice compared to man.

List of Publications, Posters and Presentations

Publications

Khan AR, Dovedi SJ, Wilkinson RW, Pritchard DI. Tumor infiltrating regulatory T cells: tractable targets for immunotherapy. International Reviews of Immunology. 2010 Oct;29(5):461-84.

Published Abstracts

Turning off the tumour immunity 'handbrake'. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Journal of Pharmacy and Pharmacology 2010, Vol 62 (10), Special Issue Sp Iss S1

The Tumour Immunology Tube Map. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Journal of Pharmacy and Pharmacology 2010, Vol 62 (10), Special Issue Sp Iss S1

Turning off the tumour immunity 'handbrake'. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Immunology 2010, Vol 131, Special Issue

The Tumour Immunology Tube Map. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Immunology 2010, Vol 131, Special Issue

Posters

The Role of Regulatory T cells in Cancer: Isolation and Expansion. **Khan AR**, Blount DG, Watson SA, Grabowska A, Wilkinson RW, Pritchard DI.

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The Tumour Immunology Tube Map. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Presented at British Association of Cancer Research Annual Conference – ‘The Hallmarks of Cancer’, Edinburgh 2010

The Tumour Immunology Tube Map. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Presented at the EPSRC Doctoral Training Centre Conference, Warwick 2010

Turning off the tumour immunity ‘handbrake’. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Presented at the UK Pharmaceutical Sciences Conference 2010

The Tumour Immunology Tube Map. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Presented at the UK Pharmaceutical Sciences Conference 2010. *Won best poster prize*

Turning off the tumour immunity ‘handbrake’. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Presented at the British Society of Immunology Annual Congress, Liverpool 2010.

The Tumour Immunology Tube Map. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Presented at the British Society of Immunology Annual Congress, Liverpool 2010.

Oral Presentations

Turning off the tumour immunity 'handbrake'. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Presented at the UK Pharmaceutical Sciences Conference 2010

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Turning off the tumour immunity 'handbrake'. **Khan AR**, Watson SA, Wilkinson RW, Pritchard DI. Presented at the AstraZeneca PhD Review Day.
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List of Abbreviations and Nomenclature

ADCC	Antibody Directed Cell Cytotoxicity
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
ATRA	All Trans-Retinoic Acid
BDCA	Blood Dendritic Cell Antigen
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CAF	Cancer Associated Fibroblast
cAMP	Cyclic Adenosine Monophosphate
CCL	(C-C) motif Ligand
CCR	(C-C) motif Receptor
CD	Cluster of Differentiation
CD40L	Cluster of Differentiation 40 Ligand
CDK4	Cyclin-dependent Kinase 4
cDNA	Complementary Deoxyribonucleic Acid
CFSE	Carboxyfluorescein succinimidyl ester
COX-2	Cyclo-oxygenase-2
CpG ODN	Cytosine-poly Guanine Oligodeoxynucleotide
CRT	Calreticulin
CSC	Cancer Stem Cell
CSF-1	Macrophage Colony Stimulating Factor
CT	Cancer Testis

CTLA-4	Cytotoxic T-Lymphocyte Antigen
CXCL	C-X-C motif Ligand
CXCR	C-X-C motif Receptor
CX3CR	C-X-C-C-C motif Receptor
DAMPs	Damage Associated Molecular Pattern Molecules
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
DTT	Dithiothreitol
EBV	Epstein-Barr virus
ECD	Electron Coupled Dye
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAM	6-Carboxyfluorescein
FasL	Fas Ligand
FCS	Fetal Calf Serum
FCϵRI	High affinity IgE receptor
FGF	Fibroblast Growth Factors
FITC	Fluorescein Isothiocyanate
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

G-CSF	Granulocyte colony-stimulating factor
GITR	Glucocorticoid-induced Tumour Necrosis Factor Receptor
GLUT1	Glucose Transporter 1
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
gp-100	Melanocyte Lineage-specific Antigen gp 100
HGF	Hepatocyte Growth Factor
HIF	Hypoxia-inducible Factors
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HSP-90	Heat Shock Protein 90
ICAM-1	Inter-Cellular Adhesion Molecule 1
ICER	inducible cAMP Early Repressor
iDC	immature Dendritic Cell
IDO	Indoleamine-2,3-dioxygenase
IFNs	Interferons
IGF	Insulin-like Growth Factor
IL	Interleukin
ING1	Inhibitor of Growth Protein
IRF	Interferon Regulatory Factor 1
KIR	Killer-cell Immunoglobulin-like Receptors
LAGE-1	L-Antigen
LAP	LRR And PDZ domain proteins
LFA-1	Lymphocyte function-associated antigen-1
LHRH	Luteinizing Hormone-Releasing Hormone

LHRHR	Luteinizing Hormone-Releasing Hormone Receptor
MAGE	Melanoma-Associated Antigen
MAP	Mitogen-activated protein kinase
MART-1	Melan-A
MCA	3-Methylcholanthrene
mDC	Myeloid Dendritic Cell
MDSC	Myeloid Derived Suppressor Cell
MHC	Major Histocompatibility Complex
MIC	Major Histocompatibility Complex (MHC) class I-related Chain
MMP	Matrix Metalloproteinase
MMTV	Mouse Mammary Tumour Virus
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUC1	Mucin1
MyD88	Myeloid Differentiation Primary Response Gene (88)
NCR	Natural Cytotoxicity Receptors
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer Cell
NKG2D	NKG2-D type II Integral Membrane Protein
NKT	Natural Killer T Cell
NOD	Non-obese Diabetic
NY-ESO-1	New York Esophageal Squamous Cell Carcinoma
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pDC	Plasmacytoid Dendritic Cells

PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PE	Phycoerythrin
PE/Cy5	Phycoerythrin/Cyanine 5
PEGMA	Poly(ethylene glycol) Methacrylate
PerCP/Cy5.5	Peridinin Chlorophyll Protein Complex/ Cyanine 5.5
PGE₂	Prostaglandin-E ₂
PHA	Phytohaemagglutinin
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PRR	Pattern Recognition Receptor
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RANKL	Receptor Activator of Nuclear Factor kappa-B Ligand
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SCC	Squamous Cell Carcinoma
SDF-1	Stromal Derived Factor-1
SIT	Allergen-specific Immunotherapy
TAA_s	Tumour-Associated Antigen
TAM	Tumour-Associated Macrophage
TAP	Transporter-Associated with Antigen Processing
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-beta
Th	T Helper

TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
TNFSF	Tumour Necrosis Factor Superfamily
Tr1	T Regulatory 1 Cell
TRAF6	TNF Receptor Associated Factor 6
TRAIL	TNF-Related apoptosis-Inducing Ligand
Treg	Regulatory T cell
UK	United Kingdom
USA	United States of America
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
β2M	Beta-2 Microglobulin

Chapter 1

General Introduction

1.1 Ovarian Cancer

Ovarian cancer is fatal in the majority of cases. The lifetime risk of ovarian cancer is approximately 2% and it is the fourth most common cause of cancer-related death in women within the UK, although its incidence worldwide is slightly less (1). In 2009 almost 7,000 cases of ovarian cancer were diagnosed, and it is considered one of the most common forms of gynaecological malignancy. The incidence of ovarian cancer appears to be on the rise (2), with rates of 15 and 17 per 100,000 women in 1975 and 2005 respectively, which may simply reflect the proportionally older population in the UK compared with 30 years ago (3). Ovarian cancer is predominately a disease of older, postmenopausal women. The incidence increases rapidly after the age of 50, with over 85% of ovarian cancers occurring above that age (4). Less than 1% of epithelial ovarian cancers occur below the age of 20, with two thirds of the ovarian malignancies in these young patients being germ cell tumours (5). Since germ cell tumours represent less than 5% of ovarian cancers and tend to present at an earlier stage, they make minimal impact on incidence and mortality rates (4).

The impact of age in ovarian cancer is not limited to incidence alone, increasing age is coupled to decreasing chemotherapeutic performance status which may limit the extent to which the most aggressive treatments can be initiated or sustained. Hence, increasing age and reduced

chemotherapeutic performance status have been linked with poor prognosis (1), which is independent of other prognostic factors (6).

Worldwide there are almost 200,000 new cases of ovarian cancer annually (4). Incidence rates vary considerably with the highest rates in Northern Europe and the USA and lowest rates in Africa and Asia. The Office for National Statistics recorded an incidence rate of 16.7 cases per 100,000 women in the United Kingdom (2).

1.2 The Hallmarks of Cancer

In order for a tumour to first develop, and secondly progress, dysregulation of a number of pathways, aptly termed the “hallmarks of cancer”, are necessary. Originally, these included sustaining proliferative signalling, resisting cell death, evading growth suppressors, inducing angiogenesis, enabling replicative immortality and activating invasion and metastasis (7). However, recent additions to these ‘hallmarks’ include deregulating cellular energetics and avoiding immune destruction (8) (Figure 1.1).

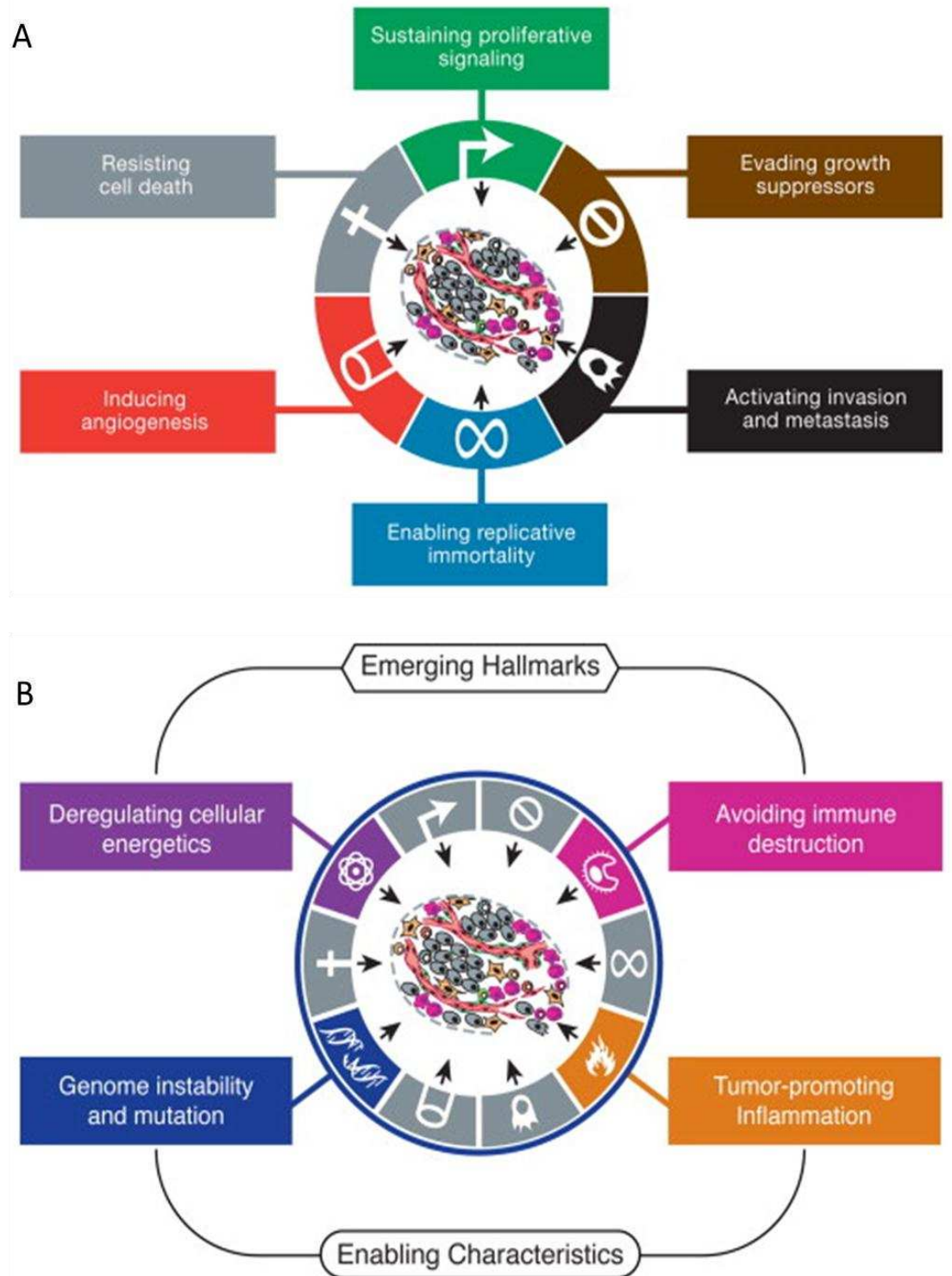


Figure 1.1 – The Hallmarks of Cancer. **A** – The original ‘hallmarks’ as described by (7). **B** – Addition of two extra ‘hallmarks’, deregulating cellular energetics and avoiding immune destruction as described by (10). Reprinted with permission (License number 2812690309460), Elsevier Ltd.

1.2.1 Sustaining Proliferative Signalling

One of the most obvious properties of cancer cells is the capacity for uncontrolled growth. Tumour cell independence from normal growth factor control is in part due to the generation of its own growth factors. This is in contrast to normal cells which only respond to exogenous growth factor stimuli (7). There are three molecular strategies by which tumours achieve growth autonomy. These include alteration in extra cellular growth signals, receptor transducers of these signals and alteration of intracellular pathways which translate these signals into cellular events. Two examples of tumours secreting and responding to their own growth factors in a positive feedback loop involve platelet derived growth factor and tumour growth factor-alpha (8). Receptor over-expression may enable tumour cells to be hypersensitive to haemostatic levels of growth factor. For example, epidermal growth factor receptor 1 is over expressed in ovarian cancer, with a subsequent negative impact on prognosis (9).

Downstream cytoplasmic pathways can be altered, resulting in abnormally prolonged stimulation of cells following receptor signalling which is often related to the Ras pathway. The Ras pathway involves numerous proteins involved with cell proliferation (10).

1.2.2 Evading Growth Suppressors

Within normal tissue, multiple anti-proliferative signals operate to maintain cellular homeostasis. The retinoblastoma protein acts as a central coordinator of anti-proliferative signals. Retinoblastoma protein blocks proliferation by sequestering and inactivating transcription factors from the E2F group of

genes which are involved in cell cycle regulation. These control the expression of genes essential for progression from G1 into S phase (11).

Disruption of the retinoblastoma protein pathway liberates E2F, thus allowing cellular proliferation and rendering cells unresponsive to anti-proliferative signals. Transforming growth factor-beta (TGF- β) represents a widely studied anti-proliferative factor, which prevents phosphorylation and subsequent inactivation of retinoblastoma protein. TGF- β blocks phosphorylation of retinoblastoma protein by stimulation of p15INK4B and p21, which block cyclin:CDK complexes which are responsible for pRb phosphorylation (12). Loss of TGF- β responsiveness has been demonstrated in tumours via down-regulation of TGF- β receptors (8).

1.2.3 Resisting Cell Death

Apoptotic machinery is present in all cells and is divided into sensors and effectors. The sensors include cell surface receptors that bind to survival (e.g. IGF-1, IGF-2 and IL-3) and death factors (Fas ligand, TNF- α and TRAIL) (13). Many apoptotic signals converge on the mitochondria which produce cytochrome c, a potent catalyst of apoptosis. The release of cytochrome c is controlled through the Bcl-2 family of proteins which have either pro-apoptotic (Bax) or anti-apoptotic effects (Bcl-2 and Bcl-XL) (14). The ultimate inducers of apoptosis are the caspases which can be stimulated through cytochrome c or death receptors.

Tumour cells all develop strategies to avoid apoptosis. One of the most common involves mutations and ultimately inactivation of p53, which is seen in over 50% of human cancers (15). Physiologically, p53 is a potent

coordinator of apoptosis resultant from DNA damage, hypoxia and oncogene overexpression (12). Defects in the TRAIL pathway, the major p53 independent pathway for apoptosis, can also occur with disruptions to the TRAIL receptor and downstream signalling pathways (16). In addition, over activity of anti-apoptotic pathways can inhibit cell death. For example, the PI3kinase-AKT/PKB pathway can be over-stimulated through extracellular factors such as IGF1/2 and IL-3 and intracellular factors, for example Ras and via loss of pTEN (a tumour suppressor gene) (17). A mechanism that inhibits cell death through Fas, involving upregulation of decoy death receptors, has also been demonstrated in tumour cells (18).

1.2.4 Enabling Replicative Immortality

Normal cells have a limited number of possible cell divisions before they enter a state of senescence. Tumour cells have been shown *in vitro* to be immortalised with limitless replicative potential. This is thought to be in part due to defects in the retinoblastoma protein and p53 tumour suppressor systems (19), although effects on telomere maintenance may play a more significant role. Telomeres represent the ends of chromosomes and essentially act as the counting device for cell division. Each cell division leads to progressive loss of base pairs from the telomere and this erosion eventually leads to loss of protection of the chromosomal DNA.

This in turn leads to fusion of chromosomal ends producing severe karyotype abnormalities resulting in cell death (20). Telomere maintenance is seen in most cancer cells, with the upregulation of telomerase enzymes which add base pairs to the telomere.

This preservation of the telomere effectively inactivates the cells usual counting mechanism for cell division (21).

1.2.5 Inducing Angiogenesis

The oxygen and nutrients supplied by tissue vasculature are crucial for cell function and survival, necessitating that all cells are within a short distance of a blood capillary. The process by which new vessels form to ensure adequate blood supply to developing tissue, angiogenesis, is controlled by stimulatory signals such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).

The rapid growth of tumours requires a high level of angiogenesis. This has been demonstrated to be strongly associated with VEGF, since neutralising antibodies to VEGF inhibited tumour angiogenesis in mice models (22). Tumours appear to activate the angiogenic switch through increased expression of VEGF and FGF with reduced expression of angiogenesis inhibitors such as thrombospondin-1 (23).

1.2.6 Activating Tissue Invasion and Metastasis

A characteristic of tumour cells, which makes them different from normal cells, is the ability to invade tissue stroma and metastasise. Tumours utilise extra-cellular proteases and changes to the physical coupling of cells to achieve this. The multistep process of invasion and metastasis has been described as a sequence of discrete steps, often termed the invasion-metastasis cascade (24,25). This depiction envisions a succession of changes, beginning with local invasion, then intra-vasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and vascular systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extra-vasation), the formation of small nodules of cancer cells (micrometastasis),

and finally the growth of micrometastatic lesions into macroscopic tumours, this last step being termed “colonisation.”

1.2.7 Deregulating Cellular Energetics

The chronic and often uncontrolled cell proliferation that represents the essence of malignant disease involves not only deregulated control of cell proliferation but also, corresponding adjustments of energy metabolism in order to fuel cell growth and division. Under aerobic conditions, normal cells process glucose, first to pyruvate via glycolysis in the cytosol and thereafter to carbon dioxide in the mitochondria; under anaerobic conditions, glycolysis is favoured and relatively little pyruvate is dispatched to the oxygen-consuming mitochondria. The altered metabolism of cancer cells has been documented since the 1930s. Otto Warburg first observed an anomalous characteristic of cancer cell energy metabolism (26,27): even in the presence of oxygen, cancer cells can reprogram their glucose metabolism, and thus their energy production, by limiting their energy metabolism largely to glycolysis, leading to a state that has been termed “aerobic glycolysis”. The existence of this metabolic switch in cancer cells has been substantiated in the ensuing decades. They do so, in part, by upregulation of glucose transporters, notably GLUT1, which substantially increases glucose uptake into the cytoplasm (28-30). Indeed, markedly increased uptake and utilisation of glucose have been documented in many human tumour types, most readily by non-invasively visualising glucose uptake using positron emission tomography (PET) with a radiolabelled analogue of glucose (¹⁸F-fluorodeoxyglucose, FDG) as a reporter. Tumours, being heterogeneous, have been found to contain multiple populations of cells that differ in their energy-generating pathways. One

subpopulation consists of a glucose-dependent sub-population of cells that secrete lactate, whereas cells of the second sub-population preferentially import and utilise the lactate produced by their neighbours as their main energy source, employing part of the citric acid cycle to do so (31-33). These two populations evidently function symbiotically: the hypoxic cancer cells depend on glucose for fuel and secrete lactate as waste, which is imported and preferentially used as fuel by their better-oxygenated brethren. Additionally, it is becoming apparent that oxygenation, ranging from normoxia to hypoxia, is not necessarily static in tumours but instead fluctuates temporally and regionally (34). This is a likely result due to the instability and chaotic organisation of the tumour-associated vasculature.

1.3 Avoiding Immune Destruction – Principles of Immunoediting

Cancer immunoediting is an extrinsic tumour suppressor mechanism that engages after cellular transformation has occurred and intrinsic tumour suppressor mechanisms have failed. The notion that the immune system not only protects the host against tumour formation but also shapes tumour immunogenicity is the basis of the cancer immunoediting hypothesis, which stresses the dual host-protective and tumour-promoting actions of immunity on developing tumours. It has been suggested that immunoediting exists in three phases that have been termed “elimination”, “equilibrium”, and “escape” (Figure 1.2) (35).

1.3.1 Elimination

The elimination phase is best described as when the immune system detects the presence of a developing tumour and destroys it before it becomes clinically apparent. The mechanisms by which the immune system is alerted

to the presence of a developing tumour are not fully understood. Among the possibilities are the classical “danger signals” such as Type I IFNs (e.g. IFN- α) as originally described by Matzinger (36), which exist during early tumour development. These cytokines activate dendritic cells and promote induction of the adaptive anti-tumour immune responses. Damage-associated molecular pattern molecules (DAMPs) such heat-shock 90 protein could also be a trigger because they are released directly from dying tumour cells (37,38). The induction of an immune response on the basis of tumour cell death is of significant interest in this thesis and is highlighted as such in Chapter 2 and Chapter 5.

Stress ligands such as RAE-1 and H60 (mouse) or MICA/B (human) are frequently expressed on the surface of tumour cells. Such ligands bind to activating receptors on innate immune cells, leading to the release of pro-inflammatory and immune modulatory cytokines, which in turn establish a microenvironment that facilitates the development of a tumour-specific adaptive immune response (39). In literature, the most effective cancer anti-tumour immune responses require the additional expression of tumour antigens capable of propagating the expansion of CD4⁺ and CD8⁺ effector T cells. Therefore, it can be suggested that an enhancement of an adaptive immune response is needed to protect the host against a developing tumour.

1.3.2 Equilibrium

In the equilibrium phase, the immune system maintains residual tumour cells in a functional state of dormancy, a term used to describe latent tumour cells that may reside in patients for decades before eventually resuming growth as either recurrent primary tumours or distant metastases (40). Equilibrium thus represents a type of tumour dormancy in which the growth of tumours is

specifically controlled by immunity. Studies with different mouse tumour models confirmed the capacity of the immune system to control the growth of primary carcinomas and metastases for extended periods of time (41,42). In the low-dose 3'-methylcholanthrene system, equilibrium appears to be the result of both the growth inhibitory and cytotoxic effects of the immune response against residual tumour cells (43). However, there could be some doubt as to the duration of an equilibrium state as chemo/radio/immunotherapeutic regimens are more than likely to alter the balance of the tumour-immune infiltrate and thus drive the development of either an elimination or escape phase.

1.3.3 Escape

In the escape phase, tumour cells that have acquired the ability to circumvent immune recognition and/or destruction emerge as growing, visible tumours. Progression from equilibrium to the escape phase is likely to occur because the tumour cell population changes as there is increased cancer-induced immune suppression or immune system deterioration. At the tumour cell level, alterations leading to reduced immune recognition (such as a loss of antigens) promote tumour growth. Loss of tumour antigen expression is one of the best-studied escape mechanisms, and it can occur in at least three ways: (i) through emergence of tumour cells that lack expression of strong rejection antigens, (ii) through loss of major histocompatibility complex (MHC) class I proteins that present these antigens to tumour-specific T cells, or (iii) through the loss of antigen processing function within the tumour cell (10,44).

Alternatively, escape may result from the establishment of an immune suppressive milieu within the tumour microenvironment (45). Tumour cells

can promote the development of such a state by producing immune suppressive cytokines such as VEGF, TGF- β , galectin, or expressing indoleamine 2,3-dioxygenase and/or by recruiting regulatory immune cells that function as mediators of effector T cell proliferation. Regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs) are two major types of immune suppressive cell that play key roles in inhibiting host-protective anti-tumour immune responses (45).

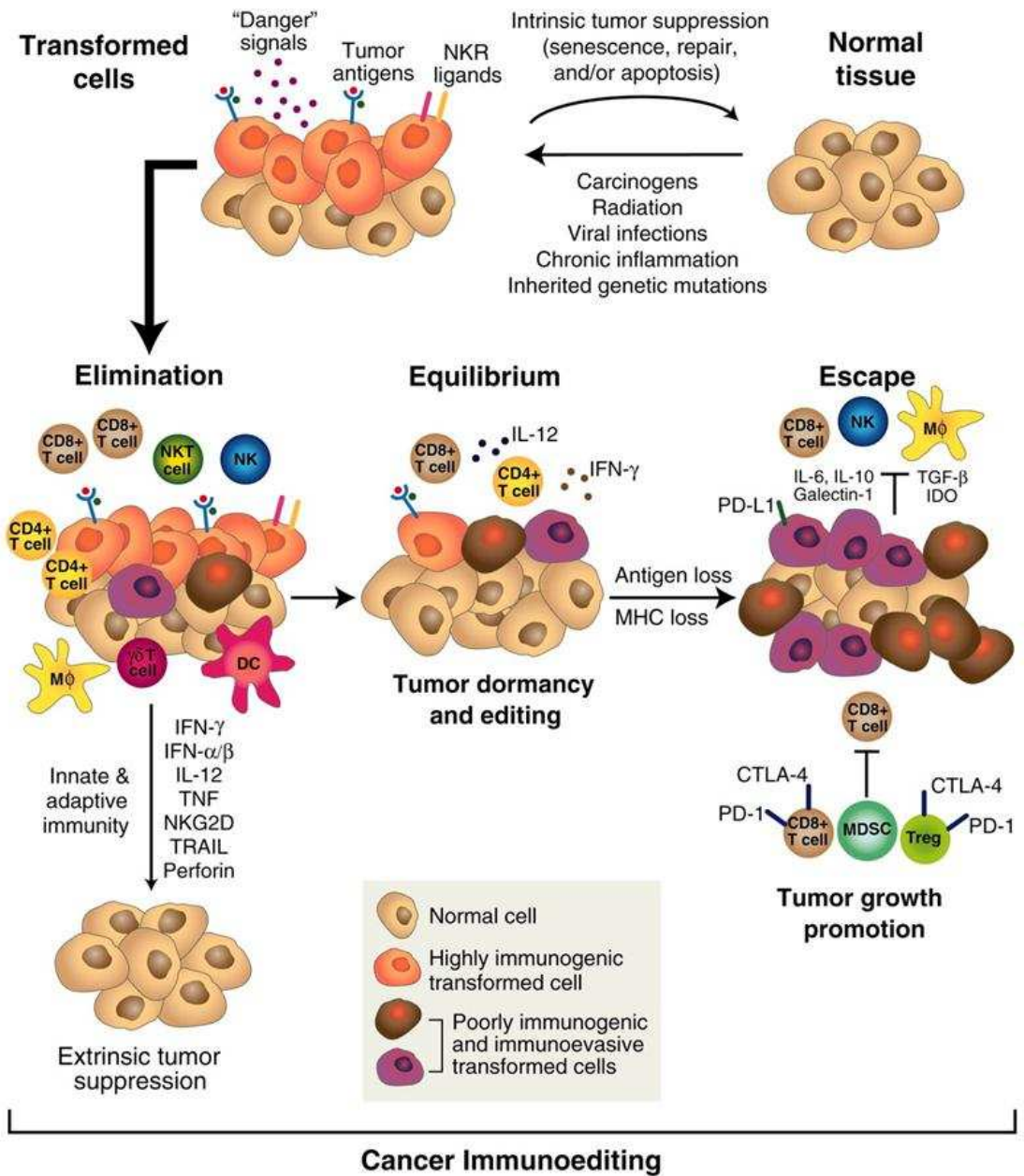


Figure 1.2 - The Cancer Immunoediting Concept. In its most complex form, cancer immunoediting consists of three sequential phases: elimination, equilibrium, and escape. In the elimination phase, innate and adaptive immunity work together to destroy developing tumours long before they become clinically apparent. If, however, a cancer cell is not destroyed in the elimination phase, it may then enter the equilibrium phase, in which its growth is prevented by immunologic mechanisms. Editing of tumour immunogenicity occurs in the equilibrium phase. Over time, tumour cell variants may emerge that (i) are no longer recognised by adaptive immunity (antigen loss variants or tumours cells that develop defects in antigen processing or presentation), (ii) become insensitive to immune effector mechanisms, or (iii) induce an immunosuppressive state within the tumour microenvironment. These tumour cells may then enter the escape phase, in which their outgrowth is no longer blocked by immunity. Adapted from (35). Reprinted with permission (License number OP-00023894), AAAS Ltd.

1.4 Evidence for the Role of Immunoediting Against Human Ovarian Cancer

Evidence of the role of immunoediting in human ovarian cancer comes from the presence of tumour-infiltrating lymphocytes, which correlated positively and strongly with patient survival. Zhang *et al.* (46) assessed the distribution of tumour infiltrating lymphocytes in 186 frozen specimens from stage III or IV ovarian cancers and conducted clinical outcome analyses. In this study, CD3⁺ T cells were detected within tumour-cell islets in 102 of the 186 tumours. They also assessed the number of CD4⁺ and CD8⁺ T cells in 30 tumours. The data showed that intratumoural CD4⁺ and CD8⁺ cells were either both present or both absent. Patients whose tumours contained tumour infiltrating lymphocytes had five-year survival rates of 38%, whereas patients whose tumours lacked this only had a five-year survival rate of 4.5%. The five-year progression-free survival rates for patients whose tumours had tumour infiltrating lymphocytes was 31%. In comparison, those that were tumour infiltrating lymphocyte negative had a progression free survival rate of just 8.7%. Other studies have confirmed that the intraepithelial CD3⁺ T cell count was a significant prognostic factor in epithelial ovarian cancer. Tomsova *et al.* (47) showed improved overall survival among 116 patients with higher versus lower counts of intraepithelial CD3⁺ T cells. Thus, overall and progression-free five-year survival rates were significantly prolonged in the patients whose tumours contained higher numbers of tumour infiltrating lymphocytes compared to the patients whose tumours did not contain these cells.

1.5 The Tumour Immunology ‘Tube Map’: The Immune System in Cancer Explained

As described earlier, the immune system has a significant role in the development of tumours. Within the structure of a tumour, stromal cells including immune cells (35), often outnumber cancer cells. The complex immune milieu which is present at the tumour site influences both the potential for immune elimination or immune escape. Extensive characterisation using immunohistochemical, cytofluorometric and micro array techniques have been performed on multiple human carcinomas. These studies suggest that dendritic cells, M1 macrophages, cytotoxic CD8⁺ T cells, Th1 CD4⁺ T cells, NK cells and Th17 CD4⁺ T cells present in the tumour bed can induce immune-mediate tumour elimination. In contrast, neutrophils, M2 macrophages, myeloid-derived suppressor cells, Th2 CD4⁺ T cells and CD4⁺FOXP3⁺ Treg are suspected to aid tumour-immune escape. The interactions between these different populations can be represented as a ‘map’ (Figure 1.3). The intricate relationship between both tumour-eliminating or tumour-escaping immune populations means that selective targeting of a particular cell type or receptor can be either circumvented by the tumour or lead to adverse systemic effects on the patient. The ‘map’ below has the capacity to highlight areas which may provide avenues for improved immunotherapy.

1.5.1 Tumour Cells

Tumours contribute to the immune suppressive milieu in a variety of ways. These include alterations to antigen presentation machinery, defects in proximal TCR signalling, secretion of immune suppressive and pro-apoptotic factors, activation of negative regulatory pathways and specific recruitment of regulatory cell populations (48-51). These mechanisms work synergistically in advanced stages of cancer to attenuate both native and therapy-induced anti-tumour immune responses (52).

One of the best studied mechanisms used by tumours to avoid T cell recognition is the impairment of antigen presentation (53). It has been observed that the continuous generation of tumour variants by an increased frequency of mutations and/or genetic deletions can result in escape from T cell recognition (50). In this regard, cancer cells that no longer express the tumour antigen may escape destruction by cytotoxic T cells and grow progressively. However, there are indications that cytotoxic T cells may indirectly eliminate these tumour variants when tumour cells express sufficient antigen to be effectively cross-presented (54).

In addition to the generation of antigen loss variants, downregulation of antigen processing machinery has been documented extensively at different levels in a wide variety of tumours and has been considered as the most common strategy exploited to escape T cell control (48,53). Complete absence of MHC-I expression caused by mutations of the β 2 microglobulin (β 2M) gene or decreased MHC-I expression due to transcriptional regulation (55-58) prevents recognition by cytotoxic T cells. Furthermore, point mutations and genetic deletions lead to selective loss of individual HLA alleles (59), which further facilitates immune evasion from T cells. These changes

have been frequently observed in renal cell carcinoma, colorectal carcinoma, melanoma (53,56) and ovarian cancer (60).

Tumours also alter the extracellular environment in order to establish immune suppression. The immune-regulatory enzyme indoleamine 2,3-dioxygenase (IDO) catalyses the degradation of the essential amino acid tryptophan, via the kynurenine pathway (61). The lack of tryptophan and the subsequent increase in kynurenine derived metabolites leads to attenuated T cell proliferation (62). This effect was confirmed by *in vitro* observations showing inhibition of T cell proliferation and antagonism of cell cycle progression by tryptophan depletion (63). The fact that IDO is also expressed by different tumour cells prompted Uyttenhove *et al.* (64) to investigate the role of IDO in the establishment of tumour-immune escape. They demonstrated that immunogenic tumours engineered to overexpress IDO grew more aggressively in immune competent hosts and this effect correlated with a decreased accumulation of activated T cells at the tumour site (64). Since this work, the prognostic significance of IDO has been investigated with changes in prognosis seen in prostate (65), leukaemia (66), endometrial (67) and ovarian cancer (68). Importantly, *in vivo* administration of the IDO inhibitor 1-methyltryptophan resulted in reduced tumour mass and stimulation of anti-tumour cytotoxic immune responses (64).

Whether IDO plays a physiological role in peripheral T-cell tolerance still remains to be elucidated. In contrast to other homeostatic mediators such as CTLA-4, IDO-deficient mice do not display autoimmunity (62), suggesting that IDO might be predominantly involved in the generation of local immune tolerance at selected sites of immune privilege, such as the tumour

microenvironment. Thus, pharmacological antagonism of IDO may be an important component of combinatorial immunotherapy strategies.

The disordered growth of an expanding tumour often outstrips the development of a supportive vascular bed, which leads to a reduction in oxygen levels throughout much of the tumour mass (69,70). For example, the hypoxic fraction in squamous cell carcinomas of the cervix and head and neck can be as high as 20-32% (71). Whereas in ovarian cancer, an increase in tumour hypoxia has been linked with increased resistance to Paclitaxel therapy (72). Adenosine is present at elevated levels in hypoxic tissues because of increased intracellular adenosine production and release from the cells. This is the result of oxygen deprivation and cellular ATP depletion (73) by activation of the 5'-nucleotidase pathway (74) and inhibition of adenosine kinase (75). Bidirectional nucleoside transporters in the membrane are responsible for exporting intracellular adenosine to the extracellular compartment (76).

As expected, hypoxia has been shown to stimulate adenosine production in cultures of 3LL Lewis lung carcinoma cells (77). Moreover, analysis of mouse and human colorectal cancer has shown that the concentration of extracellular adenosine is 10-20-fold higher than those measured in surrounding normal tissue (78,79). It is important to note that extracellular adenosine levels in solid tumours can be further supplemented or modified by ecto-enzymes that mediate adenosine production or degradation at the cell surface. Adenosine-producing ecto-enzymes that are expressed by both lymphocytes and cancer cells include NTPDase 1 (CD39) and ecto-5'-nucleotidase (CD73) (80-84).

1.5.2 Tumour-associated Macrophages

Macrophages are highly versatile, multifunctional cells that are characterised by their ability to engulf invading microbes or cell debris from injured sites, secrete a wide array of immune-modulatory cytokines, present antigens to T cells and act as accessory cells in lymphocyte activation. They display a high degree of plasticity, altering their phenotype to suit the microenvironment in which they reside. The conventional understanding is that macrophages can be subdivided into M1 (classically activated) or M2 (alternatively activated) phenotypes. M1 macrophages exhibit a pro-inflammatory phenotype and are activated by lipopolysaccharide and interferon- γ (IFN- γ) to secrete bactericidal factors and promote Th1 responses. In contrast, M2 macrophages have an immune suppressive phenotype and release cytokines that promote Th2 responses (85).

Macrophages in tumours — usually termed tumour-associated macrophages (TAM) — often express many of the characteristics typical of the M2 phenotype (86,87) and have therefore been described as ‘M2-skewed’. However, recent evidence has suggested that the phenotype of TAM varies with the stage of tumour development, with M1-like cells often predominating at sites of chronic inflammation where tumours can develop, then switching to a M2-like phenotype as the tumour begins to invade, vascularise and develop (88,89). There are usually higher numbers of TAM in malignant tumours than surrounding normal tissues (90). These cells initially enter the tumour vasculature as monocytes from the blood, starting to differentiate into TAM as they do so (91). Monocyte recruitment is driven by chemokines secreted by both malignant and stromal cells in tumours as discussed below.

TAM have a profound influence on the regulation of tumour angiogenesis. Several clinical studies have shown a correlation between a high number of TAM in human tumours and increased micro-vessel density, suggesting that these cells might promote tumour angiogenesis (92-96). Depletion of macrophages in a transgenic mouse mammary tumour virus model that expresses Polyoma Middle T antigen (MMTV-PyMT) resulted in a 50% reduction in vascular density, causing delayed tumour progression and metastasis. Reintroduction of macrophages into these knockout mice led to a significant increase in vascular density and enhanced tumour progression (97). Further evidence of the importance of TAM has come from studies in which monocytes were removed from the circulation using Clodronate liposomes. This was shown to significantly reduce TAM numbers and angiogenesis in Lewis lung carcinoma xenografts (98).

TAM express many pro-angiogenic and angiogenesis-modulating factors *in vitro*, such as VEGF, basic fibroblast growth factor (bFGF, also known as FGF2), tumour necrosis factor (TNF), interleukin 1 (IL-1), chemokine (C-X-C motif) ligand 8 (CXCL8; also known as IL-8), cyclooxygenase-2 (COX-2), plasminogen activator, urokinase, platelet derived growth factor (PDGF), matrix metalloproteinase 7 (MMP7), MMP9 and MMP12. The tumour microenvironment is now known to stimulate the pro-angiogenic functions of macrophages. For example, TNF secreted by ovarian tumour cells enhances the release of VEGF, MMP9 and other important pro-angiogenic factors by macrophages (89).

A number of recent findings have shown that in such hypoxic areas TAM have a marked effect on tumour angiogenesis. Macrophages are known to respond to hypoxia by up-regulating hypoxia-inducible transcription factors

(mainly hypoxia-inducible factors HIF1 and HIF2) (99-101), the activation of which leads to increased transcription of many genes that regulate cell proliferation, metabolism and angiogenesis (102). Moreover, human TAM express VEGF almost exclusively in hypoxic and/or peri-necrotic areas of breast carcinomas (103,104).

The fact that TAM also upregulate the pro-angiogenic matrix metalloproteinase MMP7 in hypoxic areas of tumours (100) might have relevance not only to tumour angiogenesis but also the newly identified role of TAM in metastasis. Recent work has indicated that TAM (possibly in conjunction with other inflammatory cells such as T cells) can express high levels of RANKL (also known as TNFSF11) that directly stimulate tumour cells to express a more metastatic phenotype (105). MMP7 cleaves an active form of RANKL from the cell surface (106), so increased expression of MMP7 could enhance the release of RANKL by macrophages and T cells and drive tumour progression.

Recent studies have also suggested that monocytes can differentiate into endothelial cells when exposed to the sustained stimulation by angiogenic growth factors (107,108). Whether monocytes newly recruited into tumours or differentiated into TAM directly contribute to the formation of new tumour blood vessels in this way remains to be determined.

1.5.3 Dendritic Cells

Dendritic cells (DC) are developed in the bone marrow from hematopoietic progenitor cells under the control of a complex network of soluble and cell-bound molecules produced and expressed by bone marrow stroma (109). Most DC differentiate along the myeloid lineage, and precursors of DC

include common myeloid progenitor cells and immature myeloid cells. DC can also differentiate from common lymphoid progenitor cells. The proportion of lymphoid DC is substantially lower than that of myeloid DC, and most of these cells belong to a relatively rare subset of DC known as plasmacytoid DC (pDC). pDC express lymphoid antigens and produce large amounts of type-I IFN in response to viruses. They have a lower ability to process and present antigen and stimulate T cells than myeloid DC. In humans, myeloid DC are characterised by a lack of expression of lineage-specific markers and the expression of CD11c, whereas pDC do not express lineage-specific markers or CD11c, but express a receptor for IL-3 (CD123). *In vitro*, myeloid DC are dependent on Granulocyte Macrophage-colony stimulating factor (GM-CSF), whereas pDC are dependent on IL-3 and IFN- α for survival (110). DC that leave the bone marrow are defined as immature DCs (iDC). iDC have little or no expression of co-stimulatory molecules such as CD80, CD86 and CD40 on the surface and produce little or no IL-12, which is required to support T cell proliferation.

However, these cells are capable of taking-up and processing various molecules and micro-organisms. In tumour tissues, antigens may come from dying tumour cells. DC can engulf apoptotic or necrotic tumour cells, and process and present tumour-associated antigens on their surface. This effect is clearly manifested by upregulated expression of MHC-II and co-stimulatory molecules, increased IL-12 production and enhanced stimulation of antigen-specific T-cell responses (111,112). DC are critically important for the generation and maintenance of anti-tumour immune responses (109). It is now well established that tumour cells contain a large number of antigens that can be recognised by the host immune system. DC can take up, process, and present tumour antigens to activate a tumour-specific T cell response.

However, this does not happen in most types of cancer or in animal models with spontaneously arising tumours. Data from many laboratories obtained during the past few years indicates that defects in the DC system are one of the main factors responsible for tumour escape, which contributes in various ways to the T cell defects seen in cancer. These abnormalities manifest in several major phenomena.

1.5.3.1 Lack of Competent DC

Tumour-bearing mice have been shown to have decreased numbers and function of DCs in lymph nodes, spleen and skin (113,114). Large numbers of studies in ovarian (115), breast (116,117) and prostate cancer (118) patients have found similar effects. In these studies, both myeloid and lymphoid populations of DCs were investigated. A significant decrease was observed only in the myeloid population of DC, whereas pDC were not affected. Several clinical studies have provided clear evidence that surgical removal of tumours can increase the number of DCs in the peripheral blood of patients with cancer. Almand *et al.*(119) demonstrated an increase in the total DC population in patients with breast and prostate cancer after surgery. The functional consequences of a decreased number of functionally competent DC in patients with cancer are obvious: a decreased number of DC makes immune stimulation less effective. However, it is likely that other defects in DC differentiation and activation have more of a profound effect on anti-tumour immune responses.

1.5.3.2 Accumulation of Immature DC

Immature DC have reduced capacity to capture antigens and elicited poor proliferation and IFN- γ secretion by T cells (120). In patients with cancer, it has been repeatedly shown that tumours contain cells with the phenotype of iDC. Data from renal cell carcinoma (121), melanoma metastases (122) and basal-cell carcinoma (123) suggests that DC are not recruited to tumours in large numbers and those that are recovered from the tumour site have low levels of co-stimulatory molecules and have reduced T cell stimulatory activity. Importantly, the addition of GM-CSF and TNF- α , or CD40L to *in vitro* cultures, all of which are normally potent stimulators of CD80/CD86 expression, did not induce CD80 expression on tumour-infiltrating DC. This indicates that the lack of CD80/CD86 expression does not simply result from a lack of activation of these cells in the tumour microenvironment but might be caused by defects in cell differentiation (124).

Consistent with these observations, an increased proportion of iDC with reduced expression of co-stimulatory molecules was found in the peripheral blood of patients with breast, head and neck, lung, and oesophageal cancers (114,118-126). Immature DC are unable to induce anti-tumour immune responses and can induce T-cell tolerance. It has been shown that if APCs fail to provide an appropriate co-stimulatory signal for T cells, tolerance or anergy can develop (27, 140). DC derived from colon cancer tissue or melanoma-associated DC were not only significantly less potent inducers of T cell proliferation, but also induced T cell anergy (122-124). This suggests that DC can significantly contribute to the anergic environment found within the tumour.

1.5.3.3 Accumulation of Suppressive DC

In addition to accumulation of iDC, a number of studies reported the presence in tumour tissues of subsets of DC with potential to suppress T cells. Most prominent of them are pDC (127). As described above, in contrast to myeloid DC, the number of circulating pDC in patients with different types of cancer was not altered. Furthermore, accumulation of pDC were found inside ovarian tumours (115,128). This accumulation was attributed to stromal-derived factor-1 (SDF-1) secreted by malignant cells (115,129). Tumour associated pDC induced IL-10 production by T cells (115,130,131). The ability of these pDC to produce IFN- α was diminished. Tumour-induced down-regulation of TLR9 was identified as one mechanism probably contributing to impaired pDC function within the tumour environment (132). Accumulation of pDC was also found in peri-tumoural areas of primary melanomas (133). It has been shown that mouse tumour-draining lymph nodes contained a subset of pDC that constitutively expressed immunosuppressive levels of the enzyme IDO (134). This may prevent the clonal expansion of T cells and promote T cell anergy. The accumulation of pDC in ovarian cancer is of interest and is discussed in greater detail later in this thesis.

1.5.4 Natural Killer Cells

Natural Killer cells (NK) are a type of lymphocyte defined by the expression of CD56 and the absence of CD3. NK play an important role in innate immunity by mediating direct cytotoxicity and secreting cytokines such as IFN- γ , IL-10, IL-13 and TNF- α (135,136). NK activity is dependent on a complex balance between inhibitory and stimulatory receptors which interact through MHC class molecules on potential target cells. Ultimately NK will be triggered or inhibited depending on the balance of these signals. The loss or absence of

HLA class I molecules on the surface of the target cell tends to produce triggering of NK (135). Once activated NK trigger apoptosis in the target cell through initiation of the caspase cascade. Apoptosis may also be induced through interaction of FasL and TNF Related Apoptosis Inducing Ligand (TRAIL) with the respective death receptors (137). NK are able to respond to human tumours through NKG2D and its related ligands MICA and MICB. The activation of human NK by tumours also largely depends on the natural cytotoxicity receptors (NCR), which include three members: NKp46, NKp44 and NKp30 (138,139). The NCR is unique in its expression pattern and is almost exclusively confined to NK. However, very little is known about the identity of NCR ligands, particularly with respect to tumours. Recently, it has also been suggested that the DNAX accessory molecule 1 may also play a role in tumour immune surveillance as either an activating receptor or co-stimulatory molecule (140).

Killer immunoglobulin-like receptors (KIR) of NK activity include the killer-like immunoglobulin family and the CD94-NKG2 complexes that bind to specific MHC-HLA alleles. Since NK activity is controlled by a balance of inhibitory and activating receptor signalling, the inhibitory receptors represent targets to effectively enhance NK cytotoxicity against tumours. Antibodies that inhibit KIR/MHC binding have been minimally studied in mice (141) and has only just entered clinical trials (142). Interestingly, specific inhibitory KIR/HLA ligand pairs decrease the risk of developing human cervical neoplasia while the presence of the activating KIR3DS1 results in increased risk of disease (143). It is therefore essential to thoroughly understand what role NK are playing in each neoplastic disease (inflammatory versus protective) before considering intervention. The effector molecules, TRAIL, perforin/granzymes, and the cytokine IFN- γ have all been implicated in tumour suppression by NK cells

(144). However, until the means to specifically and conditionally mutate genes in NK become available, the importance of these pathways cannot be elegantly tested. With the knowledge gained in recent years regarding suppressive tumour-immune infiltrates e.g. Treg and MDSC, it is important to recognise the potential for the attenuation of NK efficacy (145,146). Collectively, these mechanistic insights into how NK contribute to tumour suppression provide attractive new targets to formulate potential cancer therapies based in part upon NK activation.

1.5.5 Cytokines and Chemokines

Mounting evidence supports the idea that immune cells and inflammatory mediators (cytokines, chemokines) within the tumour microenvironment, can either be beneficial or detrimental for tumour progression (Figure 1.4) (147). Initially, tumour cells and cells of the tumour microenvironment, respond to tumour hypoxia and necrosis secondary to excessive tumour cell proliferation, by releasing a number of growth factors and cytokines that are chemoattractive for monocytes and macrophages, including colony stimulating factor-1 (CSF-1), GM-CSF, TGF- β and chemokines (e.g. CCL2, CCL3, CCL4, CCL5) (148). In turn, recruited macrophages secrete growth factors that affect tumour cell behaviour (e.g. induction of motility), activate tumour endothelium and propagate inflammation (149-151). Factors released by recruited monocytes/macrophages include VEGF, basic fibroblast growth factor (bFGF), tumour necrosis factor (TNF), hepatocyte growth factor (HGF), epidermal growth factor (EGF) family members, platelet- derived growth factor (PDGF), and chemokines such as CXCL12 and IL-8 (148,152,153). Monocytes and macrophages also bring in much of the cyclooxygenase-2 (COX-2) present in the tumour environment (154,155). COX-2 expression and

prostaglandin production within the tumour environment stimulate tumour cell proliferation, survival and motility but also tumour angiogenesis (154-157).

Chemokines play an important role in coordinating the stromal response to cancer, including the polarisation of the immune responses to the tumour, the determination of the composition of the cellular infiltrate, and the induction of angiogenesis. Chemokine receptors have been detected on cancer cells and the relevant ligands were found expressed at the primary tumour site and at sites of tumour metastasis, suggesting a direct role for chemokines/chemokine receptors in tumour growth and metastasis (158). Inflammatory insults lead to upregulation of CCL22, a macrophage-derived chemokine that is an attractant for Treg (159). This particular migratory pathway is of interest in this thesis.

TGF- β is a pleiotropic immune suppressive cytokine that inhibits T cell activation, proliferation and differentiation (160,161). In addition to TGF- β , other cytokines are present in the tumour microenvironment and have been shown to impair immune cell function including IL-10 (162), prostaglandin-E2 (163) and sialomucins (164). IL-10 is abundant in the tumour microenvironment, impairs DC functionality (162,165,166) and protects tumour cells from cytotoxic T cell-mediated cytotoxicity by downregulating TAP1 and TAP2 (162). However, in contrast to previous assumptions, recent evidence indicates that IL-10 may also be immune-stimulatory. Unexpectedly, overexpression of IL-10 in the tumour microenvironment synergizes with other cytokines to promote tumour rejection instead of inducing immune suppression (167,168).

Such unresolved chronic inflammation is associated with increased conversion of normal cells to pre-neoplastic foci. Accumulation of somatic

mutations can change pre-neoplastic foci into foci of fully transformed cells with tumour initiation capacities. It is this constant chronic inflammation that contributes to the development of tumour cell variants which can “escape” immune detection (35).

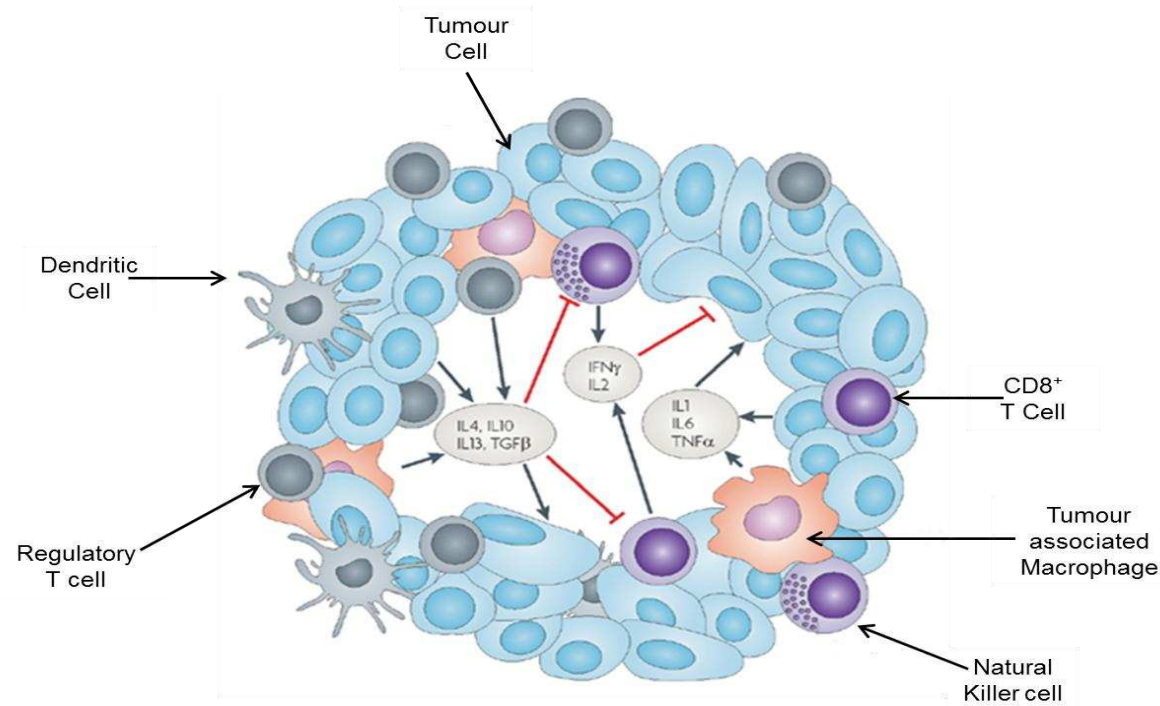


Figure 1.4 - Role of Major Cytokines in the Tumour Microenvironment. Tumour cells co-exist with immune cells: tumour-associated macrophages (TAM), regulatory T cells (Treg) and dendritic cells (DC). TAM and Treg are major sources of anti-inflammatory Th2 cytokines (e.g. IL-4, IL-10 and IL-13) and transforming growth factor- β (TGF- β), which suppress the anti-tumour immune response which is mediated by natural killer cells, cytotoxic CD8⁺ T cells and pro-inflammatory cytokines (e.g. IL-2 and interferon- γ). Simultaneously, tumour cells directly exploit activated immune cells for their growth and development. In an immunosuppressed environment various cytokines produced by innate immune cells and cancer cells directly promote the growth of cancer cells. Adapted from (267). Reprinted with permission (License number 2812710846009), Nature Publishing Group.

1.5.6 T Cells

The role of T cell infiltrates in tumour immunoediting has been widely discussed. T cells can be divided into two main groups; those expressing CD8 molecules, cytotoxic T lymphocytes, and those expressing CD4 molecules, helper T cells (Th). CD4⁺ and CD8⁺ T cells are the principal helper and effector cells, respectively, of adaptive cellular immunity, and many immunotherapy strategies are aimed at activating these cells to promote anti-tumour immunity and long-term immune memory against the recurrence of primary disease or development of metastases. T helper 1 CD4⁺ T cells (Th1) facilitate tissue destruction and tumour rejection by providing help to cytotoxic CD8⁺ T cells, while T helper 2 CD4⁺ T cells (Th2) facilitate antibody production by B cells and polarise immunity away from a beneficial cell-mediated anti-tumour response.

1.5.6.1 Cytotoxic CD8⁺ T Cells

These cytotoxic T-cells have a T cell receptor which binds to HLA class I molecules on the surface of cells displaying peptides which are typically from viral infection or abnormal cellular functioning. Their mechanism of cell killing is similar to NK (e.g. Perforin/Granzyme, IFN- γ , TRAIL, FasL). They are seen as key prognostic indicators in several disease settings (169-173).

1.5.6.2 Helper T Cells

T (Th) cells, are the key players in steering the immune responses. Th cell differentiation is characterised by the acquisition of cytokine production. Since the establishment of the Th1–Th2 paradigm, the function and regulation of effector T cells has been a subject of intense investigation. The Th1 cell, one of the first described Th cells that produces IFN- γ , TNF- α and TNF- β to stimulate innate and T-cell immune responses. The most important function of

Th1 cells is to promote cell-mediated immunity characterised by CD8⁺ T cell induced-cytolytic activity. Th1 cells are important in protection of the host from obligate intracellular pathogens. Over-exuberant pro-inflammatory activities of Th1 cells cause tissue damage and elicit unwanted inflammatory disease and self-reactivity including inflammatory bowel disease (174) and graft-versus-host disease (175).

T helper type 2 cells were identified at the same time as Th1 cells in the early 1980s. They are defined as producers of IL-4, IL-5, IL-9, IL-10 and IL-13. The Th2 response is often associated with the humoral response and is important in resistance against extracellular forms of pathogens. Th2 cells are also important for mucosal immunity in the lung. Aberrant elevation of the Th2 response often leads to chronic inflammatory airway diseases, such as atopic asthma and allergy (176-178).

In the peripheral blood of patients with bladder and colorectal cancer, the proportion of Th1 cells, identified by intracellular production of IFN- γ or IL-2, was markedly reduced, whereas the proportion of Th2 cells producing IL-4, IL-6 and/or IL-10 were significantly elevated, as compared with the proportion of Th1 and Th2 in otherwise healthy patient populations (179,180). In human cervical carcinomas, CD3⁺ tumour infiltrating T cells display enhanced Th2 cytokine profiles, specifically increased IL-4 and reduced IFN- γ production (181).

CD4⁺ Th17 cells (182,183), may have a role in the anti-tumour immune response. Th17 cells are induced by IL-23, a cytokine closely related to IL-12 (184). Upon activation by IL-23, Th17 cells produce IL-17 which exacerbates inflammation by inducing IL-6, TNF- α , G-CSF, and other acute phase proteins (185). Controversy surrounds the role of Th17 cells in tumour immunity

(186,187). IL-23 itself, has been shown to reduce CD8⁺ T cell infiltration into tumours, thereby promoting tumour growth (184). Recent work however, has shown that Th17 cells could be beneficial in propagating an anti-tumour response (183,188,189). This ambiguity may be explained by a recent study showing that Th17-induced IL-6 inhibits Treg function (189-191). Additional experiments are clearly necessary to clarify the roles of IL-17, IL-23 and the plasticity between Th17 cells and Treg in tumour progression.

Despite the mounting evidence of CD8⁺ T cell, Th1/Th2/Th17 and NK cell tumour infiltration; there is still minimal tumour elimination. This is due to the immune suppressive environment which is generated by the tumour. There is intense debate as to how tumour-T cell tolerance is achieved. It was initially considered that the lack of effector T cell efficacy in controlling tumour growth just reflected “tumour ignorance”, since antigen-specific cytotoxic responses, proliferation and cytokine production could be measured upon *in vitro* re-stimulation or secondary immunisation against tumour antigen (192,193). Other studies have indicated that CD8⁺ T-cells are, indeed, rendered tolerant to tumour antigens (194-197). The delivery of large amounts of antigen could be deleterious for mounting reactive effector T cells. It has been shown that there is a direct correlation between the amount of antigens that are expressed in the periphery, the degree of T cell proliferation and the number of antigen-specific CD8⁺ T cells in the draining lymph nodes (198).

Given the profound impairment in the function of tumour-antigen specific T cells, as determined by their blunted proliferation and cytokine production, it was assumed that the whole tumour-T cell infiltrate was rendered anergic by the growing malignancy. However, a more detailed analysis revealed a more complex picture of tumour-immune responses. In particular, the emergence of

Treg in tumour immunology and its subsequent role in conferring tolerance on a tumour is of major interest.

1.6 Introduction to Regulatory T Cells

As mentioned above tumours develop an environment which is conducive to their survival. In addition, this tumour-associated milieu supports a network of immunosuppressive adaptations favouring the generation of a subset of regulatory T lymphocytes (Treg); potent suppressors of CD8⁺ T cell-mediated cytotoxicity (199).

Several subtypes of Treg have been described (Table 1.1) and represent an important control mechanism of the adaptive immune response functioning to restrict the duration and intensity of an acute immune response, preventing the induction of autoimmunity and limiting the development of chronic inflammation (200,201). This thesis will focus on CD4⁺CD25⁺FOXP3⁺ 'natural' Treg as these have been implicated in the suppression of anti-tumour immunity and have been repeatedly identified in several clinical settings to correlate with negative outcome (202). Forkhead box P3 (FOXP3) is a key transcription factor and marker of Treg in mouse and man (203,204). In contrast to experiments in the mouse, the expression of FOXP3 alone does not confer a regulatory phenotype in man (205) as FOXP3 is also expressed transiently on activated T cells that do not exhibit regulatory function (206).

An increase in tumour infiltrating Treg has been well documented in several disease settings, including oesophageal cancer, gastric cancer (207), hepatocellular carcinoma (208), leukaemia (209), lung cancer (210), lymphoma (209,211) and melanoma (212) (Table 1.2). In a study of 104 patients with ovarian cancer Curiel *et al.* (213) demonstrated that specific

recruitment of Treg via the chemokine CCL22 supported tumour growth and predicted reduced survival. This study demonstrated that at later stages of disease CD4⁺ CD25⁺ FOXP3⁺ cells accumulated in tumours but rarely migrated to draining lymph nodes. Further, approximately 75% of Treg in the tumour masses were in proximity to infiltrating CD8⁺ T cytotoxic cells. Several other studies have examined the prognostic significance of Treg in cancer (summarised in Table 1.2), with the vast majority demonstrating that high Treg numbers have a negative impact on disease free- and overall survival. It is because of this mounting evidence that Treg are the main focus of this thesis.

Table 1.1 – Immune Suppressive T cell Populations.

Subsets of regulatory T cells	Cellular targets of suppression	Present in : -	Reference
CD4 ⁺ CD25 ⁻ Tregs	T and B cells	Rat, Mice	(214,215)
CD4 ⁺ CD25 ⁺ FOXP3 ⁺ Tregs	T cells	Rat, Mice, Human	(199,216,217)
Qa-1-restricted CD8 ⁺ Tregs	Antigen-activated T cells differentially expressing Qa- 1-self-peptide complexes.	Mice	(218)
CD8 ⁺ CD28 ⁻ Tregs	Dendritic cells	Human	(219)
CD8 ⁺ FOXP3 ⁺ Tregs	CD4 ⁺ CD25 ⁻ T cells	Human	(220)

Table 1.2 – Prognostic Significance of Treg in Cancer

Cancer Type	Study Size (n =)	Treg population	Other Observations	Prognosis	Ref.
Breast	299 (pure ductal carcinoma in situ, n = 62; invasive breast cancer, n = 237)	Increase in FOXP3 ⁺ cells in invasive tumours compared to control	High Treg numbers within oestrogen receptor positive tumours identified high risk patients	Higher intratumoural Treg numbers correlate to decreased overall survival and relapse-free survival	(221)
	28 (Stage I + II, n = 12; Stage III + IV, n = 16)	Increased CCR6 ⁺ FOXP3 ⁺ T cells found within tumour masses	CCR6 ⁺ Treg appear to be the major population within tumour	Increased intratumoural CCR6 ⁺ Treg predicts reduced survival	(222)
Ovarian	360 (FIGO stage I-IV)	Increase in FOXP3 ⁺ T cells in omental metastases compared to intratumoural ovarian tissue	High CD45RO ⁺ linked to increased disease specific survival	High CD8 ⁺ /Foxp3 ⁺ correlates with increased disease specific survival.	(223)
	99 (FIGO stage I, n = 18; stage II, n = 4; stage III, n = 62; stage IV, n = 15)	Increased FOXP3 ⁺ expression in tumour tissue compared to healthy tissue	High IFN- γ /FOXP3 ⁺ ratio associated with improved overall survival	High intratumoural FOXP3 ⁺ T cell number associated with poor overall survival and disease free survival	(224)
	117 (FIGO stage I, n = 5; FIGO stage II, n = 7; FIGO stage III, n = 93; FIGO stage IV, n = 12)	Increased intratumoural CD25 ⁺ FOXP3 ⁺ T cell number	High CD8 ⁺ T cell infiltrate associated with improved overall survival	High CD8 ⁺ /FOXP3 ⁺ correlates with increased disease specific survival	(225)

Renal cell	125 (clear cell)	Higher CD4 ⁺ CD25 ⁺ FOXP3 numbers found in peritumoural areas compared to intratumoral sites	Increase in Treg also correlated with an increase in COX-2 expression and associated with TNM stage and tumour size	High peritumoral Tregs associated with decreased overall survival and disease-free survival	(226)
Colorectal	967 (stage II, n = 593; stage III, n = 374)	Treg density higher in tumour tissue compared with normal colonic mucosa	Lower CD8 ⁺ and CD45RO ⁺ cell densities in tumour compared to normal colonic mucosa	High Treg density in normal mucosa associated with worse prognosis. In contrast, a high density of Treg in tumour tissue was associated with improved survival	(227)
	94 (stage I+II, n = 43; Stage III + IV, n = 51)	High FOXP3 ⁺ number compared to normal tissue	High TGF- β expression correlated with high FOXP3 ⁺ cells	High CD8 ⁺ /FOXP3 ⁺ ratio correlates with improved survival and longer disease free period	(228)
	40 (stage I, n = 1; stage II, n = 20; stage III, n = 6; stage IV, n = 13)	Higher FOXP3 ⁺ number compared to normal tissue	Tumour infiltration by Treg higher in limited disease compared to metastatic disease	No correlation found between high Treg number or high CD8 ⁺ /FOXP3 ratio and survival	(229)
	160 (stage II, n = 24; Stage III, n = 136)	High FOXP3 ⁺ number within tumour intraepithelia and stroma compared to normal tissue	Reduced CD3 ⁺ T cell density associated with reduced disease free survival	Low CD3 ⁺ /FOXP3 ⁺ cell ratio predicted reduced disease free survival	(230)
Anal squamous cell carcinoma	38 (stage I/II, n = 24; stage III/IV, n = 14)	High intratumoural FOXP3 ⁺ number	Increase numbers of granzyme B ⁺ cytotoxic cells has significant negative prognostic effect	No correlation found between Treg number and prognostic outcome	(231)

Endometrial	368 (FIGO stage I-IV)	High FOXP3 ⁺ cell number in stage I cancers compared to stage II	High intratumoral CD45R0 ⁺ infiltration linked to improved overall survival	High intratumoral FOXP3 ⁺ number associated with decreased survival in type I cancers. High CD8 ⁺ /FOXP3 ⁺ ratio correlates with improved survival and longer disease free period	(232)
Liver	123 (stage I, n = 32; stage II, n = 29; stage III, n = 62)	High intratumoural and circulating CD4 ⁺ CD25 ⁺ FOXP3 ⁺ T cell number compared to normal tissue	Low CD8 ⁺ T cell tumour infiltrate. Low tumoural Perforin, Granzyme A/B expression on tumoural CD8 ⁺ T cells	Increased intratumoural and circulating CD4 ⁺ CD25 ⁺ FOXP3 ⁺ T cell number lead to increased mortality and reduction in disease free survival	(233)
	302 (stage I, n = 50; stage II, n = 105; stage III, n = 100; stage IV, n = 47)	Higher FOXP3 ⁺ cell number in peritumoural regions compared to intratumoural	Intratumoural Treg associated with tumour invasiveness	High CD8 ⁺ /Treg ratio correlates with improved disease free survival and overall survival	(234)
Follicular Lymphoma	97(stage I, n = 24; stage II, n = 57, stage III, n = 16)	Reduced FOXP3 ⁺ number in diffuse tumours compared to follicular	FOXP3 ⁺ number decreases with disease severity	High FOXP3 ⁺ correlated to overall improved survival	(235)
Classical Hodgkins Lymphoma	98	No significant increase in FOXP3 ⁺ cell number around Hodgkin/ReedSt ernberg cells	High GranzymeB/FOXP3 ⁺ ratio linked to improved overall survival	High FOXP3 ⁺ number associated with improved prognosis	(236)

Gastric	110 (stage II, n = 46; stage III, n = 64)	High FOXP3 ⁺ number in tumour compared to normal gastric tissue	Increased FOXP3 ⁺ number linked to increased vascular invasion	High FOXP3 ⁺ number associated with decreased relapse free survival and overall survival	(237)
	133 (stage I, n = 38; stage II, n = 28; stage III, n = 59; stage IV, n = 8)	Higher FOXP3 ⁺ intratumoural versus peritumoural sites	CD4 ⁺ and CD8 ⁺ tumour infiltrating lymphocytes not associated with overall survival	Low intratumoural FOXP3 ⁺ cell number linked to improved disease free survival. High CD8 ⁺ / FOXP3 ⁺ ratio linked to improved disease free survival	(172)

Key – FIGO (Federation Internationale de Gynecologie et d'Obstetrique)

1.6.1 Mechanisms of Treg-mediated Immune Suppression

Treg can influence the activity of diverse effector cell populations including, CD8⁺ T lymphocytes, DC, NK, natural killer T (NKT) cells, and B lymphocytes (238). Several mechanisms of Treg-mediated immune suppression have been identified and can be categorised into cell contact-dependent and contact-independent suppression respectively.

It is currently unclear whether Treg primarily suppress through cell-cell contact or through expression/secretion of paracrine factors. Strong *in vitro* evidence suggests that Treg fail to suppress when cell-cell contact is prevented i.e in a transwell system (239,240). Work by *Rudensky et al.* (241) in IL-10^{-/-} mice, demonstrated that IL-10 produced by Treg is not required for limiting systemic autoimmunity but is necessary for restraining immunological hyperactivity at environmental interfaces. In contrast, CTLA-4 ablation in Treg cells resulted in systemic lymphoproliferative syndrome and severe pancreatic lesions, whereas the colon and skin remained largely unaffected (242). These results strongly suggest that Treg utilise multiple non redundant or partially redundant mechanisms to limit the immune response with individual suppressor mechanisms potentially operating in a particular tissue or inflammatory setting (Figure 1.5).

1.6.1.1 Cell Contact-dependent Suppression

Cytotoxic T lymphocyte-antigen 4 (CTLA-4) is constitutively expressed by Treg under the direct control of FOXP3 (203,243). Antagonism of CTLA-4 has been shown to abrogate Treg-mediated suppression (244,245) indicating an important role in the regulatory capacity of Treg. CTLA-4 has a much higher binding affinity for CD28 than that of either of the co-stimulatory molecules

CD80 and CD86 (246,247). In addition CTLA-4 can also bind CD80 and CD86 adding further competition against their successful interaction with the co-stimulatory molecule CD28 (248). Interaction of CTLA-4 with professional antigen-presenting cells (APC) leads to down regulation of the co-stimulatory molecules CD80 and CD86 (249); crucial for activation of naïve and memory T lymphocytes (250). Interaction of CTLA-4 with CD80/86 has also been shown to induce DC expression of IDO, the rate limiting enzyme in the catabolism of tryptophan; essential for the proliferation/survival of activated T cells (251,252).

Membrane bound TGF- β on Treg has been shown *in vitro* to mediate cell-contact dependent immunosuppression. *Nakamura et al.* (253) demonstrated that high membrane expression of TGF- β was responsible for suppression by Treg. In this system, soluble TGF- β was found to be low; however treatment with a depleting anti-TGF- β 1 antibody blocked Treg-mediated suppression (253). Following TCR stimulation, TGF-beta receptor II expression on effector T cells is induced. Experiments utilising a double negative TGF-beta receptor II B6 mouse model of type I diabetes demonstrated that antigen activated CD8⁺ T cells could not be suppressed by adoptive transfer of membrane bound TGF-beta positive Treg, suggesting that this axis is important in regulating immune responses (254).

Treg can transfer cAMP through gap junctions, formed with responder cells (255) inhibiting key proliferative pathways; notably STAT5 which is responsible for IL-2 receptor transduction (256). Further, over-expression of cAMP-induced ICER (inducible cAMP early repressor), has been identified in both murine and human Treg (257,258). ICER can bind NFAT/AP1 sites within the IL-2 promoter suppressing transcription (259). Interestingly, this

inhibitory complex is also up-regulated in CD4⁺ T cells upon contact with Treg (255,258).

CD8⁺ T cells and NK use perforin and granzyme B to mediate cytotoxicity against target cells. Similarly, activated Treg cells express granzyme A and perforin, permitting lysis of effector T cells and APCs (260). Additionally, experiments using Granzyme B and perforin deficient mice demonstrate that Treg can mediate immune suppression through a granzyme B dependent but perforin independent mechanism (261).

1.6.1.2 Cell Contact-independent Suppression

Elevated levels of a plethora of immune-modulators including, vascular endothelial growth factor (129), PGE₂ (262), IL-10 (155,263) and TGF-β (264) have been characterised in human cancers. This tumour-associated milieu, in addition to supporting tumour cell survival and proliferation is potently immune suppressive (265-267). Treg, through the production of IL-10 (268), TGF-β (269), IL-35 (270) and adenosine (84) contribute to this immune suppressive microenvironment. Activation of DC in the presence of Treg has been shown to induce a suppressive phenotype characterised by high IL-10 expression (271) and expression of B7-H4; a negative regulator of effector T cell function (166).

Due to their elevated expression of CD25, Tregs consume local IL-2, depriving actively dividing effector T cells of the IL-2 required for their survival and expansion (272,273). However, *Zambricki et al.* (274) suggested that the *in vitro* relevance of IL-2 sequestration on T cell functionality is unclear with IL-15 able to compensate for the loss of IL-2. Further, T cells persist in IL-2^{-/-}

mice suggesting alternative survival factors (275). Yates *et al.* (276) showed that IL-4, IL-7 and IL-15 were able to maintain the suppressive potency of human Treg *in vitro*. In addition to this, *in vitro* studies monitoring IL-2 and IFN- γ gene expression and production in human peripheral blood lymphocytes demonstrated that Treg-induced suppression occurred independent of IL-2 consumption (258).

Mounting evidence suggests that regulatory T cells play a major role both in the development of malignancy and in the response to anti-cancer therapies by mediating tumour-immune escape. Successful management of Treg through inhibition of the function, restriction of tumour trafficking or enhancement of cytotoxic T cell activity can influence the local immunological milieu which in turn may determine the outcome of local and systemic immune responses.

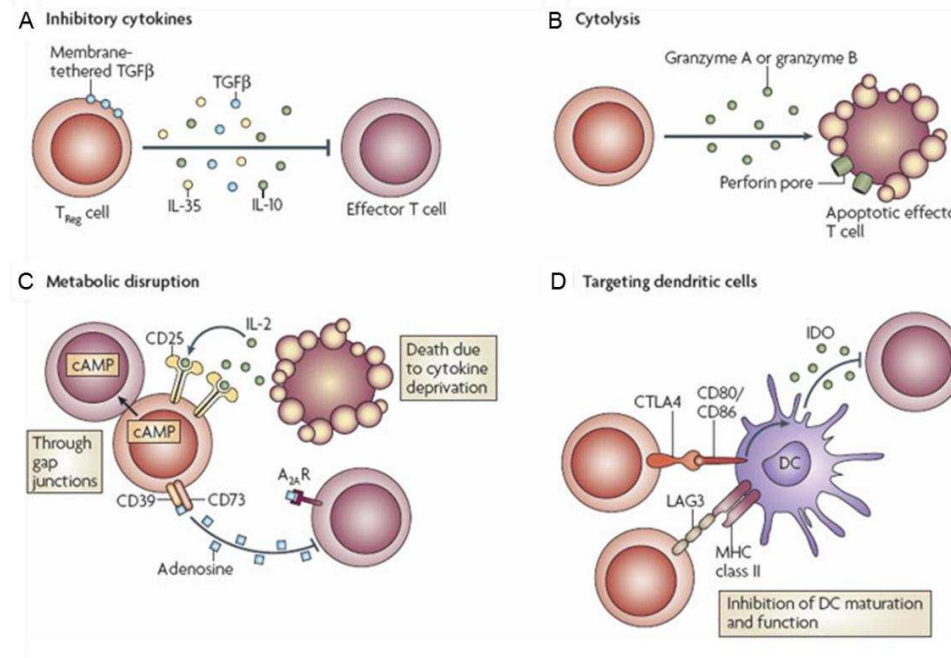


Figure 1.5 – Proposed Mechanisms of Treg Suppression. **A** – Inhibitory cytokines include IL-10, IL-35 and TGF β . **B** - Cytotoxicity includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. **C** - Metabolic disruption includes high-affinity CD25 (also known as IL-2 receptor α) -dependent cytokine-deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A (A_{2A}R)-mediated immunosuppression. **D** - Targeting dendritic cells (DCs) includes mechanisms that modulate DC maturation and/or function such as lymphocyte-activation gene 3 –MHC-class-II-mediated suppression of DC maturation, and CTLA4–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs. Adapted from (268). Reprinted with permission (License number 2812720344296), Nature Publishing Group Ltd

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Chapter 2

Project Aims

The evolution of surgical techniques and chemotherapy regimens over the past three decades has resulted in improvements in ovarian cancer treatment (1,2). Debulking surgery remains key in ovarian cancer treatment while adjuvant chemotherapy improves both overall and progression-free survival in all patient subgroups. Evidence from several randomised controlled clinical trials has now established that the platinum–paclitaxel combination regimen as first-line treatment for advanced ovarian cancer, yielding response rates of over 80% and 40–60% complete responses (1,3-7). However, these patients will eventually relapse with a median progression-free survival of 18 months (8).

However, the response rates to treatment decrease with each subsequent relapse following the development of drug resistance. To overcome the poor prognosis of ovarian cancer, immunotherapeutic strategies have been devised such as the use of an anti-CTLA-4 antibody in melanoma.

Although various immunotherapeutic approaches have been examined for the treatment of ovarian cancer, it remains true that no such therapy has entered into the clinical arena. This is due to several challenges that need to be overcome. When patients are diagnosed with cancer, by definition, the tumour has “escaped” the immune system, having passed the phases of “elimination” and “equilibrium”. Although there is no shortage of ovarian cancer antigens due to genomic instability and accumulation of mutated genes at this point, the generation of immune responses against these

antigens is likely to be unproductive in the later stages of disease, due to multiple immune tolerance mechanisms as described in Chapter 1.

At the outset of this work, careful consideration was needed to select tumour immune suppressive mechanisms that could be targeted with existing compounds. This thesis explores, in principle, a strategy to propagate an anti-ovarian cancer immune response by targeting three different facets of ovarian cancer immunity; Regulatory T cell (Treg) migration, poor release of the tumour-associated antigen, MUC1 and reduced cytotoxic T cell (CTL) proliferation (Figure 2.1).

As mentioned above tumours develop an environment which is conducive to their survival. In addition, this tumour-associated milieu supports a network of immunosuppressive adaptations favouring the accumulation of Treg; potent suppressors of CD8⁺ T cell-mediated cytotoxicity (9). An increase in tumour infiltrating Treg through the CCR4-CCL17/CCL22 chemokine axis has been well documented in progressive ovarian cancer (10,11). Courtesy of AstraZeneca, a CCR4 antagonist, AZ1, designed for use as an adjuvant in Asthma, blocks receptor internalisation and thus downstream activation of the cell. Inhibiting CCR4 activation on Treg could provide a method by which to restrict Treg accumulation at the tumour site.

However, it is important to assume that inhibition of Treg migration would not be sufficient in inducing an anti-tumour immune response. Therefore, other targets must be employed to fully enhance any anti-tumour activity. The availability of antigen is the basis for a T cell response (12). As tumours develop, less antigen is presented for immune detection. Current evidence suggests that tumour cell death could be immunogenic, with a release of tumour antigen being observed (13). It would, therefore, be logical to use

chemotherapy, in combination with immunotherapeutic adjuvants to reduce tumour burden. However, current chemotherapeutic agents, such as Camptothecin, are well-known for possessing severe lymphopaenic adverse effects. This would be counter-productive when attempting to enhance the anti-tumour immune response. To overcome this, targeted delivery of chemotherapeutic agents is required. This can be achieved through encapsulation of the drug in polymeric micelles, which can 'direct' the drug to the site of action whilst 'shielding' it from off-target effects. In doing so, tumour cell death and tumour-associated antigen release would increase while the tumour immune infiltrate and peripheral immune population would remain intact.

In order to take advantage of the increase in tumour-associated antigen and the debulking of the tumour mass, it is critical to induce an effective and prolonged enhancement of CTL proliferation. Toll-like receptors are well known for initiating immune responses. Of particular interest in this thesis is the distribution of toll-like receptor 7 (TLR7). TLR7 is expressed on CTLs and on plasmacytoid DC (pDC), a type of dendritic cell which accumulates in ovarian cancer (14). Crucially, Treg are described as also possessing this receptor. The TLR7 agonist Imiquimod has been used to treat basal cell carcinoma and malignant melanoma and has been shown to enhance not only CTL (15) and pDC (16) activity but reduce Treg suppressive activity (17).

In order to develop and validate this strategy, Treg needed to be purified from whole blood and demonstrate their suppressive properties (Chapter 3). Proportionally, Treg represent a small fraction of the total PBMC population and as such their use in downstream experimentation is hampered by lack of numbers. Treg expansion has been a burgeoning field of interest due to its

potential in auto-immune disease (18). Thus a Treg expansion protocol was conceptualised. Isolated and expanded Treg demonstrated their suppressive activity against CD8⁺ and CD4⁺CD25⁻ T effector cells (key mediators of any anti-tumour immune response (19)).

Chapter 4 shows the development of a suitable cell migration assay to reproduce the infiltrating capacity of Treg via the CCR4-CCL17/CCL22 axis. The demonstration of CCR4 expression on a variety of immune cells and tumour cells was critical in determining the specificity of this target. Conversely, cancer cells needed to show the capacity to produce CCL17/CCL22. Once chemokine-dependent Treg migration had been demonstrated the effects of the CCR4 antagonist, AZ1, were investigated.

In Chapter 5, the effect of Camptothecin on the release of the tumour-associated antigen MUC1 from ovarian cancer cells and its lymphopaenic adverse effects were assessed. This was then compared to the effects of Camptothecin incorporated into polymeric micelles to determine whether 'targeted delivery' of chemotherapeutics would be a successful way of enhancing antigen release while retaining immune cell function.

Finally, in Chapter 6 the effect of the TLR7 agonist, Imiquimod, on CTL and pDC stimulation as well as Treg suppressive activity was assessed. Activation with Imiquimod produced a pro-inflammatory cytokine milieu as well as induce pDC to cross-present antigen (20). Using the evidence of enhanced pDC activation, an attempt was made to generate MUC1-specific cytotoxic T cells.

In summary, this thesis explores, in principle, the potential of polypharmacy to treat ovarian cancer using existing compounds. The treatment stratagem suggested herewith, aims to demonstrate a reduction in the immune

suppressive and chemotactic behaviour of Treg as well as a reduction in tumour cell migration. It is intimated that the combination of chemo- and immunotherapy aides tumour debulking, releases the tumour-associated antigen MUC1 and improves the subsequent presentation and generation of antigen specific-cytotoxic T cells (Figure 2.1). The stratagem highlights the potential benefit of carefully selecting immune suppressive mechanisms that can be taken advantage of using readily available compounds rather than developing costly new drugs. The efficacy of existing drugs can be enhanced when used synergistically and therefore provides a unique perspective on cancer immunotherapy.

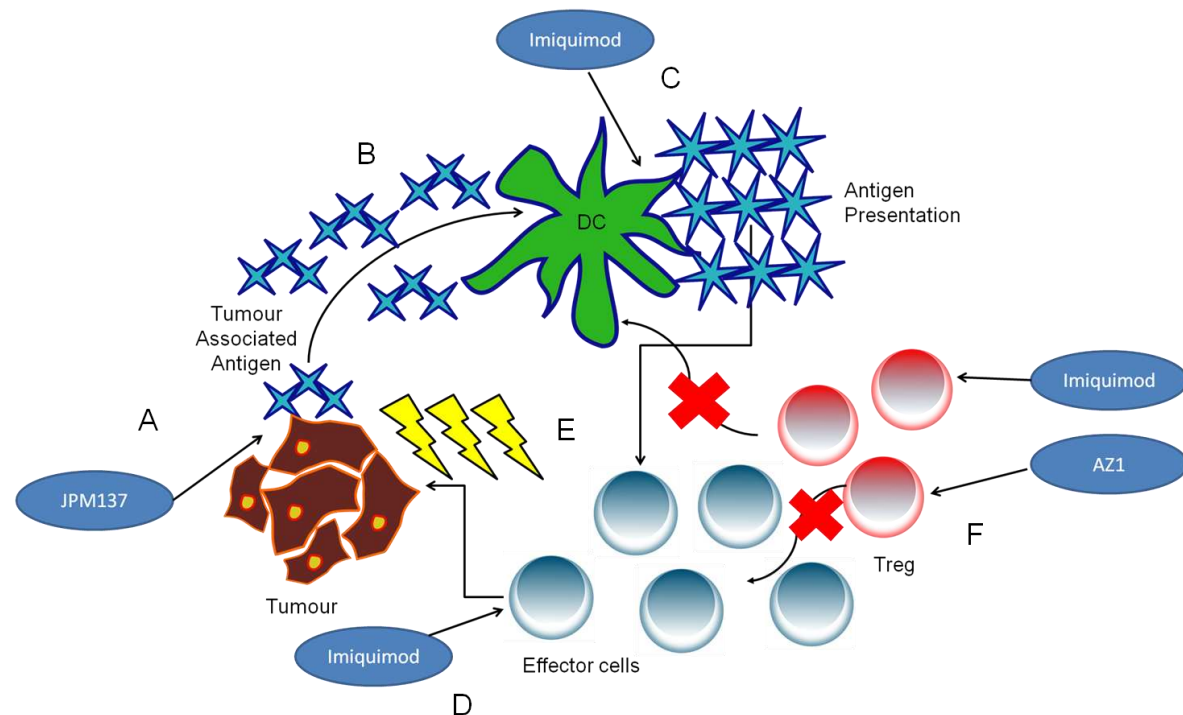


Figure 2.1 – The Suggested Effect of JPM137, Imiquimod and AZ1 on Tumour Immunity. **A** – JPM137 will localise at the tumour site and cause cytolysis. **B**- Tumour cell death will lead to the release of tumour associated antigen. **C** – Imiquimod activates dendritic cells at tumour draining- and peripheral- lymph nodes causing antigen uptake, improved antigen presentation and generation of antigen specific cytotoxic T cells. **D** – Imiquimod induces the proliferation of CD8⁺ T cells and improves the production of pro-inflammatory cytokines (E). **F** – Imiquimod reduces the suppressive effects of Treg while AZ1 blocks their migration to the tumour site (denoted by red crosses). The net result would be a reduction in tumour burden and a durable anti-tumour immune response.

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Chapter 3

Isolation, Expansion and Functionality of Regulatory T cells

3.1 Introduction

Several subsets of T regulatory cells have been described in humans. The CD4 derived major Treg populations include natural T regulatory cells (Treg) which originate in the thymus (1-5), induced Treg (iTreg) derived from naive CD4⁺ T cells in the periphery (6,7), Tr1 cells which secrete predominantly IL-10 (8,9) and Th3 cells which secrete predominantly TGF- β (10). Other cell populations such as NKT cells and CD8 T suppressor cells can also mediate immune regulation. As discussed previously, natural Treg play a key role in inducing and maintaining immunological tolerance, immune homeostasis and tumour immunity (1-3,5,11). These cells have been implicated in the suppression of anti-tumour immunity and have been repeatedly identified in several clinical settings to correlate with negative outcome (Table 1.2). Natural Treg constitute a minor population in peripheral blood with a frequency of 1–2% of total circulating CD4⁺ T cells (12). In order to progress the work in this thesis, Treg needed to be isolated from human blood and, due to their small number, expanded to a suitable population size for experimentation.

Expanded CD4⁺ T cells should ideally have the characteristics of Treg i.e. a

stable phenotype of CD25^{hi}FOXP3⁺ expression and a retention of their functional ability to suppress immune reactive T cells. To accomplish the goal of expanding Treg *ex-vivo* for experimental purposes, it is critical to begin with the appropriate starting cell population and to use culture conditions that selectively favour the expansion of Tregs with properties characteristic of a Treg lineage.

3.1.1 The Definition of Treg?

In humans there is no Treg-specific cell surface marker which would allow for their isolation and no clear understanding of how they function to control immune responses *In vivo*. The majority of CD4⁺CD25⁺ Treg are produced by the thymus with a repertoire of antigen specificities that are as broad as that of naïve T cells. They are capable of recognising both self and non-self antigens and control various immune responses such effector T cell proliferation and DC activation (1-3). A specific role of FOXP3 in the development and function of natural CD4⁺CD25⁺ Treg has been described (13-15). Mutations of the human gene FOXP3, similar to the gene mutated in scurfy mice (*Foxp3*) were found to be the cause of IPEX syndrome which has X linked inheritance and is characterised by polyendocrinopathy, enteropathy and immune dysregulation (16,17). However, expression of FOXP3 is not sufficient to designate cells as Treg and FOXP3⁺CD4⁺ cells may in fact be composed of a mixture of Treg derived from the thymus, iTreg generated in the periphery from CD4⁺ naive cells and activated non-regulatory CD4⁺ cells which may not have suppressor activity. Therefore the functional characteristics that differentiate true Treg from other subsets are critical for defining Treg that are used in subsequent assays during this project.

For the magnetic isolation of Treg, peripheral CD4⁺ cells with a high expression of CD25 were utilised as the main markers. The choice of markers on which to isolate human Tregs for expansion has been controversial. As FOXP3 is an intracellular protein it cannot be used to isolate viable cells. CD25 represents the α chain of the IL-2 receptor that is essential for the generation and maintenance of Treg. The high expression of CD25 is frequently taken advantage of in protocols for isolating as well as targeting peripheral Treg. However, CD25 is also upregulated upon cellular activation, thus recently activated effector CD4⁺ T cells may be confused with Treg and iTreg. Nevertheless there are differences between CD4⁺CD25⁺ Treg and activated T cells with respect to the characteristics of CD25 expression. Human and mouse CD4⁺ cells with potent regulatory properties express high and sustained levels of CD25, whereas recently activated T cells express transient and low levels of CD25 (11,12). Thus a stable and high expression of CD25 is an essential characteristic of Treg. Throughout the course of this thesis, the expanded Treg population were derived from the CD4⁺CD25⁺ cell fraction which maintained a stable, high expression of CD25.

Other markers, such as latency-associated peptide (LAP) and IL-1 receptor type I & II (CD121a/CD121b) have also been used for Treg. These markers are not expressed on resting FOXP3⁺Treg, but are rapidly induced and expressed for a short time period after TCR-mediated activation (18). Thus these markers can only isolate TCR activated FOXP3⁺ Treg but not resting, peripheral FOXP3⁺ Treg. Other markers have been ascribed to natural Treg, e.g. CTLA-4 and GITR but, currently, the most reliable marker for natural Treg is FOXP3 when applied in conjunction with the other properties of Treg.

A feature that is increasingly used for isolating Treg from blood is the absence of CD127, the IL-7 receptor alpha subunit, which is abundantly expressed on naïve cells. There are several reasons to suggest that CD127 negativity should not be used to select the initial starting population for Treg expansion. Firstly, there is the potential to eliminate thymic derived resting precursors of Treg which may express CD127. Recent data of Treg expansion using umbilical cord blood which is enriched in naïve cells support this. Umbilical cord blood Treg isolated by positive selection using either AutoMACS or CliniMACS based on CD4⁺CD25⁺ expression and not on the absence of CD127 expression, were cultured with anti-CD3/CD28 mAb coated Dynabeads with IL2 and Rapamycin (19,20) and showed approximately 100-fold (19) to 199-fold (20) expansion. Foxp3 expression was 72.6% in one report (20) and they exhibited potent suppressor activity of ~95% (18) and 58 +/- 11% (20) respectively in allogeneic mixed lymphocyte reaction. In freshly isolated CD4⁺CD25⁺ populations, the expression of CD127 was approximately 1%, and the final expanded population was negative for CD127.

This suggests that CD127 negativity may be more useful for characterising functional expanded Treg rather than for initial selection of the population to be expanded. Another reason against using the absence of CD127 expression for selecting the initial population is that CD127 negativity as a biomarker cannot discriminate between Treg and T effector cells (9,21). Upon cellular activation CD127 is down-regulated in CD4⁺ cells including CD4⁺CD25⁺ Treg. Thus when CD127 low/- expression is used in combination with CD25⁺ expression for isolating Treg, it can concentrate a heterogeneous subpopulation of cells consisting of Treg, iTreg and activated CD4⁺CD25⁺ non-Treg which can

transiently express FOXP3. Thus, the CD4⁺CD25⁺CD127^{low/-} population may have greater potential for differentiating into cytokine secreting effector cells. A previous study that has used FACS sorting for isolating CD4⁺Treg based on CD25 expression and CD127 negativity and subsequent expansion resulted in contamination with effector cells based on their cytokine profile (22). In that report, the expanded cells, despite showing FOXP3 expression of ~95%, manifested substantial cytokine producing cells. This is an important criterion because the mechanism of natural Treg function is by cell-to-cell interaction and not via secretion of cytokines IL-10 and TGF- β which are rarely found in the supernatants of *in vitro* Treg assays and that the use of anti-IL-10 or anti-TGF- β antibodies fails to abrogate suppression (3). Another distinct subset of regulatory T cells (Tr1) suppresses immune responses via cell-to-cell interactions and/or the production of IL-10 and TGF- β (8,9) for a variety of antigens (9). IL-10 is also secreted by other cells like Th2 cells (23,24), macrophages (25,26), monocytes (27) and dendritic cells (28).

3.1.2 Expansion of Treg

The expansion of Treg both *in vivo* and *in vitro* provides useful information pertaining to their ontogeny and existence within the tumour microenvironment.

3.1.2.1 In vivo

As Treg are present in the immune system as a functionally distinct and mature population with a diverse TCR repertoire, mere clonal expansion of Treg through appropriate pathways of antigenic stimulation leads to induction of antigen-specific immune suppression. For example, Treg specific for islet antigen were

shown to be more potent in suppressing diabetes in NOD mice than polyclonally activated Treg (29,30).

Allergen-specific immunotherapy (SIT), which consists of repeated subcutaneous or sublingual administration of allergen, has been widely used and proved effective for the management of allergies (31). The mechanism of the therapeutic effect of SIT is not completely understood (32) but might involve the activation and expansion of Treg (33,34) along with the induction of adaptive Treg, such as IL-10-secreting Tr1 cells (35,36). Regarding the mechanism of SIT, it is of note that Treg can be activated to exert suppression at a much lower concentration of antigen than required for activation of naive T cells (37).

As mentioned above, Treg bear a T-cell receptor (TCR) repertoire that is as broad and diverse as that of naive T cells yet is more self-reactive than the latter. This means that the TCRs of Treg bear a higher affinity than other T cells for the class II MHC/self-peptide ligands, positively selecting them in the thymus (2). They also express a higher level of accessory molecules, including adhesion molecules, such as lymphocyte function associated 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) (38).

These unique immunological features of Treg make them well suited for controlling self-reactive T cells efficiently and swiftly during episodes when self-antigens are aberrantly or excessively presented in the immune system.

By exploiting the immunologic characteristics that differentiate Treg from other T cells, the expansion of antigen-specific Treg cells can be achieved by a controlled exposure to antigen, and alteration of the cytokine milieu while suppressing the activation and expansion of effector T cells. The immune

privileged site of the tumour microenvironment could provide the basis for the generation of tumour antigen specific Treg (39). It is suggested that before the development of systemic metastasis, antigenic cancer cells (i.e. those that express tumour antigen) are embedded in the solid tumour. The stroma of the tumour prevents the efficient release of TAAs (40) which are ignored in conventional sites of antigen priming and presentation — the draining lymph nodes (41,42).

Initially, tumour antigen is thought to be efficiently released, which propagates anti-tumour responses. However, several tolerogenic mechanisms, as discussed before, would already be upregulated in the surrounding microenvironment. This would cause the abrogation of the functions of APCs and other effector populations (43). This in turn allows tumour development to occur unchecked, leading to stroma development. The generation of dysfunctional APCs can not only induce T cell tolerance through soluble factors and tryptophan depletion but can also generate antigen specific Treg (39,44).

3.1.2.2 Polyclonal Expansion of Treg In vivo

In addition to the antigen- or TCR-based approaches to Treg expansion discussed above, control of Treg and effector populations via soluble factors or pharmacological agents can be conducive to establishing Treg cell-mediated dominant tolerance. This would occur either by expanding Treg, reducing effector T cells, or both.

As mentioned previously the immune suppressive milieu which is associated with malignancy can lead to enrichment in the Treg fraction of tumour-immune infiltrates. Naïve CD4⁺CD25⁻ cells can be 'converted' to an almost identical Treg

phenotype through a combination of cytokines such as IL-10 and TGF- β (45), a lack of IL-2 (46) and induced anergy via tryptophan depletion (47) and increased extracellular adenosine (48).

Several pharmacological treatments can lead to increased numbers of Treg. Administration of IL-2, a key cytokine for the development and, in particular, the maintenance of Treg might facilitate the expansion of antigen-stimulated Treg cells in the periphery or at the site of inflammation. IL-2 is pleiotropic in function; in addition to maintaining Treg, it induces activation-induced cell death in activated T cells, maintains CD8⁺ cytotoxic T cells, and inhibits Th17 cell differentiation (49). Drugs that facilitate immune suppression such as the cancer chemotherapeutics, Cyclosporine and Rapamycin lead to a deletion in proliferating effector populations thus leaving a proportionally greater Treg fraction (50).

3.1.2.3 In vitro Expansion of Treg

Approaches to activate and expand Treg *ex-vivo* are now well established. Most involve the use of anti-CD3 plus anti-CD28 mAb co-stimulation together with IL-2. More recently Rapamycin, a macrocyclic lactone commonly used in organ transplantation to prevent acute rejection (51) has been used as an adjuvant in Treg expansion. The enrichment of Treg by Rapamycin treatment can be attributed to their relative resistance to apoptosis/necrosis because of different modes of activation in the phosphoinositide 3-kinase/Akt/mammalian target of the Rapamycin (mTOR)-signaling pathway in Treg cells and effector T cells (52).

Retinoic acid, a derivative of vitamin A, plays an important role in T cell function and trafficking and has been postulated as an alternative to Rapamycin to promote the expansion of Treg. All-Trans Retinoic Acid (ATRA) produced by DC facilitates *de-novo* generation of FOXP3⁺ T regulatory cells from CD25⁻ T cell populations in mice (53,54). Two non-mutually exclusive mechanisms have been proposed to account for the ATRA-promoted induction of suppressive T cells. One set of data indicates that ATRA augments TGF- β mediated signaling (55,56), while other investigators report that ATRA suppresses the ability of memory T cells to block the induction of FOXP3 expressing Treg (57). Many studies have tried to mimic the *in vivo* conversion of CD4⁺CD25⁻ T effector cells to Treg by ATRA *in vitro* as a means to rapidly generate suppressive T cells (58-60). Two studies have reported that human CD4⁺CD25⁻ T cells derived from adult peripheral and cord blood were converted to suppressive cells in the presence of TGF- β and ATRA (61,62). One of these groups also performed a series of experiments examining how ATRA affected the expansion and function of natural Treg and concluded that ATRA augmented their suppressive activity and should therefore be considered for use in Treg-based therapy (62).

Studies have also indicated that Treg can be generated from CD4⁺CD25⁻ T cells through Foxp3 induction in response to a variety of stimuli *in vitro* (63-65). One group has described the generation of antigen-specific Treg from human CD4⁺CD25⁻ T cells. This group had previously shown that anti-CD3 activation of human CD4⁺CD25⁻ cells in the presence of APC lead to the induction of FOXP3 and suppressive activity in a population of cells that remained CD25^{high} (66). Further work went on to show that stimulation of human CD4⁺CD25⁻ cells with APC and HA peptide resulted in the generation of a population of HA-specific

CD25^{high} cells that expressed Foxp3 (67). Antigen-specific cells were then isolated using HLA class II tetramers presenting the HA peptide. These cells required cognate antigen to activate suppressive function but once activated were capable of bystander suppression *in vitro*.

3.1.3 Functionality of Treg

With both isolated and expanded populations it is important to ascertain the suppressive activity of Treg against effector populations. Reports of standard assays for Treg suppression look at their impact on the proliferation of CD4⁺CD25⁻ effector T cells (68). This is a good starting point in the first instance as it enables optimisation of the assay conditions as well as providing useful data on the potency of Treg against other T helper cells. However, with regards to tumour immunity and the project as a whole, a large proportion of clinical data looks at CD8⁺/FOXP3⁺ ratio as a prognostic indicator for many cancers including ovarian malignancies (69). Therefore, assessing Treg function against CD8 T cells is also important.

The cytokine profile of Treg suppression assays can not only corroborate the observations seen during proliferation but also provide insight into the molecular basis of Treg activation when different stimuli are used. Critically, these assays lay the foundation for probing Treg function and gaining an understanding of the potential mechanism by which they operate.

3.1.4 Experimental Objectives described in Chapter 3

- Isolation of Treg from whole blood
- Demonstration of Treg suppressive activity against both CD4+CD25- and CD8+ T cells
- Development of a viable and cost effective Treg expansion protocol
- Demonstration of expanded Treg functionality

3.2 Methodology

3.2.1 Isolation of Regulatory T cells via Magnetic Selection (Dyna)

A maximum volume of 100 ml of peripheral blood was obtained from healthy donors in accordance with local ethical committee approval (EC# BT/04/2005). Peripheral blood mononuclear cells (PBMC) were separated via a density-gradient centrifugation over Histopaque-1077 (Sigma) twice, firstly to isolate PBMCs with the second density gradient used for the removal of platelets. The resultant cells were then washed and resuspended at 5×10^7 cells per 500 μ l isolation buffer containing phosphate buffered saline (PBS) + 1% w/v bovine serum albumin (BSA) (Sigma) + 1 mM EDTA (Sigma) before viability counting via Trypan Blue exclusion (Fluka). 200 μ l of CD4 Human Antibody Mix (Invitrogen) and 100% v/v foetal calf serum (Sigma) (FCS) were incubated with PBMC for 20 minutes. This was followed by the addition of 1 ml Depletion MyOne Dynabeads (Invitrogen) before magnetic removal of bead-bound non-CD4⁺ cells, leaving CD4⁺ cells in solution.

CD4⁺CD25⁺ T cells were isolated by positive selection using 200 μ l CD25 Dynabeads per 1.5×10^7 cells. The CD4⁺CD25⁺ Treg population was liberated from the CD25 Dynabeads using Detach-a-bead solution (Invitrogen) and were then used in downstream applications. The remaining CD4⁺CD25⁻ effector T cells were either used in T cell suppression assays or placed in RPMI-1640 (Sigma) + 10% v/v FCS (Sigma) + 5 mM L-Glutamine (Sigma) + 100 IU/ μ g/ml Pencillin/Streptomycin (Sigma) and 10% v/v DMSO (Sigma) before undergoing controlled freezing at -80 °C with prolonged storage in liquid nitrogen.

A small volume of cells (10 μ l) was taken at each isolation step for analysis via flow cytometry.

3.2.2 Isolation of Regulatory T cells via Magnetic Selection (MACS)

Peripheral blood mononuclear cells (PBMC) were separated via a density-gradient centrifugation as described previously. The resultant cells were then washed and resuspended at 1×10^7 cells per 90 μ l of isolation buffer containing phosphate buffered saline (PBS) + 1% w/v bovine serum albumin (BSA) (Sigma) + 1 mM EDTA (Sigma) before viability counting via Trypan Blue exclusion (Fluka). PBMC were then labeled with 10 μ l anti-biotin cocktail followed by 20 μ l anti-biotin Microbeads. The cell/bead mixture was applied to a primed 'LD' magnetic column which was subsequently washed several times before the enriched, bead-free CD4⁺ T cell population was eluted.

CD4⁺CD25⁺ T cells were selected via addition of CD25 Microbeads. The labeled cell suspension was then applied to a primed 'MS' magnetic column. The unlabeled CD4⁺CD25⁻ T cells were collected were either used in T cell suppression assays or placed in RPMI-1640 (Sigma) + 10% FCS (Sigma) + 5 mM L-Glutamine (Sigma) + 100 IU/ μ g/ml Pencillin/Streptomycin (Sigma) and 10% DMSO (Sigma) before undergoing controlled freezing at -80 °C with prolonged storage in liquid nitrogen. The bead bound CD4⁺CD25⁺ T cells were eluted from the column for downstream applications. A small volume (10 μ l) of cells was taken at each isolation step for analysis via flow cytometry.

3.2.3 Isolation of CD8⁺ T cells via Magnetic Selection

Peripheral blood mononuclear cells (PBMC) were separated via density-gradient centrifugation as described previously. The resultant cells were then washed and resuspended at 1×10^7 cells per 80 μ l of isolation buffer containing phosphate buffered saline (PBS) + 1% w/v bovine serum albumin (BSA) (Sigma) + 1 mM EDTA (Sigma) before viability counting via Trypan Blue exclusion (Fluka). Cells were labeled with 20 μ l CD8 Microbeads (Miltenyi Biotec) per 1×10^7 cells for 15 minutes before washing in isolation buffer. The cell/bead mixture was applied to a primed 'LD' magnetic column which was subsequently washed several times before the enriched, bead-free CD8⁺ T cell population was eluted. A small volume (10 μ l) of cells was taken for counting and phenotypic analysis via flow cytometry.

3.2.4 Flow Cytometry Analysis

Cells were stained with cell surface markers CD4-ECD (1.25 μ l) (clone SFC112T4D11 - Beckman Coulter), 2.5 μ l CD8-FITC (Clone HIT8a – BioLegend) CD25-PE (7.5 μ l) (Clone BC96 - BioLegend), CCR4-PerCP/Cy5.5 (2.5 μ l) (Clone TG6 - BioLegend). Cells were incubated with fluorescently-conjugated antibodies for 15 minutes before fixation with 3% v/v formaldehyde (Sigma). Cells were then permeabilised using FOXP3 Perm Buffer (BioLegend) before incubation with Foxp3-AlexaFluor488 (Clone 259D – BioLegend) for 30 min. Cells were then resuspended in FACS wash buffer (BioLegend) and read via a Beckman Coulter FC 500 flow cytometer. Analysis involved gating on lymphocyte populations from forward/side-scatter plots before selecting regions of interest.

3.2.5 Suppression Assays

T cell proliferation assays were set-up in triplicates in 96-well, round-bottomed plates with stimulated and non-stimulated divisions for comparison where possible. Briefly, CD4⁺CD25⁻ or CD8⁺ effector T cells were either resuscitated from frozen stocks or freshly isolated from whole blood. These were plated out at 5×10^4 cells/well in X-vivo-15 + 5% v/v Human AB serum. Treg were then added to each well to provide Teff:Treg ratios of 1:5, 1:10, 1:20, 1:40 to bring volume per well to 100 μ l. Cells were stimulated with 10 μ g/ml anti-CD3 (Clone-OKT, Biolegend) and 5 μ g/ml anti-CD28 (Clone – 28.2, Biolegend) or 10 μ g/ml of PHA (Sigma). The final volume was adjusted to 200 μ l using X-vivo-15 + 5% Human AB serum. After 72 hours, 100 μ l of cell supernatant was taken and stored at – 40 °C, the plate was then pulsed with 1 μ Ci of [³H]-labelled thymidine per well (TRA120, Amersham). The plate was left to incubate for a further 18 hours before being harvested onto a microscintillation plate (Perkin-Elmer). After repeated washes with de-ionised water, the plate was allowed to dry for an hour. 20 μ l of scintillation fluid (Microsint 0, Perkin-Elmer) was added per well before the plate was covered with optical tape (Perkin Elmer). Thymidine incorporation was quantified using a Packard TopCount NXT Microplate Scintillation counter.

3.2.6 Expansion of CD4⁺CD25⁺ T Cells

Isolated CD4⁺CD25⁺ T cells were divided into three experimental groups and placed into culture using X-Vivo-15 + 5% Human AB Serum (Lonza). The final cell concentration was maintained at 1×10^6 cells/ml. The first experimental group was supplemented with 1000 IU/ml recombinant IL-2 (Novartis) and stimulated

with 10 $\mu\text{l/ml}$ αCD28 superagonist (AnCell). The second group was supplemented with 1000 IU/ml recombinant IL-2 and stimulated with plate bound 1 $\mu\text{g/ml}$ αCD3 and soluble 1 $\mu\text{g/ml}$ αCD28 antibodies (Biolegend). The third group was as a negative control with no media supplementation or stimulation.

After days 3, 10 and 17, those cells being expanded were supplemented with 500 IU/ml recombinant IL-2. At days 7, 14 and 21, cells were collated and counted via Trypan Blue (Fluka). A sample was taken for analysis via flow cytometry. The cells were then re-suspended in culture media and re-stimulated as described previously.

3.2.7 Enzyme linked-immunosorbant Assay (ELISA)

Cytokine production was measured by ELISA. The cytokines of interest were IFN- γ and IL-10. Polystyrene 96-well plates (MaxSorp, Nunc) were coated with the relevant capture antibody (2 $\mu\text{g/ml}$ IFN- γ ; Clone MD-1 – BioLegend, 2 $\mu\text{g/ml}$ IL-10; Clone JES3-12G8, BioLegend) overnight. The plate was then blocked with 5% w/v skimmed milk powder in PBS (pH 7.2) + 0.05% v/v Tween (PBS/Tween) to prevent non-specific binding. The plate was washed in PBS/Tween using an automated plate washer and tapped dry. The relevant recombinant cytokine (PeproTech) and samples were added to the plate. After 2 hours, the plate was washed in PBS/Tween before the appropriate biotinylated detection antibody (1 $\mu\text{g/ml}$ IFN- γ , 0.5 $\mu\text{g/ml}$ IL-10; BioLegend) was diluted in PBS, 1% w/v BSA, and 0.1% v/v Tween 20 and incubated for 2 h at room temperature before being washed. 50 μl of 1 $\mu\text{g/ml}$ streptavidin-horseradish peroxidase (BD-Pharmingen) was added each well. After 30 minutes incubation at room temperature, the plate was washed and bound horseradish peroxidase

was visualised with 0.1 mg/ml tetramethylbenzidine (Sigma) and 2 ul of 30% v/v hydrogen peroxide (Sigma) diluted in 3 M sodium acetate buffer (Sigma). The colour reaction was stopped with addition of 2 M sulphuric acid (Sigma), and absorbance was measured at 450 nm using a MRX spectrophotometer (Dynex Technologies). The concentrations of samples were calculated from standard curves of each recombinant cytokine. ELISA detection limits were determined as follows (IFN- γ – 10 pg/ml, IL-10 – 2 pg/ml).

3.3 Results and Discussion

3.3.1 Magnetic Isolation of Treg from PBMC

Key to the outcome of this project was the successful isolation of Treg from healthy volunteers. Although several methods for the isolation of Treg were available, it was decided that magnetic isolation would be the most cost-effective approach. Magnetic isolation involves the selective labeling of target cell populations using antibody-bead complexes. Using magnetic fields, these cells can be separated from PBMC populations relatively easily with a generally high level of purity. Isolation systems from two companies, Invitrogen (Dyna) and Miltenyi Biotec (MACS) were assessed for their ease of use, cell purity and cost effectiveness.

In order for Treg to be isolated by this method, sequential magnetic separations are required. Due to Treg being an inherently small population (1 – 2% of total PBMC) (70), an enriched CD4⁺ T cell population is required. This can be achieved by either positive or negative selection of CD4⁺ cells. A further positive selection step is required to isolate those CD4⁺ cells which also highly express CD25. Figure 3.1 demonstrates the purification process of Treg from PBMC in a step-wise manner. Treg were strictly analysed as those cells which were CD4⁺CD25^{hi}FOXP3⁺. By maintaining such a strict analysis, it appears that the cells isolated are of a low purity. However, this ensures improved accuracy when attempting to quantify those Treg which have been expanded (Figure 3.9).

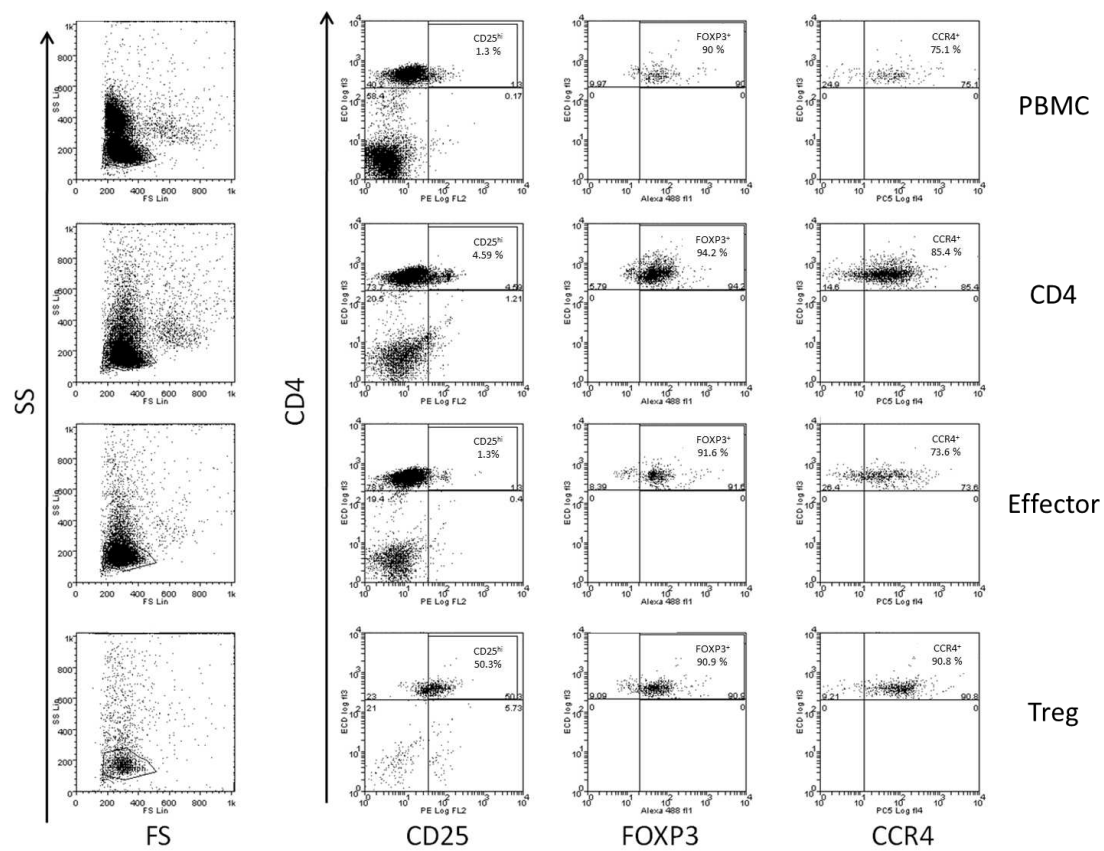


Figure 3.1 - Flow Cytometry Analysis of Cells Isolated using Magnetic Selection. Cells were stained for CD4, CD25, FOXP3 and CCR4 expression. Lymphocytes were identified from forward/side scatter plots before populations of interest were gated upon. Cells gated as CD4⁺CD25^{hi} were analysed for FOXP3 and CCR4 expression respectively. Analysis confirms CD4⁺CD25^{hi} Treg proportion as approximately 4% of total CD4⁺ T cells which agrees with those percentages found in literature.

3.3.2 Assessment of Vendors for Treg Isolation Kits

As previously mentioned, two vendors Invitrogen (Dyna) and of Miltenyi Biotec (MACS) of Treg magnetic selection kits were compared for ease of use, cell purity and cost effectiveness. Figure 3.2 represents the Treg purity obtained from the same donor using these two different kits. To calculate the purity of Treg the percentage of CD4⁺CD25^{hi} cells was multiplied by percentage of CD4⁺FOXP3⁺ cells. The purity of Treg (CD4⁺CD25⁺FOXP3⁺) isolated using Dyna was calculated to be 68.5% (CD4⁺CD25^{hi} – 74.9 x CD4⁺FOXP3⁺ - 91.6) compared to MACS which provided a purity of 40.7% (CD4⁺CD25^{hi} – 85 x CD4⁺FOXP3⁺ - 47.9).

These results meant that Dyna from Invitrogen was used as the main method for isolating Treg.

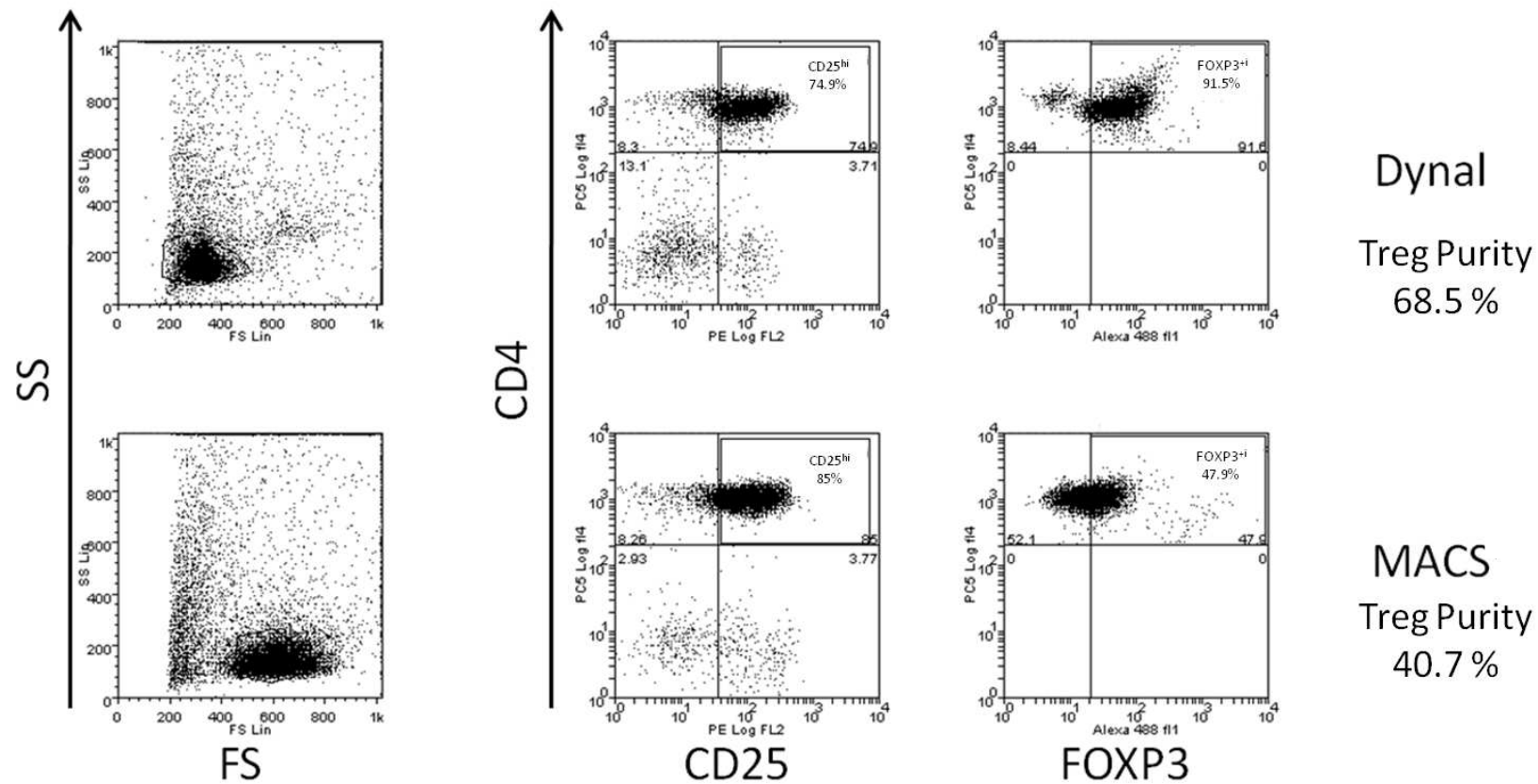


Figure 3.2 - Comparison of Treg Isolated from Dynal® and MACS® Regulatory T cell Magnetic Selection Kits. Cells were taken from the same donor and phenotyped as described previously for CD4, CD25 and FOXP3. Differences in the purity of isolated Treg (CD4⁺CD25⁺FOXP3⁺) were found with Dynal achieving 68.5 % purity compared to 40.7 % isolated via MACS. On the basis of this result and the respective costs involved when using each kit, Dynal were selected as the Treg isolation kit of choice.

3.3.3 Isolated Treg Suppress Effector T cell proliferation

The major characteristic of Treg is their ability to suppress the proliferation of multiple effector T cell populations. This can be demonstrated through the use of T cell proliferation assays. Figure 3.3 and Figure 3.5 show that these isolated Treg have suppressive activity against CD4⁺CD25⁻ T cells and CD8⁺ T cells which have been stimulated with PHA or anti-CD3/anti-CD28 respectively. Treg demonstrated a dose-dependent effect in suppressing CD4⁺CD25⁻ T cells which was statistically significant up to a 1/10 fraction (Figure 3.3; Effector vs. **1/10** (cpm) – 4086 vs. **1385**, $n = 6$; $p < 0.05$). With regards to CD8⁺ T cells, Treg again demonstrated a dose-dependent effect in suppressing proliferation which was statistically significant up to a 1/10 fraction (Figure 3.5; CD8 vs. **1/10** (cpm) – 19420 vs. **13375**, $n = 3$; $p < 0.05$).

Suppression at a 1/20 fraction was not significant for both CD4⁺CD25⁻ T cells (Figure 3.3; Effector vs. 1/20 (cpm) – 4086 vs. 4007, $n = 6$; $p < 0.05$) or CD8⁺ T cells (Figure 3.5; CD8⁺ vs. 1/20 (cpm) – 19420 vs. 17224, $n = 3$; $p < 0.05$). This suggests that there is a limit to which Treg can suppress effector T cell populations. Therefore it can be hypothesised that reducing the numbers of Treg within a co-inhabited system e.g. the tumour microenvironment, would allow greater effector T cell proliferation.

The supernatants of the suppression assays were analysed for classical pro-inflammatory (IFN- γ) and suppressive (IL-10) cytokines (Figure 3.4). Previous evidence dictates that effector populations should produce more IFN- γ and less IL-10 as Treg number decreases, and that Treg should not readily produce either IFN- γ or IL-10 when stimulated alone. The cytokine profiles seen when effector

populations have their TCR engaged with anti-CD3/anti-CD28 appear to corroborate with existing evidence. However when these cells are stimulated with PHA, there appears to be an increase in IFN- γ and a subsequent decrease in IL-10 with increasing Treg number. PHA stimulated Treg demonstrated significantly higher IFN- γ production compared to CD4⁺CD25⁻ T cells (Figure 3.4; **Treg vs. Effector** (ng/ml) **5.39** vs. 3.01, $n = 6$; $p < 0.05$).

This counter intuitive result could be explained by the existence of different molecular activation pathways in Treg compared to effector T cells. Recently, there have been several advances in defining how the intracellular signalling events in Treg differ from those in effector populations, particularly in regard to TCR activation. It is well defined that in order for T cells to be fully activated, proliferate and acquire effector functions, they must receive two activation signals. One signal via the TCR and the other via one of several co-stimulatory molecules expressed by APCs e.g. CD80/CD86. However, Treg remain hyporesponsive to TCR engagement, which is demonstrated by the lack of cytokine production seen when stimulated with anti-CD3/anti-CD28 (71,72) (Figure 3.7). This is due to reported differences in the Ras and phosphokinase C pathways where FOXP3 restricts the translocation and transcription of NF- κ B which leads to cytokine production (73) (Figure 3.8).

Yet, with mitogenic stimulation, such as the use of PHA, there is a bypassing of these pathways such that NF- κ B is activated (74), thus promoting production of cytokines such as IFN- γ and IL-10 which corroborates with the cytokine profile of PHA stimulated Treg (Figure 3.7). This suggests that NF- κ B induction could be advantageous in modulating Treg cytokine output.

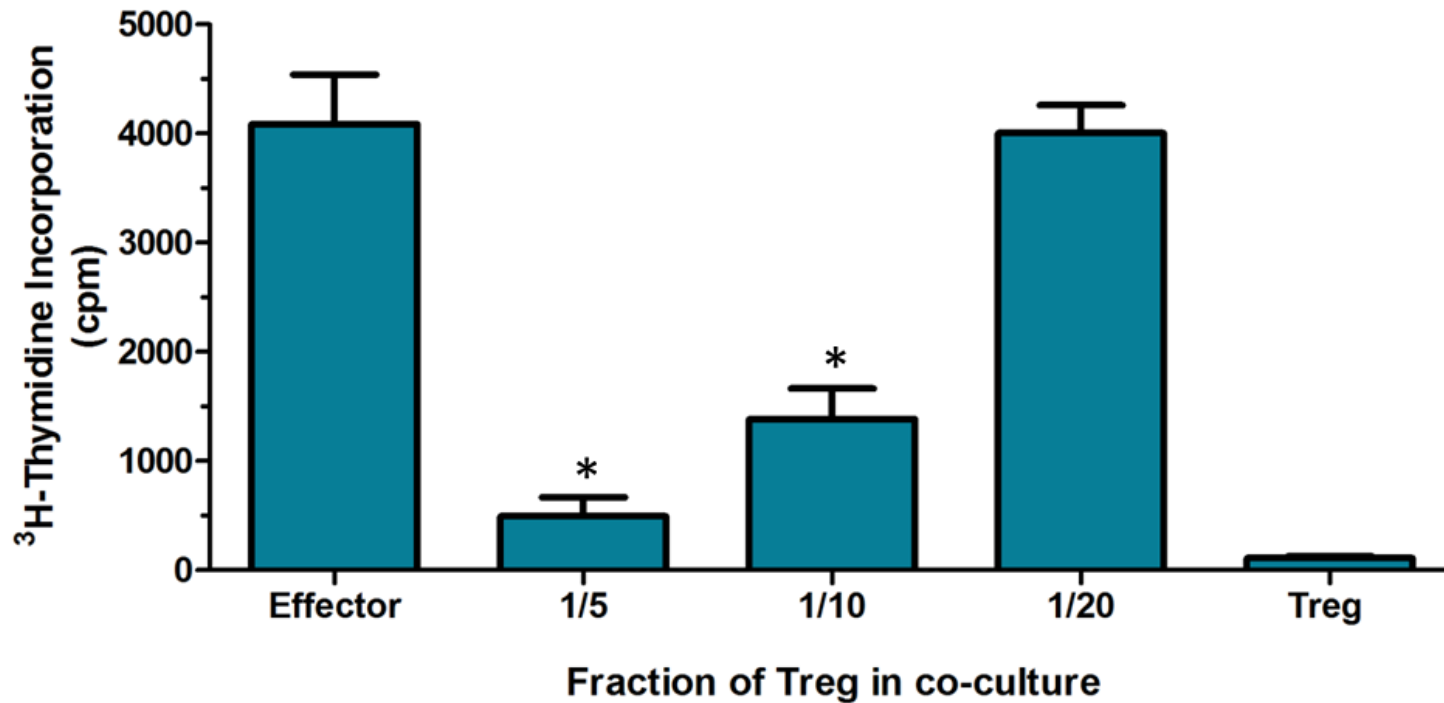


Figure 3.3 - Treg Suppress the Proliferation of PHA-stimulated CD4⁺CD25⁻ Effector T cells in a Dose-dependent Manner. Figure represents the means of six independent experiments from six different donors. Treg demonstrate suppression of CD4⁺CD25⁻ T cells where Treg were present at a 1/5 and 1/10 fraction of total co-culture. $n = 6$, $*=p<0.05$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

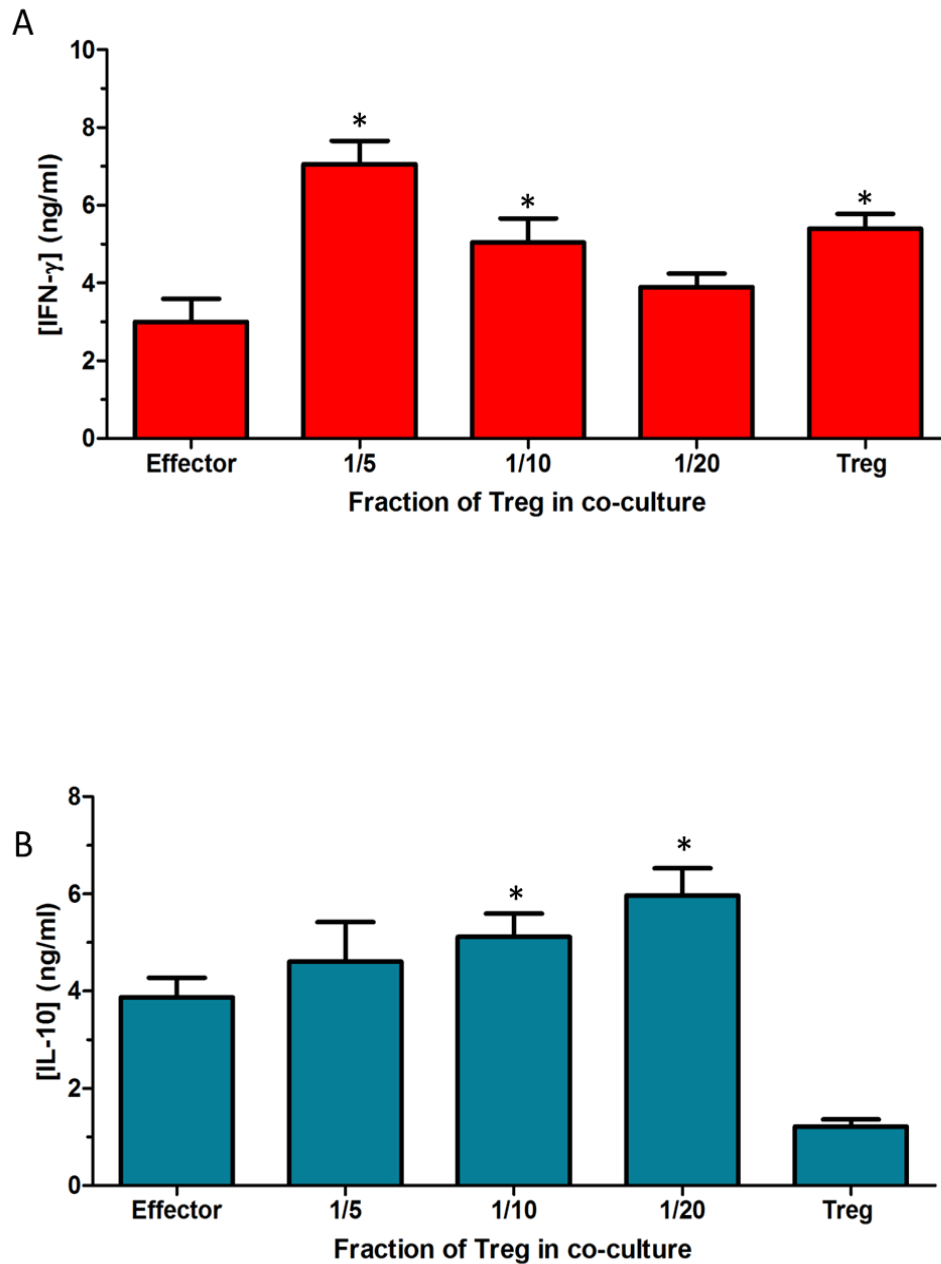


Figure 3.4 - Production of IL-10 and IFN- γ from T cell Suppression Assays Stimulated with PHA. Treg demonstrate an increase in IFN- γ and a decrease in IL-10 when stimulated with PHA. **A** - IFN- γ production. **B** - IL-10 production. Figures represent the means of six independent experiments from six different donors. Error bars represent standard deviations. *= $p < 0.05$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Detection limit = 40 pg/ml.

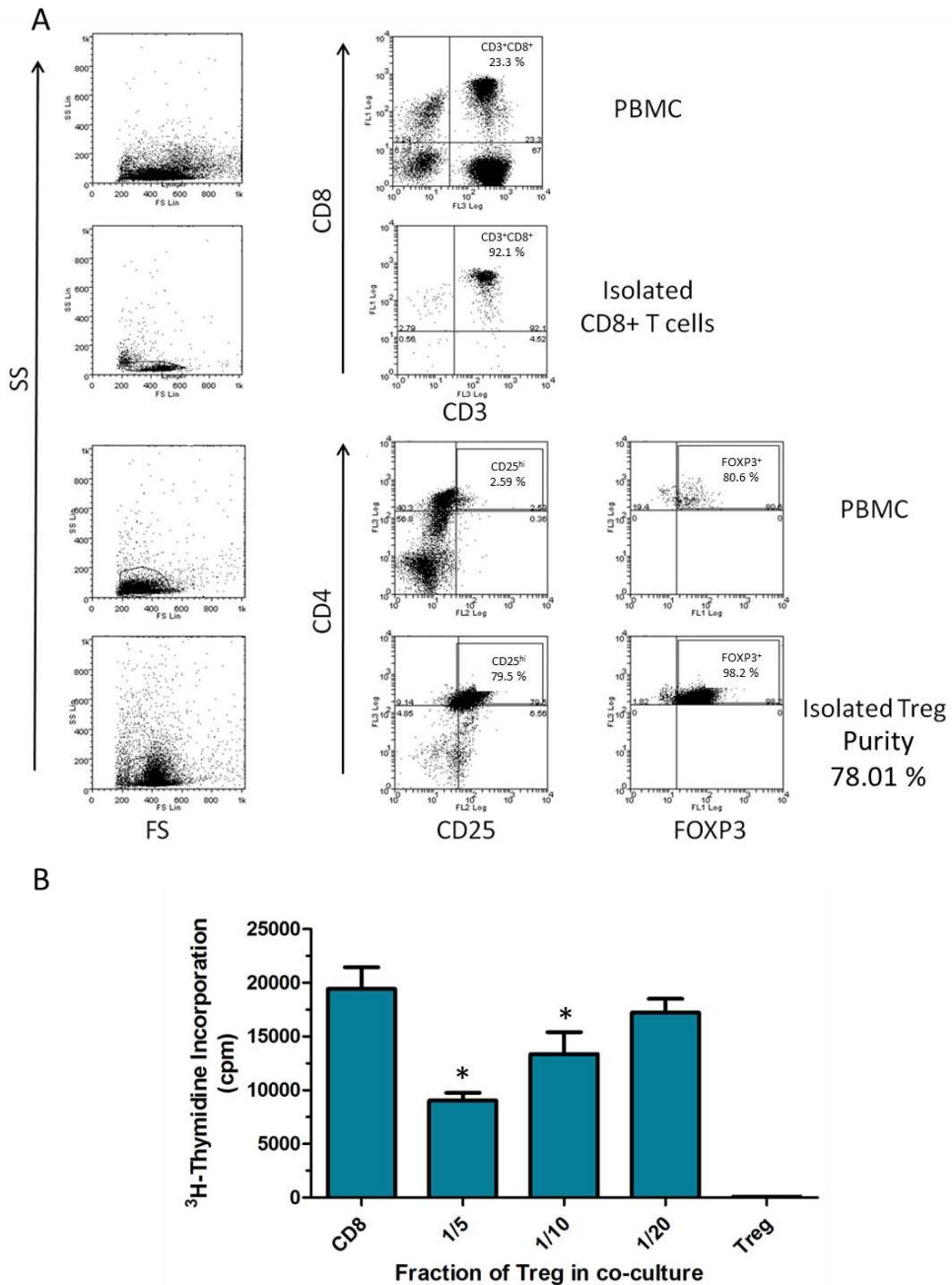


Figure 3.5 - Treg Suppress the Proliferation of anti-CD3/anti-CD28-stimulated CD8⁺ T cells in a Dose-dependent Manner. **A** – FACS analysis of CD8⁺ and CD4⁺CD25⁺ T cell isolations. Purity of CD3/CD8 isolation was calculated to be 93.3 % (92.1 – 95.7 %; *n* = 3). Purity of CD4⁺CD25⁺ T cells calculated to be 78.01 % (77.3 – 79 %; *n* = 3). **B** – CD8 T cell proliferation suppressed by Treg. Figure representative of the means of three independent experiments from three different donors. Treg demonstrate suppression of CD8⁺ T cells where Treg were present at a 1/5 and 1/10 fraction of total co-culture. *n* = 3 *=*p*<0.05. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

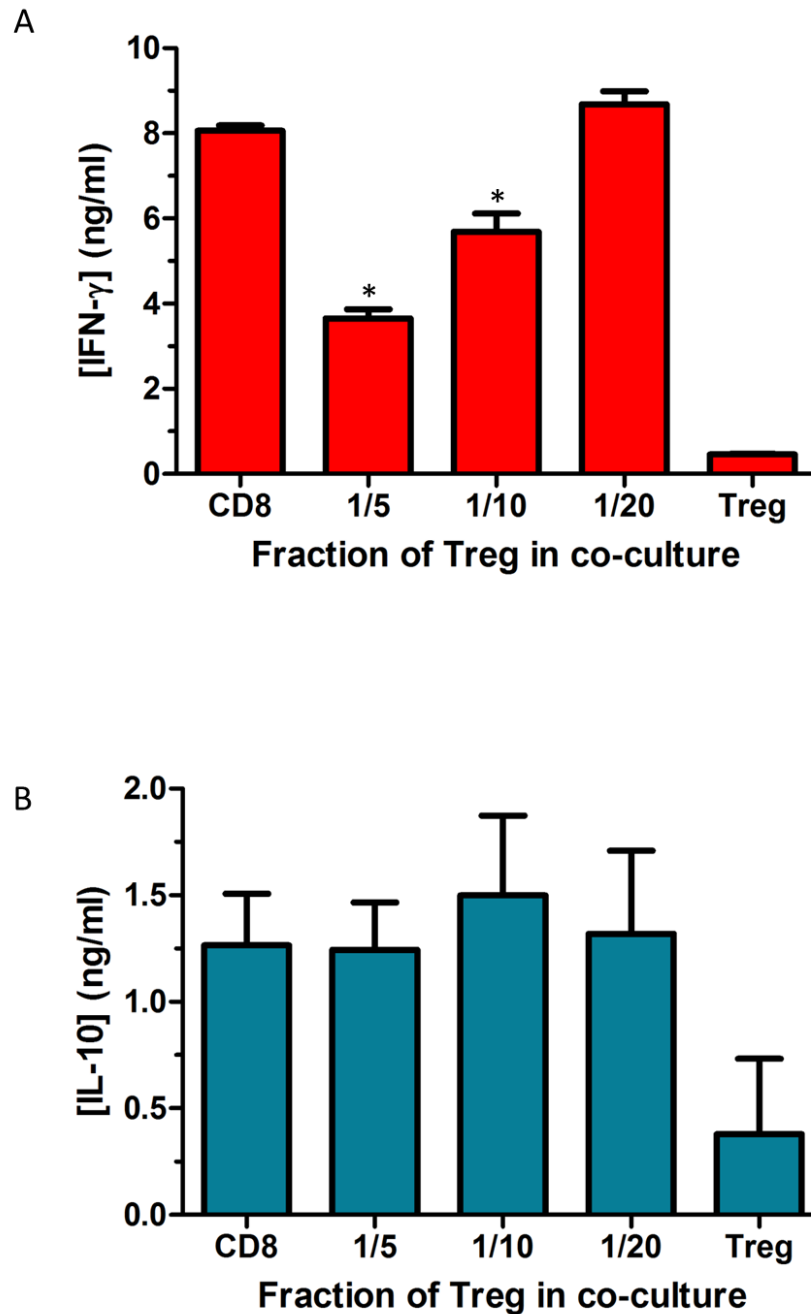


Figure 3.6 - Production of IL-10 and IFN- γ from CD8⁺ T cell Suppression Assays Stimulated with anti-CD3/anti-CD28. Treg restrict CD8⁺ T cell production of IFN- γ . No significant change in IL-10 production detected. **A** - IFN- γ production. **B** - IL-10 production. Figures representative of the means of three independent experiments from three different donors. Error bars represent standard deviations. $n = 3$, $*=p<0.05$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Detection limit = 40 pg/ml.

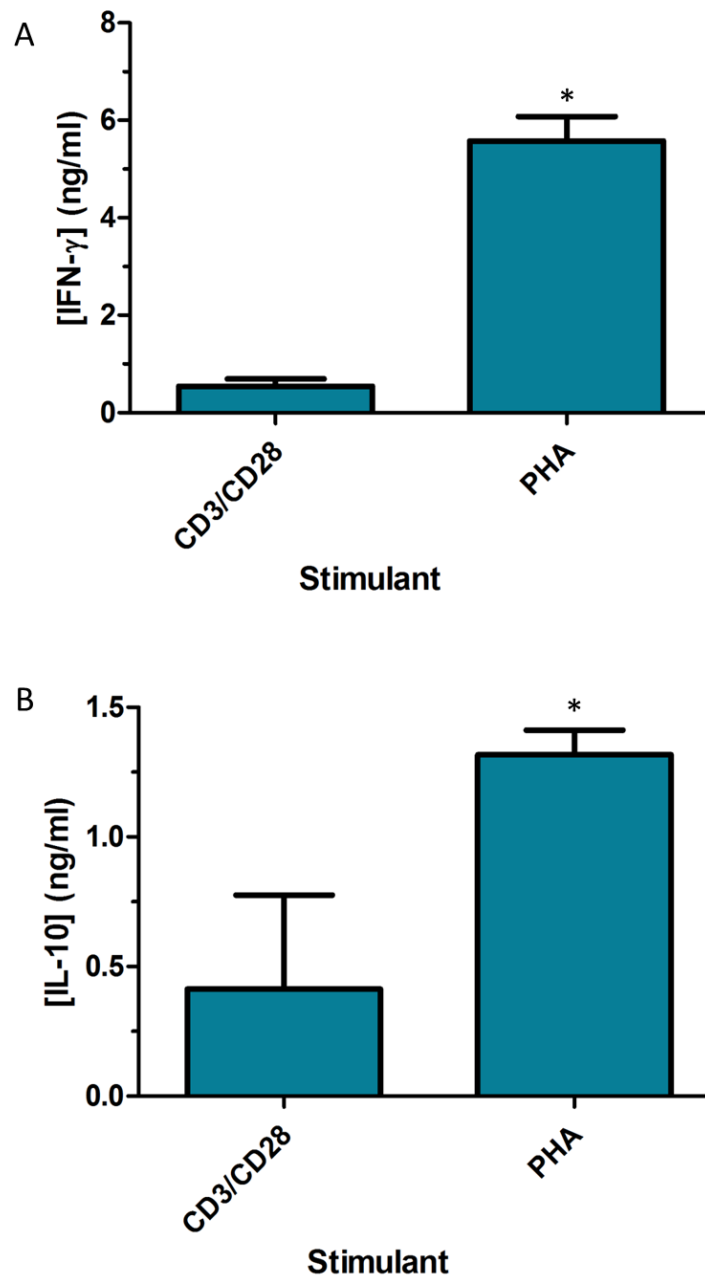


Figure 3.7 - Production of IL-10 and IFN- γ from Treg stimulated with PHA or anti-CD3/anti-CD28. Treg demonstrate an increase in **A** - IFN- γ and **B** - IL-10 when stimulated with 10 μ g/ml PHA compared to stimulation of Treg with 10 μ g/ml α CD3 and 5 μ g/ml α CD28. Figures representative of the means of three independent experiments from the same donor. Error bars represent standard deviations. $n = 3$, $*=p<0.05$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Detection limit = 40 pg/ml

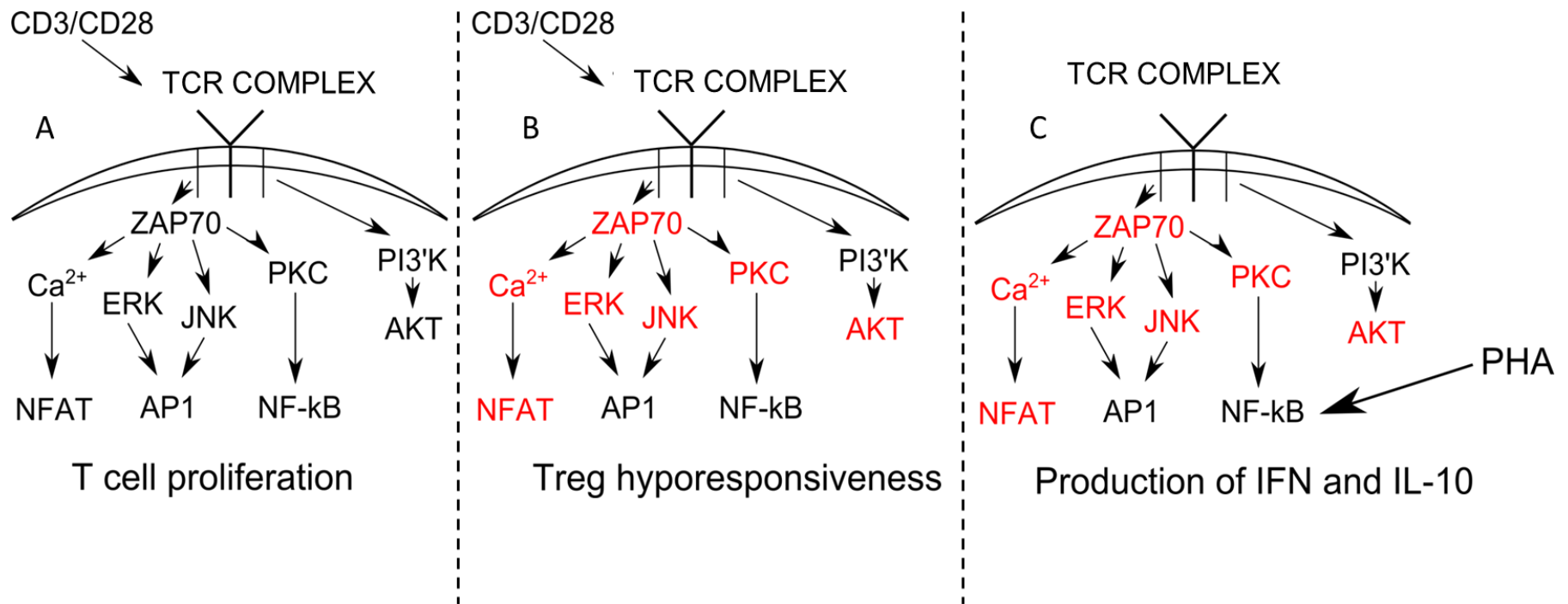


Figure 3.8 - Differences between Effector T cell and Treg Activation. **A** – Effector T cells, once TCR engagement occurs, have multiple proliferative pathways engaged which causes the activation of NF-κB and AKT, leading to production of IFN-γ and other cytokines as well as inducing cell proliferation. **B** – Treg have several signaling components whose activation/phosphorylation have been demonstrated to be defective, thus TCR engagement causes Treg hyporesponsiveness and thus very little IFN-γ and IL-10 as seen in Figure 3.7. **C** – Treg stimulated with PHA, see a direct innervation by the mitogen on NF-κB activation thus leading to production of IFN-γ and IL-10.

3.3.4 Expansion of Treg

Due to the high cost of isolating Treg, their small cell number and ethical restriction on the amount of blood that could be taken from healthy donors, it became essential to develop a cost effective expansion protocol for these cells. This would allow the generation of enough Treg for use in experimentation. The expansion protocol is made up of two distinct components. The first is T cell stimulation.

Classic T cell expansion work suggests the use of α CD3/ α CD28 stimulation either in the form of soluble antibodies or coated expander beads (9,21,68,75). This approach, although validated, was not cost effective. An alternative stimulus was found in the form of an α CD28 superagonist (76). This form of stimuli provides durable activation of T cells without the need for further TCR engagement. This method proved to be cost effective and thus was incorporated into the expansion protocol. Work by Battaglia *et al.* (21) and Pahwa *et al.* (75) demonstrated that Rapamycin, in conjunction with α CD3/ α CD28 stimulation, selectively expands murine and human CD4⁺CD25⁺FOXP3⁺ *in vitro*. This is due to Rapamycin selectively blocking the expansion and proliferation of CD4⁺CD25⁺ effector T cells thus sparing and promoting growth of Treg. However, using Rapamycin, as suggested, continuously in culture was too expensive.

The other component of Treg expansion is growth factor supplementation. As discussed previously, IL-2 is considered to be one of the key growth factors in Treg homeostasis. However, other cytokines can be used as well. Garden *et al.* (68) showed that several other cytokines, namely IL-4, IL-7 and IL-15 can help maintain Treg phenotype and function. IL-35, a cytokine reportedly secreted from

Treg, also aides their proliferation (77). Due to the high cost of supplementing Treg with these growth factors, it was decided that only IL-2 would be used.

One constant throughout the literature on Treg expansion was the high concentration (1000 IU/ml) of IL-2 provided to cells. It has been suggested that high concentrations of IL-2 in culture assist in the breaking of Treg anergy and that contaminating CD4⁺CD25⁻ T cells can be peripherally converted to become CD25⁺ (78). The ability to obtain Aldesleukin (recombinant IL-2 used in Renal Cell Carcinoma) cheaply meant that supplementing Treg with high concentrations of IL-2 was not an issue.

Comparative analysis of α CD3/ α CD28 versus α CD28 superagonist as the stimulus in Treg expansion showed that anti-CD28 superagonism provided a significantly faster rate of cell growth over 42 days of culture (α CD3/ α CD28 vs. **α CD28SAg** – 3.3×10^6 vs. **4.6×10^6** , $n = 3$) (Figure 3.9). Figure 3.10 indicates that not only did expansion with α CD28SAg provide more cells but that it also produced a population of Treg that was of a significantly higher purity (Figure 3.10; % CD4⁺CD25⁺FOXP3⁺; **α CD28Sag** vs. α CD3/ α CD28, **88.5** vs. 82.9 % - starting population = 78.04 %, $n = 3$) and as such was adopted as the stimuli of choice in the expansion protocol. The increase in purity could be due to expansion of Treg clones and an increase in CD25⁺ due to high levels of IL-2. However, it must be noted that FOXP3 can be seen on activated T cells and so it is a possibility that the increased purity could be due to a greater proportion of T cells being activated.

The reproducibility of Treg expansion with anti-CD28SAg was seen with six different donors (Figure 3.11). Starting populations were 3×10^5 ($n = 6$) which

were expanded over 70 days. The rate of expansion appeared to be reproducible. This suggests that the protocol was sufficient for the needs of the project.

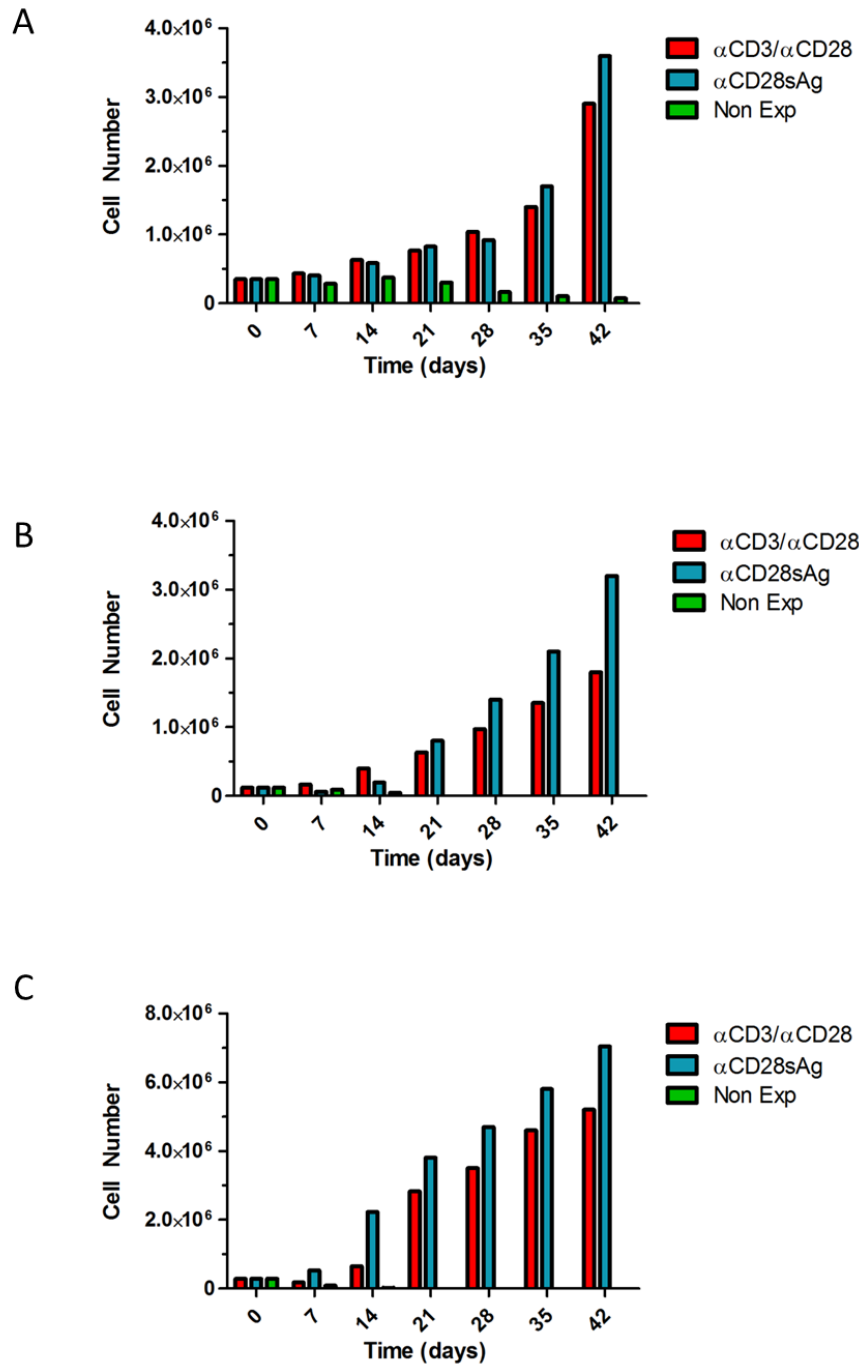


Figure 3.9 - Comparison of Treg Expansion Methodologies. Treg from each donor (**A** – Donor 4, **B** – Donor 5, **C** – Donor 6) were separated into three experimental groups. First group (**red**) were expanded using 10 μ g/ml α CD3 and 1 μ g/ml α CD28 mAbs. Second group (**blue**) were expanded using 1 μ g/ml α CD28 super agonist. IL-2 concentration remained constant at 1000 IU/ml. Third group (**green**) acted as the non-expanded control. Data represents three donors with Treg expanded over 42 days.

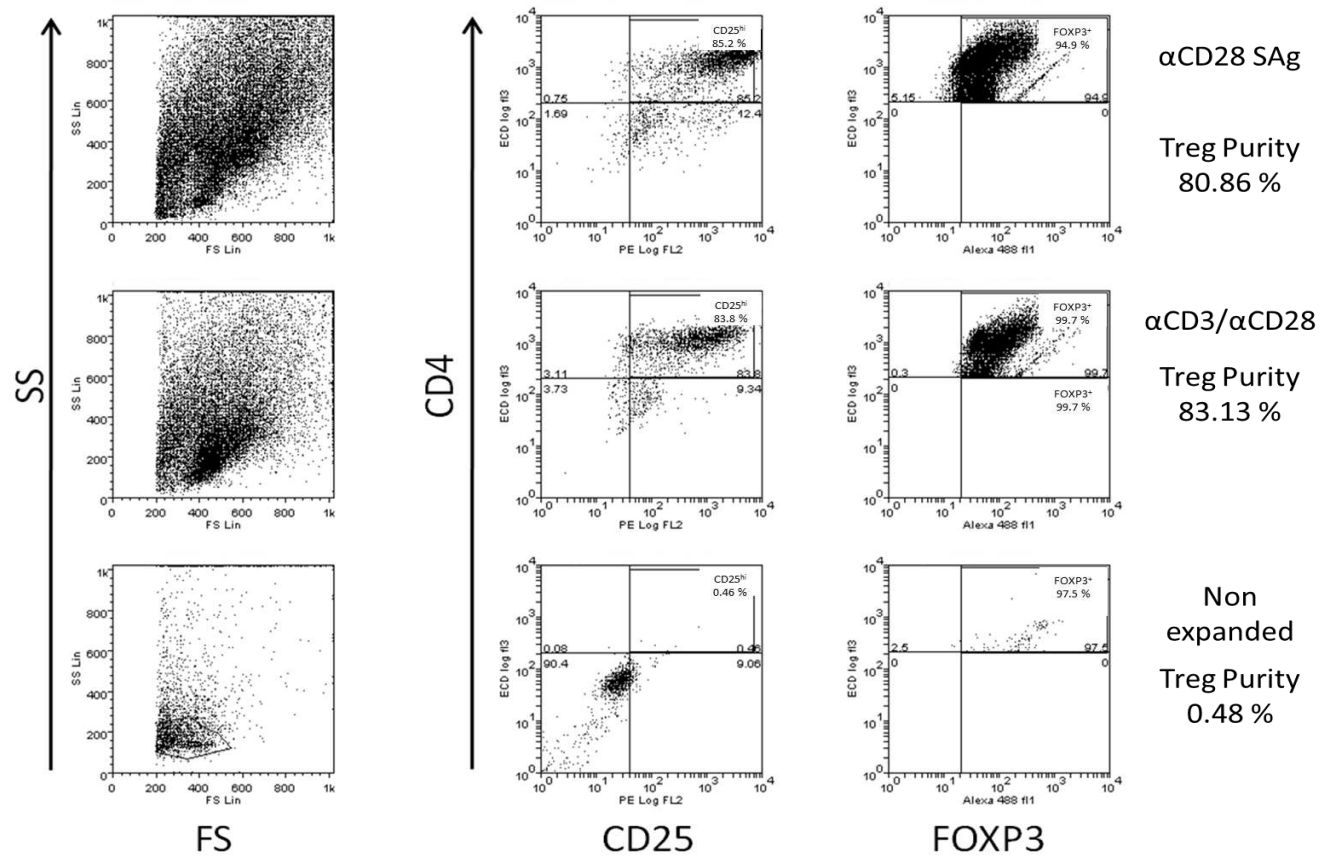


Figure 3.10 - Comparison of Expanded Treg Purity after Six Weeks. The expression of CD4, CD25 and FOXP3 were assessed using flow cytometry. Treg expanded by anti-CD28 superagonist produced a cell purity of 88.5 % (80.6 – 97.0 %; $n = 3$) compared to anti-CD3/anti-CD28 expanded purity of 82.9 % (82.7 - 83.1 %).

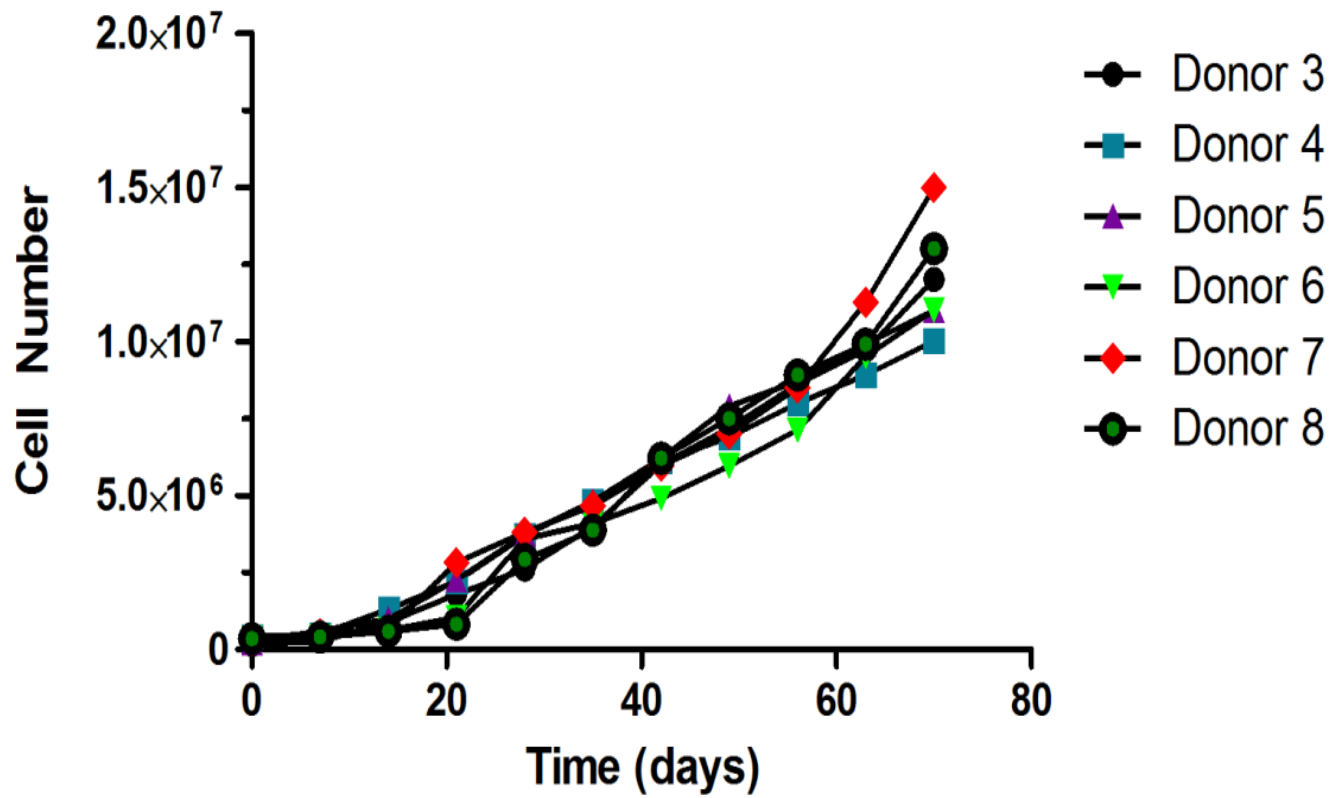


Figure 3.11 - Treg Expansion Curve. Treg were expanded using 1 $\mu\text{g/ml}$ anti-CD28 superagonist and 1000 IU/ml IL-2 for 70 days.

Expanded Treg demonstrated suppressive behavior, similar to that of natural naïve Treg (see Figure 3.12b). Combined with phenotypic analysis of the expanded cells (Figure 3.12a) there is sufficient evidence to suggest that these cells maintain 'Treg-like' properties (i.e. CD4⁺CD25⁺FOXP3⁺ and can suppress effector cell proliferation). On further analysis of the suppression data in Figure 3.12b, there is a case for suggesting that the expanded Treg were more suppressive than those isolated from whole blood. Suppression with expanded Treg was significant at fractions as low as 1/40 (Figure 3.12; Effector vs. **Expanded Treg** vs. Isolated Treg, cpm; 1960 vs. **930** vs. 2132, $n = 3$, $p < 0.05$). These assays provided a similar cytokine profile to those seen with other PHA-stimulated proliferation assays (Figure 3.4) and thus do not follow the conventional evidence of Treg-mediated suppression. However, as explained previously this is could be due to the mechanism by which PHA activates Treg.

Figure 3.10 highlighted the efficiency of the expansion protocol by showing that Treg phenotype is maintained after six weeks. However, Treg could not be expanded continuously as seen in Figure 3.13. After prolonged stimulation, Treg began to lose their classical phenotype with a decrease in CD25, FOXP3 and CCR4. Although still viable, these cells exhibited a loss in suppressive function after 56 days which meant that Treg were only expanded for a maximum of 42 days to avoid loss of function and phenotype. This loss could be attributed to the accumulation of pro-apoptotic factors within the culture environment over time during expansion (79).

The loss of Treg suppressive function combined with a simultaneous loss of CD25 and FOXP3 expression is an interesting observation. Although there are

documented instances of T cell suppression via cells which are CD25⁻ and FOXP3⁻, the loss of suppressive function suggests that they are important in the maintenance of Treg (15). Furthermore, pre-clinical and clinical data of CD25 antagonism does suggest a potential amelioration Treg number and function (80,81). The loss of FOXP3 in man is less well defined and as discussed earlier in this chapter does not lend itself to be being an entirely accurate marker for phenotyping Treg.

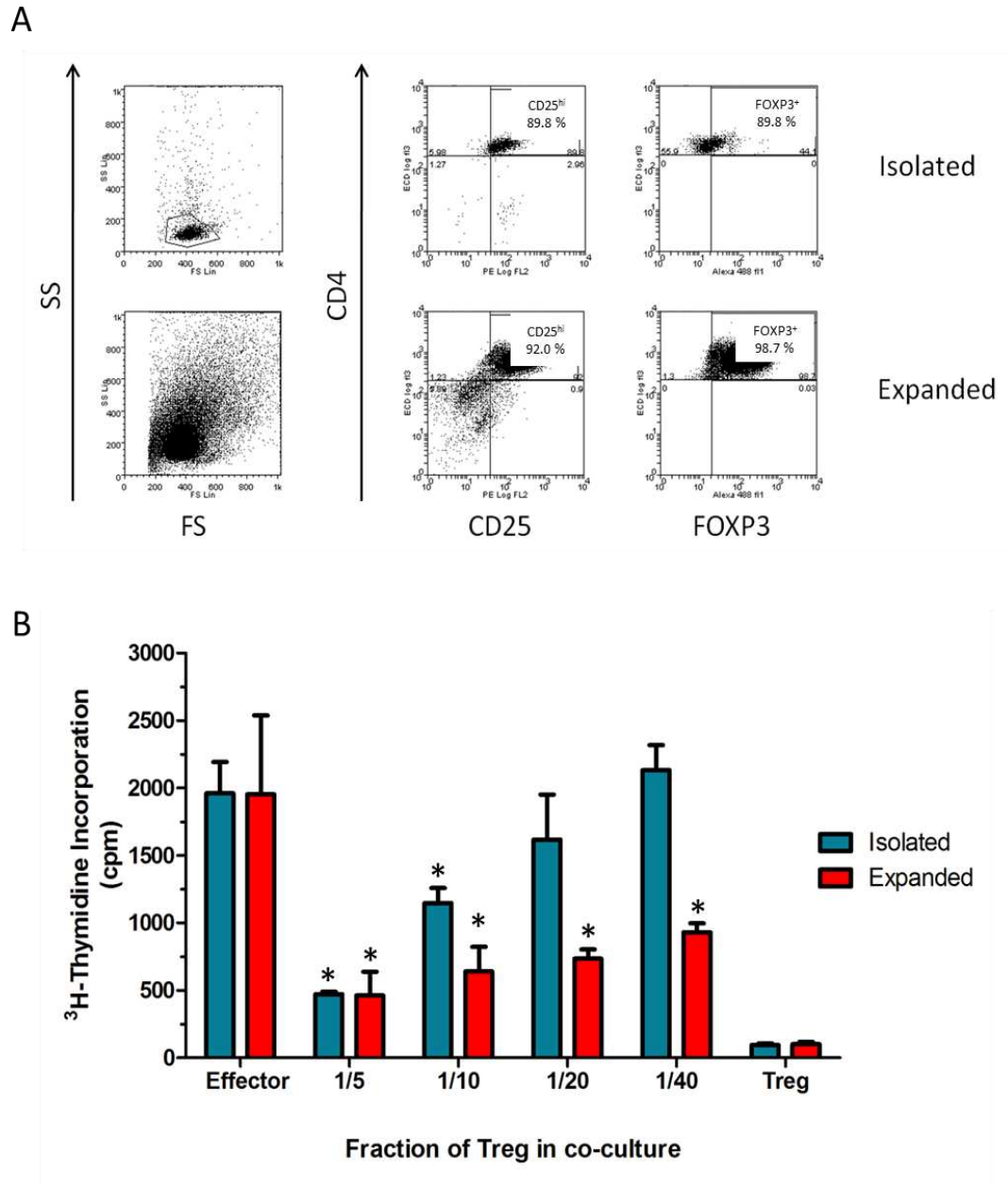


Figure 3.12 - Comparison of the Suppressive Function of Isolated and Expanded Treg. **A** - Flow cytometry analysis of CD4, CD25 and FOXP3 expression. One representative experiment of three is presented. **B** - T cell proliferation assay. Figure representative of the means of three independent experiments from the same donor. Error bars represent standard deviations. $n = 3$; $*=p<0.05$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

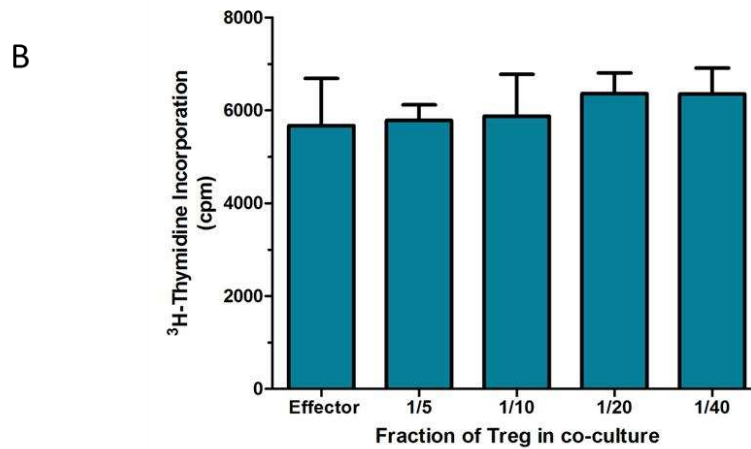
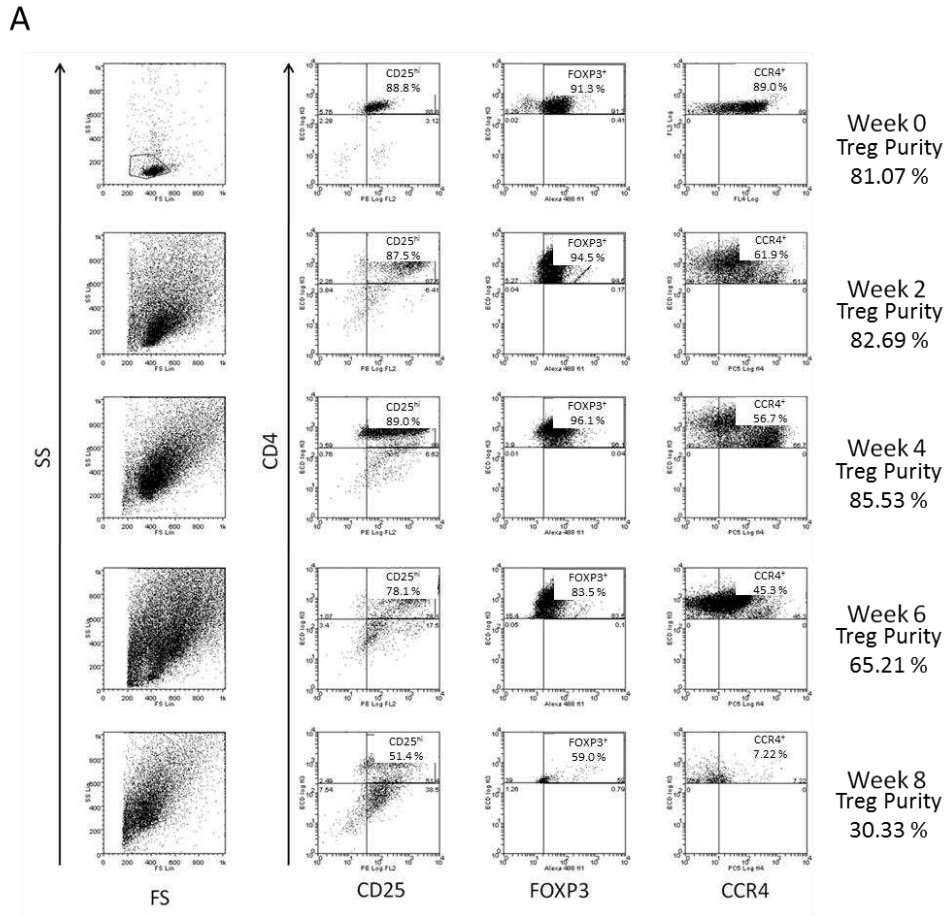


Figure 3.13 - Assessment of Treg Phenotype over Expansion Period. A - Flow cytometry analysis of Treg undergoing expansion over 56 days. One representative experiment of three is presented. Plots represent analysis at weeks 0, 2, 4, 6 and 8. **B** - T cell proliferation assay. Figure representative of the mean of three independent experiments from the same donor. Error bars represent standard deviations. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. $n = 3$; $*p < 0.05$

3.4 Conclusions

The studies conducted above were used to develop a suitable method by which to characterise Treg both functionally and phenotypically. Although there is still much debate surrounding a truly reliable human Treg phenotype it was decided that CD4⁺CD25⁺FOXP3⁺ cells were to be used in all experiments on the basis that these were commonly used markers within the literature. Due to the high running costs of the project, Treg were isolated via Dynal magnetic beads. This was compared to MACS but this was found to be too expensive to use on a regular basis. Critical to the success of the project was the expansion of these cells to larger populations than those obtained on isolation from whole blood. After comparing the cost/purity benefits of different stimuli used in expansion it was decided that an α CD28 superagonist would be used alongside high concentrations of IL-2. This allowed breakage of the anergic phenotype of Treg and thus provided suitably pure and large populations of cells with which downstream experiments could be conducted. The expansion of Treg is limited to 42 days as continued stimulation led to a down-regulation of key Treg markers as well as a loss of suppressive activity.

Treg demonstrated dose dependent suppression of both CD4⁺CD25⁻ and CD8⁺ T cells. This provides the basis for a functional tool with which to alter the suppressive effects of Treg pharmacologically. Interestingly, the cytokine milieu generated by Treg suppression can be substantially altered, depending on the stimuli added. Mitogenic stimulation leads to the production of IFN- γ and IL-10 from Treg whereas TCR engagement with α CD3/ α CD28 does not. This could be attributed to the altered proliferative pathways that exist within Treg. It also

indicates that induction of certain molecular pathways, in particular the NF- κ B pathway may be of interest in altering Treg function.

After the establishment of Treg suppression, it became apparent that the less Treg present within the environment, the greater the proliferation of the effector population. This observation, together with those found in literature suggests that strategies to either manipulate Treg function or decrease the number of Treg within the tumour microenvironment will be of clinical benefit (82). The following chapters of this thesis will seek to alter the suppressive and migratory capacity of Treg as well as enhance the generation of a tumour antigen-specific immune response.

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Chapter 4

The CCR4-CCL17/CCL22 axis and its role in Tumour Immunology

4.1 Introduction

It is generally agreed that Treg are involved in generating an immunosuppressive milieu within the tumour microenvironment. As such, blocking the mechanism of tumour recruitment of Treg through chemokine/chemokine receptor interactions could prove to be of clinical importance. This chapter aims to investigate this.

Chemokines are a group of cytokines with chemotactic properties that have been recognised in regulating leukocyte trafficking and positioning in both homeostatic and inflammatory conditions. Currently, more than 40 chemokines have been identified. These are classified according to the configuration of cysteine residues near the N-terminus into CC-, CXC-, C- and CX3C chemokines. They can also be classified into 'inflammatory/inducible' and 'homeostatic/lymphoid' chemokines based on the site of production and the eliciting stimuli.

A number of studies have examined Treg chemotaxis and receptor expression profiles (Figure 4.1). Multiple axis' of this chemokine network regulate Treg migration during both normal tissue homeostasis and pathological conditions and there is strong evidence for Treg migration into sites of inflammation (1). Solid-

tumour microenvironments have an altered expression profile of an extensive network of chemokine and chemokine receptors (2). Tumour associated chemokines are thought to facilitate multiple roles in the biology of primary and metastatic disease including; survival and metastatic seeding of tumours, the regulation of intra-tumoural trafficking by different immune cell sub-populations - which in turn influences the outcome of the immunological response to TAA exposure and facilitates the development of an immune suppressive microenvironment. This immune suppressive chemokine milieu favours angiogenesis (3) and acts as a source of autocrine/paracrine tumour-survival and proliferative factors (4,5).

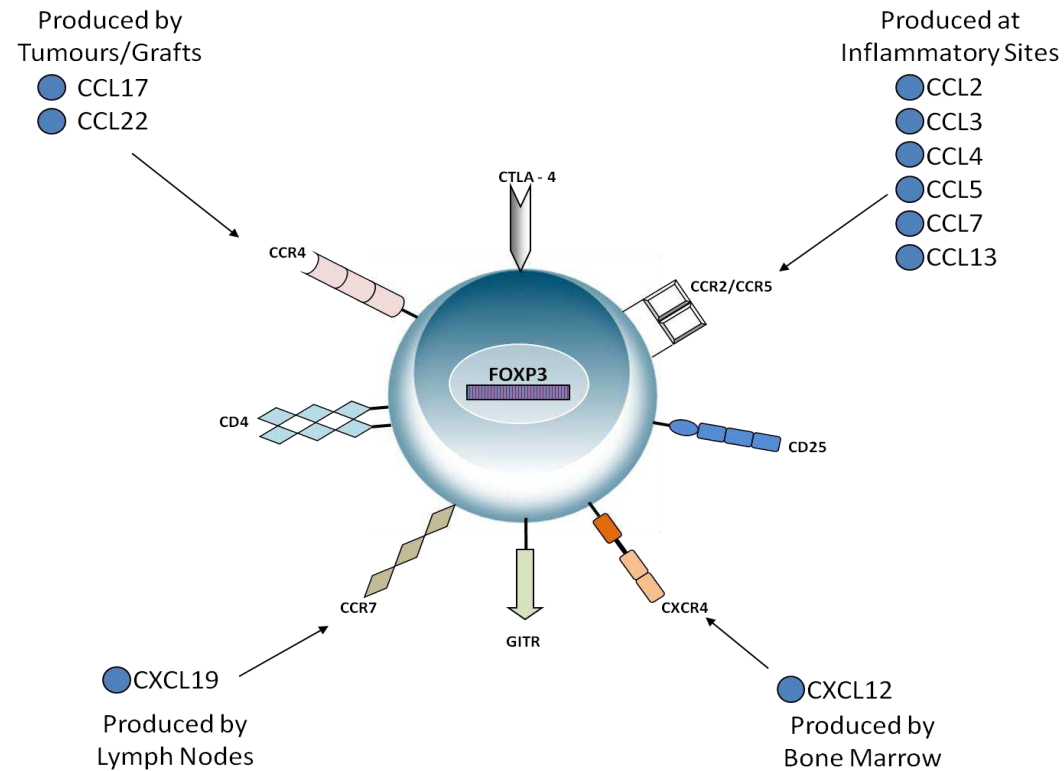


Figure 4.1 – Treg and Chemotaxis. Some of the key markers present on Treg include CD4, CD25, FOXP3, cytotoxic T lymphocyte antigen-4 (CTLA-4) and glucocorticoid induced tumour necrosis factor receptor (GITR). Distinct chemokine receptors are implicated in Treg organ/tissue migration. Bone marrow-derived CXCL12 mediates Tregs into bone marrow. CCL22 supports Treg migration into human ovarian cancer and mouse cardiac grafts. CCR7 may facilitate lymphoid movement of Treg cells. Certain CC chemokines may mediate Treg migration into inflammatory tissues/organs.

4.1.1 Direct and Indirect Roles of Chemokines on Tumours

Recent work has shown a much broader involvement of chemokine function in tumour biology. One of the first recognised mechanisms of chemokine function different from cell chemotaxis, was their effect on angiogenesis. Chemokines have important implications in the regulation of the angiogenic switch in tumours, either directly (through receptors expressed on endothelial cells) or indirectly, by recruiting leukocytes that provide angiogenic factors (6). Endothelial cells express CXCR4 and its triggering by CXCL12 induces endothelial cell migration and proliferation; moreover CXCR4 acts synergistically with VEGF to enhance neo-angiogenesis in human ovarian cancers (7). CXCL12 also promotes tumour angiogenesis by the local recruitment of circulating or bone marrow-derived endothelial precursors (8). Both CXCR4 and CXCL12 are targets of the hypoxia transcription factor HIF-1 α . During tumour-induced hypoxia both molecules are up-regulated in tumour cells, TAM and vessels. These all participate in the building of a vascular network that is essential for tumour progression (9,10).

In the complexity of the chemokine system, other ligands are characterised as inhibitory mediators of angiogenesis. The CXC chemokines (CXCL9, CXCL10 and CXCL11) inhibit endothelial cell proliferation (11) and suppress tumour angiogenesis in several different tumour types (12-14). Therefore, the balance of angiogenic vs. angiostatic chemokines produced in the tumour microenvironment may determine the rate of angiogenesis within a tumour tissue and thus the consequent clinical outcome.

Inflammatory chemokines are also potent activators of matrix-metalloproteases (MMPs), enzymes that digest the extracellular matrix. TAMs in the tumour stroma

produce MMPs and other proteolytic enzymes that affect matrix degradation. The constant remodelling of the stroma; which is a characteristic of solid tumours has two major effects. Firstly, the release of active growth factors and the promotion of tumour cell invasion. Chemokines have been shown to induce gene expression and functional activation of various MMPs, in particular MMP-9 (15).

4.1.2 Tumour Cell Survival and Proliferation

Since the discovery that tumour cells express chemokine receptors and may functionally respond to ligands, there has been an increased effort to identify the direct effect of chemokines on neoplastic cells. Earlier studies highlighted that some tumour cell lines were able to migrate in response to CXCL8, and that antibodies against CXCR2 inhibited melanoma cell growth *in vitro* (16). In the last decade several studies have provided evidence that tumour cells express a wide panel of chemokine receptor (17,18). In general, receptor engagement enhances cancer cell resistance to apoptotic stimuli and proliferation through the activation of the MAP/Erk and PI3K pathways (17,19).

Most tumours express CXCR4 at levels higher than the normal corresponding tissues (17,20); other investigated receptors are for instance: CCR6 and CX3CR1 in colorectal and pancreatic cancer (21,22), CXCR6 in prostate cancer (23), CXCR2 in melanoma (24), and oesophageal cancer cells (23), CCR7 in squamous cell carcinoma of the head and neck (25) and CCR10 in melanoma (26). In ovarian cancer cells, the small CXCR4 antagonist CTCE-9908 caused cell death via a mechanism that was not apoptotic but involved damage of DNA checkpoint proteins and cell cycle arrest (27).

4.1.3 Tumour Cell Invasion and Migration to Distant Organs

There is now ample evidence that chemokines can serve as cues for the secondary localisation of tumour cells. The most frequently over-expressed chemokine receptor on tumour cells is CXCR4. In general, CXCR4 is associated with tumour progression and metastasis (28,29). In a seminal paper, Muller *et al.* (30) demonstrated the expression of CXCR4 and its involvement in metastasis in a model of breast cancer. Leukaemic cells expressing CXCR4 migrate to bone marrow and localise at sites where stromal cells secrete CXCL12 (19). In glioblastoma, several CXC receptors have been reported, with CXCR4 being the most frequently described with an associated increase in the aggressiveness of the disease as well as poor patient survival (31-33).

CXCR4 is the principal chemokine receptor identified on cancer stem cells (CSC). CXCR4⁺ CSC have been isolated from glioblastoma (31) and pancreatic tumours (34). In the latter case, a distinct subpopulation of CD133⁺/CXCR4⁺ CSC was identified at the invasive front of the tumour and determined the metastatic phenotype of individual tumours (34). Other CXC-receptors have been implicated in the malignant dissemination to distant organs. For instance, CXCR1, CXCR2 and CXCR3 in malignant melanoma (35,36); CXCR3 in B-cell chronic lymphocytic leukaemia cells (37); CXCR5 in liver metastasis of colorectal carcinoma (38). The CX3CR1 receptor is implicated in the perineural invasion frequently occurring in pancreatic adenocarcinoma (22) and in metastasis to bone of prostatic tumours (39).

Secondary lymphoid organs are a primary site of metastasis; in several tumours (e.g. breast, melanoma, gastric, non-small cell lung cancer, head and neck tumours, colorectal carcinoma), CCR7 is upregulated and mediates tumour cell dissemination to lymph nodes (40-45). In a recent study, brain infiltration by T cell leukaemic blasts was mediated by CCR7 (46). Members of the CCR family are also used by tumour cells to spread to specific tissues such as the skin, the gut and the liver. CCR6 plays a role in organ selective liver metastasis of colorectal cancer (47,48). The skin-homing receptors CCR4 and CCR10 were found expressed together with CCR3 in cutaneous lymphoma (49-51). CCR9 was associated with intestinal melanoma metastasis (52), and CCR10 with spread to LN (53). CCR5 is expressed by Hodgkin lymphoma (54), in prostate cancer (55) [115] and by mammary tumours (56).

Overall, the above studies have indicated a strong involvement of the chemokine system in metastasis dissemination. This was more precisely demonstrated *in vivo* with mouse tumour models where receptor-transduced tumour cells metastasised more than parental cells (57,58).

Another important aspect in the generation of a chemical gradient is the chemokine production by non-tumoural adjacent cells (e.g. endothelial cells and fibroblasts, as well as macrophages). Cancer-associated fibroblasts (CAF) have been extensively studied in more recent years and found to be a source of CCL2, CCL5 and CXCL-chemokines; indeed there is 'crosstalk' between tumour cells and CAF (59,60). A positive correlation has been reported between stromal expression of CXCL12 and a high tumour proliferative index (61) and, in another

study, with proliferation of CD44⁺CD24⁻ breast cancer stem cells (62). A notable example was provided in breast cancer, where tumour cells induced CCL5 secretion in newly recruited mesenchymal cells; stromal-derived CCL5 then interacted with CCR5-positive tumour cells enhancing their growth *in vivo* and metastatic ability (56).

4.1.4 Treg Migration

Treg cells express a number of chemokine receptors such as CCR2, CCR4, CCR5, CCR7, CCR8 and CXCR4 and are able to migrate in response to a variety of chemokines such as CCL2, CCL5, CXCL12, CCL17 and CCL22 (63).

CCL2 is produced by many stromal cells including T cells and monocyte-derived cells and also by tumour cells. It is a chemoattractant principally for macrophages (64), but also for activated CD8⁺ and CD4⁺ T cells (65), and NK cells (66) thereby influencing the tumour microenvironment at many levels. CCL2 has also been implicated in driving the metastatic seeding of prostate cancer and non-small cell lung cancer cells to bone (67-69). In addition to the primary role of the CCR2/CCL2 axis in directing monocyte populations it has also been implicated in the migration of Treg into human gliomas (70) and prostate cancers (71). Interestingly, high expression of CCL2 has been described in patients with breast cancer (72) and malignant myeloma (73) and correlates with poor prognosis in melanoma (74). Although this data does not directly implicate Treg infiltration/activity, further studies are required to delineate the cellular consequences of the CCR2/CCL2 signalling axis in cancer.

In pancreatic cancer patients, intratumoural Treg cells expressed high-levels of CCR5 and respond to CCL5 produced by pancreatic cancer cells (66). Interestingly, disruption of CCR5-dependent homing of Treg cells by abolishing CCL5 expression in pancreatic tumour cells or antagonism of CCR5 on intratumoural Treg cells by CCR5 antagonists inhibits tumour growth in a murine model of pancreatic cancer (66).

In addition to the well documented role of the CXCL12–CXCR4 axis in the seeding of tumour cells to metastatic sites (75,76), Treg also express CXCR4 and can transmigrate along a CXCL12 gradient (77). In a syngeneic murine glioma model Grauer *et al.*(78) were able to show a systematic increase in CXCR4⁺ Treg as the tumour developed. In a trial of 31 cisplatin resistant ovarian cancer patients undergoing IL-2 therapy, CXCR4 expression was found to be elevated on Treg with concurrent expression of CXCL12 within tumours (79). These data suggest that Treg may be involved in compensatory adaptation of the tumour micro-environment during IL-2 therapy thereby limiting effector T cell reactivity. This may in part explain the poor response rates of IL-2 therapy in this patient population.

As discussed previously it is clear that a Treg response is an integral part of an inflammatory process. Human intestinal epithelial cells express CCL22 following exposure to TNF- α and IFN- γ (80). This proclivity has also been observed in ovarian cancer cells (81) suggesting that tumours may actively recruit Treg during an acute inflammatory response. In a mouse model of ovarian cancer, tumour and tumour associated macrophage (TAM) production of CCL22 were found to be responsible for selective recruitment of Treg expressing CCR4 (81).

Clinical evidence of tumour-associated CCL22 production has been described in lung cancer (82,83), Epstein-Barr virus-positive B-cell lymphoproliferative disorder (84), oesophageal squamous cell carcinoma (85) and breast cancer (86). In addition, another CCR4 ligand, CCL17 is also implicated in the migration of Treg (87,88). *In vitro* evidence suggests that CCL22 is the dominant chemokine in terms of governing Treg migration (89). In a clinical study involving 163 treatment naïve Hodgkin lymphoma patients 82 % and 57 % demonstrated expression of CCL22 and CCL17, respectively, which was associated with disease severity (63). Pleural effusions taken from patients with lung cancer showed elevated levels of CCL22 and reduced levels of CCL17 compared to matched samples taken from serum. To determine the effect of the chemokine profile on Treg migration the effusion samples were treated *in vitro*, with neutralising anti-CCL22 or -CCL17 antibodies. Treg migration was partially abrogated by anti-CCL22 but not by anti-CCL17 (83).

In addition to its affinity for CCR4, CCL17 also binds CCR8 (90) which is expressed on both human Treg (89) and macrophages (91). A landmark study by Curiel *et al.* (92) in 2004 detailed the existence of CD4⁺CD25⁺FOXP3⁺ Treg cells in 104 individuals affected with ovarian carcinoma. Not only did they demonstrate that Treg were associated with a high death hazard and reduced survival but that these cells preferentially migrated to and accumulate in tumours and ascites, but rarely entered draining lymph nodes in later stages of disease. On investigation of the tumour microenvironment they found that CCL22 was produced by both tumour cells and tumour-associated macrophages (92).

Essentially, tumours could be said to use migratory chemokines as a defence mechanism. Berin *et al.*(80) looked at inducing the production of CCL22 from human intestinal epithelial cells by exposing them to pro-inflammatory chemokines such as TNF- α and IFN- γ (80). Their results showed a significant increase in CCL22 production upon stimulation. This was also demonstrated *in vitro* in ovarian cancer cells which suggests that immune assault upon tumours may be short-lived as the tumour produce chemokines that actively recruit Treg. Tumour-associated macrophages and DC have also been implicated in the production of CCL22. This collective induction of chemotactic pathways leads to increased migration of Treg. Although the CCL22-CCR4 axis is not exclusive to Treg, this 'selective' recruitment might represent another mechanism by which tumours may induce immune privilege by affecting the balance between Treg and effector T cells.

4.1.5 Chemokine Network Targeting

Manipulation of the chemokine network to modulate pathological inflammatory conditions is an area of active interest both pre-clinically and clinically.

As described previously the role of CCL2 in the tumour microenvironment is diverse and is chiefly implicated in monocyte trafficking. CCR2, the receptor for CCL2, is also expressed on Treg (93). Blocking antibodies to CCL2, in combination with an anti-CCL12 antibody and either HPV-E7 or Listeria mesothelin vaccines were administered in three murine non-small cell lung cancer models (TC1, LKR and AE17). In these models a combination of both vaccine and anti-CCL2/CCL12 resulted in decreased tumour burden, increased intratumoural CD8⁺ T cell numbers and decreased intratumoural and splenic

Treg numbers. However, no change in TAM or splenic MDSC was observed, suggesting that the effects on tumour burden were dependent on Treg-tumour trafficking and prevention of Treg-mediated immune suppression (94). Several CCR2 inhibitors have been trialled in non-cancer indications including MK0812 (Merck) and INCB-8696 (Pfizer/Incyte) in rheumatoid arthritis and multiple sclerosis, BMS-741672 (Bristol Myers Squibb) and CCX140 (Chemocentrix) in Type II diabetes and vascular restenosis (95). A neutralising antibody for CCL2, ABN912 has also been trialled in rheumatoid arthritis (96). Selective inhibitors of the CCL2/CCR2 axis may have clinical utility in the immunotherapy of cancer.

The CXCR4 antagonist Plerixafor (AMD-3100; Genzyme Corp.) has recently been licensed in the United States for use in stem cell transplantation and is currently in a phase III trial for malignant myeloma (97). Azab *et al.* (98) have shown that the compound enhanced the sensitivity of myeloma cells to multiple therapeutic agents *in vitro* by disrupting their adhesion to bone marrow stromal cells. Although effects on Treg migration were not assessed in this study, preclinical data demonstrate that Treg express CXCR4 and are able to migrate along a CXCL12 gradient (7). It is possible that ongoing clinical trials will provide clinical evidence that inhibition of the CXCR4/CXCL12 axis affects Treg migration.

Bayry *et al.*(99) have designed a series of CCR4 antagonists which show specificity and restrict Treg migration for use as vaccine adjuvants which could have applications in an oncology setting. Additionally, Andrews *et al.* (100) have also designed a series of antagonists for both CCR4 and CCR5 for use in asthma. siRNA targeting of CCL17 and CCL22 expression in DCs has been

used to reduce Treg and increase CD8⁺ tumour infiltration in xenograft models of human breast cancer (101). Preclinical data using the 4T1 metastatic breast cancer model, demonstrated that selective targeting of CCR4 expressing Treg with a CCL17-Pseudomonas exotoxin (PE38) fusion protein significantly enhanced NK cell activity preventing lung metastasis (102,103). KW-0761 (Kyowa Hakko Kirin), a humanised defucosylated antibody to CCR4 is currently in a phase II trial in CCR4 positive cutaneous T cell lymphoma and acute T-cell lymphoblastic leukaemia (104). Preclinical data suggests that efficacy is linked to NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) (105). Clinical evaluation of the potential of KW-0761 to selectively target CCR4-expressing Treg in solid tumours is clearly warranted as a selective method for Treg depletion.

The lead CCR4 antagonist designed by Andrews *et al.* was kindly donated by AstraZeneca for use in this project. The primary aim was to use this compound as a means to abrogate CCR4-mediated Treg migration. In conjunction with this, it is important to consider the potential for CCR4-mediated ovarian cancer cell migration. If this is observed then the rationale for using a CCR4 antagonist as part of an immunotherapeutic regimen would gain further credence. It is also critical to assess the effect of the drug on Treg suppressive activity. This is to ensure that CCR4 antagonism does not impinge on peripheral Treg function.

4.1.6 Experimental Objectives described in Chapter 4

- Development of a robust chemotaxis assay
- Demonstration of CCR4 expression on Treg, CD4⁺CD25⁻ T cells, CD8⁺ T cells and ovarian cell lines.
- Confirmation of CCL17 and CCL22 production from ovarian cancer cells due to pro-inflammatory stimuli.
- Use a specific CCR4 antagonist to abrogate the chemotactic effect of CCL17 and CCL22 on Treg and ovarian cancer cells.

4.2 Methodology

4.2.1 Isolation of Regulatory T cells via Magnetic Selection (Dyna)

A maximum volume of 100 ml of peripheral blood was obtained from healthy donors in accordance with local ethical committee approval (EC# BT/04/2005). Peripheral blood mononuclear cells (PBMC) were separated via a density-gradient centrifugation over Histopaque-1077 (Sigma) twice as previous, firstly to isolate PBMCs with the second density gradient used for the removal of platelets. The resultant cells were then washed and resuspended at 5×10^7 cells per 500 μ l isolation buffer containing phosphate buffered saline (PBS) + 1 % w/v bovine serum albumin (BSA) (Sigma) + 1 mM EDTA (Sigma) before viability counting via Trypan Blue exclusion (Fluka). 200 μ l of CD4 Human Antibody Mix (Invitrogen) and 100 % v/v foetal calf serum (Sigma) (FCS) were incubated with PBMC for 20 minutes. This was followed by the addition of 1 ml Depletion MyOne Dynabeads (Invitrogen) before magnetic removal of bead-bound non-CD4⁺ cells, leaving CD4⁺ cells in solution.

CD4⁺CD25⁺ T cells were isolated by positive selection using 200 μ l CD25 Dynabeads per 1.5×10^7 cells. The CD4⁺CD25⁺ Treg population was liberated from the CD25 Dynabeads using Detach-a-bead solution (Invitrogen) and were then used in downstream applications. The remaining CD4⁺CD25⁻ effector T cells (Teff) were either used in T cell suppression assays or placed in RPMI-1640 (Sigma) + 10 % v/v FCS (Sigma) + 5 mM L-Glutamine (Sigma) + 100 IU/ μ g/ml Pencillin/Streptomycin (Sigma) and 10 % v/v DMSO (Sigma) before undergoing controlled freezing at -80 °C with prolonged storage in liquid nitrogen.

A small volume of cells (10 μ l) was taken at each isolation step for analysis via flow cytometry.

4.2.2 Expansion of CD4⁺CD25⁺ T cells

Isolated CD4⁺CD25⁺ T cells were divided into three experimental groups and placed into culture using X-Vivo-15 + 5% Human AB Serum (Lonza). The final cell concentration was maintained at 1 x 10⁶ cells/ml. The first experimental group was supplemented with 1000 IU/ml recombinant IL-2 (Novartis) and stimulated with 10 μ l/ml α CD28 superagonist (AnCell). The second group was supplemented with 1000 IU/ml recombinant IL-2 and stimulated with bound 1 μ g/ml α CD3 and soluble 1 μ g/ml α CD28 antibodies (Biolegend). The third group was as a negative control with no media supplementation or stimulation.

After days 3, 10 and 17, those cells being expanded were supplemented with 500 IU/ml recombinant IL-2. At days 7, 14 and 21, cells were collated and counted via Trypan Blue (Fluka). A sample was taken for analysis via flow cytometry. The cells were then re-suspended in culture media and re-stimulated as described previously.

4.2.3 Suppression Assays

T cell proliferation assays were set-up in triplicates in 96-well, round-bottomed plates with stimulated and non-stimulated divisions for comparison where possible. Briefly, CD4⁺CD25⁻ T cells were either resuscitated from frozen stocks or freshly isolated from whole blood. These were plated out at 5 x 10⁴ cells/well in X-vivo-15 + 5 % v/v Human AB serum. Treg were then added to each well to provide Teff:Treg ratios of 1:5, 1:10, 1:20, 1:40 to bring volume per well to 100 μ l. Cells were stimulated with 10 μ g/ml of PHA (Sigma). The final volume was

adjusted to 200 µl using X-vivo-15 + 5 % v/v Human AB serum. After 72 hours, 100 µl of cell supernatant was taken and stored at – 40 °C, the plate was then pulsed with 1 µCi of [³H]-labelled thymidine per well (TRA120, Amersham). The plate was left to incubate for a further 18 hours before being harvested onto a microscintillation plate (Perkin-Elmer). After repeated washes with de-ionised water, the plate was allowed to dry for an hour. 20 µl of scintillation fluid (Microsint 0, Perkin-Elmer) was added per well before the plate was covered with optical tape (Perkin Elmer). Thymidine incorporation was quantified using a Packard TopCount NXT Microplate Scintillation counter.

4.2.4 MTT Cell Proliferation Assay

To quantitatively assess the number of cells, 5 mg/mL MTT solution were added to each well. The plates were incubated for 4 hours at 37 °C and formazan crystals were dissolved by the addition of 100 % v/v DMSO. Absorbance was measured at 570 nm. The amount of cells migrated was deduced from a standard curve of known cell number.

4.2.5 CFSE Cell Staining

To quantitatively assess the chemotaxis of cells, CFSE was used (Sigma). Briefly, cells were resuspended in PBS at a concentration of 2×10^7 cells/ml. An equal volume of PBS containing CFSE was added to the cells to provide a final CFSE concentration of 2.5 µM. Cells were washed repeatedly in phenol red-free X-Vivo-15 (Lonza) to remove excess stain.

4.2.6 T cell Chemotaxis Assay

Recombinant human CCL22 was made up to various concentrations (0.0 – 100 ng/ml) using phenol-red free X-vivo-15. 50 µl of these chemokine solutions were plated in triplicate into 96-well ChemoTX™ microplates (5 µm pore size, 6 mm diameter well; Neuroprobe Inc). The chamber was sealed with 7.5×10^4 cells being placed in the upper chamber in a final volume of 50 µl. After two hours of incubation, the microplate was taken from the chamber. The suspension from the upper chamber was removed before the plate was incubated at room temperature with a 5 mM solution of EDTA (Fluka) for 30 minutes to detach cells from the filter. The chamber was washed with PBS before the contents of the lower chamber were transferred to a black fluorescence plate. Visual checks were made on both the filter and the lower chamber to ensure no residual cells. The plate was read using a Dynex MFX micropate fluorimeter (excitation 490 nm, emission detection 520 nm). The number of migrated cells was calculated from a standard curve of labeled cells and compared against a non-chemokine control.

4.2.7 Flow Cytometry Analysis

Cells were stained with cell surface markers CD4-ECD (1.25 µl) (clone SFC112T4D11 - Beckman Coulter), CD25-PE (7.5 µl) (Clone BC96 - BioLegend), CCR4-PerCP/Cy5.5 (2.5 µl) (Clone TG6 - BioLegend). Cells were incubated with flourochrome-conjugated antibodies for 15 minutes before fixation with 3 % v/v formaldehyde (Sigma). Cells were then permabilised using FOXP3 Perm Buffer (BioLegend) before incubation with FOXP3-AlexaFluor488 (Clone 259D – BioLegend) for 30 min. Cells were then resuspended in FACS wash buffer (BioLegend) and read via a Beckman Coulter FC 500 flow cytometer. Analysis

involved gating on lymphocyte populations from forward/side-scatter plots before selecting regions of interest.

4.2.8 Induction of CCL22 in A2780

Cell lines A2780 (EACC) and IGROV-1 (Marco Negri Institute, Milan) were grown to confluence in six well plates using phenol red free RPMI-1640 (Sigma) + 10 % v/v FCS, L-Glutamine and 100 IU/ml Penicillin/Streptomycin at 1.5 ml per well. Recombinant tumour necrosis factor-alpha (Peprotech) and interferon-gamma (Peprotech) were added at various concentrations (5 ng/ml, 10 ng/ml, 20 ng/ml) both separately and concurrently. The cells were then incubated for twenty four hours after which, the supernatant was taken off and stored at – 40 °C. The cells were then prepared for mRNA isolation.

4.2.9 mRNA isolation

mRNA was isolated using the Qiagen RNeasy Spin Kit. Briefly, cells were disrupted by adding 600 µl Buffer RLT (Qiagen) to wells. This was then placed into 15 ml centrifuge tubes. The lysate was homogenised for thirty seconds before being mixed with an equal volume of 70 % v/v ethanol. 700 µl of sample was transferred to an RNeasy spin column (Qiagen) placed in a 2 ml collection tube. The tube was spun for fifteen seconds at full speed after which the flow-through was discarded. 700 µl of Buffer RW1 (Qiagen) was added to the column which was then centrifuged at full speed for fifteen seconds. The flow-through was again discarded. 500 µl of Buffer RPE (Qiagen) was then added to the column which was centrifuged twice at full speed, once for fifteen seconds and then for two minutes. The RNA was eluted by adding 30 µl RNase-free water (Ambion) on the spin column membrane and centrifuging for one minute at full

speed. RNA clean-up was carried out using the Turbo DNA-free kit (Ambion). 3 μ l of 10x Turbo DNA (Ambion) and 1 μ l Turbo DNase (Ambion) were added to the RNA mixture and incubated for thirty minutes at 37 °C. DNase Inactivation Reagent (Ambion) was added, with the entire mixture left for two minutes before centrifugation at full speed for two minutes. The resultant supernatant containing the RNA was removed and placed in a fresh tube. Optical density of RNA was measured using the Gene Meter UV spectrophotometer (AB). Ratios of absorbance at 260 and 280 nm were taken as an indicator of purity (Ratio approximately 2).

4.2.10 cDNA preparation

1 μ g of RNA made to a volume of 10 μ l using RNase-free water. To this, 1 μ l random hex primers (Roche) was added. The mixture was heated at 70 °C for ten minutes. During this time, a buffer mix was made using 4 μ l 5 \times First strand buffer (Invitrogen), 2 μ l 0.1 M DTT (Invitrogen), 1 μ l 10 mM dNTPs (Invitrogen) and 1 μ l RNase out (Invitrogen). The buffer mix was added to the RNA mixture and incubated at 25 °C for 10 min. 1 μ l of Superscript II (Invitrogen) was added to this and incubated at 42 °C for 50 minutes followed by incubation at 70 °C for 15 min. Following a quick spin, the resultant cDNA was made up to 100 μ l, using RNase-free water, to provide a final cDNA concentration of (10 μ g/ml).

4.2.11 qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was run on a TaqMan System. Inventoried TaqMan probe for CCL22 was purchased from Applied Biosystems. cDNA (5 μ l) was incubated with 12.5 μ l of TaqMan MasterMix (Applied Biosystems) containing the fluorescent dye 6-

carboxyfluorescein (FAM) and 1.25 μ l TaqMan probe. The reaction mixture was brought up to a final volume of 25 μ l with RNase-free water. GAPDH was used as a housekeeping gene. The amplification was carried out and analysed in the Bio-Rad iCycler iQ real time polymerase chain reaction detection system. To rule out contamination from buffers and tubes, a negative control with water instead of the cDNA template was used. Thermal cycler parameters included two minutes at 50 $^{\circ}$ C followed by ten minutes at 95 $^{\circ}$ C and 45 cycles of 95 $^{\circ}$ C for fifteen seconds and 60 $^{\circ}$ C for one minute. The change in expression was calculated by the method of Pfaffl.

4.2.12 Inhibition of Cell Chemotaxis

Cells were incubated with AZ1 for 60 min in a humidified incubator at 37 $^{\circ}$ C before addition to the chemotaxis assay as described above. For a measurement of background activity, 30 μ L of assay buffer in the presence of 0.1 % v/v DMSO was added to some of the wells.

4.2.13 CCR4 Internalisation Assay

Treg were incubated in fresh medium at a concentration of 1×10^6 /ml in the presence or absence of 500 ng/ml CCL22 and incubated for 30 min at 37 $^{\circ}$ C. Internalisation was blocked on ice and an aliquot of cells were kept on ice for 30 min during chemokine stimulation to inhibit receptor internalisation as a positive control. AZ1 treatment was conducted as described previously. CCR4 expression was then evaluated by staining and FACS.

4.2.13 ELISA

Chemokine production was measured by ELISA. The cytokines of interest were CCL17 and CCL22. Polystyrene 96-well plates (MaxSorp, Nunc) were coated

with the relevant capture antibody (2 µg/ml CCL17; Clone - #54026, 2 µg/ml CCL22; Clone - #57226, R&D Systems) overnight. The plate was then blocked with 5 % skimmed milk powder in PBS (pH 7.2) + 0.05 % v/v Tween (PBS/Tween) to prevent non-specific binding. The plate was washed in PBS/Tween using an automated plate washer and tapped dry. The relevant recombinant cytokine (PeproTech) and samples were added to the plate. After 2 hours, the plate was washed in PBS/Tween before the appropriate biotinylated detection antibody (1 µg/ml CCL17, 125 ng/ml CCL22; R&D systems) was diluted in PBS, 1 % w/v BSA, and 0.1 % v/v Tween 20 and incubated for 2 hours at room temperature before being washed. 50 µl of 1 µg/ml streptavidin-horseradish peroxidase (BD-Pharmingen) was added each well. After 30 min incubation at room temperature, the plate was washed and bound horseradish peroxidase was visualised with 0.1 mg/ml tetramethylbenzidine (Sigma) and 2 ul of 30 % v/v hydrogen peroxide (Sigma) diluted in 3 M sodium acetate buffer (Sigma). The colour reaction was stopped with the addition of 2 M sulphuric acid (Sigma), and absorbance was measured at 450 nm using a MRX spectrophotometer (Dynex Technologies). The concentrations of samples were calculated from standard curves of each recombinant cytokine. ELISA detection limits were determined as follows (CCL17 - 100 pg/ml, CCL22 – 100 pg/ml).

4.3 Results and Discussion

4.3.1 Method Development – MTT vs CFSE

An important part of the chemotaxis assay was to identify an economical, reliable and sensitive assay for detecting cell migration. Two methods of assessing cell number were compared for their reliability and sensitivity. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan in living cells. A solubilisation solution (isopropanol) is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent cell staining dye. CFSE was originally developed as a fluorescent dye that could be used to label lymphocytes and track their migration within animals.

Literature on migration suggests that either MTT or CFSE are used when looking at cell migration (87,106). It was therefore decided to compare the two methods via a curve of known cell number. Cells were stained and measured according to their protocol. Figure 4.2 shows the standard curves generated by both MTT and CFSE assays.

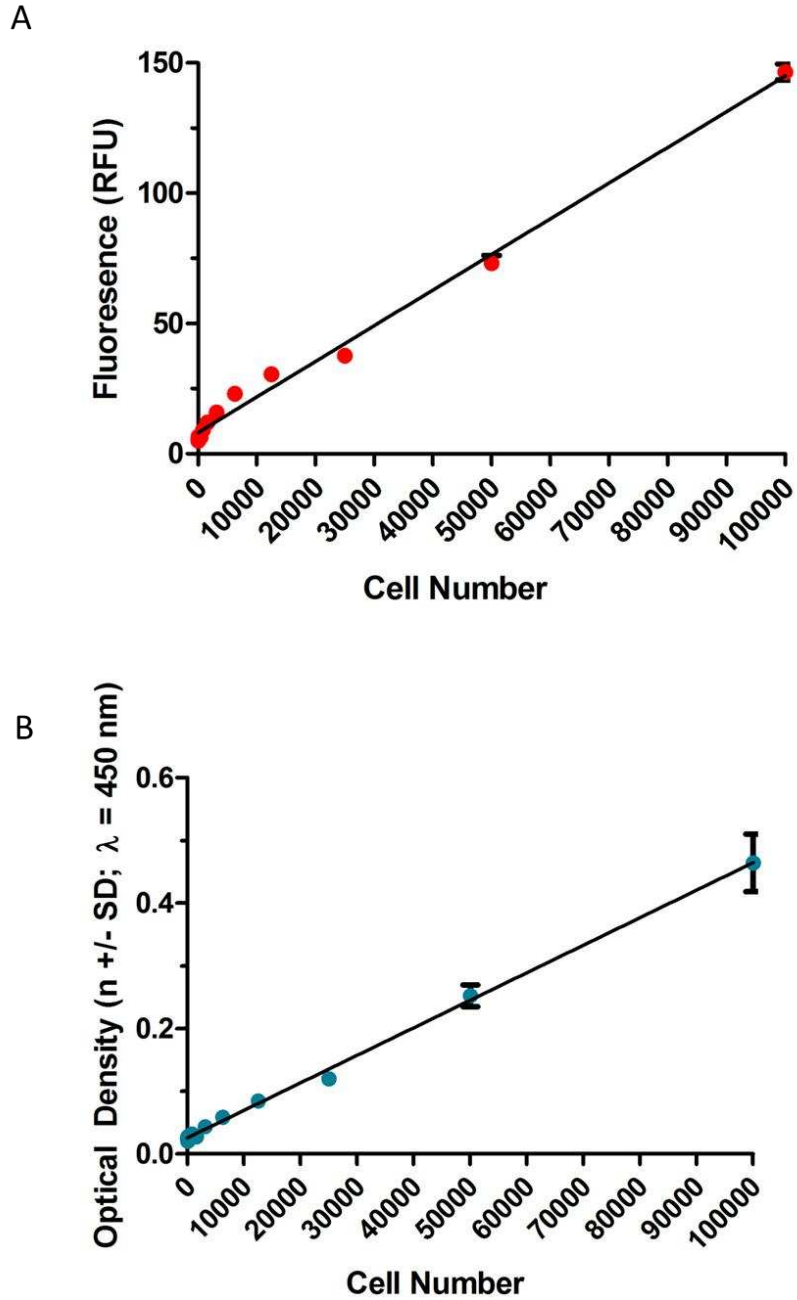


Figure 4.2 – Output of Standard Cell Number Curves for CFSE and MTT Assays. A standard cell number curve was set up to determine assay sensitivity (cell number = 0 – 100,000). **A** – CFSE (Fluorescence; $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$). **B** – MTT ($\lambda = 450$ nm). Both methods provide a good correlation between cell number and signal. CFSE demonstrated a greater sensitivity compared to MTT.

Although both methods demonstrated good correlation in cell number (Figure 4.2; r^2 ; **CFSE** vs. MTT – **0.9919** vs. 0.9912), there was a difference regards the sensitivity of each assay. The MTT assay was able to detect cell numbers to approximately 3000 cells ($n = 2$; $p < 0.05$), however in comparison, using CFSE enabled the detection of cell numbers to approximately 400 cells ($n = 2$; $p < 0.05$). Any slight changes in cell numbers seen with chemotaxis assays require a sensitive detection method. CFSE provides this sensitivity whilst remaining cost effective and was thus used in all chemotaxis experiments.

4.3.2 CCR4 Expression

The specificity of CCR4 as a target for immunotherapy has been widely debated. It is critical to understand which cell populations would be affected, should CCR4 antagonism be employed. Flow cytometry was used to detect CCR4 on different cell populations (Figure 4.3). From Figure 4.3, there is definitive expression of CCR4 on Treg (89.1 % (79.2 – 99.1), $n = 6$), this indicates that Treg would be susceptible to CCR4 antagonism. Other cell populations were assessed for their CCR4 expression. CD8⁺ T cells (18.2 % (15.3 – 21.4), $n = 6$) and CD4⁺CD25⁻ effector T cells (30.4 % (19.7 – 36.2), $n = 6$) demonstrated significantly lower CCR4 expression compared to Treg. Ovarian tumour cells also demonstrated significantly lower CCR4 expression compared to Treg (A2780; 5.5 % (4.3 – 6.3) and IGROV1; 41.9 (39.6 – 44.2); $n = 3$). Literature suggests that CCR4 tends to be expressed on those cell types which provide a ‘regulatory’ function such as Treg (107) and M2 macrophages (108). Effector immune populations such as CD8 T cells and CD4⁺CD25⁻ effector T cells are reported to express CCR3 and CCR5 at higher degrees (109).

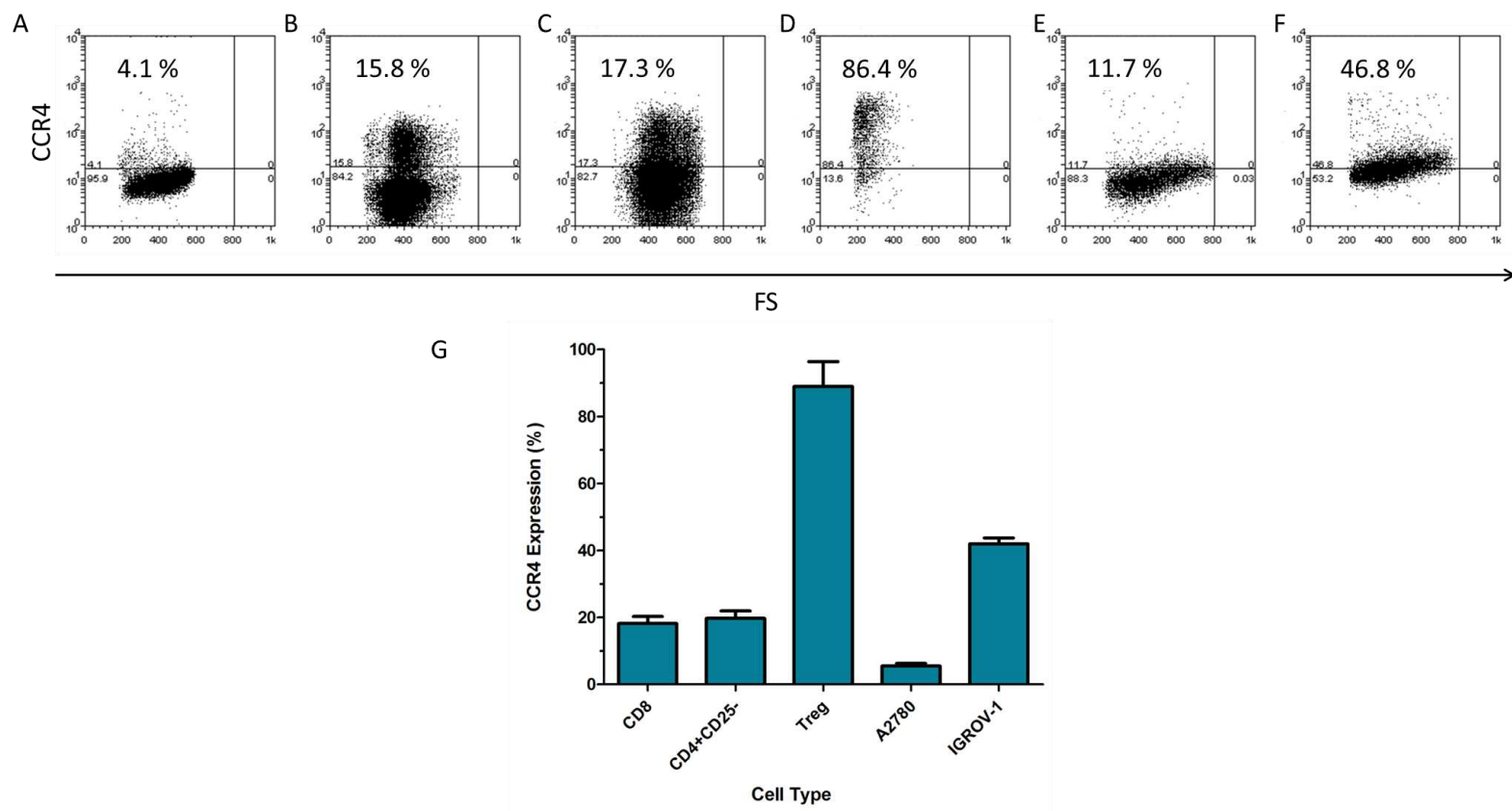


Figure 4.3 – CCR4 Expression on Different Cell Populations. FACS analysis for CCR4 expression. **A-** Isotype Control, Mouse IgG1-FITC, **B –** CD8⁺ T cell, **C-** CD4⁺CD25⁻ T cell, **D –** Treg, **E –** A2780, **F –** IGROV1, **G –** Percentage CCR4 expression; *n* = 6.

4.3.3 Induction of CCL17/CCL22

The role the tumour plays in causing this migration needs to be established *in vitro*. Work conducted by Berin *et al.* (110) demonstrated the upregulation of CCL22 at both the genetic and protein level by human intestinal epithelial cells. This upregulation was caused by the addition of the cytotoxic cytokines TNF- α and IFN- γ to monolayers of intestinal epithelial cells.

Work by Curiel *et al.* (92) has suggested that CCL22 is a driver for Treg migration in ovarian tumours, with increased levels found in tumour ascites. This led to the concept of CCL22 acting as a regulatory element to cytotoxic immune attack. If effector T cells recognise tumour associated antigens, they mount an immune response towards the target, which in part is the release of cytotoxic cytokines like TNF- α and IFN- γ (111). This in turn could cause the increased production of CCL22, which induces Treg migration and thus aids suppression of the cytotoxic immune response.

In order to replicate this effect, the ovarian cancer cell line A2780 was exposed to varying concentrations of TNF- α and IFN- γ . Responses were measured at the genetic level using qRT-PCR (Figure 4.4) and at the protein level using ELISA (Figure 4.4).

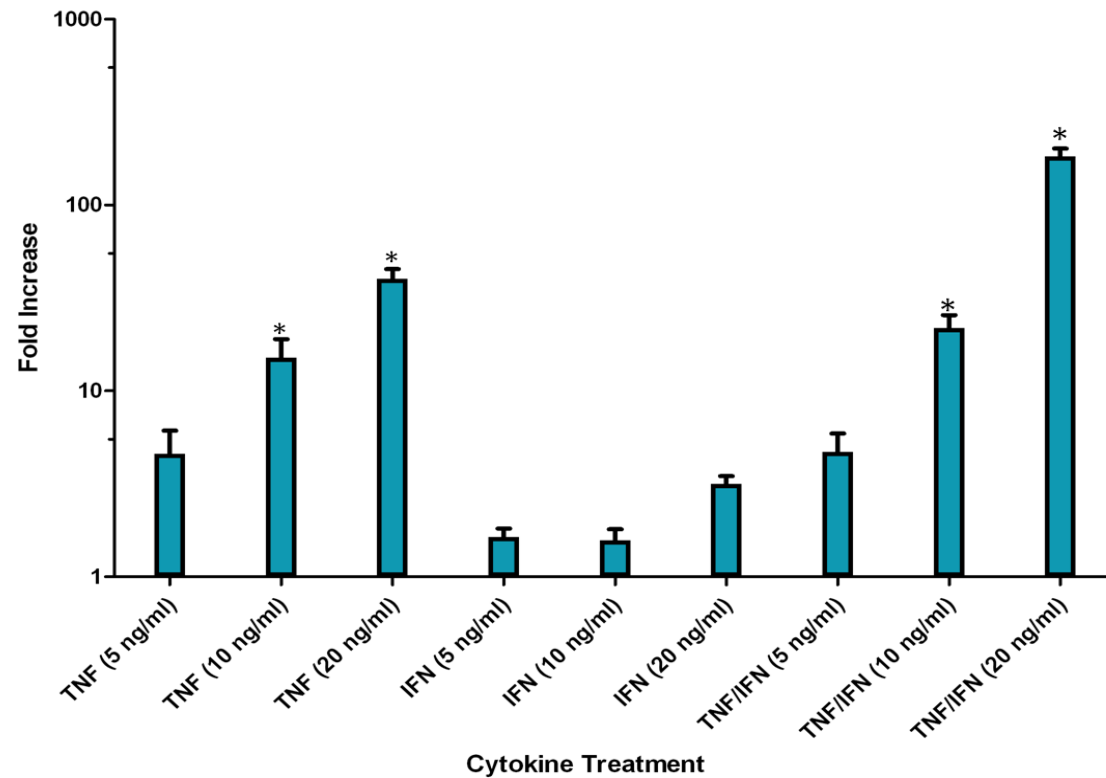


Figure 4.4 – Fold increase in Expression of CCL22 from A2780 on addition of TNF- α and IFN- γ . Cytokines were added at concentrations of 5, 10 and 20 ng/ml as individual treatments and as a synergic mixture. CCL22 mRNA expression was assessed by real-time PCR after stimulation with the indicated cytokines for 24 h. Fold increase in gene expression compared to non-stimulated samples. GAPDH was used as a housekeeping gene. Results shown are mean values of triplicates ($n = 3$, $p < 0.05$). Error bars represent standard deviation. $*=p<0.05$. TNF - tumor necrosis factor-alpha; IFN – interferon-gamma.

From Figure 4.4 there is an increase in CCL22 expression with increasing pro-inflammatory cytokine concentration (E.g. Figure 4.4, TNF- α , 5/10/20 ng/ml; (fold increase, 1.74/2.36/3.26). There is no significant increase in expression with low concentrations of IFN- γ Figure 4.4, IFN- γ , 5/10 ng/ml; (fold increase, 1.71/2.31, $n = 3$, $p > 0.05$). However, treatment with a combination of 20 ng/ml IFN- γ and 20 ng/ml TNF- α led to a 183 (+/- 31) fold increase in gene expression. This expression is translated into increases in actual CCL22 production. Figure 4.5 shows a correlation with the gene expression data. With the addition of pro-inflammatory cytokines, there is a statistically significant increase in CCL22 production compared to non-stimulated samples (Figure 4.5; no-stim; ng/ml, 0.841 +/- 0.188, $n = 3$). However, there is no statistical difference between treatment regimens ($p < 0.05$). This could be due to the lack of translation of the upregulated gene to protein or the robustness of the assay. CCL17 was also shown to be upregulated on pro-inflammatory stimulus of cells (Figure 4.5; no-stim; ng/ml, 0.037 +/- 0.006, $n = 3$). Although CCL17 is a major consideration for CCR4 related migration, only protein expression was assessed. This was due in part to cost and work by Mariani *et al.* (112) who suggested that CCL22 acted as the dominant chemokine i.e. more readily produced in an inflammatory environment.

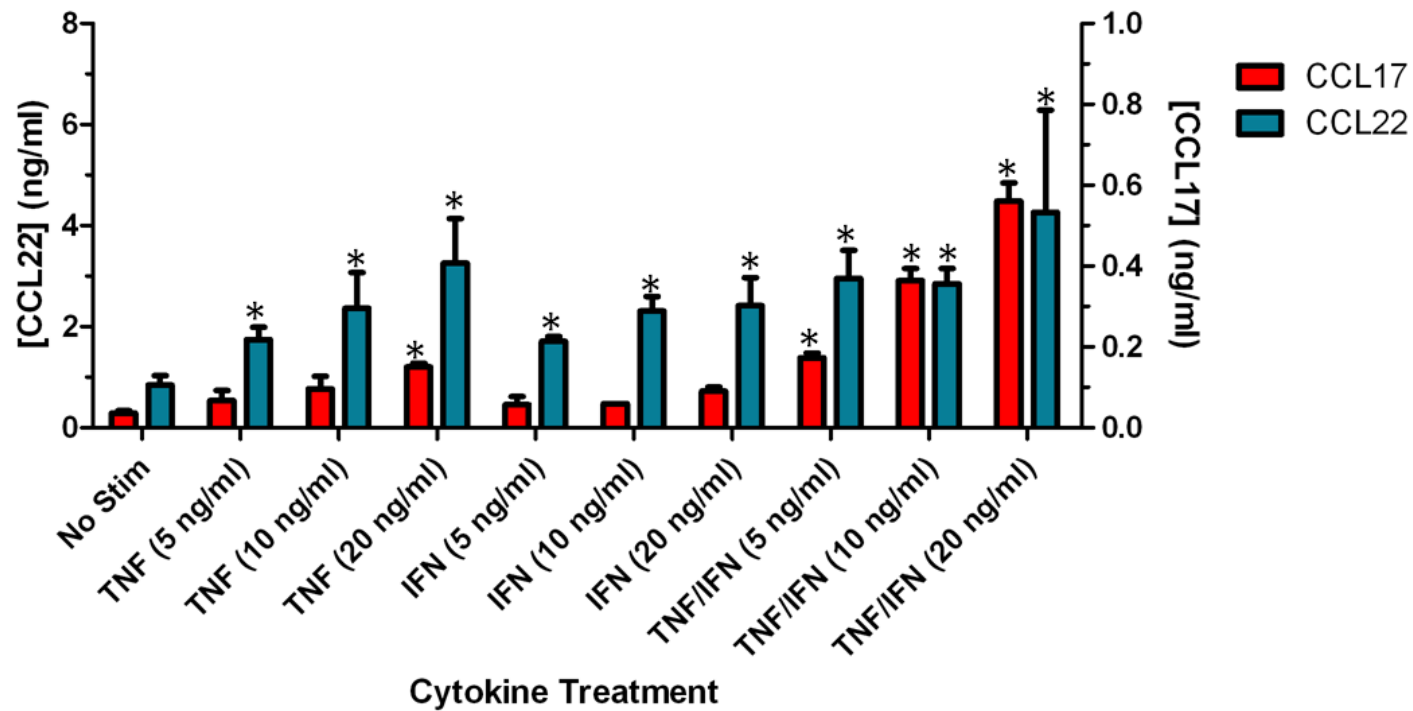


Figure 4.5 – Concentration of CCL17 and CCL22 Produced by A2780 on Treatment with Varying Concentrations of TNF- α and IFN- γ . A2780 cells were grown to confluence and left unstimulated (control) or were stimulated with TNF- α or IFN- γ at 5, 10 and 20 ng/ml respectively. CCL17 and CCL22 in culture supernatants were determined by ELISA. Error bars represent standard deviation. Values are means of triplicates. ($n = 3, p < 0.05$) *= $p < 0.05$. TNF - tumor necrosis factor-alpha; IFN – interferon-gamma.

4.3.4 Cell Migration

The migration of Treg and ovarian cancer cells via CCL17 and CCL22 has been studied *in vitro* by several groups (81,87,102,113). The assay involved the use of a transwell-like system made of an upper and lower chamber, separated by a filter. In the lower chamber, chemokine solutions were placed whilst CFSE-labelled cells were then placed in the upper chamber. The system was then incubated for two hours at 37 °C before the contents of the lower chamber were counted using a fluorescent microplate reader. The number of cells that had migrated was extrapolated from a standard curve of CFSE labelled cells. The major limitation with this type of assay was the number of cells required to provide statistical strength i.e. $n = 3$ replicates. The ability to expand Treg populations which maintain their phenotype and function, as described in Chapter 3, allowed experiments to be carried out more readily. Figure 4.6 shows the migration of Treg to CCL17 and CCL22.

Figure 4.6 demonstrates that both CCL17 and CCL22 are chemotactic factors for Treg compared to non-chemokine controls ($p < 0.05$). This dose response data highlighted concentrations at which Treg migration was at its highest (Figure 4.6; CCL17, control vs. **1 ng/ml**; 9827 +/- 1525 vs. **33933 +/- 2033**; CCL22, control vs. **10 ng/ml**; 9216 +/- 941 vs. **36274 +/- 2620**). These concentrations corroborated with published literature (87,89) and were used in inhibition studies (Figure 4.8).

Figure 4.7 shows that ovarian cancer cells can also migrate under the influence of CCL17 and CCL22. This was evaluated due to evidence of breast cancer

metastases seeding in the lung via CCR4/CCL17 (103). Therefore, it could be possible for ovarian cancer to metastasise via the same mechanism to distant sites. However, it was noted that higher concentrations of both chemokines (100 ng/ml) were required to induce a statistically significant migration ($p < 0.05$). The lower expression of CCR4 has a significant impact on the degree to which these cells migrate. A2780 exhibits a significantly lower degree of migration with both chemokines compared to IGROV-1 at 100 ng/ml (Figure 4.7 (CCL17; A2780 (9230 +/- 191) vs. **IGROV-1 (19345 +/- 1199)** and CCL22; A2780 (12384 +/- 1525) vs. **IGROV-1 (24136 +/- 2620)**). The exhibition of CCR4-mediated migration suggests that antagonism of the receptor would be advantageous in reducing ovarian cell migration.

Despite demonstrating CCR4 expression, the chemotactic activity of CD8⁺ and CD4⁺CD25⁻ effector T cells were not assessed as literature suggests that the dominant chemokines in their migration to tumours are CCL3 and CCL5, which bind to CCR1/CCR3 and CCR5 respectively (114).

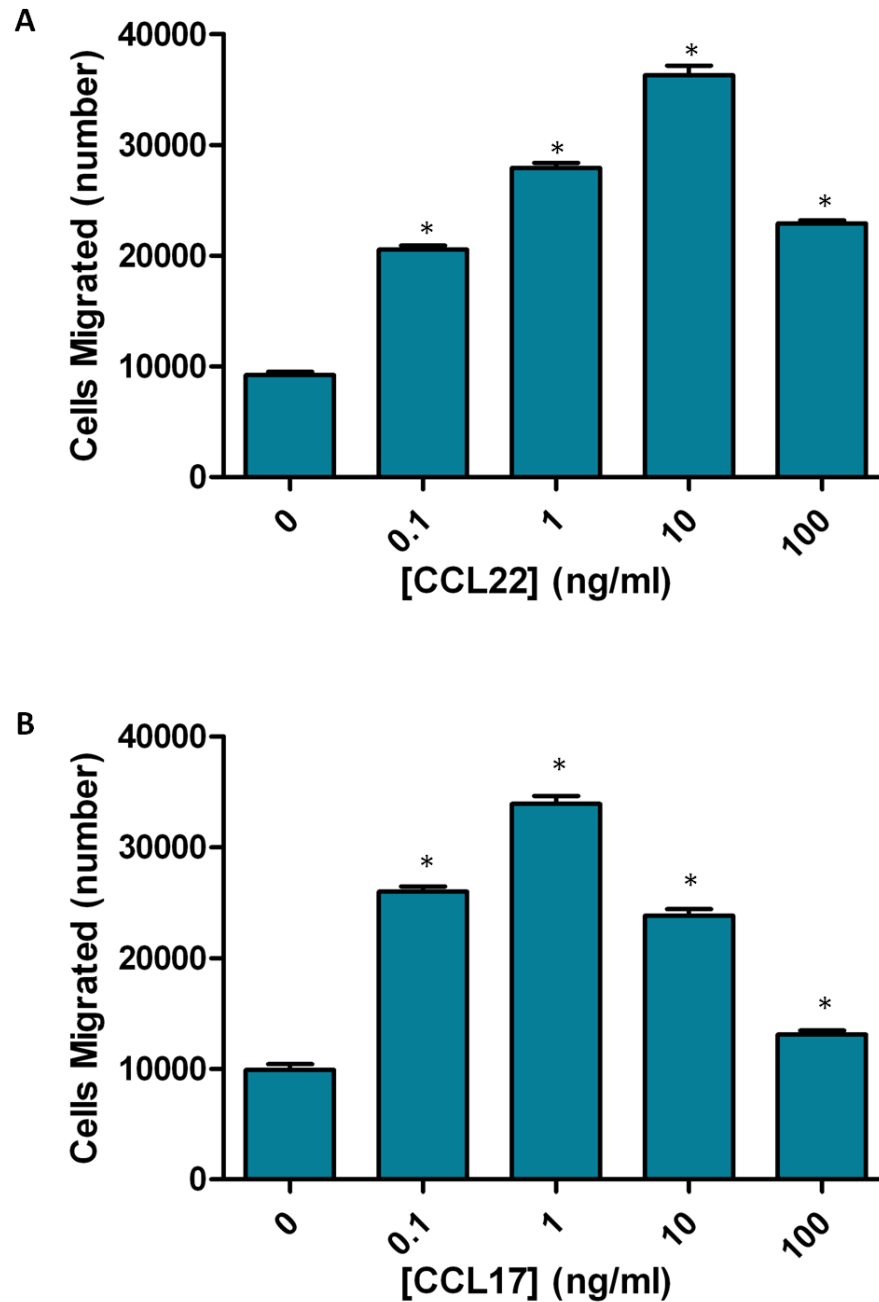


Figure 4.6 - Chemotaxis of Treg. A – to CCL22, B – to CCL17. Treg demonstrate migration to CCL17 and CCL22. Peak Treg migratory chemokine concentrations were determined. CCL17; 1 ng/ml and CCL22; 10 ng/ml. Results shown are mean values of triplicates ($n = 3$, $p < 0.05$). Significance of data compared against 0 ng/ml migration using Dunnett's Multiple comparison test. Error bars represent standard deviation. $*=p<0.05$

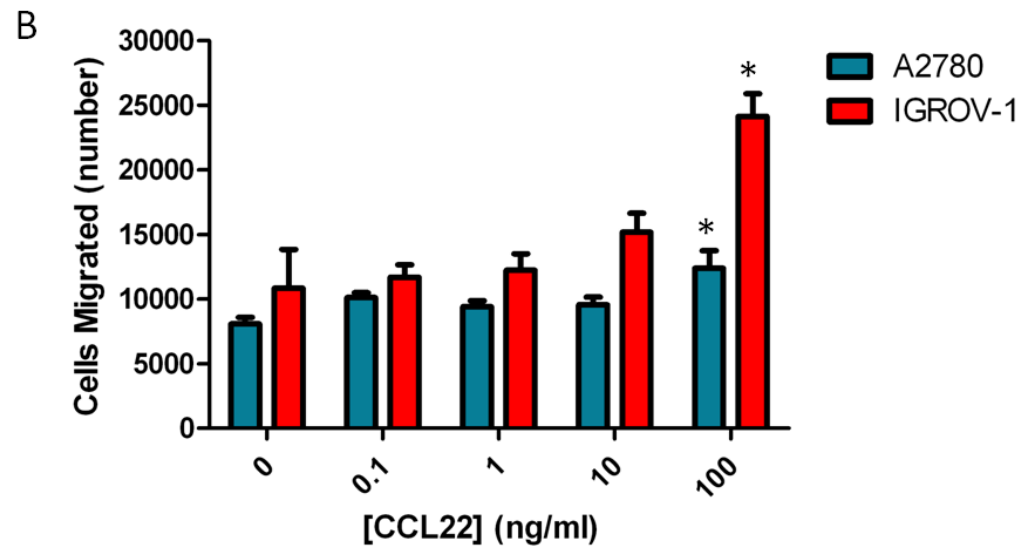
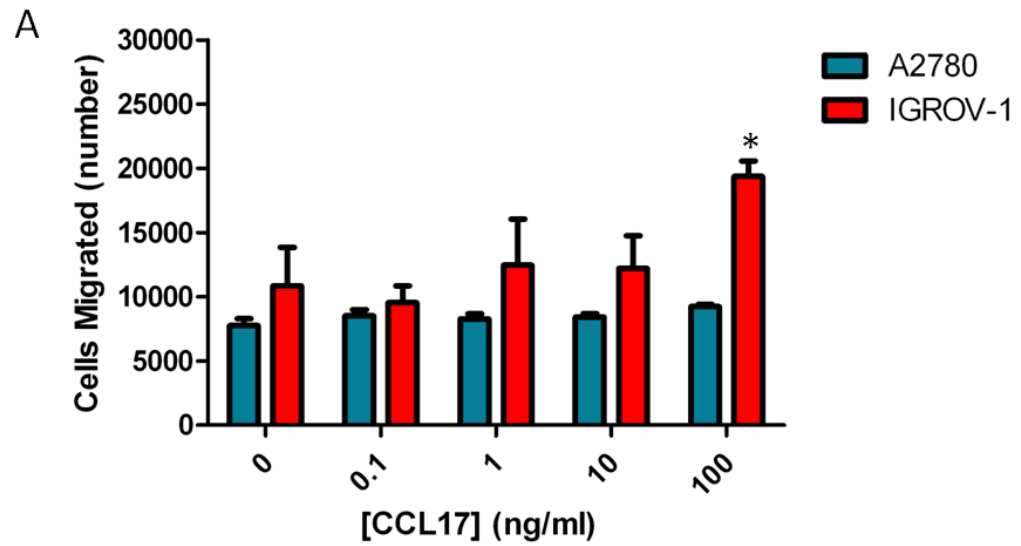


Figure 4.7 - Chemotaxis of A2780 Ovarian Cancer Cells. A – to CCL17. B – to CCL22. IGROV-1 ovarian cancer cells migrate via CCL17 and CCL22. Peak cell migratory chemokine concentrations were determined. CCL17; 100 ng/ml and CCL22; 100 ng/ml. Results shown are mean values of triplicates ($n = 3$, $p < 0.05$). Significance of data compared against 0 ng/ml migration using Dunnett's Multiple comparison test. Error bars represent standard deviation. $*=p<0.05$

4.3.5 Chemotactic Antagonism

Once the migration of Treg was established, AZ1 was employed as a CCR4 inhibitor to investigate its effects on cell chemotaxis. Figure 4.8 highlights the effect on Treg migration to CCL22 (10 ng/ml; 4.8a), CCL17 (1 ng/ml; 4.8b) and a combination of both CCL17 and CCL22 (1 and 10 ng/ml respectively; 4.8c). Cells were treated with AZ1 at a concentration of (0 – 10000 nM).

AZ1 affected Treg migration in a dose dependent manner to CCL17 (100 – 10000 nM, $n = 3$; $p < 0.05$) and CCL22 (10 – 10000 nM, $n = 3$; $p < 0.05$). A combination of both chemokines at their optimal migratory concentrations for Treg (CCL17 = 1 ng/ml; CCL22 = 10 ng/ml) were also restricted by AZ1 (100 – 10000 nM, $n = 3$; $p < 0.05$). The increased migration of Treg seen with a combination of chemokines could be attributed to the ability of CCL17 to bind to CCR8, another chemokine receptor present on Treg (89). However, this was not substantiated due to time and cost.

With regards to ovarian cancer cell line migration (Figure 4.9), AZ1 restricted cell migration to 100 ng/ml CCL22 (Figure 4.9a; 10 – 10000 nM, $n = 3$; $p < 0.05$) and 100 ng/ml CCL17 (Figure 4.9b; 10 – 10000 nM, $n = 3$; $p < 0.05$) and as well as a combination of both (Figure 4.9c; 10 – 10000 nM, $n = 3$; $p < 0.05$) in a dose dependent manner. Trypan Blue staining revealed that AZ1 did not affect cell viability at any concentration. The results indicate that AZ1 could be effective in restricting the recruitment of Treg and ovarian cancer cells via CCR4 antagonism.

The concentration range of AZ1 was partially determined by the specificity of the compound. Previous work has demonstrated that there is a region of high

homology between the chemokine receptors CCR4 and CCR5 (100). AZ1 has been shown to demonstrate affinity for CCR5 at concentrations above 10000 nM. As CCR5 is present on CD8⁺ and CD4⁺CD25⁻ effector T cells (115,116) it would be counter intuitive to dose at such high concentrations as this may restrict effector cell migration to tumour sites.

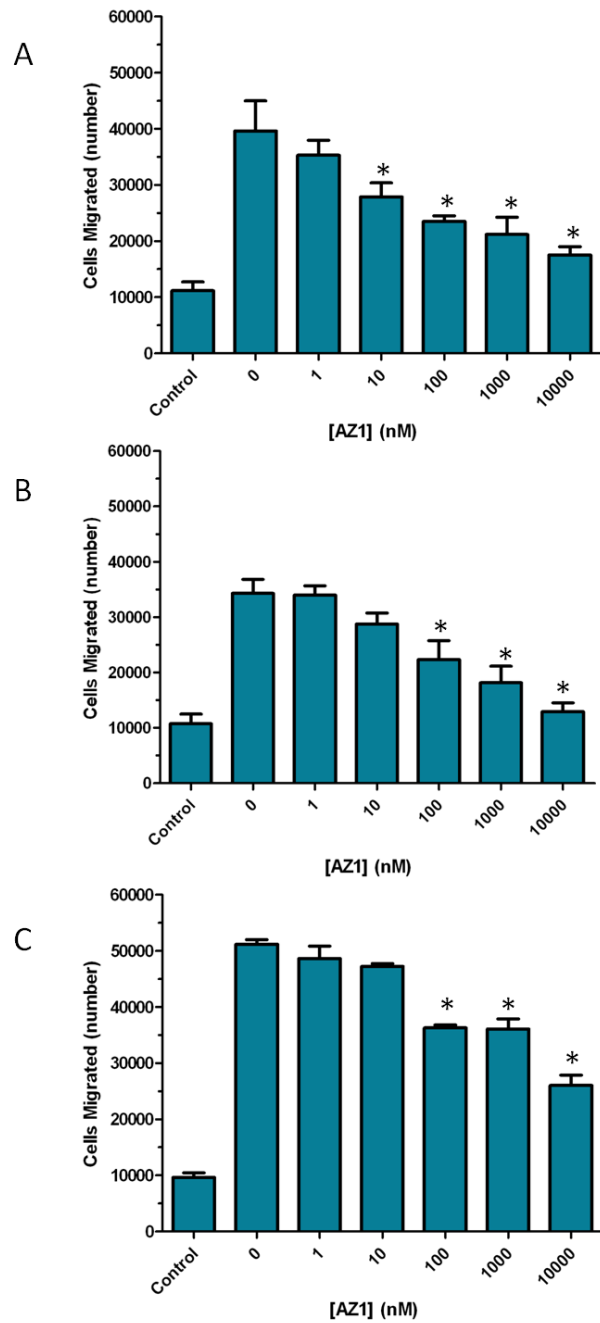


Figure 4.8 - Chemotaxis of AZ1-treated Treg. **A** – against CCL22 (10 ng/ml), **B** – against CCL17 (1 ng/ml), **C** – CCL22 (10 ng/ml) and CCL17 (1 ng/ml). Results shown are mean values of triplicates ($n = 3$, $p < 0.05$). Significance of data compared against 0 ng/ml containing 0.1 % v/v DMSO migration. Control non-chemokine control. Significance of data compared against 0 ng/ml migration using Dunnett's Multiple comparison test. Error bars represent standard deviation. *= $p < 0.05$

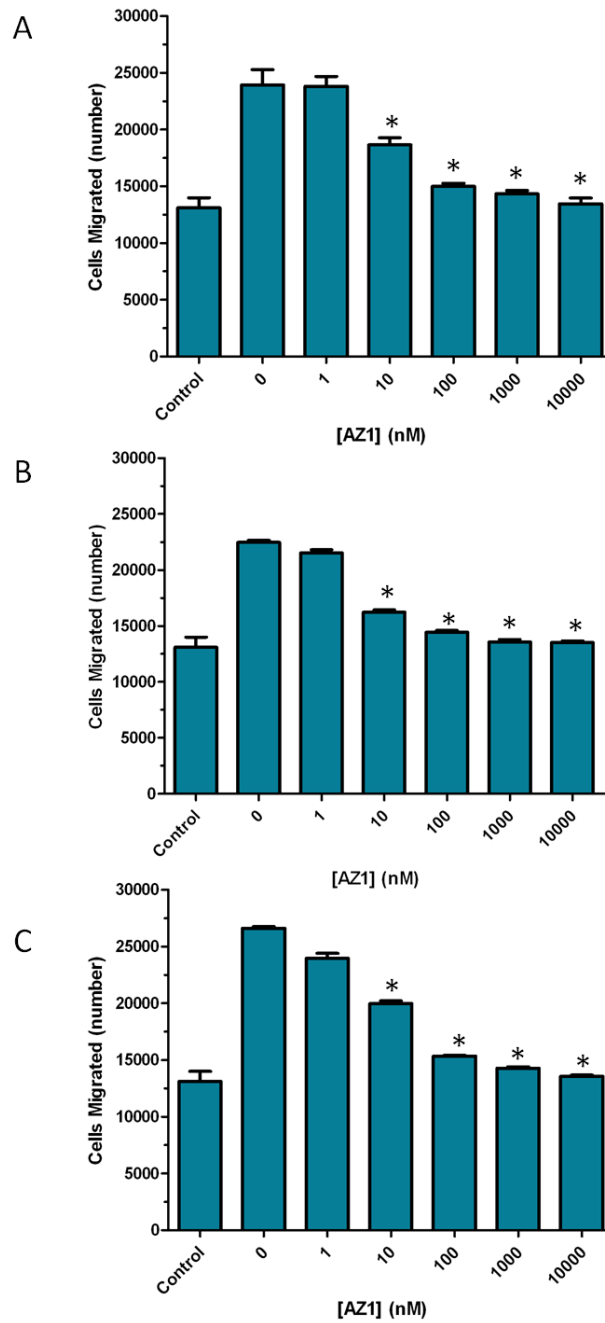


Figure 4.9 - Chemotaxis of AZ1-treated IGROV-1. **A** – to CCL22 (100 ng/ml), **B** – to CCL17 (100 ng/ml), **C** – CCL22 (100 ng/ml) and CCL17 (100 ng/ml). Results shown are mean values of triplicates ($n = 3$, $p < 0.05$). Significance of data compared against 0 ng/ml containing 0.1 % v/v DMSO migration. Control non-chemokine control. Significance of data compared against 0 ng/ml migration using Dunnett's Multiple comparison test. Error bars represent standard deviation. *= $p < 0.05$

As described previously, pro-inflammatory stimuli can lead to the production of CCL17 and CCL22 from ovarian tumour cells. The supernatant analysed from cells stimulated with 20 ng/ml IFN- γ and TNF- α (CCL17 concentration = 0.56 +/- 0.046 ng/ml; CCL22 concentration = 4.26 +/- 3.01 ng/ml) in Figure 4.5 was used in a Treg migration assay (Figure 4.10). Treg migrated under the influence of CCL22 derived from stimulated ovarian cancer cells. Using AZ1 in Figure 4.10, this effect was abrogated (1 – 10000 nM, $n = 3$; $p < 0.05$) which adds further evidence for the ability of AZ1 to restrict Treg migration to ovarian tumours.

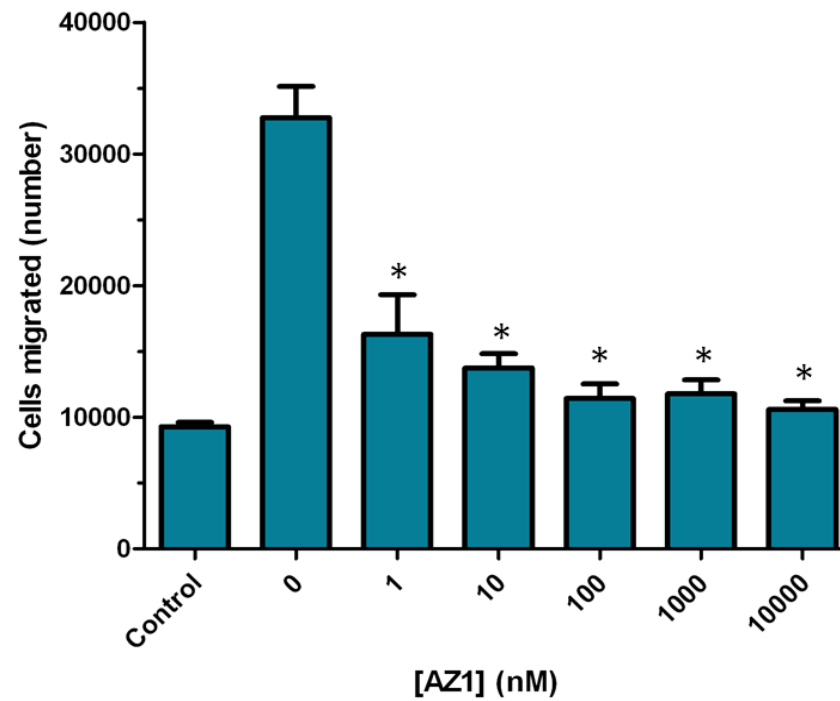


Figure 4.10 - Chemotaxis of AZ1-treated Treg to A2780 cancer cell derived CCL22. Results shown are mean values of triplicates ($n = 3$, $p < 0.05$). Significance of data compared against 0 ng/ml containing 0.1 % v/v DMSO migration. Control non-chemokine control. Significance of data compared against 0 ng/ml migration using Dunnett's Multiple comparison test. Error bars represent standard deviation. *= $p < 0.05$

4.3.6 CCR4 Internalisation

Elegant work by Mariani *et al.* (112) has demonstrated the functional consequences of chemokine-chemokine receptor binding. They describe the rate at which the CCR4 receptor is internalised and then recycled after CCL22 engagement. CCL17 did not demonstrate similar phenomena. As described by Andrews *et al.* (100) AZ1, a pyrazinyl-sulphonamide, requires access to the cytoplasm for its activity, suggesting that the compound acts via an intracellular site on CCR4 which is could involve part of the C-terminus. This interaction between compound and receptor leads to not only a significant reduction in the affinity of chemokine binding but the degree of receptor internalisation and thus a reduction in downstream calcium signalling (100). It was therefore decided that a demonstration of the pharmacological activity of AZ1 on CCR4⁺ Treg would be important in highlighting its potential use as a chemokine receptor antagonist and subsequent immunotherapeutic adjuvant.

Figure 4.11 demonstrates the effect of AZ1 on CCR4 internalisation. Treg were treated with a range of concentrations of AZ1 prior to incubation with CCL22. A high dose of CCL22 (500 ng/ml) was used to promote rapid internalisation as described by Mariani *et al.* (112) the surface expression of CCR4 was then assessed using FACS analysis. Ideally, intracellular staining for CCR4 would also have been conducted. Unfortunately, the expansion of a suitably large, high CCR4 expressing Treg population to cover all experimental groups was unachievable. However, the changes in surface expression of CCR4 on Treg provide an insight into the effects of CCR4 antagonism.

On addition of CCL22 to Treg, CCR4 surface expression fell significantly from 95.73 % (94.2 – 97.2) to 18 % (15.6 - 20.3); $p < 0.05$, which is suggestive of receptor binding and internalisation. The pre-treatment of Treg with various concentrations of AZ1 showed a significant impairment of this internalisation event. At concentrations as low as 1 nM (66.93 % (66.1 – 68.3) vs. control (18 % (15.6 - 20.3); $p < 0.05$), there is still appreciable retardation of receptor internalisation. This adds sufficient strength to the argument of functional CCR4 expression on Treg, whose activity can be impeded through the use of AZ1.

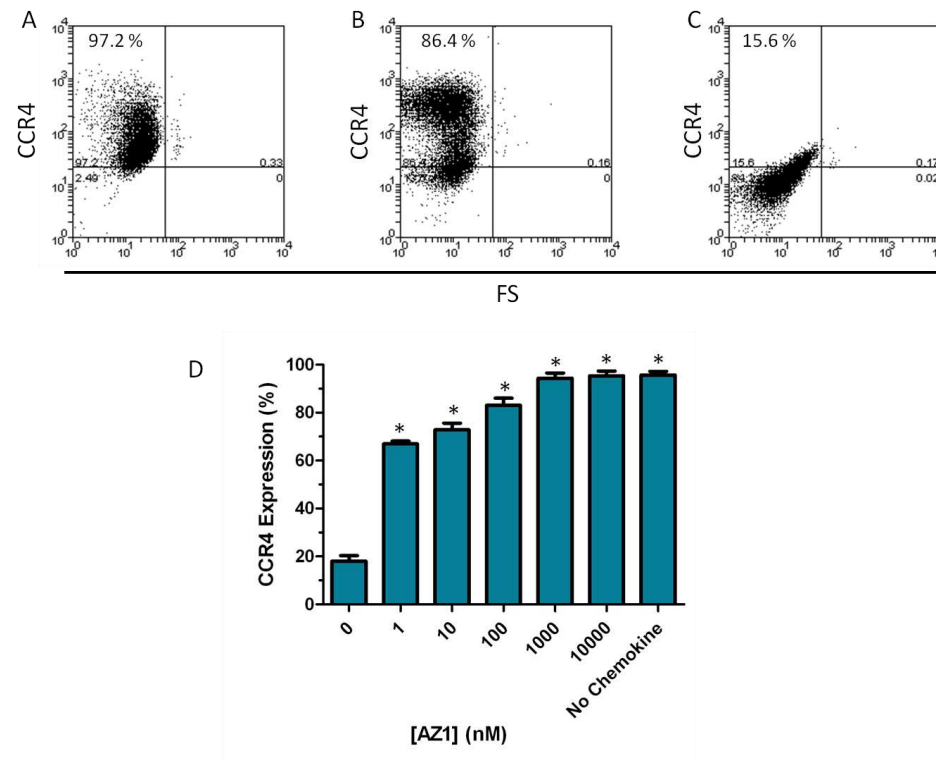


Figure 4.11 – Internalisation of CCR4 on Treg-treated with CCL22 and AZ1. FACS Plots represent CCR4 expression on Treg. **A** – Incubated on ice. **B** – Treated with 500 ng/ml and 100 nM AZ1, **C** – Treated with 500 ng/ml CCL22, **D** – Percentage CCR4 surface expression. Results shown are mean values of triplicates ($n = 3$, $p < 0.05$). Significance of data compared against 0 nM antagonist. Significance of data compared against 0 nM migration using Dunnett's Multiple comparison test. Error bars represent standard deviation. *= $p < 0.05$

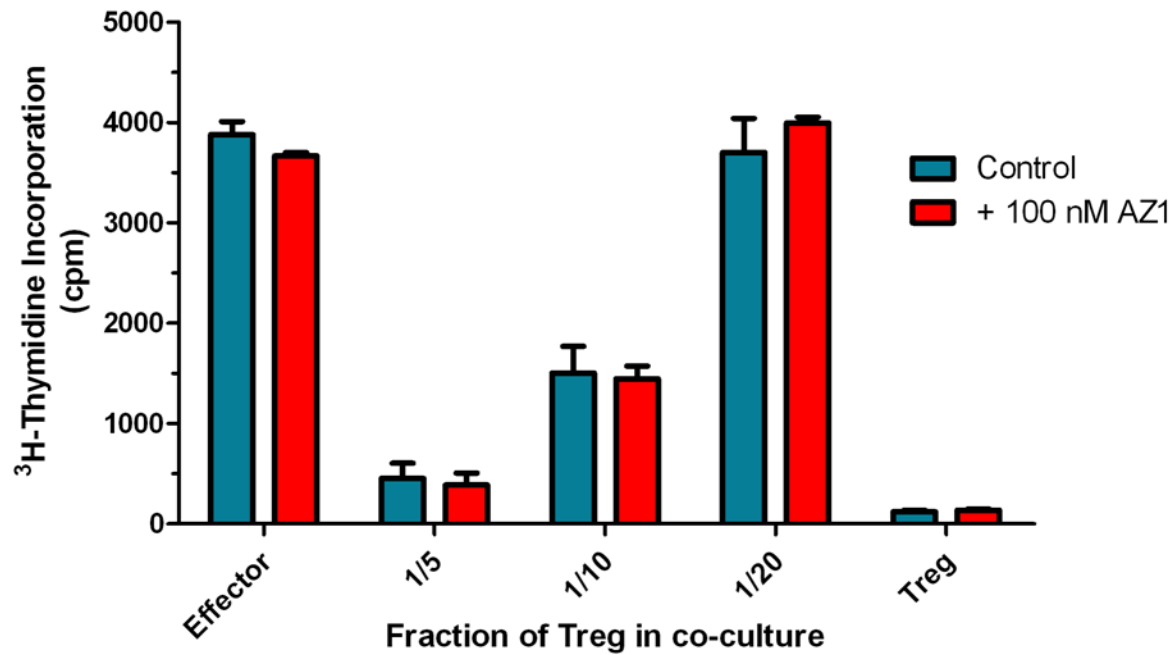


Figure 4.12 – Treg Suppression of CD4⁺CD25⁻ Effector T cells in the Presence of 100 nM AZ1 Treg demonstrate suppression of CD4⁺CD25⁻ T cells where Treg were present at a 1/5 and 1/10 fraction of total co-culture. AZ1 has no effect on the suppressive activity of Treg. Figure representative of three independent experiments from the same donor. Error bars represent standard deviation. * $p < 0.05$

4.3.7 Effect of AZ1 on Treg Suppressive Function

An important characteristic of Treg is their ability to suppress multiple arms of the immune system. In an immune competent individual, Treg homeostasis is critical in regulating auto-immunity (117-119) and so it is important to assess whether AZ1 has any detrimental effect on this ability. As stated previously, Treg cells remained viable after treatment. Figure 4.12 shows how treatment of Treg with 100 nM of AZ1 does not alter their suppressive phenotype. A concentration of 100 nM was used as this is the IC_{50} of the compound (100). This suggests that AZ1 could be used as a therapeutic moiety in ovarian cancer without compromising peripheral Treg function.

4.4 Conclusions

As part of the immunotherapeutic strategy which has been conceptualised in this thesis, the migration of both Treg and ovarian cancer cells has been studied. The clinical significance of increased Treg infiltration in progressively poorly prognostic ovarian cancer has been, in part, attributed to the chemokine-chemokine receptor axis CCL17/CCL22-CCR4. The expression of CCR4 and its specific chemokines has also been identified as a means for certain tumour types to undergo migration and metastatic seeding at distant sites. This evidence suggests that this method of cell 'recruitment' may be a target for therapeutic intervention.

Based on the findings above, CCL17 and CCL22 are both implicated in Treg migration. Optimal migratory concentrations of both chemokines (CCL17, 1 ng/ml; CCL22, 10 ng/ml) were obtained and used in subsequent inhibition assays. The propensity of ovarian cancer cells to produce CCL17 and CCL22 in response to pro-inflammatory stimuli was also observed. This suggests that the immunological time course of events within the solid tumour microenvironment starts with a pro-inflammatory period, i.e. an anti-tumour response, followed by an ensuing regulatory, or anti-inflammatory, response. The release of CCL17 and CCL22 into the microenvironment could be considered part of that anti-inflammatory response; inducing the migration of Treg to dampen any local pro-inflammatory event. It should be noted that tumour cells are not the sole contributor to CCL17/CCL22 production within the tumour microenvironment and that tumour-associated macrophages have also been shown to produce these chemokines.

The use of AZ1, a specific CCR4 antagonist has demonstrated efficacy in reducing both Treg and ovarian cell migration to both chemokines. The proposed mechanism of action of the compound is through association with the cytoplasmic tail of the chemokine receptor, thus rendering it unable to internalise and activate downstream pathways to facilitate migration. AZ1 ably demonstrates this on Treg as shown above. With no change in cell viability or the suppressive function of Treg, AZ1 has the potential to be used as part of an immunotherapeutic regimen. Despite its potential, AZ1 cannot be used as a standalone therapeutic. This is due to the therapeutic window under which it would be administered. In general, tumour therapy begins after the establishment of a solid tumour. During this time, Treg may have already migrated from the peripheral to the tumour site. Evidence suggests that as the disease progresses there is an increase in Treg infiltration (1,92). This means that abrogating the chemotactic function of CCL17 and CCL22 would reduce Treg infiltrate over time as the disease progressed. However, it would not limit the immune suppressive effects of Treg that have already migrated and thus, embedded themselves in the tumour microenvironment architecture.

To account for this effect, the next two chapters of this thesis look at restricting the suppressive function of Treg as well as simultaneously enhancing the generation of an anti-tumour immune response.

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Chapter 5

Chemotherapeutic induction of Tumour-associated Antigen Release

5.1 Introduction

Chemotherapy remains the treatment of choice for most advanced cancers. However, for solid tumours in particular, it is rarely curative. Immunotherapy is a less conventional form of therapy and is also rarely curative. Chemotherapy and immunotherapy have usually been regarded as unrelated or, more commonly, opposing forms of therapy. This is mainly due to the assumption that a) cell death by chemotherapeutic agents is non-immunogenic or aides immune tolerance and b) haematological adverse effects such as lymphopaenia have been assumed to be antagonistic toward mounting an immune response. However, more recent evidence has suggested the case for a combination of both chemo- and immunotherapy (1).

5.1.1 Current Treatment

Treatment of ovarian cancer is based on the integration of surgery and chemotherapy. Chemotherapy plays a major role both in the adjuvant treatment and in the care of patients with advanced disease. Moreover, since most of the patients suffer a recurrence after front-line surgical and medical therapy, further chemotherapy is required for the treatment of the relapse.

Several clinical and prognostic factors are taken into account before starting an anti-cancer treatment for ovarian cancer. Histological type, degree of differentiation, patient's age, type of surgery and residual disease after primary cytoreduction are all important variables that influence therapeutic strategy.

Due to the unavailability of effective screening programmes, ovarian cancer is diagnosed at an early stage (I-IIA) in about 20 % of cases. In general, surgery is able to cure the disease although recurrences are reported in up to 25 % of patients (2). However, according to the results of two studies from the International Collaborative Ovarian Neoplasm group and the European Organisation for Research and Treatment of Cancer, chemotherapy is able to reduce the risk of recurrence in patients with stage I to IIA and prolong overall survival (3).

The standard of care for patients with advanced ovarian cancer is maximal surgical cytoreduction followed by systemic platinum-based chemotherapy. This treatment schedule is considered to yield a 5-year survival rate of 40 % and 20 % for women diagnosed with ovarian cancer at stage III and IV, respectively (4).

Over the years, experts and research groups have experimented with different combinations of drugs in order to improve the prognosis of ovarian cancer. In the 1970s, the treatment of women with advanced ovarian cancer was based on alkylating agents such as Melphalan, Cyclophosphamide, Chlorambucil and Thiotepa, all given as single drugs; response rates were limited with a complete clinical response documented in approximately 20 % of patients, and a median survival of 10–14 months for responding patients (5). The platinum-based drug, Cisplatin was introduced in the early 1980s and

demonstrated that either alone, or in combination with other chemotherapeutic agents, an improvement in overall survival compared to those regimens without platinum (6,7). 1990 saw the introduction of Paclitaxel, an active constituent of bark of the Pacific Yew tree, *Taxus brevifolia* (8,9). Two randomised trials, the GOG 111 and the OV-10, compared Cisplatin/Paclitaxel with Cisplatin/Cyclophosphamide and showed additional clinical benefit when Cyclophosphamide was replaced by Paclitaxel as first-line therapy (10,11).

In the last 20 years studies have been performed in order to improve the outcome of first line therapy such as the possibility to deliver drugs in ovarian cancer through the intra-peritoneal route. The biology of epithelial ovarian cancer, in that it arises from the epithelial surface of the ovary with intra-abdominal spread to the peritoneal cavity, is a strong rationale for the attempt to treat the cancer within the abdominal cavity (12).

In the late 1970s, Robert Dedrick *et al.* (13) predicted that for anti-neoplastic agents possessing particular biological properties there would be a major pharmacokinetic advantage for exposure of the drug to cancer present within the peritoneal cavity following regional delivery, compared to systemic administration of the same agent. Paclitaxel, Cisplatin, and Carboplatin have shown a significant pharmacological advantage when given through an intraperitoneal route (14).

Although the majority of countries still administer drugs intravenously this highlights the importance of localising chemotherapeutic agents at the site of interest while attenuating systemic adverse and pharmacokinetic effects.

5.1.2 New Therapies

The larger expectation for improved prognosis in ovarian cancer is related to the use of the new biological agents. Sustained research has led to a greater fundamental understanding of some key molecular targets such as growth factor receptors, signal transduction pathways, cell cycle regulators and angiogenic mechanisms.

One of the most investigated and promising drugs in ovarian cancer is Bevacizumab, a monoclonal antibody directed against VEGF. Several phase II studies have shown that Bevacizumab is active in recurrent ovarian cancer (15,16). A phase III trial has recently evaluated the role of Bevacizumab in first-line chemotherapy as an adjunct to Carboplatin and Paclitaxel. The GOG 218 trial is a multi-centre, placebo-controlled trial with the primary end point to determine whether the addition of Bevacizumab to standard cytotoxic therapy is able to prolong progression-free survival after primary cytoreductive surgery. Preliminary data has been presented at the American Society of Clinical Oncology annual congress 2010 showing that Bevacizumab plus chemotherapy followed by Bevacizumab maintenance therapy is able to prolong progression-free survival by approximately 4 months (10.7 months versus 14.1 months) compared to Carboplatin–Paclitaxel alone. The survival data for this trial are not yet available (17,18).

Pazopanib is a potent and selective multi-targeted receptor tyrosine kinase inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR- α/β , and c-kit that blocks tumour growth and inhibits angiogenesis. Recently approved for use in renal cell carcinoma, it is now thought to be of use in ovarian cancer. A recent phase II trial saw 11 out of 36 patients respond to the drug (19). Another tyrosine kinase inhibitor, Erlotinib which targets epidermal growth factor

receptors is currently indicated for metastatic non-small cell lung cancer and advanced pancreatic cancer. Phase II trials of Erlotinib have yielded mixed results with inconclusive data suggesting that the drug is of benefit in those patients that are platinum sensitive (20,21) but causes unexpected gastrointestinal adverse effects (22).

Abagovomab is a murine monoclonal antibody that functionally imitates the tumour-associated antigen, CA-125. It has been shown to be well tolerated and to induce a sustained immune response in initial Phase I and II clinical trials. A Phase III trial (MIMOSA) completed its double-blind period in December 2010 and will compare Abagovomab maintenance therapy to placebo, which will determine its efficacy in patients with ovarian cancer (23,24).

5.1.3 Chemotherapy and the Immune System

As mentioned previously, cytotoxic chemotherapy is an effective and common modality for the treatment of ovarian cancer. These drugs exploit the rapid proliferation of malignant cells and typically act to disrupt the cell cycle (Cyclophosphamide), inflict damage by a range of mechanisms including prevention of microtubule function (Paclitaxel), disruption of purine and pyrimidine supply (Gemcitabine), and DNA damage (Cisplatin) resulting predominantly in apoptotic cell death. While such therapy has shown success by directly killing tumour cells, the resulting apoptosis was previously considered to be a non-immunological event, and chemotherapy itself is considered to have an immune suppressive effect on the host immune system.

During normal cell turnover, apoptotic bodies are cleared through macrophage-mediated phagocytosis and do not induce an immune response, due to release of potent anti-inflammatory mediators. However, there is increasing evidence that cytotoxic drugs result not only in tumour cell death but are able to stimulate the immune system (25-30). It is proposed that massive tumour cell apoptosis releases a flood of tumour-associated antigen which may be processed by DC for cross-presentation to naïve T cells in tumour- and peripheral- draining lymph nodes (29). This increase in antigen can also result in the loading of tumour stroma allowing bystander killing of local tumour cells by activated tumour-specific CD8⁺ T cells resulting in tumour eradication (31). Interestingly, and somewhat more controversially, cytotoxic chemotherapy has been shown to modulate the anti-tumour immune response directly by altering the context of dying tumour cells and indirectly, by reducing immune suppressive pathways (1,26). The well-known haematological adverse effects of cytotoxic drugs can affect the balance of pro- and anti-tumour immune cell populations and it is probable that this contributes to the therapeutic efficacy of some cytotoxic drugs.

The importance of the immune system in the anti-tumour effects mediated by cytotoxic agents has been studied for many years. In the 1960s, Mihich *et al.* (32) examined the possibility that the curative effects of anti-tumour agents resulted from synergy between the drug itself and the immune system of the host. Since then, the availability of transgenic animal models bearing defects in genes encoding immune functions have been key to showing that the clinical efficacy of some cytotoxic drugs relies on an intact immune system. The removal of the IFN- γ response or depletion of CD8⁺ T cells abrogated the therapeutic effect of Oxaliplatin against EL4 thymoma and CT26 colon carcinoma (33) and abolished the anti-tumour effects of Doxorubicin against

CT26 cells, MCA-induced fibrosarcomas or rat PROb colon carcinomas (33,34). Gemcitabine has also been shown to require an intact immune system for its full anti-tumour function (35). Given this, extensive studies have been performed over recent years to identify the ways in which cytotoxic drugs utilise the immune system for their clinical effectiveness.

5.1.4 Immunogenic Chemotherapy-induced Tumour Cell Death

Chemotherapy may interfere with several steps in the induction of anti-tumour immunity. The final effect of chemotherapy is thus dependent on the overall effect on several steps from initial apoptosis induction to the development of immunological memory. Scheffer *et al.* (36) compared the immune response against apoptotic or necrotic tumour cells. This study showed that the cell death mechanism is important for the immunogenicity of malignant cells. Furthermore, cytotoxic drugs often act by inducing apoptosis in malignant cells, and in a recent experimental study Casares *et al.* (34) described that the apoptotic phenotype was important for induction of anti-tumour immunity. These authors compared the two DNA-binding and apoptosis-inducing drugs Doxorubicin and Mitomycin in an experimental animal model and demonstrated that malignant cells could take different pathways to apoptosis. Death by Doxorubicin triggered an immune response, whereas the apoptotic cells killed by Mitomycin did not. The authors demonstrated that colon-carcinoma (CT26) cells, killed by Doxorubicin were first endocytosed by dendritic cells which led to the subsequent induction of anti-tumour CD8⁺ T cells. Direct injection of Doxorubicin into the tumours caused immune-dependent regression (34,36). In contrast, neither dendritic cell uptake nor tumour regression was observed by using Mitomycin. Therefore it was

suggested that the Doxorubicin-induced apoptosis and the development of tumour-specific immunity was critical for outcome.

Some chemotherapeutic agents have been shown to alter the context of antigen presentation from the dying tumour cell such that it becomes immunogenic (37-39). Obeid *et al.* (38) performed a systematic screening of chemotherapeutic compounds for their ability to induce immunogenic tumour cell death by treating CT26 colon carcinoma cells with a panel of cytotoxic agents known to induce apoptosis and assessing their capacity to generate a productive T cell response in a vaccine setting. While the majority of apoptosis-inducing agents failed to induce immunogenic cancer cell death, Doxorubicin, induced a potent protective anti-tumour immune response, an effect which was also observed in EL4 thymoma and MCA205 sarcoma (38). Further study revealed that this effect was mediated by translocation of the endoplasmic reticulum resident calreticulin (CRT) and ERp57 to the plasma membrane of dying cells (38,40). Importantly, CRT/ERp57 exposure appeared to function as an 'eat-me' signal which was required for optimal phagocytosis of dying tumour cell material by DC, resulting in a protective anti-tumour immune response (37).

These initial studies led to the discovery of other substances associated with cellular damage which provide immunogenic signals. High mobility group box protein-1 (HMGB-1) is a nuclear protein released from damaged and necrotic cells which triggers an inflammatory response (41). Doxorubicin and Oxaliplatin-induced apoptosis of tumour cells has been associated with release of HMGB-1 into the extracellular milieu (37,42). Subsequent interaction of HMGB-1 with its receptor, TLR-4, on DCs is reported to facilitate efficient processing and cross-presentation of tumour-associated antigens.

However, despite interactions with DC and the resulting CTL response, neither HMGB-1 nor CRT/ERp57 was sufficient to induce maturation of DC. In contrast, chemotherapy-mediated damage of tumour cells may also induce heat-shock proteins such as HSP-90, which can stimulate the immune system by (a) interacting with DC via the scavenger receptor CD91 causing activation and (b) chaperoning tumour-specific peptides into antigen presentation pathways leading to efficient T cell activation (39). HSP-90 expression on the surface of Bortezomib-treated myeloma cells enabled phagocytosis and activation of autologous DC (39). Delivery of this activation signal was dependent on cell–cell contact between DC and dying tumour cells and was mediated by Bortezomib-induced expression of HSP-90. This effect was not observed when myeloma cells were treated with γ -irradiation or steroids (39). Thus, the type of stress response induced depends on the cytotoxic drug of choice. In addition to TLRs, nucleotide-binding domain, leucine-rich repeats containing proteins (NLR) can recognise danger signals associated with chemotherapy-induced cell damage. A recent study by Ghiringhelli *et al.* (33) identified the importance of the NLR receptor family purine domain containing the NLRP3 inflammasome, in the DC-mediated immune response to dying tumour cells.

The ramifications of these findings for human disease have not been extensively studied; however, emerging data suggests that some of these pathways play a crucial role in mediating the clinical efficacy of anti-cancer chemotherapy. The clinical relevance of the HMGB-1/TLR-4 interaction was shown recently in Doxorubicin-treated breast cancer patients, where the loss of functional TLR4 due to polymorphism correlated with poor survival following chemotherapy or radiotherapy (37). The same polymorphism was also seen in patients with metastatic colorectal cancer treated with Oxaliplatin

(42). Conversely, patients bearing normal expression showed increased progression free survival compared with those bearing the mutant allele. This effect was unique to those treated with Oxaliplatin as CRC patients who underwent curative surgery with no chemotherapy did not differ in their progression-free survival in terms of their TLR4 expression (42). These initial studies have shown that selective immune defects in cancer patients can compromise the clinical efficacy of some anti-cancer drug regimens and thus provide proof of principal that an intact immune system helps mediate the chemotherapeutic response to some drugs.

5.1.5 Chemotherapeutic-induced Immune Modulation

While cytotoxic drugs exploit the increased proliferative activity of cancerous cells relative to other cells, they have a narrow therapeutic index, and toxicity to more rapidly proliferating normal cells, in particular, haematopoietic cells, produces potentially unwanted side effects including neutropaenia, lymphopaenia, thrombocytopaenia, and anaemia. This was traditionally thought to be detrimental to the immune system as such conditions rendered cancer patients more susceptible to infection. However, recent opinion has changed, indicating that chemotherapy may actually prime anti-tumour T cell responses and thus it is now less clear how this depletion of immune cells may affect tumour-specific CD8⁺ T cells. Transient lymphopaenia following treatment of solid tumours is rarely sufficient to cause infective complications and may, in fact, lead to elimination of cells that suppress anti-tumour T cell responses and activate homeostatic proliferation of tumour-specific CD8⁺ T cells due to the increased availability of IL-7, IL-15, and IL-21 (43). Turtle *et al.* (44) showed that some memory CD8⁺ T cells survive chemotherapy and replenish the memory T cell pool during lymphocyte recovery. In addition, lymphodepletion may enhance T cell homing to the tumour site and intra-

tumoural proliferation of effector cells. However, the clinical relevance of these effects is not clear, especially in terms of their requirement for the therapeutic efficacy of chemotherapy.

While most cytotoxic compounds are associated with non-discriminatory lymphodepletion, some drugs, when used at certain doses, are able to selectively eradicate suppressive cell populations, most notably Treg.

As discussed previously, Treg depletion or abrogation of Treg function leads to an improved clinical outcome in a variety of cancers (45) and has become an therapeutic strategy of great potential.

Cyclophosphamide, can impair the proliferation and effector function of Treg in both humans and mice (27,46,47). It also induces a robust type I IFN response that can modulate the activation of DC and T cells enhancing the generation of effector T cell immunity (48). In animal studies, these effects translate into protection against developing tumours and regression of established tumours (27).

Several mechanisms have been suggested for this Cyclophosphamide-induced impairment of Treg functionality including the downregulation of the suppressive molecules FOXP3, GITR and CTLA-4 (49) and inhibition of inducible nitric oxide synthase (50). More recently, their sensitivity has been attributed to low levels of intracellular ATP (51). Given the widespread use, availability, and known toxicity of drugs like Cyclophosphamide, Treg depletion through these means is a distinct possibility.

Several small studies have been conducted in man to assess the capacity of Cyclophosphamide to impair Treg function and augment effector T cell responses. Low-dose cyclophosphamide has been shown to decrease the

proportion of Treg (46,52), augment delayed type hypersensitivity reactions, and enhance vaccine protocols (52). In a study by Ghiringhelli *et al.* (46), low-dose oral Cyclophosphamide administered to 9 patients with advanced disease led to a selective reduction in the proportion, number, and suppressive function of peripheral blood Treg. This correlated with an enhanced cytotoxic capacity of peripheral blood NK cells and T cells following treatment. More recently, the immune-modulatory effects of low-dose Cyclophosphamide was studied in 13 patients with advanced hepatocellular carcinoma which yielded valuable information about the dose-dependent effect of cyclophosphamide-mediated Treg depletion (53). Patients were enrolled consecutively in three cohorts of four patients with a dose escalation of Cyclophosphamide (150, 250, 350 mg/m²). The authors observed a reduction in the number, frequency, and suppressive activity of peripheral blood Treg in patients receiving 150 and 250 mg/m² but not at the higher dose of 350 mg/m².

Paclitaxel, has also recently been investigated for its capacity to augment the function and number of Treg. Paclitaxel treatment of the renal cell carcinoma, RENCA, and an EG7 thymoma led to delayed tumour growth which was partially abrogated following depletion of CD8⁺ T cells and enhanced the anti-tumour effect of the TLR9 agonist, CpG (54). To substantiate this evidence, paclitaxel-based chemotherapy was shown to reduce the proportion of peripheral blood Treg in a cohort of patients with stage III and IV non-small cell lung cancer (55).

The above observations, in conjunction with data suggesting that sub-cytotoxic doses of Paclitaxel, Doxorubicin, Mitomycin C, Methotrexate, Vincristine, and Vinblastine were found to increase expression of maturation

and co-stimulation markers on DC (CD40, CD80, CD83 and CD86) and increase antigen presentation to specific T cells (56,57), suggests that classical chemotherapeutic approaches indirectly boost anti-tumour immunity (Figure 5.1). However, there is still doubt as to whether chemotherapy-driven lymphopaenia allows for generation of a robust anti-tumour response. Interestingly, Muller *et al.* (58) noted that the Treg fraction increased on lymphodepletion in mice, while Winstead *et al.* (59) highlighted changes in the constituents of the T cell pool on immune reconstitution. Future work in this area needs to understand the kinetics of immune repopulation especially in the context of the immune suppressive milieu created by solid tumours (60).

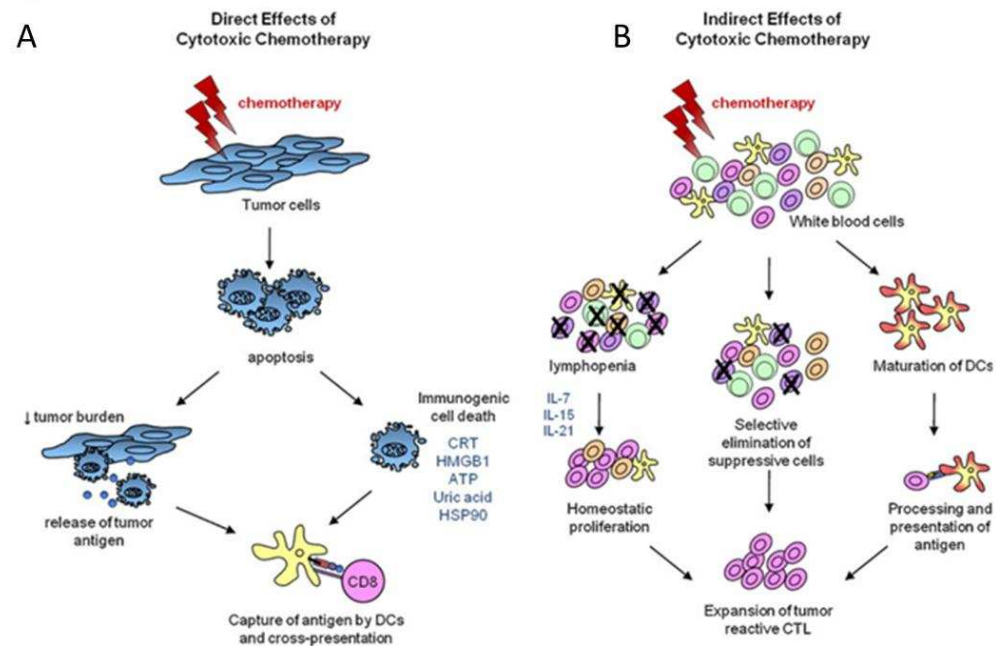


Figure 5.1 – The Direct and Indirect Effects of Chemotherapy. Mechanisms by which chemotherapy may enhance the anti-tumour CD8⁺ T cell response. **A** Cytotoxic chemotherapy acts directly on tumour cells to (1) reduce the physical burden of disease making it a smaller target for immune attack, (2) increase the range of available tumour antigen, and (3) cause immunogenic cell death. **B** - Some cytotoxic drugs stimulate the immune system by causing lymphopenia followed by homeostatic proliferation of immune effectors, activation of DC, or the selective elimination of suppressive subsets, such as Treg. Adapted from (127). Reprinted with permission (License number 2813871408369), Springer Ltd

5.1.6 Release of Tumour-associated Antigens

The rate limiting step in the development of an anti-tumour immune response is the detection and processing of tumour associated antigens (61). The development of approaches for analysing humoral (62) and cellular (63) immune reactivity to cancer in the context of the autologous host has led to the molecular characterisation of tumour antigens recognised by autologous CD8⁺ T cells (64) and/or antibodies (65). Some of these approaches include serological analysis of recombinant cDNA expression libraries (SEREX) (66), differential gene expression analysis, T-cell epitope cloning (TEPIC) (67,68) and bio-informatics (69,70). As a consequence of these advances, human tumour antigens defined to date can be classified into one or more of the following categories: (i) differentiation antigens (that are restricted to very defined tissues), e.g. tyrosinase (71) Melan-A/MART-1 (72) and gp-100 (73) (ii) mutational antigens (that are altered forms of proteins), e.g. CDK4 (74), β -catenin (75), caspase-8 (76), and p53 (77), (iii) amplification antigens, e.g. Her2/neu (78) and p53 (77), (iv) splice variant antigens, e.g. NY-CO-37/PDZ-45 (79) and ING-1 (80), (v) glycolipid antigens, MUC1 (81) (vi) viral antigens, e.g. HPV, (82) EBV (83) and (vii) cancer-testis (CT) antigens (that are not are restricted in expression to the germ line and tumours) e.g. MAGE (67), NY-ESO-1 (66) and LAGE-1 (84).

5.1.7 MUC1

An interesting tumour-associated antigen is Mucin1 (MUC1), because it is overexpressed in most adenocarcinomas (85-87). MUC1 is a protein expressed on the apical surface of most simple, secretory epithelia and on a variety of haematopoietic cells (88). MUC1 is a transmembrane mucin with an

extracellular domain made up largely of 20 amino acid tandem repeats. After translation, the MUC1 protein becomes modified by extensive O-glycosylation. Within each tandem repeat, two serines and three threonines represent five potential O-glycosylation sites. The extent of glycosylation depends mainly on the expression of tissue-specific glycosyltransferases (86). Importantly, it has been demonstrated that in most cancer types, including ovarian cancer, MUC1 is aberrantly glycosylated, has lost its apical expression and is secreted into the circulation (89).

Mucins have numerous functions in the glycocalyx. Their high degree of glycosylation provides lubrication, prevents dehydration, and offers protection from proteolysis. Microbial challenge is frequent in most mucous membranes, and mucins protect against attack by inhibiting microbial access to the cell surface through steric hindrance. Bacterial adhesins bind mucin carbohydrates at the cell surface (90,91), a process that normally protects against infection. In addition to this, MUC1 and MUC4, modulate cell-cell and cell-extracellular matrix interactions (92,93).

The MUC1 cytoplasmic tail has been shown to associate with β -catenin (94), as well as with other signalling molecules, e.g. Grb2/Sos (95), suggesting a potential role for MUC1 in cell signalling (86). In the mammary gland, MUC1 expression increases markedly during lactation along with increased MUC1:erbB1 interactions (96). Tyrosine phosphorylation of the MUC1 cytoplasmic tail occurs in both intact MUC1 and chimeric molecules consisting of CD8 ecto-domains and the MUC1 cytoplasmic tail (97,98). It is not clear if MUC1 phosphorylation or interactions with signal transducing proteins change in response to physiological stimuli. Activation of erbB1 with EGF induces tyrosine phosphorylation of the MUC1 cytoplasmic tail (96,99)

and activation of ERK 1/2 [13]. Moreover, EGF mediated activation of ERK 1/2 is drastically enhanced in the presence of high levels of MUC1 in the mouse mammary gland [13]. Thus, potential stimuli, including growth factors or cytokines may affect MUC1 stability, localisation at the cell surface, or phosphorylation state. Direct interactions with the MUC1 ecto-domain, e.g. by microbes or selectins, also could conceivably trigger signalling events. In this regard, increased tyrosine phosphorylation of the MUC1 cytoplasmic tail is associated with cell-substratum adhesion (97). Thus, MUC1, and perhaps other mucins, have the potential to function as receptors either alone or in cooperation with known signal transducing proteins.

Studies in tumour cells indicate that the amount and type of MUC1 expressed modulate immune responses to these cells. MUC1 is differentially glycosylated in many cancerous cells, exposing tumour-specific epitopes that may trigger an immune response (100,101); however, MUC1 also has been shown to protect cancer cells from immune cell attack (102,103), indicating both immune stimulatory and immune suppressive functions. Several reports demonstrated that MUC1-specific CD8⁺ T cells could be generated from a variety of tumour settings (104-106). This, in conjunction with evidence that MUC1 could be found in the systemic circulation (107-109) suggests that this could be an antigen of interest in attempting to generate an immune response against ovarian tumours.

From the previous work in this thesis and the evidence described above, it became obvious that restricting the migration of Treg to the ovarian tumour microenvironment, as seen in Chapter 4, would not be sufficient to induce an anti-tumour immune response. Therefore, a more complex, multi-faceted treatment modality would need to be implemented which would account for

the potentiation of anti-tumour immune responses. To achieve this, the use of the classical chemotherapeutic, the topoisomerase I inhibitor, Camptothecin, and an immune stimulatory adjuvant, the TLR7 agonist Imiquimod would be used as an additional two-step procedure. First, Camptothecin would cause a significant degree of tumour cell death, leading to the release of the antigen, MUC1. This would be followed by the use of Imiquimod which would be used to stimulate dendritic cells to endocytose and cross-present antigen as well increase CD8⁺ T cell proliferation while reducing the suppressive effects of Treg (see Chapter 6).

However, for this strategy to be successful, the lymphopaenic effect of Camptothecin therapy would need to be accounted for. Despite the evidence above regarding the unheralded ability for chemotherapeutic agents to eliminate Treg and potentiate CD8⁺ T cell activity, there remains a question mark over the durability of this effect. Therefore, any approach which modified the potential lymphopaenia caused by Camptothecin would be advantageous in this setting.

In collaboration with Dr. Johannes Magnusson and Prof. Cameron Alexander of the Division of Drug Delivery and Tissue Engineering, School of Pharmacy, University of Nottingham, Camptothecin was conjugated via disulphide bonds to a hydrophilic polymer, poly(ethylene glycol methacrylate), (PEGMA). The hydrophobic nature of the drug, in combination with the hydrophilic polymer meant that on contact with water, the polymer-drug conjugate self-assembled to form a micelle with drug encapsulated within the structure. This self-assembling phenomena was taken advantage of with the addition of a leuteinizing-hormone like peptide, conjugated to the polymer which was presented on the surface on micellisation of the construct (Figure 5.2).

Leuteinizing hormone releasing hormone receptors (LHRHR) are over-expressed in ovarian cancer compared to other tissues (110,111), thus the application of a ligand to the drug-polymer construct provided a degree of tumour localisation, which as described earlier, is of clinical benefit in the ovarian setting (14). The utilisation of disulphide bonds to conjugate the drug to the polymer also means that on internalisation of the construct into the cell, the drug would be released, under the reductive conditions of the endosome.

This chapter aims to demonstrate, *in vitro*, that treating ovarian cancer cells with Camptothecin yields the tumour associated antigen MUC1. However, it can also be shown the Camptothecin causes significant lymphodepletion which reduces the number of CD8⁺ T cells, key mediators in anti-tumour immunity. Using the novel delivery system described above, the encapsulated drug should yield a reduced adverse immune effect profile while demonstrating preferentially toxicity for ovarian cancer cells and release of MUC1 antigen.

5.1.8 Experimental Objectives described in Chapter 5

- Modification of Camptothecin to target ovarian tissue
- Demonstration of cytotoxicity of modified versus native drug
- Demonstration of MUC1 antigen release on ovarian cancer cell death
- Investigation of the potential for immune toxicity with cytotoxics
- Determination of cytotoxics to alter CD8⁺ T cell and Treg fractions in PBMC

5.2 Methodology

5.2.1 LHRHR Detection

To detect the expression of LHRHR on the cell surface of IGROV-1, A2780, CACO-2 and CALU-3, indirect immunofluorescence staining was performed. Cells were incubated with Rabbit Polyclonal LHRHR (Abcam) (500 ng/mL) or a Rabbit polyclonal IgG isotype control (Sigma) (500 ng/mL) for 20 minutes. After washing, cells were incubated with a goat anti-rabbit-FITC IgG (Sigma) for 30 minutes in the dark. The cells were washed again and re-suspended in for analysis via flow cytometry.

5.2.2 Cytotoxicity Assay

The cell lines A2780, IGROV-1, CALU-3 and CACO-2 were seeded on 96-well plates at 1×10^4 /well. Cells were then treated with a concentration range (0 – 100 μ M) of Camptothecin or JPM137, with JPM135 used a non-targeting control (i.e. no LHRH peptide present). The equivalent amounts of DMSO or PBS were used as vehicle controls. Treatment durations were 24, 48 or 72 hours. After treatment of cells 10 μ l of 5 mg/mL MTT solution (Sigma) was added to each well. The plates were incubated for 4 hours at 37 °C and formazan crystals were dissolved by the addition of isopropanol. Absorbance was measured at 570 nm. The percentage of cell viability was calculated using untreated cells as a maximal proliferation and repeatedly freeze-thawed cells as minimal proliferation.

5.2.3 Competitive Inhibition of JPM137 by LHRH

Cells were seeded as described above. A2780 and IGROV-1 cells were incubated simultaneously with 10 μ M LHRH peptide (GenScript) and a concentration range of JPM137 (equivalent to 0 – 100 μ M Camptothecin). Assays ran for 24, 48 and 72 hours. Cell viability was measured by MTT

assay as described above percentage of cell viability was calculated using untreated cells as a maximal proliferation and repeatedly freeze-thawed cells as minimal proliferation.

5.2.4 ELISA

Antigen release was measured by ELISA. The antigens of interest were MUC1 and CA-125. Polystyrene 96-well plates (MaxSorp, Nunc) were coated with the relevant capture antibody (MUC1; Clone - #ME97, Abcam, CA-125; Clone - #57226, Fitzgerald) overnight. The plate was then blocked with 5 % w/v skimmed milk powder in PBS (pH 7.2) + 0.05 % v/v Tween (PBS/Tween) to prevent non-specific binding. The plate was washed in PBS/Tween using an automated plate washer and tapped dry. The relevant purified antigen (Obtained from Fitzgerald) and samples were added to the plate. After 2 hours, the plate was washed in PBS/Tween before the appropriate horseradish-peroxidase detection antibody (MUC1; Clone - #M3A106, Abcam, CA-125; Clone - #M77161, Fitzgerald) was diluted in PBS, 1 % w/v BSA, and 0.1 % v/v Tween 20 and incubated for a further 2 hours. After incubation at room temperature, the plate was washed and bound horseradish peroxidase was visualised with 0.1 mg/ml tetramethylbenzidine (Sigma) and 2 ul of 30 % v/v hydrogen peroxide (Sigma) diluted in 3 M sodium acetate buffer (Sigma). The colour reaction was stopped with the addition of 2 M sulphuric acid (Sigma), and absorbance was measured at 450 nm using a MRX spectrophotometer (Dynex Technologies). The concentrations of samples were calculated from standard curves of each recombinant cytokine. ELISA detection limits were determined as follows (MUC1 - 2 IU/ml, CA-125 – 4 IU/ml).

5.2.5 PBMC Toxicity Assay

Peripheral blood was obtained from healthy donors in accordance with local ethical committee approval (EC# BT/04/2005). Peripheral blood mononuclear cells (PBMC) were separated via a density-gradient centrifugation over Histopaque-1077 (Sigma) twice as before, firstly to isolate PBMCs with the second density gradient used for the removal of platelets. The resultant cells were then washed in isolation buffer containing phosphate buffered saline (PBS) + 1 % w/v bovine serum albumin (BSA) (Sigma) + 1 mM EDTA (Sigma) before viability counting via Trypan Blue exclusion (Fluka). 1×10^5 /well PBMC cells were stimulated with 10 μ g/ml PHA (Sigma). The final volume was adjusted to 200 μ l using cell media. After 72 hours, 100 μ l of cell supernatant was taken and stored at -40°C . Cells were then either stained for viability using Propidium Iodide (Sigma) or pulsed with 1 μ Ci of [^3H]-labelled thymidine per well (TRA120, Amersham). The plate was left to incubate for a further 18 hours before being harvested onto a microscintillation plate (Perkin-Elmer). After repeated washes with de-ionised water, the plate was allowed to dry for an hour. 20 μ l of scintillation fluid (Microscint 0, Perkin-Elmer) was added per well before the plate was covered with optical tape (Perkin Elmer). Thymidine incorporation was quantified using a Packard TopCount NXT Microplate Scintillation counter.

5.2.6 PI Live/Dead Sorting

Treated PBMC were washed in PBS + 0.1 % w/v BSA before being incubated with 2 μ g/ml Propidium Iodide for 5 minutes. Cells were then washed and sorted by the MoFlo flow cytometer. Cells that were negative for Propidium Iodide were sorted into tubes for Treg and CD8 staining.

5.2.7 Flow Cytometry Analysis

Cells were stained with cell surface markers CD4-ECD (1.25 µl) (clone SFC112T4D11 - Beckman Coulter), 2.5 µl CD8-FITC (Clone HIT8a – BioLegend) CD25-PE (7.5 µl) (Clone BC96 - BioLegend), CCR4-PerCP/Cy5.5 (2.5 µl) (Clone TG6 - BioLegend). Cells were incubated with fluorescently-conjugated antibodies for 15 minutes before fixation with 3 % v/v formaldehyde (Sigma). Cells were then permeabilised using FOXP3 Perm Buffer (BioLegend) before incubation with FOXP3-AlexaFluor488 (Clone 259D – BioLegend) for 30 min. Cells were then resuspended in FACS wash buffer (BioLegend) and read via a Beckman Coulter FC 500 flow cytometer. Analysis involved gating on lymphocyte populations from forward/side-scatter plots before selecting regions of interest.

5.3 Results and Discussion

5.3.1 Expression of LHRHR

LHRHRs are overexpressed in several types of human cancer cells including ovarian, breast, and prostate cancers (112-116). Figure 5.3 demonstrates the expression profile of LHRHR on ovarian (A2780 and IGROV-1), lung (CALU-3) and colon (CACO-2) cancer cell lines. These cell lines were used as they were readily available. The expression of LHRHR is evident on A2780, IGROV-1 and CALU-3 with no detectable expression seen with CACO-2. The expression seen on these cells correlates with existing evidence in both ovarian (115) and lung (117). However, LHRHR is expressed at significantly higher levels compared to other organ groups (116). This differential expression allows for the use of the LHRH peptide and LHRHR as a targeting moiety and target, respectively, to direct Camptothecin specifically to cancer cells. These results demonstrate that the choice of LHRH peptide as a targeting moiety permitted the selective targeting of the tumour and, therefore, prevent damage to normal tissues.

5.3.2 Cytotoxicity of JPM137

Once expression of LHRHR had been confirmed it was important to assess the cytotoxicity of the Camptothecin/PEGMA/LHRH construct (JPM137) against a variety of cell lines in order to ascertain whether this type of delivery system would be useful as part of the treatment stratagem suggested in this thesis.

Figures 5.4, 5.5, 5.6 and 5.7 illustrate the cytotoxicity of Camptothecin, JPM137 and JPM135 (Camptothecin/PEGMA) against the cell lines A2780, CACO-2, CALU-3 and IGROV-1 respectively. These cell lines provide different tissue types which allow for a more accurate assessment of site-

specific targeting. The first observation that can be made is that in those cell lines that express LHRHR (A2780, CALU-3 and IGROV-1) the IC₅₀ values of JPM137 decreased over time. However, treatment of CACO-2 demonstrated an inverse trend with an increase in the IC₅₀.

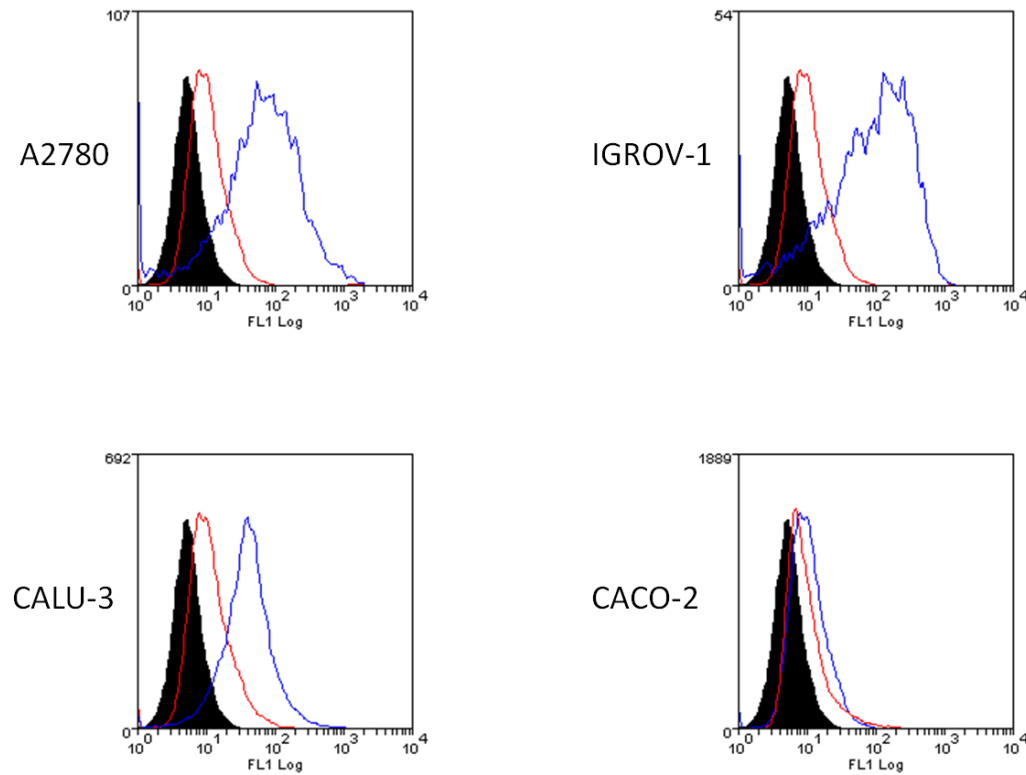


Figure 5.3 – Expression of LHRHR on Different Cell Lines. LHRHR expression measured by flow cytometry. Black represents unstained cells. Red represents Rabbit polyclonal IgG with a goat anti-rabbit IgG-FITC as isotype control. Blue represents Rabbit polyclonal LHRHR Rabbit polyclonal IgG with a goat anti-rabbit IgG-FITC IgG. Figure represents one experiment of three.

This suggests that JPM137 demonstrates greater selectivity for cells which express LHRHR (e.g. **A2780** vs. CACO-2 (72 hours) – Figure 5.4C vs. Figure 5.5C; IC_{50} (M) **8.18×10^{-8}** vs 6.78×10^{-4} , $n = 3$; $p < 0.05$). Confirmation of this observation comes from comparing JPM135 (Camptothecin/PEGMA) with JPM137 (Camptothecin/PEGMA/LHRH). The lack of targeting ligand reduces the efficacy of the construct (IGROV-1 (72 hours) – Figure 5.7C, JPM135 vs. **JPM137**; IC_{50} (M) 9.40×10^{-8} vs. **1.24×10^{-8}** , $n = 3$; $p < 0.05$). There is also a notable difference in cytotoxicity between those cells that express LHRHR at different levels. As mentioned previously, A2780 ovarian cells express LHRHR at a significantly higher degree compared to CALU-3 (112,116). This difference in expression is highlighted in the IC_{50} of JPM137 (**A2780** vs. **IGROV-1** vs. CALU-3 (72 hours), Figure 5.4C vs. Figure 5.7C vs. Figure 5.6C; IC_{50} (M) (**8.18×10^{-8}** vs. **1.24×10^{-8}** vs. 6.78×10^{-4} , $n = 3$; $p < 0.05$). The change in potency adds further evidence to the suggestion that JPM137 is site-specific.

Interestingly, the potency of Camptothecin decreases over time (e.g. A2780, Figure 5.4; 24 hours vs. **72 hours**; IC_{50} (M) 7.21×10^{-9} vs. **5.69×10^{-8}** , $n = 3$; $p < 0.05$). This decrease in potency could be attributed to the ease with which Camptothecin can hydrolyse, becoming a sodium salt which displays up to 90 % reduction in activity compared to the parent drug (118).

By focusing on the ovarian cell lines only (Figures 5.4 and 5.7), it becomes clear that JPM137 acts in a controlled release manner. The IC_{50} of JPM137 decreases over time (A2780 – Figure 5.4; 24 vs. 48 vs. 72; IC_{50} (M) (8.49×10^{-7} vs. 1.01×10^{-7} vs. 8.18×10^{-8} , $n = 3$; $p < 0.05$). Ideally, a longer time course would be run to observe whether this effect continued, however the effects of cell death through cell media depletion would make it harder to

assess such a phenomena accurately. The likely rationale for such an effect could be attributed to a two causes. The first is the degree to which LHRH is presented on JPM137. The results suggest that binding and internalisation of the construct does not occur as often, or as quickly, as first hoped. However, characterisation of JPM137 shows that more than 50 % of the micelle surface is LHRH. Higher percentages of ligand presentation could lead to steric hinderance and therefore decreased efficacy, although this needs to be investigated. The second reason is the development of 'reducing conditions' within the culture environment inducing release of drug. It is generally considered that the extracellular environment is an oxidising milieu whereas the intracellular environment is a reducing milieu (119). However, as cells proliferate there is cysteine uptake, leading to increase glutathione synthesis, export and finally, degradation to cystenine outside the cell. T cells are key contributors to this effect (120). The increased concentrations of cysteine and glutathione reduce the oxidising potential of the extracellular fluid leading to the development of reducing conditions.

As mentioned above, drug release occurs under reducing conditions. Thus, as cell proliferation occurs and the culture media is utilised, it is possible that the media became a more reductive environment which implies that extracellular drug release could occur over time. However, data below in Figure 5.8 indicates that this second reason is less likely. Nonetheless, it is important to account for such a phenomena as this could lead to a secondary complication in the generation of Camptothecin resistant cells. Metronomic treatment of tumours is known to confer cellular resistance (121), therefore a slow and steady extracellular release of drug could lead to resistance developing. A repetitive dose regimen would be needed to determine whether this would be possible.

Crucially, JPM137 does not demonstrate superior potency compared to Camptothecin over time (Figure 5.4; A2780 (72 hours) – Figure 5.2C, **CPT** vs. JPM137; IC_{50} (M) **5.69×10^{-8}** vs. 8.18×10^{-8} , $n = 3$; $p > 0.05$; Figure 5.7; IGROV-1 (72 hours) – Figure 5.5C, **CPT** vs. JPM137; IC_{50} (M) **1.02×10^{-8}** vs. 1.24×10^{-8} , $n = 3$; $p > 0.05$). The performance of JPM137 is comparable to Camptothecin and could be improved upon by increasing the presence of LHRH on the construct, thus increasing the opportunity for LHRHR binding and internalisation.

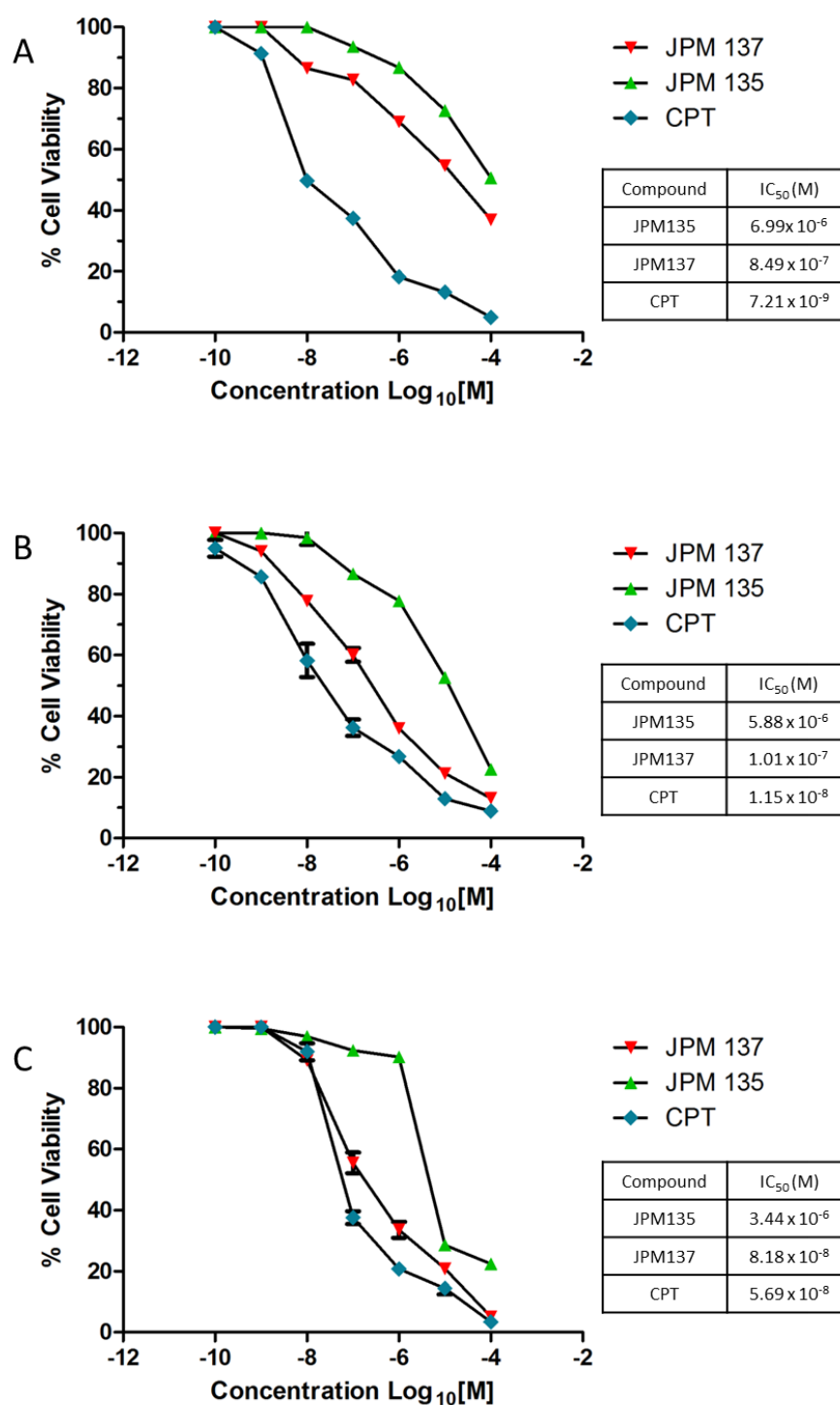


Figure 5.4 – Cytotoxicity of Compounds against A2780 Ovarian Cancer Cell Line. Cytotoxicity of JPM135 (green), JPM137 (red) and Camptothecin (blue) at **A** – 24 hours, **B** – 48 hours and **C** – 72 hours. IC₅₀ values are also presented. Figure represents the mean of three independent experiments. Error bars represent standard deviation. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

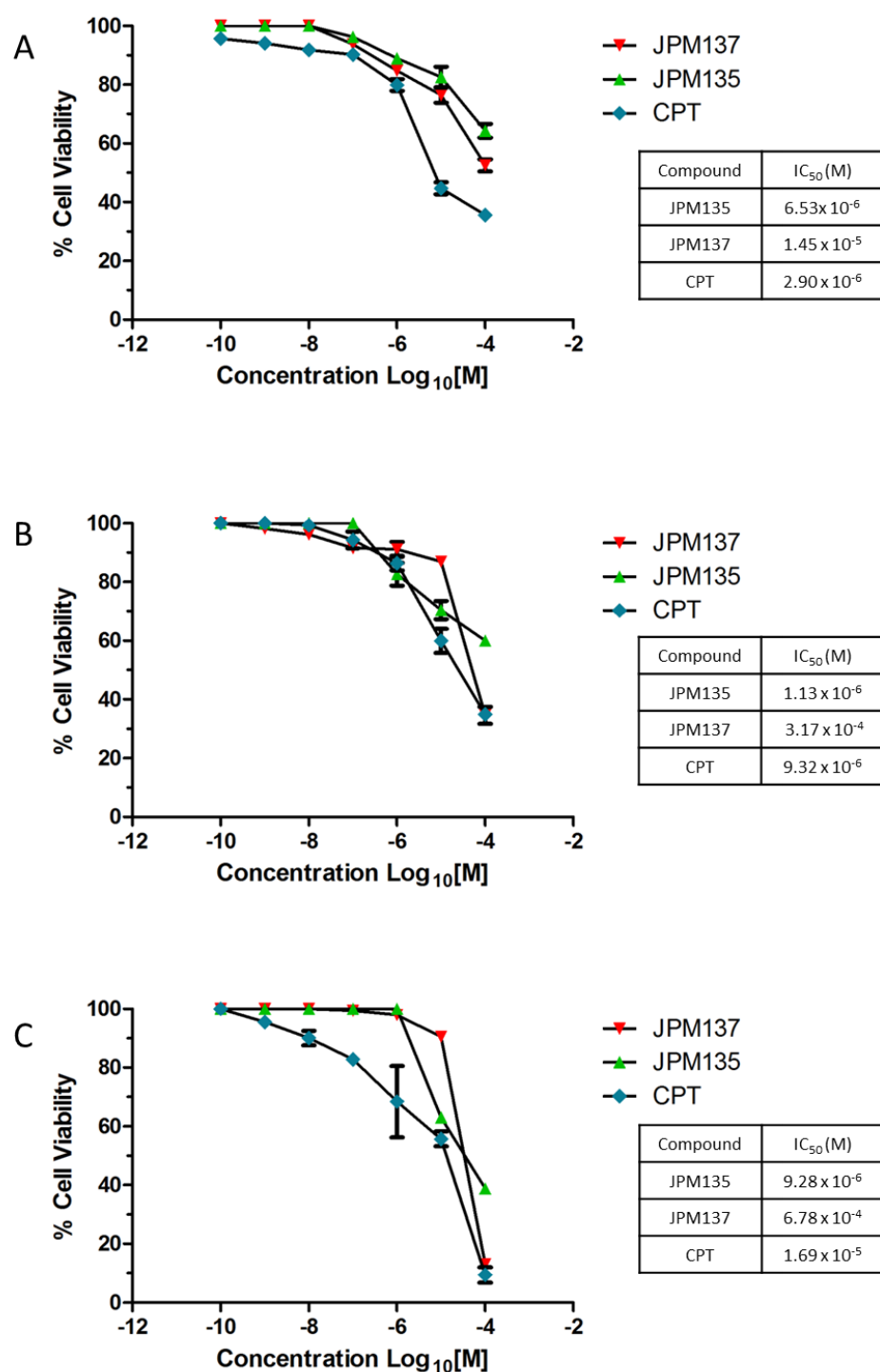


Figure 5.5 – Cytotoxicity of Compounds against CACO2 Colon Cancer Cell Line. Cytotoxicity of JPM135 (green), JPM137 (red) and Camptothecin (blue) at **A** – 24 hours, **B** – 48 hours and **C** – 72 hours. IC₅₀ values are also presented. Figure represents the mean of three independent experiments. Error bars represent standard deviation. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

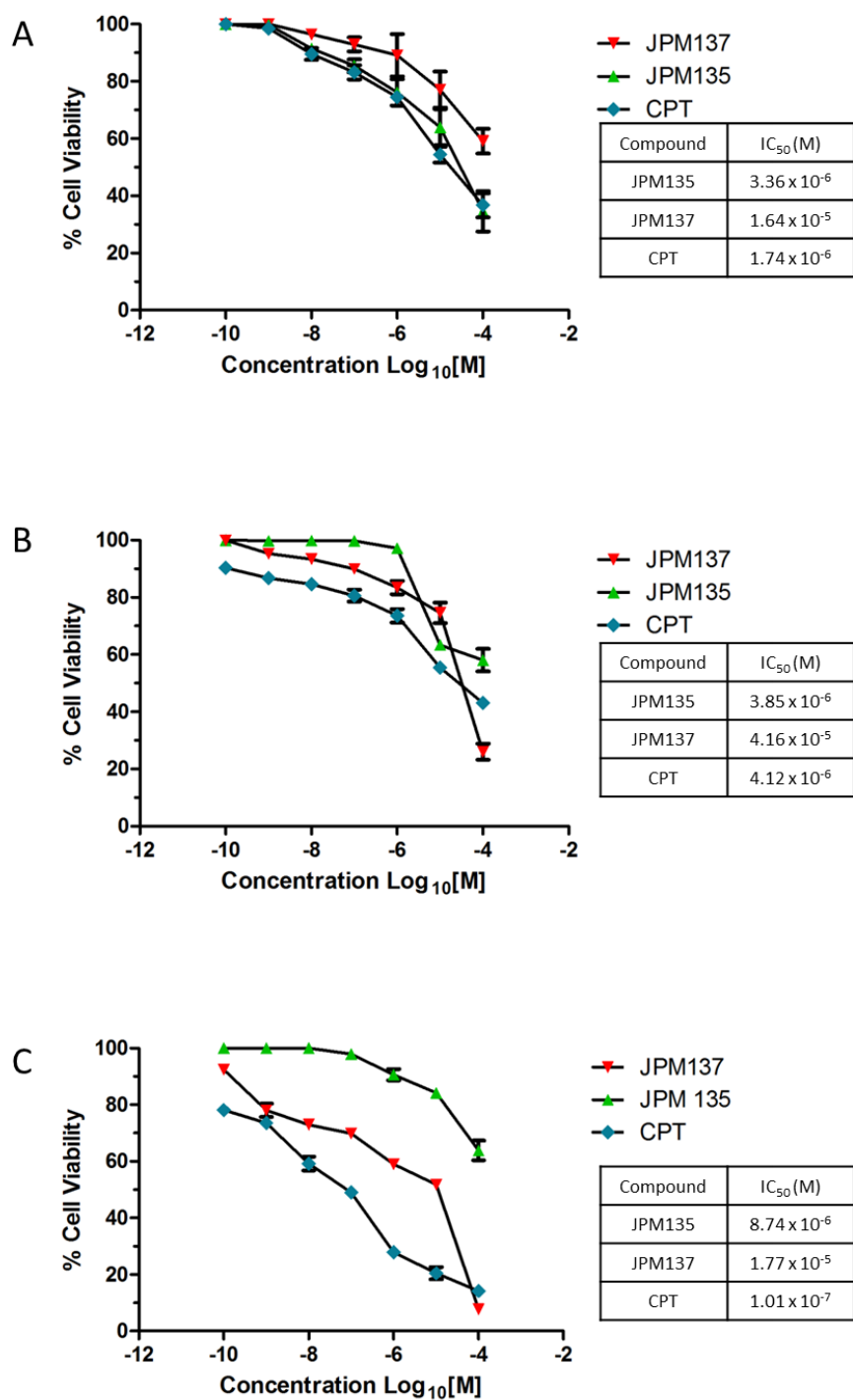


Figure 5.6 – Cytotoxicity of Compounds against CALU-3 Lung Cancer Cell Line. Cytotoxicity of JPM135 (green), JPM137 (red) and Camptothecin (blue) at **A** – 24 hours, **B** – 48 hours and **C** – 72 hours. IC₅₀ values are also presented. Figure represents the mean of three independent experiments. Error bars represent standard deviation. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

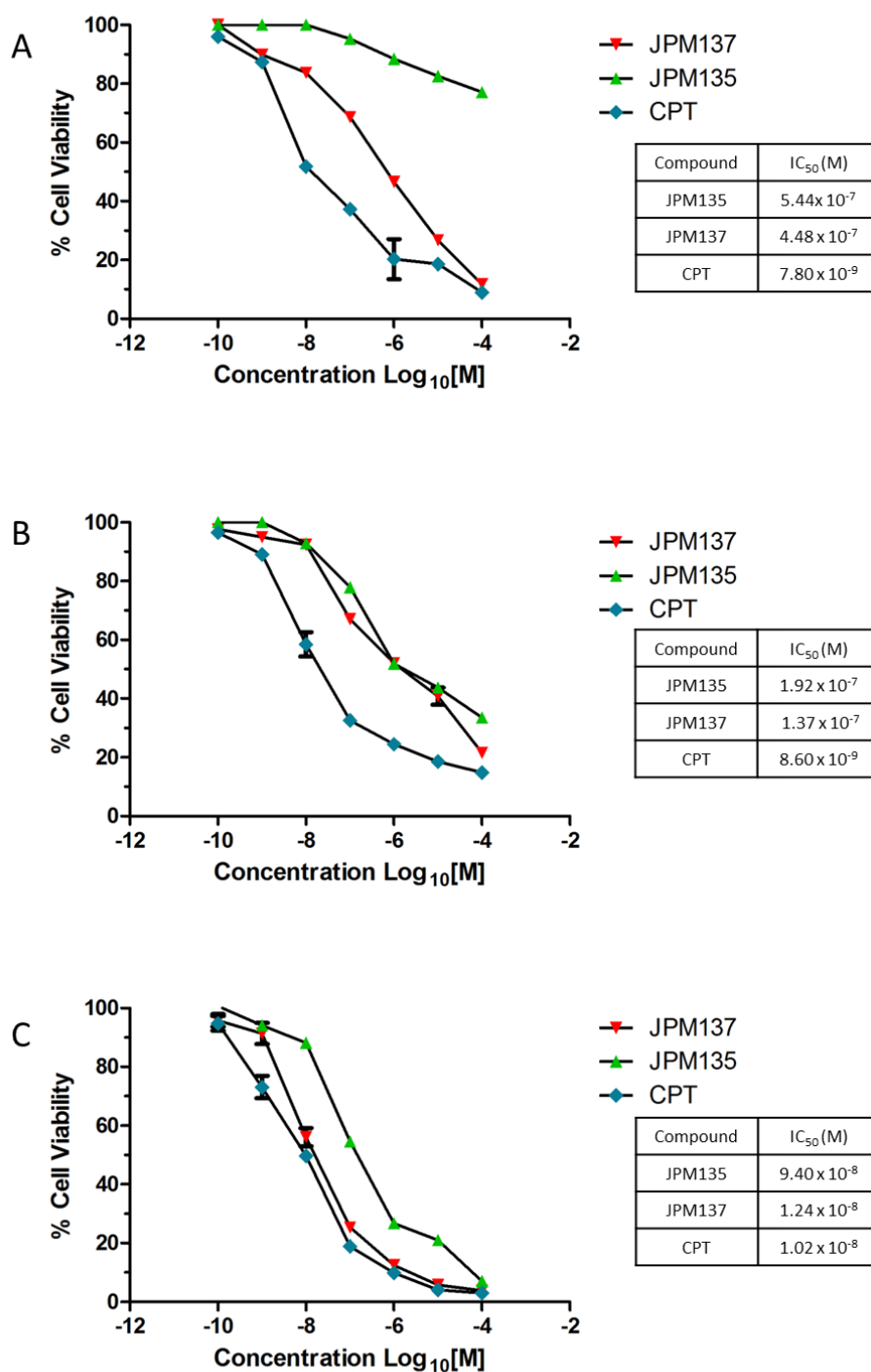


Figure 5.7 – Cytotoxicity of Compounds against IGROV-1 Ovarian Cancer Cell Line. Cytotoxicity of JPM135 (green), JPM137 (red) and Camptothecin (blue) at **A** – 24 hours, **B** – 48 hours and **C** – 72 hours. IC₅₀ values are also presented. Figure represents the mean of three independent experiments. Error bars represent standard deviation. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

5.3.3 Competitive Binding Assay

In order to further substantiate the claim that JPM137 does bind to LHRHR on ovarian cancer cells, A2780 cells were treated with a combination of 10 μ M LHRH and a concentration range (0 – 100 μ M) of JPM137 before measuring cytotoxicity over a period of time. The most accurate method for assessing the competitive binding of ligands to a receptor would be through using a radiolabelled ligand. This would allow quantitative analysis of the radio-ligand binding to the receptor (122,123). However, due to cost restrictions an alternative assay needed to be sought. Early work by Dharap and Minko used cell cytotoxicity as a way to measure whether their peptide-mediated construct would compete with for its target receptor (113). Given the access to such an assay, cell cytotoxicity was used as the end point in this set of experiments.

Figure 5.8 illustrates the effect LHRH treatment has on inhibiting the cytotoxicity of JPM137. At 24, 48 and 72 hours, LHRH treatment significantly restricts JPM137-mediated cytotoxicity (24 hours – Figure 5.8A, **JPM137 + LHRH** vs. JPM137; IC_{50} (M) **6.38×10^{-5}** vs. 8.36×10^{-7} , $n = 3$; $p < 0.05$; 48 hours – Figure 5.8B, **JPM137 + LHRH** vs. JPM137; IC_{50} (M) **1.21×10^{-5}** vs. 1.21×10^{-7} , $n = 3$; $p < 0.05$; 72 hours – Figure 5.8C, **JPM137 + LHRH** vs. JPM137; IC_{50} (M) **0.71×10^{-5}** vs. 8.3×10^{-8} , $n = 3$; $p < 0.05$).

As expected JPM137 failed to outcompete 10 μ M LHRH. This is an exceptionally high concentration of ligand used compared to competitive assays of a similar nature (124). The rationale for this high concentration was to demonstrate that LHRHR antagonism led to decreased cytotoxic activity. It also demonstrated that the cytotoxic effect of JPM137 is primarily mediated

by receptor internalisation, rather than via a controlled release 'leeching' into the extracellular environment as discussed above.

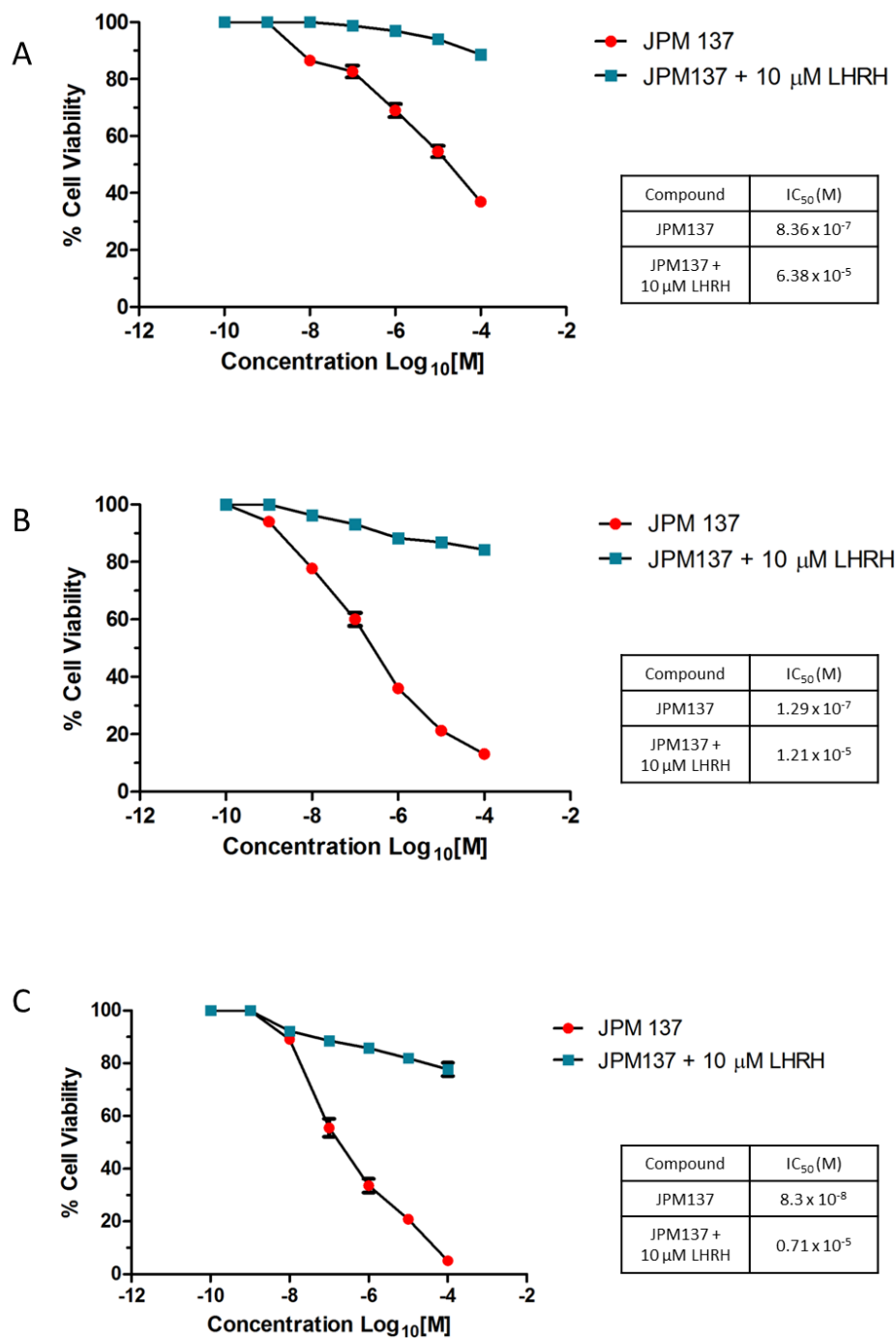


Figure 5.8 – Competitive Inhibition of JPM137 Activity using LHRH. Cytotoxicity of JPM137 (red) was compared to JPM137 incubated with 10 μM LHRH (blue) at A – 24 hours, B – 48 hours and C – 72 hours. IC₅₀ values are also presented. Figure represents the mean of three independent experiments. Error bars represent standard deviation. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

5.3.4 Release of Tumour-associated Antigen MUC1

In order for the treatment strategy proposed in this thesis to be successful, chemotherapy-induced cell death needs to be followed by subsequent tumour-associated antigen release. Much has been made of the potential for chemotherapy to induce immunogenic-cell death (1,125-127) and thus it is important to determine whether treatment of ovarian cancer cell lines with Camptothecin, and more crucially JPM137, leads to an increase in tumour-associated antigen.

As previously mentioned, this thesis will focus on the generation of anti-MUC1 immune responses. To achieve this, MUC1 needs to be released into the extracellular milieu where it will either be processed by antigen presenting cells such as dendritic cells and macrophages or be recognised by MUC1 specific T cells embedded in the tumour stroma (31,128). The supernatant of A2780 and IGROV-1 cells, after treatment with a concentration range (0 – 100 μ M) of Camptothecin or JPM137 for 72 hours, was analysed for MUC1 and CA-125. CA-125 is a tumour marker widely used to diagnose, monitor, and follow-up women with epithelial ovarian cancer (129). A2780 is a CA-125-negative cell line (130) but both have been shown to both express MUC1 (131).

Figure 5.9 illustrates the release of MUC1 in both A2780 and IGROV-1 as the concentration of drug increases (0.01 – 100 μ M, $n = 3$; $p < 0.05$). Correlating the cell death observed in Figure 5.4C (A2780) and Figure 5.7C (IGROV-1) with the data in Figure 5.7 indicates that increased cytotoxicity leads to an increase in MUC-1 release (A2780, Spearman's Rank Co-efficient; $\rho = - 0.93$, $p < 0.05$, IGROV-1, Spearman's Rank Co-efficient; $\rho = - 0.94$, $p < 0.05$). This suggests that treatment of tumours with chemotherapy leads to a significant

release in MUC1. The extra, available antigen means that there is an increased prospect of inducing a MUC1-specific anti-tumour immune response through the mechanisms mentioned above.

Figure 5.10 indicates the release of the tumour marker CA-125 from treated cells. IGROV-1 shows an increase in CA-125 release (0 – 100 μ M, $n = 3$; $p < 0.05$). Although clinically, an increase in CA-125 is considered as a marker for progressive disease (129), there is evidence of a sharp increase of CA-125 on initial treatment with chemotherapy (132). The data in Figure 5.8 mimics this increase and thus can be considered as an indicator of tumour cell death.

Interestingly in both Figures 5.9 and 5.10, there is a significant increase in the amount of antigen released when cells were treated with dimethyl sulphoxide (DMSO) as a vehicle control. When correlated with the cytotoxicity data, there is a dose-dependent increase in antigen release with DMSO (Spearman's Rank Co-efficient; $\rho = -0.98$, $p < 0.05$). The high percentage of DMSO (10 % v/v) is required due to the poor solubility of Camptothecin (133) in aqueous solutions and could thus be a contributing factor to the cytotoxic effect of Camptothecin (134). In comparison, JPM137 is soluble in phosphate buffered saline and as such demonstrates a far greater solubility profile.

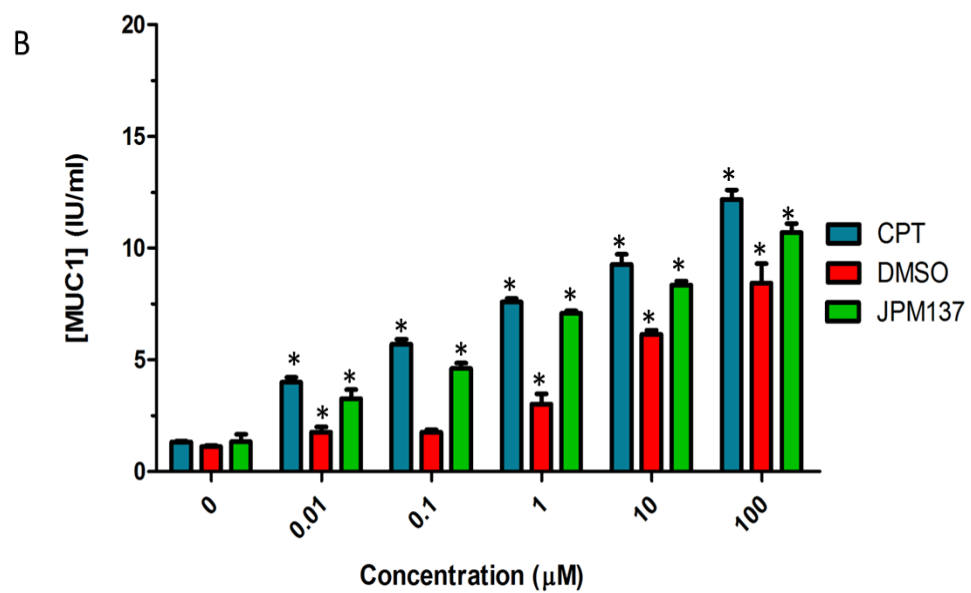
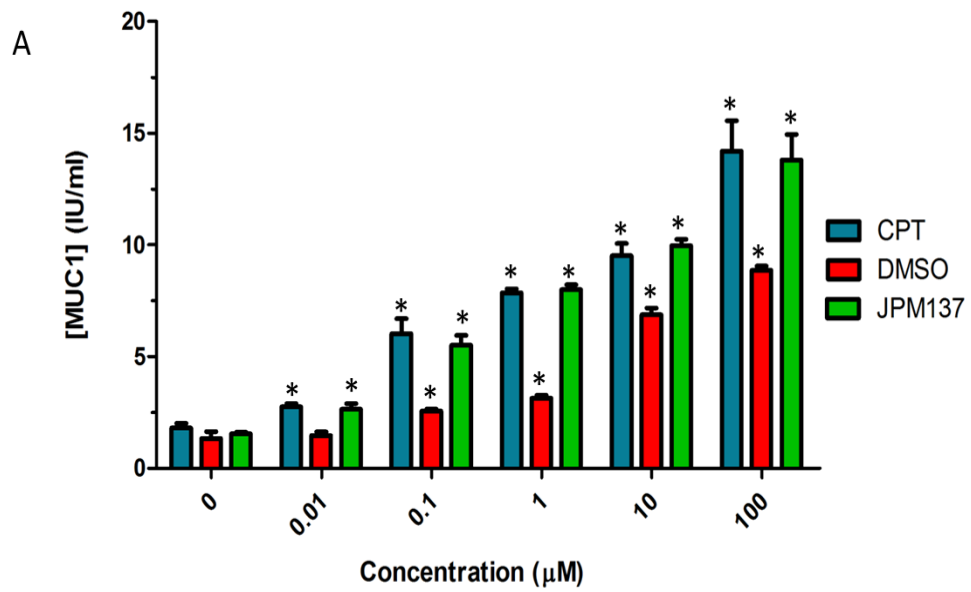


Figure 5.9 – Detection of MUC1 from Ovarian Cancer Cell Lines on Cytotoxic Treatment. Detection of antigen via ELISA from **A-** A2780 and **B-** IGROV1. Cells treated with Camptothecin (blue), JPM137 (green), and DMSO (red) as a vehicle control (blue) 72 hours. Figure representative of three independent experiments. Error bars represent standard deviation. $*=p<0.05$, $n = 3$. Detection limit = 2 IU/ml. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

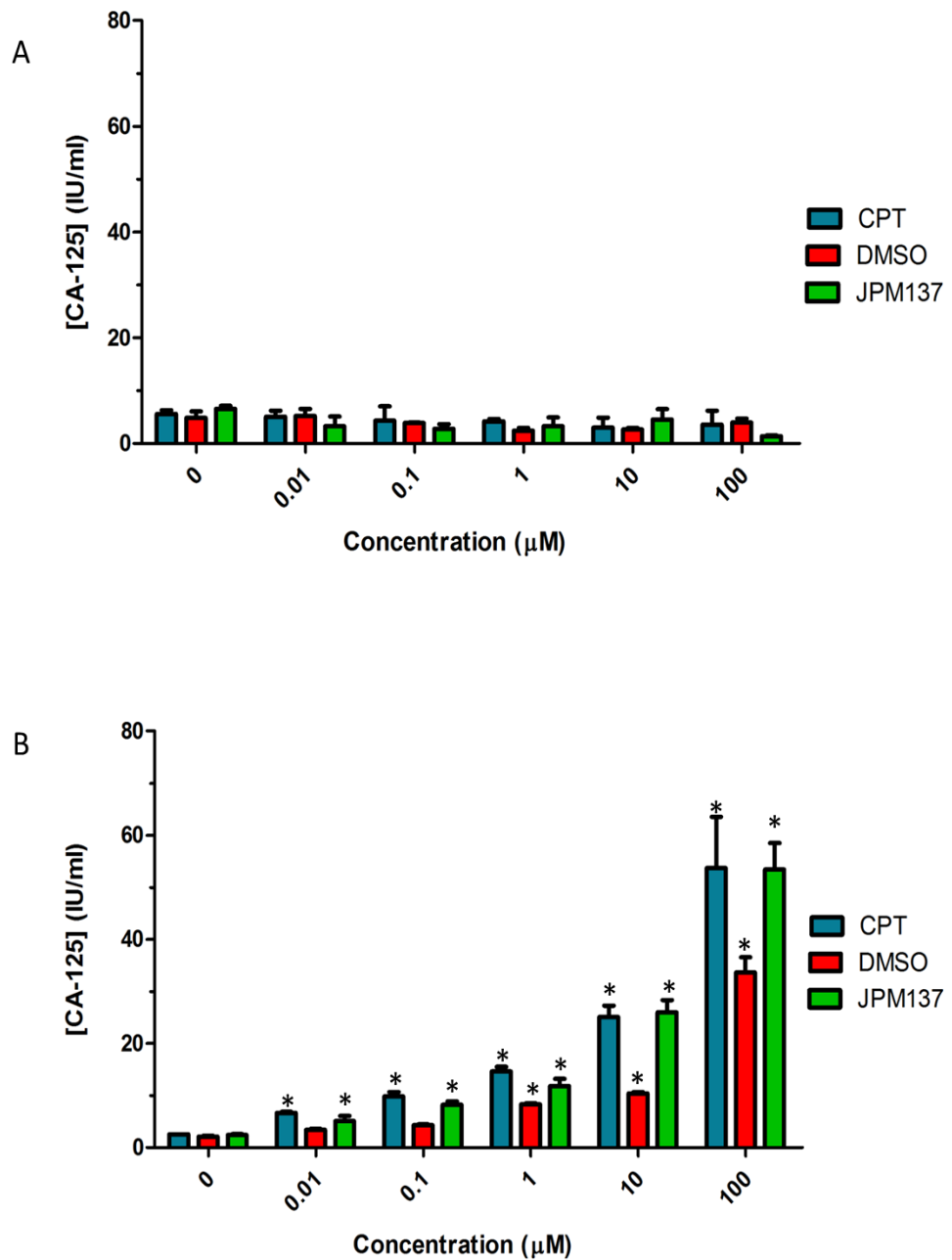


Figure 5.10 – Detection of CA-125 from Ovarian Cancer Cell Lines on Cytotoxic Treatment. Detection of antigen via ELISA from **A**- A2780 and **B** - IGROV1. Cells treated with Camptothecin (blue), JPM137 (green), and DMSO (red) as a vehicle control (blue) 72 hours. Figure representative of three independent experiments. Error bars represent standard deviation. $*=p<0.05$, $n = 3$. Detection limit = 4 IU/ml. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

5.3.5 Effect of Cytotoxics on Immune Cells

With the increase in free MUC1, it is critical to have a fully functional immune system to process, present and proliferate and thus induce a response against the antigen. However, the lymphopaenia associated with several cytotoxic agents, including Camptothecin, could render this 'liberation' of antigen inappropriate. As mentioned previously, cytotoxic agents can conceivably alter the T cell pool in either an anti-tumour or pro-tumour manner. In order to assess the effect of Camptothecin and JPM137 on the immune population, PBMC were divided into stimulated and non-stimulated groups (stimulation with the T cell mitogen, PHA) and treated with JPM137 or Camptothecin over a concentration range (0 – 100 μ M) for 72 hours. The duration of this assay was selected as at this time point the IC₅₀ of both JPM137 and Camptothecin were similar in ovarian cell cultures (Figure 5.4 and Figure 5.7). The proliferation of these cells was then measured.

Figure 5.11 highlights the change in proliferation seen on addition of each compound. From Figure 5.11A it is clear that Camptothecin almost completely abrogated stimulated PBMC proliferation (0.01 – 100 μ M, $n = 3$; $p < 0.05$). In comparison, JPM137 demonstrated a dose-dependent effect with doses of 1 μ M and higher causing a significant decrease in cell proliferation (1 – 100 μ M, $n = 3$; $p < 0.05$). In Figure 5.11B, a similar trend follows, whereby Camptothecin shows a reduction in cell proliferation, albeit to a lesser extent compared to when incubated with stimulated cells (**Camptothecin stimulated - Figure 5.11A** vs. non-stimulated – Figure 5.11B (% cell proliferation) 0.01/0.1/1/10/100 – **0.20** vs. 74.53, **0.37** vs. 79.73, **1.63** vs. 86.10, **2.64** vs. 91.2, **4.90** vs. 96.67, $n = 3$; $p < 0.05$). The marked reduction in proliferation is due the selectivity of Camptothecin to proliferating cells (135). T cell proliferation has also been documented to generate a reductive

environment (120) which could induce the release of Camptothecin from JPM137 thus explaining the effect it has on PBMC proliferation.

To quantify these observations further, cytotoxic-treated PBMC were stained with Propidium Iodide to identify those cells that had died. Live cells were collected and then phenotyped for CD8⁺ T cell and Treg populations. Figure 5.12 illustrates the percentage of PBMC that had died after treatment. There was a significant difference in the percentage of cells that died, in both stimulated and non-stimulated groups, when treated with Camptothecin in comparison to JPM137 (Non-stimulated - Figure 5.12B, **Camptothecin** vs. JPM137 (% dead cells) 0.01/0.1/1/10/100 – **20.94** vs. 15.53, **25.09** vs. 17.47, **41.91** vs. 15.83, **67.59** vs. 12.54, **81.01** vs. 50.67, $n = 3$; $p < 0.05$), (Stimulated - Figure 5.12C, **Camptothecin** vs. JPM137 (% dead cells) 0.01/0.1/1/10/100 – **84.95** vs. 15.53, **88.36** vs. 16.30, **94.10** vs. 56.90, **93.75** vs. 80.01, **84.70** vs. 93.96, $n = 3$; $p < 0.05$).

The high percentage of cell death seen in both treatment groups correlates with the inhibition in proliferation seen in Figure 5.11. This, together with the proliferation data, is a critical result as it indicates that Camptothecin impairs PBMC competency through immune cell death. This effect would be seen peripherally as well as at the tumour microenvironment level and is a major contributing factor to the adverse effects seen when using these drugs. Many pieces of literature describe how chemotherapy-induced lymphodepletion is of benefit in cancer due to the phenomena of proliferating cells during immune re-capitulation (1,25,28,136). However, it could be argued that as long as tumour cells exist they are capable of inducing several immune suppressive mechanisms as described previously. This could give

rise to a tolerogenic environment which leads to tumour progression and subsequent disease relapse.

A key factor in the recapitulation of the immune system is the phenotype of those cells which remain after cytotoxic treatment. In order to assess this, those cells that were Propidium Iodide-negative were collected and stained for CD3⁺CD8⁺ T cells and Treg. Figure 5.13 depicts the proportion of these T cell subsets within the remaining viable PBMC population. The first observation to be made is from Figure 5.13A where the percentage of Treg (CD4⁺CD25⁺FOXP3⁺) in stimulated PBMC is very high compared to non-stimulated cells (% Treg, Figure 5.13A, **stimulated** vs. non-stimulated, 0.01 μ M Camptothecin (%) **40** vs. 4, $n = 3$; $p < 0.05$). This can be attributed to the markers used to phenotype Treg.

As mentioned in Chapter 3, FOXP3 is not an accurate marker for Treg in human and can be transiently expressed in non-suppressive cells, especially upon stimulation (137-139). Therefore, it becomes difficult to accurately phenotype Treg in proliferating PBMC. CD8⁺ T cells are easily phenotyped due to their co-expression of the T cell specific marker CD3. In Figure 5.13C, the proportion of CD8⁺ T cells is significantly decreased in stimulated PBMC that are treated with Camptothecin (Figure 5.13C, Camptothecin (%CD3⁺CD8⁺) 0 vs. **0.01/0.1/1/10/100** μ M – 34.53 vs. **36.24/11.81/6.40/6.57/2.22**, $n = 3$; $p < 0.05$). Treatment with JPM137 did not alter the percentage of CD8⁺ T cells at doses below 1 μ M (Figure 5.13C, JPM137 (%CD3⁺CD8⁺) 0 vs. **0.01/0.1/1/10/100** μ M – 34.86 vs. **36.24/36.24/36.14/34.53/7.17/0.80**, $n = 3$; $p < 0.05$).

Non-stimulated PBMC yielded two key observations. Firstly, low doses of Camptothecin and JPM137 caused a statistically significant reduction in the

fraction of Treg within the viable PBMC population (Figure 5.13B, Camptothecin (% Treg) 0 vs. **0.01/0.1/1/10/100** μ M – 4.14 vs. **3.64/1.64/0.87/2.79/4.02**, $n = 3$; $p < 0.05$), (Figure 5.13B, JPM137 (%Treg) 0 vs. **0.01/0.1/1/10/100** μ M – 4.05 vs. **4.12/3.74/3.15/3.65/4.27**, $n = 3$; $p < 0.05$). This effect is well documented (53,140-143) and highlights the link between chemo- and immunotherapy. The Treg-depleting effect of JPM137 could be attributed to the ‘leeching’ of Camptothecin as described earlier in the chapter. The second observation is that JPM137 does not reduce the proportion of CD8⁺ T cells whereas there is a statistically significant reduction when treated with Camptothecin (Non-stimulated - Figure 5.13D, **Camptothecin** vs. JPM137 (%CD3⁺CD8⁺) 0.01/0.1/1/10/100 – **31.75** vs. 32.56, **30.07** vs. 34.11, **27.39** vs. 34.74, **26.62** vs. 35.64, **27.85** vs. 29.80, $n = 3$; $p < 0.05$). These results indicate that JPM137 is less toxic to immune cells. This ‘immune-protective’ property of JPM137 means that the proportion of CD8⁺ T cells within PBMC remains intact while causing a reduction in Treg. This would be of benefit within the ovarian tumour microenvironment as a reduction in tumour burden, combined with an increase in CD8⁺:FOXP3⁺ ratio (a prognostic indicator in malignancy (144-147)) would lead to greater tumour clearance.

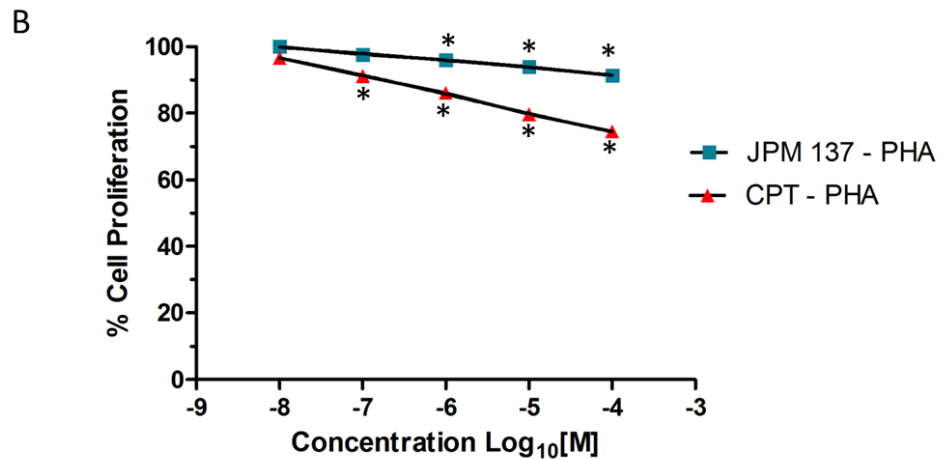
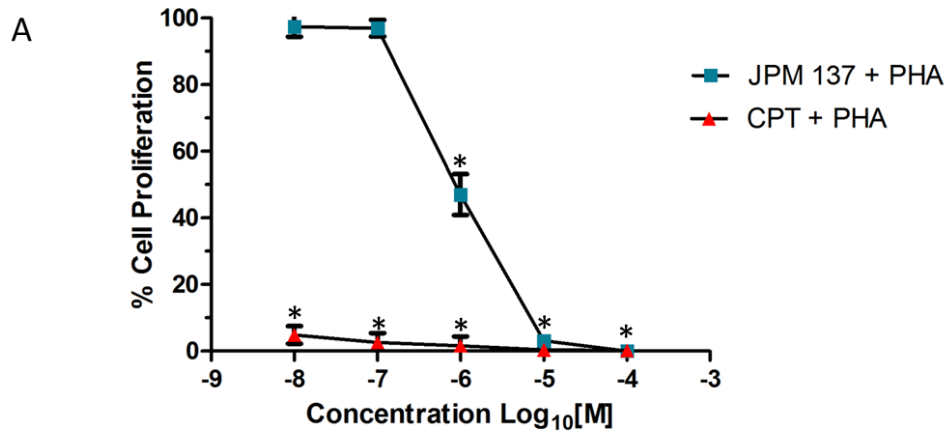


Figure 5.11 – PBMC Proliferation on Treatment with Cytotoxics. ³H-Thymidine incorporation used to assess the proliferation of PBMC when treated with cytotoxics. Cells were treated with a concentration range (0 – 100 μM) of Camptothecin (red) or JPM137 (blue) with **A** - PBMC were stimulated with 10 μg/ml PHA or **B** - PBMC were non-stimulated. Cultures were treated for 72 hours before being pulsed with ³H-Thymidine. Figure representative of three independent experiments from three different donors. Error bars represent standard deviation. *= $p < 0.05$, $n = 3$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

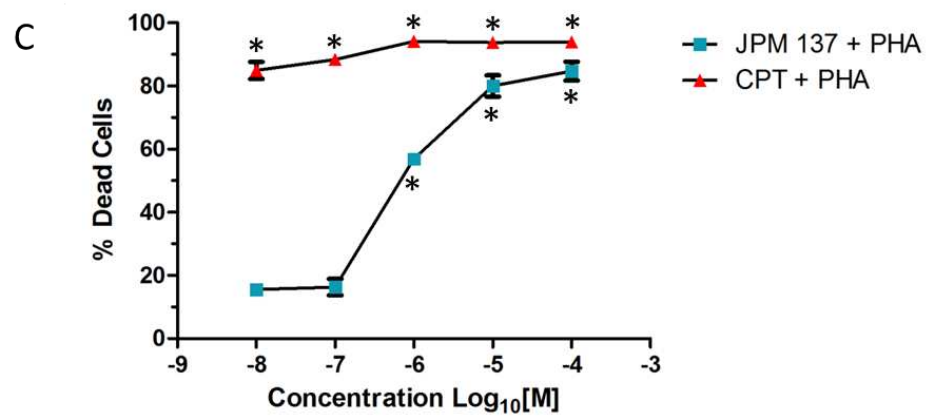
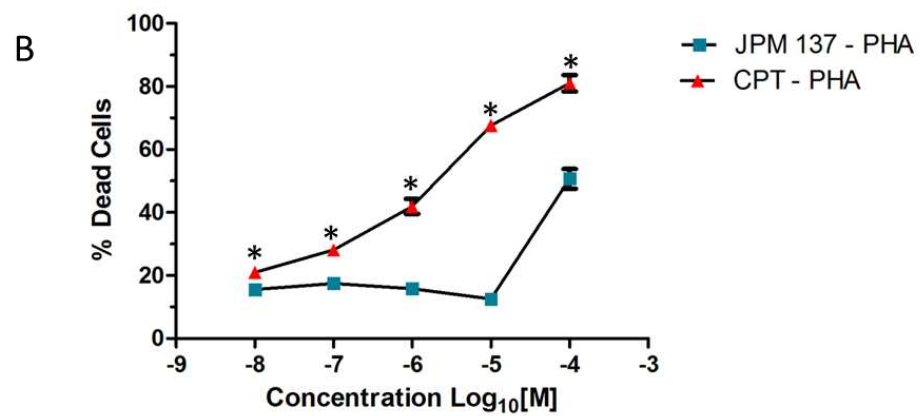
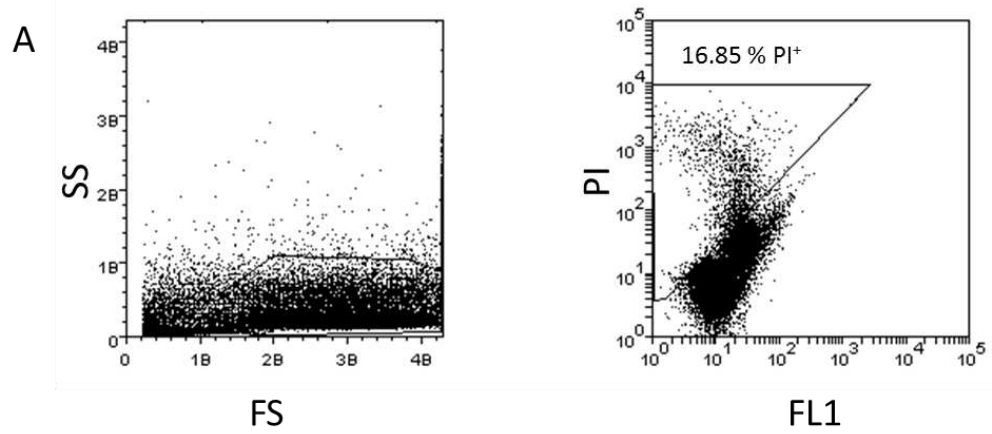


Figure 5.12 – Percentage of Dead PBMC after Treatment with Cytotoxics. Propidium Iodide used to differentiate between live and dead cells. Cells were treated with a concentration range (0 – 100 μ M) of Camptothecin (red) or JPM137 (blue) with **A** - PBMC stimulated with 10 μ g/ml PHA or **B** - PBMC non-stimulated. Cultures were treated for 72 hours before being stained with propidium iodide. Remaining viable cells (PI negative) were collected for phenotypic analysis. Figure representative of three independent experiments from three different donors. Error bars represent standard deviation. $*=p<0.05$, $n = 3$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

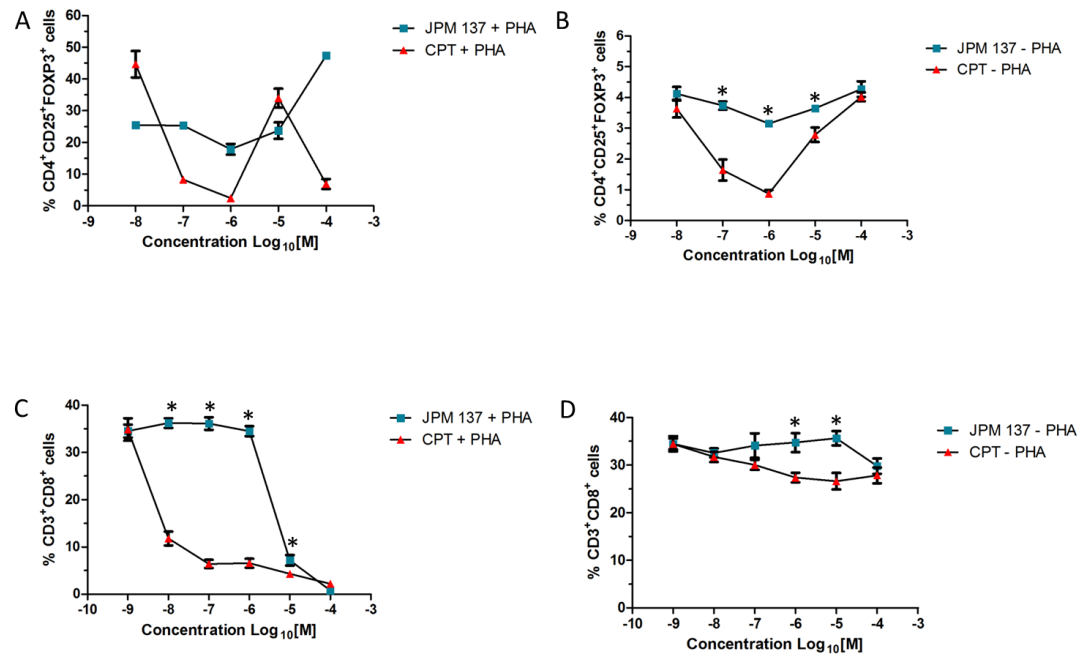


Figure 5.13 – Fraction of CD8⁺ T cells and Treg after Treatment with JPM137 or Camptothecin. Percentage populations calculated by flow cytometry. Cells were treated a concentration range (0 – 100 μM) of Camptothecin (red) or JPM137 (blue) with **A** – Fraction of Treg after treatment and stimulation with 10 μg/ml PHA. **B** – Fraction of Treg after treatment without stimulation with PHA. **C** - Fraction of CD8⁺ T cells after treatment and stimulation with 10 μg/ml PHA. **D** – Fraction of CD8⁺ T cells after treatment without stimulation. Cultures were treated for 72 hours before being staining for flow cytometry. Figure representative of three independent experiments from the same donor. Error bars represent standard deviation. *= $p < 0.05$, $n = 3$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

5.4 Conclusions

This chapter has sought to highlight the immunological importance of chemotherapy. Immune populations can be affected by treatment with cytotoxics and although there is a growing opinion that lymphodepletion is clinically beneficial it is still a controversial area. In order to improve tumour targeting and thus reduce peripheral lymphodepletion, JPM137 was created. This novel drug delivery system, encapsulated the drug within a hydrophilic polymer to improve solubility and its pharmacokinetic profile. The addition of the Leuteinizing-hormone releasing hormone ligand (LHRH) gave the construct tumour specific targeting properties.

Before the use of JPM137, several cell lines were assessed for their expression of LHRHR. Higher expression was noted in ovarian cell lines compared to lung and colon as per previous findings. The cytotoxic activity of JPM137 was then compared against Camptothecin in these cell lines. Importantly, the construct demonstrated site-specific cytotoxicity, with greater toxicity seen in ovarian cell lines compared to colon and lung. However, JPM137 had similar, rather than superior, cytotoxic activity to Camptothecin although this could be attributed to an inefficient level of LHRHR ligand on the surface of the construct.

On cell death, there was a significant increase in the tumour-associated antigen MUC1. This antigen has been shown to be highly expressed in ovarian cancer and thus its release into the extracellular environment could be advantageous in attempting to generate an anti-ovarian tumour immune response. Clinically, despite a cytoreduction in tumour burden and the increase in MUC1 availability, the lymphodepletive effect of Camptothecin would mean that generating a potent anti-tumour immune response would be

difficult. This was highlighted by the significant lack in PBMC proliferation but also in terms of a reduction in CD8⁺ T cells. Interestingly, and in concordance with current literature, low doses of Camptothecin reduced the number of Treg from PBMC. JPM137, however, demonstrated a reduction in these effects with improved PBMC proliferation, cell viability and maintenance of the CD8⁺ T cell population whilst also causing a reduction in Treg.

The data above indicates that JPM137 could localise at ovarian tumour sites, retain similar cytotoxic performance compared to Camptothecin while maintaining a more viable immune cell population. Critically, JPM137 would have an effect at the tumour site, depleting Treg while maintaining CD8⁺ T cells whereas Camptothecin would cause peripheral, as well as localised immune suppression which would not be as advantageous or clinically beneficial.

The release of the tumour associated antigen MUC1 means that there is a significant opportunity to induce an antigen specific anti-tumour immune response. The final experimental chapter, Chapter 6, attempts to take advantage of this antigen release by potentiating an immune response through the use of toll-like receptors.

5.5 References

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Chapter 6

Enhancement of immune responses against tumour-associated antigens

6.1 Introduction

As discussed previously a fundamental tenet of tumour immunology is that cancer cells express antigens that differentiate them from their non-transformed counterparts. However, it is the inability of the immune system to detect, process, present and use these antigens as a means to illicit an anti-tumour immune response. In order to improve tumour antigen presentation, it is important to understand why there is a failure to generate a significant population of antigen specific cytotoxic T cells.

6.1.1 Issues with Adequate Antigen Presentation

Models of infection have provided useful information about the process of antigen-specific T cell generation. Recent studies indicate that antigens are presented in the draining lymph nodes a few hours after the subcutaneous injection of a pathogen (1,2). The activation of naive CD8⁺ T cells is a relatively rapid process, with as little as 2.5 hours of antigen exposure required to induce clonal expansion and differentiation of CD8⁺ T cells *in vitro* (3,4). Further work by Wong *et al.* (5) has suggested that 24 hours of pathogen presence is sufficient for the induction of protective CD8⁺ T-cell responses *in vivo*, albeit in mice.

Therefore it can be said that CD8⁺ T cell priming takes place at a very early stage of infection — the acute infection phase. This rapid process seems to be crucial for effective expansion of antigen-specific T-cells, followed by the development of a memory T cell population. However, the same cannot be said of T cell priming in the context of TAA-specific T cell immunity. The development of tumours is fundamentally a slow process that, like chronic infection, might occur over several years. An acute infection may effectively activate the immune system at multiple levels. E.g. acute infection might be crucial for systemic antigen distribution and efficient induction of an innate immune response such as that from NK. It is well known that activated NK stimulate the maturation of DC and facilitate adaptive anti-tumour immunity (6).

Immune surveillance in tumours is impacted upon due to the lack of detectable antigen. It is suggested that before the development of systemic metastasis (including lymphoid metastasis), antigenic cancer cells (expressing TAA) are embedded in the solid tumour. The stroma of the tumour prevents the efficient release of TAA (7,8) which are ignored in the conventional central priming sites — the draining lymph nodes (9,10).

In the later stages of tumour development, TAA are thought to be efficiently released, which induces the protective immune system to mount an effective response (11,12). However, with tumour growth comes the development of tolerising mechanisms within the microenvironment, which limit the normal functions of APCs and effector T cells. These mechanisms include down-regulation of MHC expression (13,14), dysregulation of antigen processing machinery (15,16), secretion of immunosuppressive paracrine and endocrine factors such as transforming growth factor- β (TGF- β) (17), interleukin-10 (IL-

10) (18), prostaglandin E2 (PGE2) (19) and tumour expression of inhibitory receptors such as B7H1 (20), B7DC (21) and CTLA-4 (22).

To support this notion, studies have shown that CD8⁺ T cell responses to common melanoma epitopes are generally weak, localised, and occur mostly in patients with advanced metastatic disease (11,12). This suggests that the quality of the tumour immune response is not only due to efficient T cell priming but also due to the kinetics and temporal release of tumour associated antigen. Chapter 5 has demonstrated that increased tumour cell death using chemotherapeutics, like Camptothecin, can facilitate the release of antigen and thus should improve the possibility for effective antigen presentation occurring.

In addition to peripheral TAA priming, antigen presentation within the tumour microenvironment may also occur to some degree. Indeed, intra-tumoural TAA specific priming has been reported (23,24). However, there is a consensus that the tumour is a 'false' lymphoid organ, and T cell priming in the tumour microenvironment has at least two basic defects. Firstly, intra-tumoural antigen presenting cells are either dysfunctional or induce T cell tolerance. Secondly, the infrastructure of tumours is different from that of lymph nodes. Tumour tissues show increased interstitial fluid pressure and contain defective blood vessels. There is fibrosis and contraction of the interstitial matrix. Many tumours lack lymph vessels. These structural and haemodynamic defects would potentially contribute to poor or dysfunctional T cell priming within the tumour microenvironment. This would lend credence to the argument discussed in Chapter 5 regarding the targeting of tumours to reduce the potential immune suppressive effect exerted by a tumour mass.

6.1.2 Antigen Presenting Cells

APCs include DC, monocytes/macrophages and B lymphocytes. DC are a heterogeneous group of APCs that display differences in anatomic localisation, cell-surface phenotype, and function (25,26). Human DC are traditionally divided into two main populations: Myeloid (mDC) and Plasmacytoid (pDC) (25,27,28).

Vermi *et al.* (29) were one of the first to identify immature mDC and pDC infiltrating solid tumours. pDCs in particular appear to lack the ability to prime and induce tumour antigen specific cytotoxic T cells (29). Interestingly, they still present tumour antigens and induce IL-10-producing CD4⁺CD25⁺ Treg that contribute to the inhibition in anti-tumour immunity (30,31). Recently published work has added weight to their immune suppressive contribution in ovarian cancer (32). pDC from 44 patients were isolated and found to have their capacity for pro-inflammatory cytokine production reduced, as well as increased production of IL-10. Nevertheless, work in mice, using an anti-IL-10 mAb and CpG oligodeoxynucleotides, demonstrated that it is possible to induce a robust anti-tumour T cell response and tumour rejection *in vivo* (33,34). Functional plasmacytoid DC are found in the local tumour environment of patients with ovarian cancer (32,35), melanoma (36) and head and neck squamous-cell carcinoma (37). Tumour cells produce the chemokine ligand CXCL12 and plasmacytoid DC express CXCR4, the receptor for CXCL12 (also expressed on Treg). Tumour-derived CXCL12 mediates trafficking of plasmacytoid DC into the tumour (35,38). Their use in this project is justified due to work by Zou *et al.* (35) who identified that a large amount of pDC, but not functional mature mDC, accumulate in the tumour microenvironment.

It has been reported that pDCs pulsed with tumour antigens *in vitro* can prime IFN γ -secreting melanoma-specific CTLs (36). The synergy among DC subsets has not been fully explored in the development of anti-tumour immunity. An interesting study has shown that immunisations with a mixture of matured pDC plus mDC resulted in increased levels of antigen-specific CD8⁺ T cells and an enhanced anti-tumour response compared with immunisation with either dendritic cell subset alone (39). This is possibly due to the type I IFN secreting, particularly IFN- α , capacity of pDC (40). IFN- α not only triggers innate immunity, such as activating NK cells, but also promotes adaptive Th1-type T-cell responses. Altogether, these studies suggest that it is possible to re-establish and/or maximise an anti-tumour immune response when pDC are targeted (41,42).

6.1.3 Antigen Presentation Capacity of pDC

The antigen presenting ability of pDC has been widely debated in literature for many years. The category of antigens that pDC seem to present poorly is exogenous—antigens that have to be captured from the extracellular environment. Recently, murine pDCs have been described to have the ability to elicit *In vivo*, in naive mice, an antigen-specific CD8⁺ T cell response against endogenous antigens, as well as exogenous peptides, but not against exogenous antigens, and were capable of protecting mice from tumour challenge (43).

These are the antigens that mDC present with a higher efficiency than any other APCs. There are three features that make mDC particularly efficient at exogenous antigen presentation: 1) high endocytic activity, 2) ability to retain on their surface long-lived MHCII-peptide complexes, and 3) the capacity to cross-present. Although all three characteristics are important, the role of

pDC in tumours hinges primarily on their endocytic activity and their ability to cross-present antigen. mDC can internalise extracellular material by macropinocytosis, phagocytosis, and receptor-mediated endocytosis, the latter two facilitated by the expression of multiple types of receptors (44). This makes mDCs “multi-purpose” APCs, capable of capturing virtually any extracellular material (e.g., soluble proteins, glycosylated compounds, immunocomplexes, artificial particles, cells, bacteria, nucleic acids, etc). Overall, pDC do not appear as endocytic as mDC, but this is still a matter of contention. Several mouse and human studies concluded that pDC cannot phagocytose dead cells, or artificial particles (45-48).

The ability of mDC to cross-present antigen is well documented (44). The phenomena of cross-presentation can be described by looking at the classical model of MHC I antigen presentation. CD8⁺ T cells recognise peptide-class I MHC complexes. These complexes are generally generated after direct presentation as a result of endogenous production of the antigens in the cytosol. Exogenous antigens are internalised by dendritic cells and generally presented as MHC II. Cross-presentation is the exception to this rule whereby exogenous antigen is internalised and then processed and presented as MHC I. This allows presentation of antigens from microorganisms that do not infect DC, from autologous and importantly, in the context of this project, from tumour cells (49).

Whether pDC are able to cross-present is a controversial and unresolved matter. Several studies have shown that mouse pDC do not possess the capacity to cross-present (43,50) or that their capacity is negligible when compared to mDC (51). This is consistent with a number of reports that showed that cross-priming of CD8⁺ T cells *in vivo* against viruses or

intracellular bacteria is exerted by mDC, with no detectable involvement of pDC (44,52). The cross-presenting capacity of human pDC had only been assessed *in vitro*. The tumour antigen NY-ESO-1 in soluble form, associated to immunoglobulins or formulated with an adjuvant, was not cross-presented by human pDC, although there was MHC II presentation (53). In contrast, two studies have reported cross-presentation of lipo-peptides, cell-associated antigens, and viral particles by human pDC (54,55). At present it is difficult to give an explanation for the contrasting results of the different mouse and human studies.

Yamahira *et al.* (56) have a leukemic pDC line. By stimulation with LPS, this cell line showed enhancement in expression of antigen presentation-associated surface molecules and production of cytokines (IL-12p70 and TNF- α). The antigen presenting ability was also markedly increased. Co-culture of CD8⁺ T cells with LPS-stimulated and the WT1/CMVpp65 peptide-pulsed cell line led to the efficient generation of WT1/CMVpp65 tetramer⁺ cytotoxic T lymphocytes (56). There has been debate as to whether pDC only cross-present antigens captured via some specific receptors or if stimulated in a particular way. Interestingly, work by Mouries *et al.* (57) has demonstrated that pDC can cross-present antigen *in vivo* using the TLR 7 agonist Imiquimod as an adjuvant. They found that efficiency of antigen presentation was not compromised in presence of Treg, suggesting that their suppressive effects had been circumvented (57). This raised an interesting observation regarding the role of TLR agonists and their potential effects on the suppressive activity of Treg.

6.1.4 Toll-like Receptors

TLR are a family of 11 pattern recognition receptors (PRRs) which mediate the recognition of many pathogens through the detection of distinct pathogen-associated molecular patterns (PAMPs) (58). pDC and mDC each have a different TLR expression profile. In humans, mDC can express TLR-1, -2, -3, -4, -5, -7, and -8, while pDC express mainly TLR7 and -9 (59,60).

Transcriptional regulation of IFN β and IFN α genes on pDCs is controlled mainly by the factors IRF-3 and IRF-5/7. IRF-3 can be activated by TLR3 and TLR4, but there is no evidence of this pathway on pDC. Instead, IRF-7 has a constitutively high expression in pDC and it is recruited by myeloid differentiation primary response gene 88 (MyD88) through the adaptor molecule TRAF6 when TLR-7 or -9 is triggered (61).

Many studies have shown that exposure to synthetic TLR7 (Imiquimod and R-848 (also acts on TLR8)) or -9 (Cpg ODN) agonists induces pDC to secrete IFN- α and other pro-inflammatory cytokines such as IL-6 and TNF- α , as well causing pDC maturation which heighten their T cell stimulatory capacity (40,59,62).

However, T-cell priming not only depends on the activation status of DC but also by the activity of Treg. Although the presence of TLR on mDC and pDC has been reported, their existence and function on T cells, and Treg in particular, remains a topic of debate.

6.1.5 TLR Prevalence

Caramalho *et al.* (63) were the first to compare the TLR expression profiles of murine Treg and effector T cell subsets. Both Treg and effector T cells

expressed TLR1, TLR2 and TLR6 in equal measure. However, Treg expressed significantly more TLR4, TLR5, TLR7 and TLR8 (63). This study also showed that LPS, acting on TLR4, induced Treg activation and proliferation, resulting in an increased suppressive activity. Similar observations were made with the triggering of TLR5 with flagellin on human Treg with an increase in FOXP3 expression also seen (64). These data imply that Treg increase their suppressive capacity following TLR4 and TLR5 triggering.

Interestingly, a synthetic bacterial lipoprotein, Pam3Cys-SK4, a ligand for TLR2, induced proliferation in effector T cells and Treg, yet abrogated the suppressive activity of Treg (65). In addition to this, Foxp3 expression, a key regulator of Treg-cell function, was decreased following TLR2 stimulation of the Treg cells, thus providing a further insight into the mechanism by which TLR2 controls Treg-cell function (65).

It is not known exactly how the TLR triggering of Treg modulates their suppressive effects, and whether the triggering of different TLR occurs through the same pathway or has the same effect on Treg function. One explanation could be the upregulation or downregulation of Foxp3 expression following the different TLR stimulations (64,66), but how TLR-signaling affects Foxp3 expression is still unclear.

A key study by, Peng *et al.* (67) showed that human Treg cells express high levels of TLR8, and that the TLR8 triggering of Treg cells also prevents their suppressive phenotype. The results from their co-culture suppression assay demonstrated that suppression is abrogated by TLR8 triggering directly on Treg, and not effector T cells. It seemed that the TLR8 stimulation of human Treg did not induce proliferation. However, recent work by Forward *et al.* has

(68) suggested that TLR7 engagement on murine Treg lead to an increase in the suppressive activity. TLR7 and TLR8 share similar signalling pathways, and as such there are suggestions that the effects of TLR engagement on Treg differ on a species-by-species basis.

The most convincing clinical evidence thus far of using TLR7/TLR 8 agonists was a study by Huang *et al.*(69) who investigated the effect of Imiquimod on squamous cell carcinoma (SCC). SCC treated with Imiquimod prior to excision contained dense T cell infiltrates associated with tumour cell apoptosis and histologic evidence of tumour regression. Effector T cells from treated SCC produced more IFN- γ , granzyme and perforin and less IL-10 and TGF- β than T cells from untreated tumours. A key observation, however was that Imiquimod acted on Treg to reduce their suppressive activity. Treatment also led to an increased production of IL-6, which is known for rendering effector T cells resistant to Treg suppression (70,71).

Tumour immune vaccination has for a long time been focused on fostering T cell central priming in draining lymph nodes. As peripheral TAA-specific T cell priming is possible, although technically challenging, it could be postulated that another alternative would be to restructure the tumour into a real functional 'lymphoid organ' and to engineer 'quality' peripheral TAA-specific priming (for example, T cell priming within the tumour microenvironment), which would overcome the potential TAA ignorance in the draining lymph nodes of a tumour.

In the previous chapter of this thesis, targeted treatment of ovarian cancer cells with Camptothecin caused a release of MUC1 antigen while causing limited immune toxicity. *In vivo*, treatment with cytotoxics should alter the

structure of solid tumours, thus reducing their suppressive effect upon the surrounding milieu as well as providing an increase in TAA (72).

The above evidence, suggests that using TLR7/TLR8 agonists as an immunotherapeutic adjuvant would be highly beneficial on several fronts. TLR7 engagement would not only affect Treg but also pDC as well. The suppressive capacity of Treg would be restricted while there would be an increased potential for TAA presentation (via the cross presentation mechanisms mentioned above), CD8⁺ T cell priming and a generation of a pro-inflammatory cytokine milieu.

This final experimental chapter seeks to take advantage of the antigen release described previously by enhancing antigen presentation and cytotoxic T cell generation while reducing the suppressive effect of Treg.

6.1.6 Experimental Objectives described in Chapter 6

- Determination of TLR 7 expression on Treg, CD4⁺CD25⁻ T cells, CD8⁺ T cells, pDC and ovarian cancer cell lines.
- Demonstration of Imiquimod activity on Treg suppressive function.
- Demonstration of Imiquimod activity on pDC maturation.
- Determination of the effect of Imiquimod on MUC1⁺ Cytotoxic T cell generation

6.2 Methodology

6.2.1 Isolation of Regulatory T cells via Magnetic Selection (Dyna)

A maximum volume of 100 ml of peripheral blood was obtained from healthy donors in accordance with local ethical committee approval (EC# BT/04/2005). Peripheral blood mononuclear cells (PBMC) were separated via a density-gradient centrifugation over Histopaque-1077 (Sigma) twice, as previous, firstly to isolate PBMCs with the second density gradient used for the removal of platelets. The resultant cells were then washed and resuspended at 5×10^7 cells per 500 μ l isolation buffer containing phosphate buffered saline (PBS) + 1 % w/v bovine serum albumin (BSA) (Sigma) + 1 mM EDTA (Sigma) before viability counting via Trypan Blue exclusion (Fluka). 200 μ l of CD4 Human Antibody Mix (Invitrogen) and 100 % v/v foetal calf serum (Sigma) (FCS) were incubated with PBMC for 20 minutes. This was followed by the addition of 1 ml Depletion MyOne Dynabeads (Invitrogen) before magnetic removal of bead-bound non-CD4⁺ cells, leaving CD4⁺ cells in solution.

CD4⁺CD25⁺ T cells were isolated by positive selection using 200 μ l CD25 Dynabeads per 1.5×10^7 cells. The CD4⁺CD25⁺ Treg population was liberated from the CD25 Dynabeads using Detach-a-bead solution (Invitrogen) and were then used in downstream applications. The remaining CD4⁺CD25⁻ effector T cells were either used in T cell suppression assays or placed in RPMI-1640 (Sigma) + 10 % v/v FCS (Sigma) + 5 mM L-Glutamine (Sigma) + 100 IU/ μ g/ml Pencillin/Streptomycin (Sigma) and 10 % v/v DMSO (Sigma) before undergoing controlled freezing at -80 °C with prolonged storage in liquid nitrogen. A small volume of cells (10 μ l) was taken at each isolation step for analysis via flow cytometry.

6.2.2 Isolation of CD8⁺ T cells via Magnetic Selection

Peripheral blood mononuclear cells (PBMC) were separated via density-gradient centrifugation as described previously. The resultant cells were then washed and resuspended at 1×10^7 cells per 80 μ l of isolation buffer containing phosphate buffered saline (PBS) + 1% w/v bovine serum albumin (BSA) (Sigma) + 1 mM EDTA (Sigma) before viability counting via Trypan Blue exclusion (Fluka). Cells were labelled with 20 μ l CD8 Microbeads (Miltenyi Biotec) per 1×10^7 cells for 15 minutes before washing in isolation buffer. The cell/bead mixture was applied to a primed 'LD' magnetic column which was subsequently washed several times before the enriched, bead-free CD8⁺ T cell population was eluted. A small volume (10 μ l) of cells was taken for counting and phenotypic analysis via flow cytometry.

6.2.3 Isolation of Plasmacytoid Dendritic Cells

Peripheral blood mononuclear cells (PBMC) were separated via a density-gradient centrifugation as described previously. The resultant cells were then washed in isolation buffer containing phosphate buffered saline (PBS) + 1% w/v bovine serum albumin (BSA) (Sigma) + 1 mM EDTA (Sigma) before viability counting via Trypan Blue exclusion (Fluka). Cells were incubated with 100 μ l of FcR Blocking Reagent and 100 μ l of CD304 MicroBeads per 10^8 cells (Miltenyi Biotec) for 15 minutes before washing in isolation buffer. The cell/bead mixture was applied to a primed 'LD' magnetic column which was subsequently washed several times before the enriched, bead-free pDC population was eluted into another column. Elution was repeated to enhance cell purity. A small volume (10 μ l) of cells was taken for counting and phenotypic analysis via flow cytometry.

6.2.4 Flow Cytometry Analysis

Cells were stained with cell surface markers 2.5 µl CD3-ECD (clone UCHT1- Beckman Coulter), 1.25 µl CD4-ECD (clone SFCl12T4D11 - Beckman Coulter), 2.5 µl CD8-FITC (Clone HIT8a – BioLegend), 7.5 µl CD25-PE (Clone BC96 - BioLegend), 2.5 µl CCR4-PerCP/Cy5.5 (Clone TG6 - BioLegend), 2.5 µl CD69-PE/Cy5 (Clone FN50 – BioLegend), 2.5 µl CD80-FITC (Clone 2D10, BioLegend), 2.5 µl CD83-PE (Clone HB15e, BioLegend), 2.5 µl CD86-PE/Cy5 (Clone IT2.2, BioLegend), 2.5 µl CD123 (Clone 6H6 – BioLegend), 2.5 µl CD303-PE (Clone 446921 – R & D Systems), 10 µl HLA-A2-FITC (Clone BB7.2 – Abcam). Cells were incubated with stains for 15 minutes before fixation with 2 % v/v formaldehyde (Sigma). Cells, if required, were then permabilised using FOXP3 Perm Buffer (BioLegend) before incubation with FOXP3-AlexaFluor488 (Clone 259D – BioLegend) or TLR7-FITC (Abcam) or TLR8-PE (Abcam) for 30 min. Cells were then resuspended in FACS wash buffer (BioLegend) and read via a Beckman Coulter FC 500 flow cytometer. Analysis involved gating on lymphocyte populations from forward/side-scatter plots before selecting regions of interest.

6.2.5 Imiquimod-treated T cell Suppression Assays

T cell proliferation assays were set-up as described in chapter 3. Treg were incubated with Imiquimod at various concentrations (0 – 30 µM) for one hour. Control samples were incubated with equivalent concentrations of DMSO. The cells were then washed and added to the assay as per normal protocol.

6.2.6 CD8⁺ T Cell Proliferation Assay

5 x 10⁴/well CD8⁺ T cells were stimulated with 10 µg/ml anti-CD3 (Clone-OKT, Biolegend) and 5 µg/ml anti-CD28 (Clone – 28.2, Biolegend) or 10 µg/ml of

PHA (Sigma). The final volume was adjusted to 200 μ l using X-vivo-15 + 5% Human AB serum. After 18 hours, 100 μ l of cell supernatant was taken and stored at -40°C . Cells were then either stained for flow cytometry using CD69-PE/Cy5 (Clone – FN50, Biolegend) as a activation marker or pulsed with 1 μ Ci of [^3H]-labelled thymidine per well (TRA120, Amersham). The pulsed plate was left to incubate for a further 18 hours before being harvested onto a microscintillation plate (Perkin-Elmer). After repeated washes with de-ionised water, the plate was allowed to dry for an hour. 20 μ l of scintillation fluid (Microsint 0, Perkin-Elmer) was added per well before the plate was covered with optical tape (Perkin Elmer). Thymidine incorporation was quantified using a Packard TopCount NXT Microplate Scintillation counter.

6.2.7 pDC Stimulation Assay

1×10^4 /well pDC were treated alone or in combination with either Imiquimod (0-30 μ M), DMSO (0 – 10 % v/v) or MUC1 peptide (LLLLTVLTV – 0 -100 μ g/ml). The final volume was adjusted to 200 μ l using X-vivo-15 + 5 % v/v Human AB serum. After 18 hours, 100 μ l of cell supernatant was taken and stored at -40°C for analysis at a later date. Activation of pDC was determined by flow cytometry using CD80, CD83 and CD86 as activation markers. Donors from which cells were isolated were screened for being HLA-A2 $^+$.

6.2.8 ELISA

Cytokine production was measured by ELISA. The cytokines of interest were IFN- α , IFN- γ , IL-6, IL-10 and TNF- α . Polystyrene 96-well plates (MaxSorp, Nunc) were coated with the relevant capture antibody (1 μ g/ml IFN- α ; Clone EBI-10 – eBioscience, 2 μ g/ml IFN- γ ; Clone MD-1 – BioLegend, 1 μ g/ml IL-6;

Clone MQ2-39C3, BioLegend, 2 µg/ml IL-10; Clone JES3-12G8, BioLegend, 2 µg/ml TNF-α; Clone MAb1, BioLegend) overnight. The plate was then blocked with 5 % w/v skimmed milk powder in PBS (pH 7.2) + 0.05 % v/v Tween (PBS/Tween) to prevent non-specific binding. The plate was washed in PBS/Tween using an automated plate washer and tapped dry. The relevant recombinant cytokine (PeproTech) and samples were added to the plate. After 2 hours, the plate was washed in PBS/Tween before the appropriate biotinylated detection antibody (2 µg/ml IFN-α, eBioscience; 1 µg/ml IFN-γ, 1 µg/ml IL-6, 2 µg/ml IL-10, 1 µg/ml TNF-α; BioLegend) was diluted in PBS, 1 % w/v BSA, and 0.1 % v/v Tween 20 and incubated for 2 hours at room temperature before being washed. 50 µl of 1 µg/ml streptavidin-horseradish peroxidase (BD-Pharmingen) was added each well. After 30 minutes incubation at room temperature, the plate was washed and bound horseradish peroxidase was visualised with 0.1 mg/ml tetramethylbenzidine (Sigma) and 2 µl of 30 % v/v hydrogen peroxide (Sigma) diluted in 3 M sodium acetate buffer (Sigma). The colour reaction was stopped by addition of 2 M sulphuric acid (Sigma), and absorbance was measured at 450 nm using a MRX spectrophotometer (Dynex Technologies). The concentrations of samples were calculated from standard curves of each recombinant cytokine. ELISA detection limit were determined as follows (IFN-α - 40 pg/ml, IFN-γ – 10 pg/ml, IL-6 – 8 pg/ml, IL-10 – 2 pg/ml, TNF-α – 4 pg/ml).

6.2.9 Cytotoxic T cell Generation

5 x 10⁴/ml pDC were cultured and pulsed with 40 µg/ml MUC1 peptide (amino acids 12 – 20, LLLLTVLTV, ProImmune) for 2 hours. 1 x 10⁶/ml CD8⁺ T cells were added to the co-culture with or without 2 x 10⁵/ml Treg or 3 µM Imiquimod for seven days in the presence of 50 IU/ml IL-2. Fifty percent of the

medium was replaced on days 3 and 5 with fresh medium containing 25 IU/ml IL-2. The co-culture was harvested and analysed by flow cytometry for CD8⁺MUC1⁺ cells using an R-PE labelled Pro5 MHC Pentamer (A*02:01; ProImmune).

6.2.10 Cytotoxic T cell Expansion

Purified CD8⁺MUC1⁺ T cells were placed in culture using X-Vivo-15 + 5% Human AB Serum (Lonza). The culture was supplemented with 1000 IU/ml recombinant IL-2 (Novartis) and stimulated with 1 µg/ml αCD3 and soluble 1 µg/ml αCD28 (Biolegend). After days 3, 5 and 7, cells were supplemented with 1000 IU/ml recombinant IL-2. The cells were then re-suspended in culture media and re-stimulated on day 10 as described previously. Viability and cell number were assessed using Trypan Blue Staining.

6.2.11 Intracellular Cytokine Staining

Analysis of intracellular cytokines by flow cytometry was performed. Cytokine secretion was blocked with the addition of 10 µg/ml brefeldin A (BioLegend) for the last 6 hours of culture. Permabilisation was carried out as described previously and cells stained with IFN-γ-FITC (Clone - 4S.B3, Biolegend). Cells were then resuspended in FACS wash buffer (BioLegend) and read via a Beckman Coulter FC 500 flow cytometer. Analysis involved gating on lymphocyte populations from forward/side-scatter plots before selecting regions of interest.

6.3 Results and Discussion

6.3.1 TLR Expression Profiles

Clarification was sought on the expression of TLR7 using flow cytometry. Figure 6.1 shows the expression of TLR7 on CD8⁺ T cells, pDCs, CD4⁺CD25⁻ T cells, Treg and the ovarian cancer cell line A2780. As mentioned previously, the expression of TLR7 on various cell populations, and in particular Treg, has been debated for some time (73).

As expected, pDC demonstrated the most prominent expression of TLR7. Interestingly, CD8⁺, CD4⁺CD25⁻ and Treg also expressed TLR7. Therefore, the use of TLR7 agonists, like Imiquimod, will have an effect on several different immune populations. The existence of toll-like receptors on effector populations (CD8⁺ and CD4⁺CD25⁻ T cells) has been widely discussed, though focus in this area has tended to be on TLR1, -2,-3 and -5 (74,75). The expression of TLR7 on Treg is of great interest as earlier literature suggested that TLR7 was not present on human Treg (76). A widely cited paper in this area by Peng *et al.* (67) suggested that TLR7 was not expressed in with CD4⁺CD25⁻ T cells or Treg. However, Figure 6.1 and recent evidence has suggested otherwise (77,78).

Of all the cell populations tested, only the ovarian cancer cell line A2780 was negative for TLR7 expression. TLR expression on both normal and malignant ovarian tissue has been investigated (79) which indicates the expression of several other TLR (-2,-3,-4,-5). Cremer *et al.* (80) have demonstrated that primary human lung tumours and human lung cancer cell lines express TLR7. Interestingly, they also show that engagement of TLR7 lead to an increase in cancer cell proliferation as well as increased chemoresistance to poly-chemotherapy (Doxorubicin, Navelbine, Cycloheximide and Cisplatin).

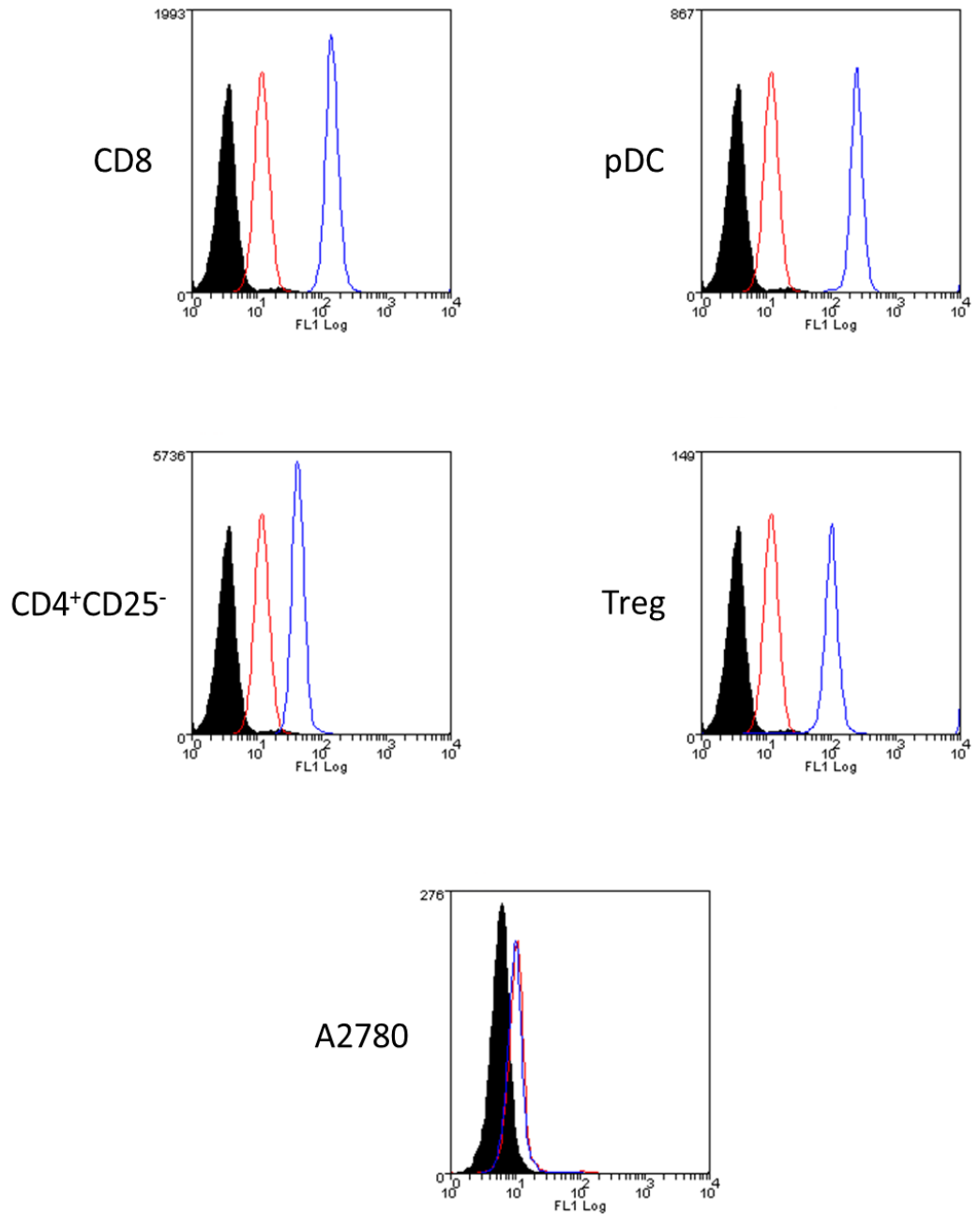


Figure 6.1 – Expression of TLR7 on Various Cell Populations – TLR7 expression measured by flow cytometry. Black represents unstained cells. Red represents Rabbit polyclonal FITC IgG as isotype control. Blue represents Rabbit polyclonal TLR7 IgG. Figure representative of three independent experiments.

6.3.2 Effect of Imiquimod on Treg Activity

The key question to now answer is the effect of TLR7 activation on Treg and other immune populations. Activation of TLR7 on CD8⁺ T cells (69,81,82) and pDC (57,83,84) has been described in detail. However, the effect of TLR7 activation on Treg is a matter of contention. Huang *et al.* (69) have suggested that TLR7 agonism leads to a decrease in FOXP3⁺ cells in squamous cell carcinoma patients. These cells, on isolation, were poorly suppressive and allowed the proliferation of IFN- γ secreting cytotoxic T cells. Despite their claim that Treg do not possess TLR7, Peng *et al.* (67) have shown that Imiquimod can significantly reduce Treg mediated CD4⁺CD25⁻ T cell suppression. Their rationale for this effect is the potential agonism of TLR8 by Imiquimod. However, key papers suggest that Imiquimod is more selective for TLR7 (40,85). As mentioned previously, Forward *et al.* (77) have described how TLR7 agonism leads to an increase in the suppressive behaviour of murine Treg. It was therefore essential to assess the effect of TLR7 agonism on Treg function.

Imiquimod was selected as the TLR7 agonist of choice. Although, its chemical progeny, Resiquimod, is reportedly up to 50 times more potent for TLR7 binding (40), Imiquimod was used as it was significantly cheaper to obtain.

Figure 6.2 shows the effect of 3 μ M Imiquimod on Treg mediated suppression of both CD4⁺CD25⁻ T cells and CD8⁺ T cells. The concentration of Imiquimod was selected on the basis of literature in the field (67,69). Treatment with Imiquimod caused a partial abrogation of Treg mediated suppression on CD4⁺CD25⁻ T cells (Figure 6.2A). There was a significant increase in CD4⁺CD25⁻ T cell proliferation at the 1/5 fraction (Figure 6.2A; 1/5; Control vs.

Treated (cpm) – 452 vs. **1387**, $n = 3$; $p < 0.05$) and the 1/10 fraction (Figure 6.2A; 1/10; Control vs. **Treated** (cpm) – 1502 vs. **2447**, $n = 3$; $p < 0.05$). There was no change in either Treg (Treg; Control vs. Treated (cpm) – 120 vs. 136, $n = 3$; $p > 0.05$) or Effector CD4⁺CD25⁻ T cell (Effector; Control vs. Treated (cpm) – 3879 vs. 3668, $n = 3$; $p > 0.05$).

Imiquimod also partially reduced the suppressive effect of Treg on CD8⁺ T cell proliferation (Figure 6.2B). Significant increases in proliferation were noted at the 1/5 fraction (Figure 6.2B; 1/5; Control vs. **Treated** (cpm) – 19980 vs. **20450**, $n = 3$; $p < 0.05$) and the 1/10 fraction (Figure 6.2B; 1/10; Control vs. **Treated** (cpm) – 12840 vs. **16030**, $n = 3$; $p < 0.05$). As with the CD4⁺CD25⁻ T cell assay, there was no change in the proliferation of Treg (Figure 6.2B; Treg; Control vs. Treated (cpm) – 67 vs. 54.33, $n = 3$; $p > 0.05$). However, there was a significant increase in CD8⁺ T cell proliferation (Figure 6.2B; CD8; Control vs. **Treated** (cpm) – 19980 vs. **24780**, $n = 3$, $p < 0.05$). The alteration in the suppressive phenotype of Treg and an increase in the proliferation of CD8⁺ T cells are key observations, both of which are advantageous in developing an anti-tumour immune response (86,87). After the above result the immediate objective was to observe whether there was a dose dependent effect on the abrogation of Treg suppressive activity.

Figure 6.3 depicts the effect of a dose titration of Imiquimod on the suppressive activity of Treg. The proliferation of PHA-stimulated CD4⁺CD25⁻ T cells in co-culture with a 1/5 fraction of Treg when treated with Imiquimod was assessed. A 1/5 fraction of Treg was used as this experimental group provided the greatest suppressive effect. Imiquimod demonstrated a dose-dependent reversal of Treg suppression (Figure 6.3; 0.3 – 30 μ M, $n = 3$; $p < 0.05$). Concentrations above 30 μ M demonstrated no effect (Figure 6.3; 0 vs.

300 (cpm) – 468 vs. 31, $n = 3$; $p > 0.05$) which could be contributed to excessive amounts of DMSO within the culture.

It is not known exactly how the TLR triggering of Treg cells modulates their suppressive effects, and whether the triggering of different TLRs occurs through the same pathway or has the same effect on Treg function. One explanation could be the upregulation or downregulation of FOXP3 expression following the different TLR stimulations (64,65), but how TLR-signalling affects FOXP3 expression is still unclear. As discussed previously, the role of FOXP3 expression in human Treg is still under scrutiny (88-90) and so its relevance in Treg-TLR signalling is to be questioned. Another possibility to explain the abrogated regulatory function but enhanced proliferative capacity of Treg after TLR7 stimulation corresponds with reports looking at TLR2 agonism indicating that Treg rapidly lose their ability to inhibit proliferation after receiving strong activation signals (91).

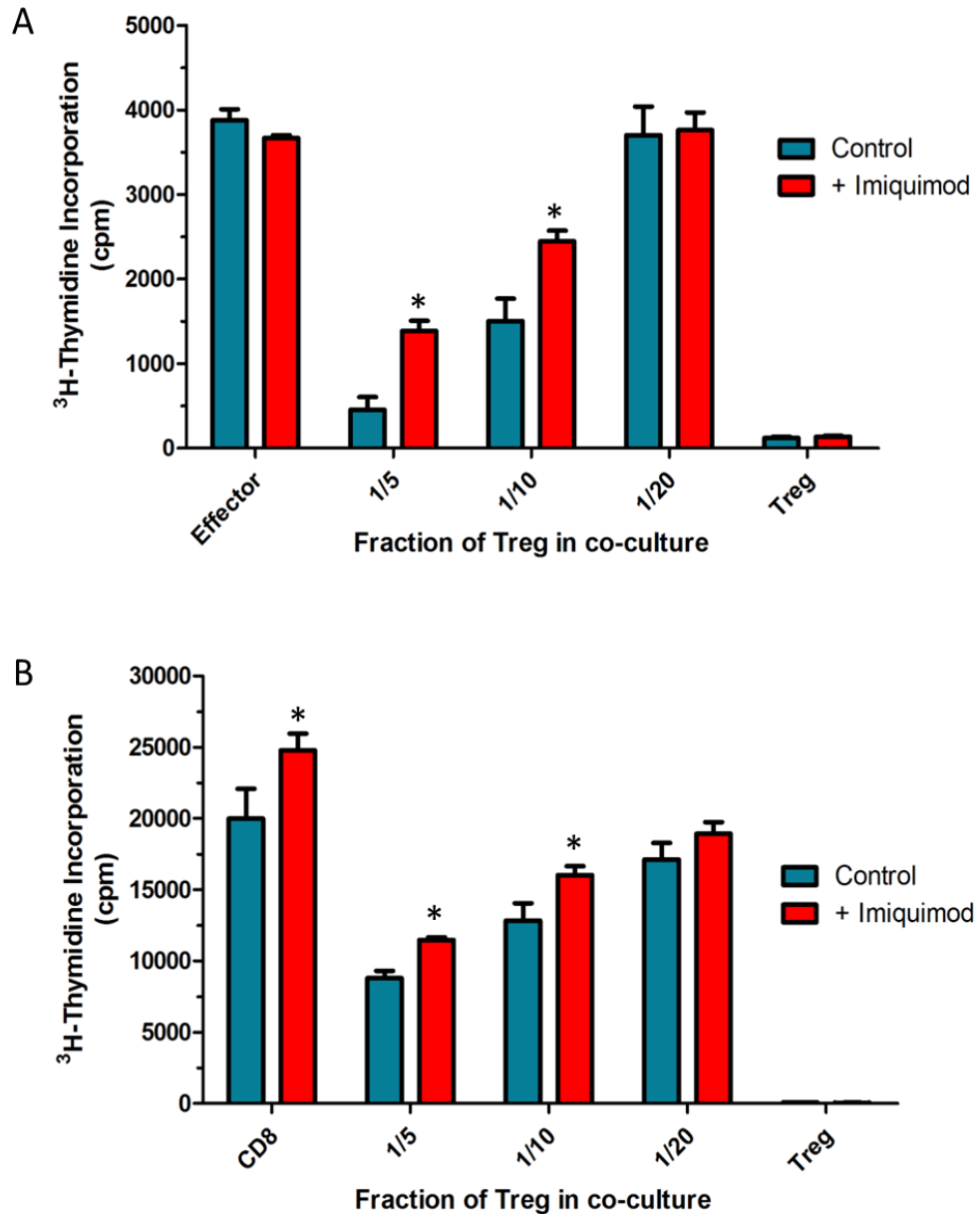


Figure 6.2 – Treg-mediated Suppression of Effector T cell Populations is Abrogated by 3 μ M Imiquimod. A - CD4+CD25- Effector T cells stimulated by PHA. **B** – CD8+ T cells stimulated by 10 μ g/ml α CD3 and 5 μ g/ml α CD28. 3 μ M Imiquimod reduces the suppressive effects of Treg where Treg were present at 1/5 and 1/10 fraction of co-culture. Figures representative of the means of three independent experiments from the same donor. *= p <0.05. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Error bars represents standard deviation.

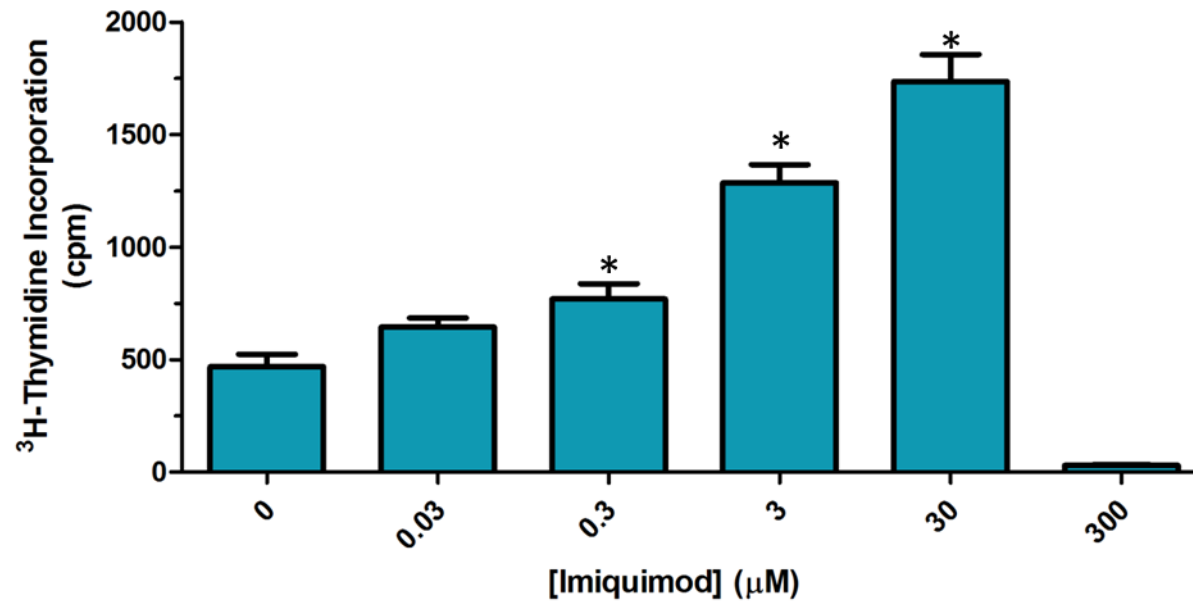


Figure 6.3 – Treg-mediated Suppression of Effector T cell Populations is Abrogated by Imiquimod in a dose-dependent manner. CD4+CD25- Effector T cells co-cultured with Treg where Treg were present at 1/5 fraction of co-culture. Suppressive effect of Treg reduced at concentrations 0.3, 3 and 30 μM . Figure representative of the means of three independent experiments from three different donors. *= $p < 0.05$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Error bars represent standard deviation.

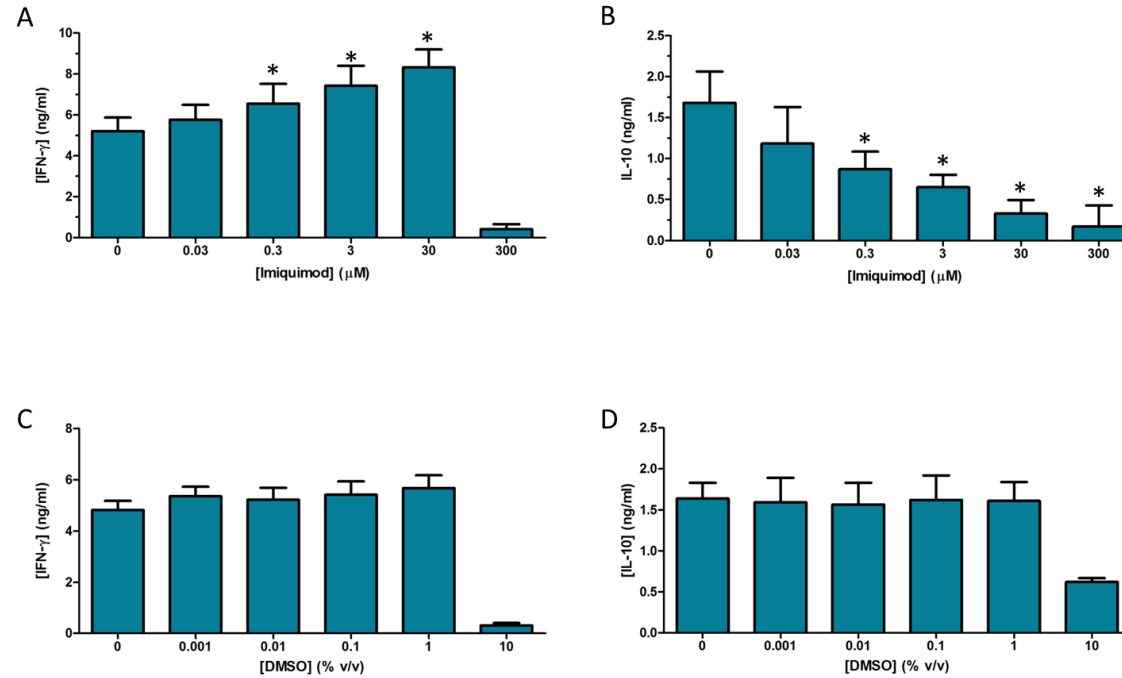


Figure 6.4 - Production of IL-10 and IFN- γ from T cell Suppression Assays Stimulated with PHA. Treatment with Imiquimod leads to an increase in IFN- γ and a decrease in IL-10 when cells were co-stimulated with PHA. **A** – IFN- γ ; cells treated with Imiquimod (0 – 300 μ M), **B** – IL-10; cells treated with Imiquimod (0 – 300 μ M). **C** - IFN- γ ; cells treated with DMSO (0 – 10 % v/v) and **D** – IL-10; cells treated with DMSO (0 – 10 % v/v). Figures representative of the means three independent experiments from different donors. Error bars represent standard deviation. *= p <0.05. Detection limit = 40 pg/ml. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

The reduction in Treg-mediated suppression meant that the cytokine milieu produced during co-culture with CD4⁺CD25⁻ T effector cells also changed, as seen in Figure 6.4. As expected, there was a significant, dose dependent increase in the amount of IFN- γ (Figure 6.4A; 0.3 – 30 μ M, $n = 3$; $p < 0.05$) and a decrease in IL-10 (Figure 6.4B; 0.3 – 30 μ M, $n = 3$; $p < 0.05$). The cytokine profile can be explained by the activation of NF- κ B, downstream of TLR7 activation (92). Interestingly, the decrease in IL-10 secretion has not previously been noted. As explained in Chapter 3, PHA acts on NF- κ B, leading to cytokine production. TLR agonism provides strong signals for pro-inflammatory cytokine production (92). It could therefore be suggested that these pro-inflammatory signals ‘out-compete’ those signals for IL-10 production, thus demonstrating the behaviour seen in Figure 6.4.

Having observed a significant change in the phenotype of Treg-mediated suppression, it was important to assess the co-culture system. This was in an attempt to delineate the mechanism by which Treg suppression was being abrogated. Figure 6.5 illustrates the proliferative effect of Imiquimod and α CD3/ α CD28 stimulation on both CD8⁺ T cells and Treg. The proliferation of Treg did not change under co-stimulation with α CD3/ α CD28 and a concentration range of Imiquimod (Figure 6.5A; 0 – 30 μ M, $n = 3$, $p > 0.05$).

Interestingly, as noted earlier in Figure 6.2B, α CD3/ α CD28-stimulated CD8⁺ T cells saw increased proliferation when treated with Imiquimod. This effect was dose dependent (Figure 6.5B; 0.03 – 30 μ M, $n = 3$, $p < 0.05$) with an Imiquimod concentration of 3 μ M providing maximal stimulation (Figure 6.5B; 0.03 vs. 0.3 vs. **3** vs. 30 μ M; (cpm) – 123 vs. 177 vs. 213 vs. **240** vs. 225, $n = 3$; $p < 0.05$). The proliferative effect of TLR7 engagement on CD8⁺ T cells has been discussed previously (62,69,93). The rationale for the lack of Treg

proliferation could be linked to the reported differences in proliferative pathways between effector populations and Treg as discussed in Chapter 3 (94) (Figure 3.8).

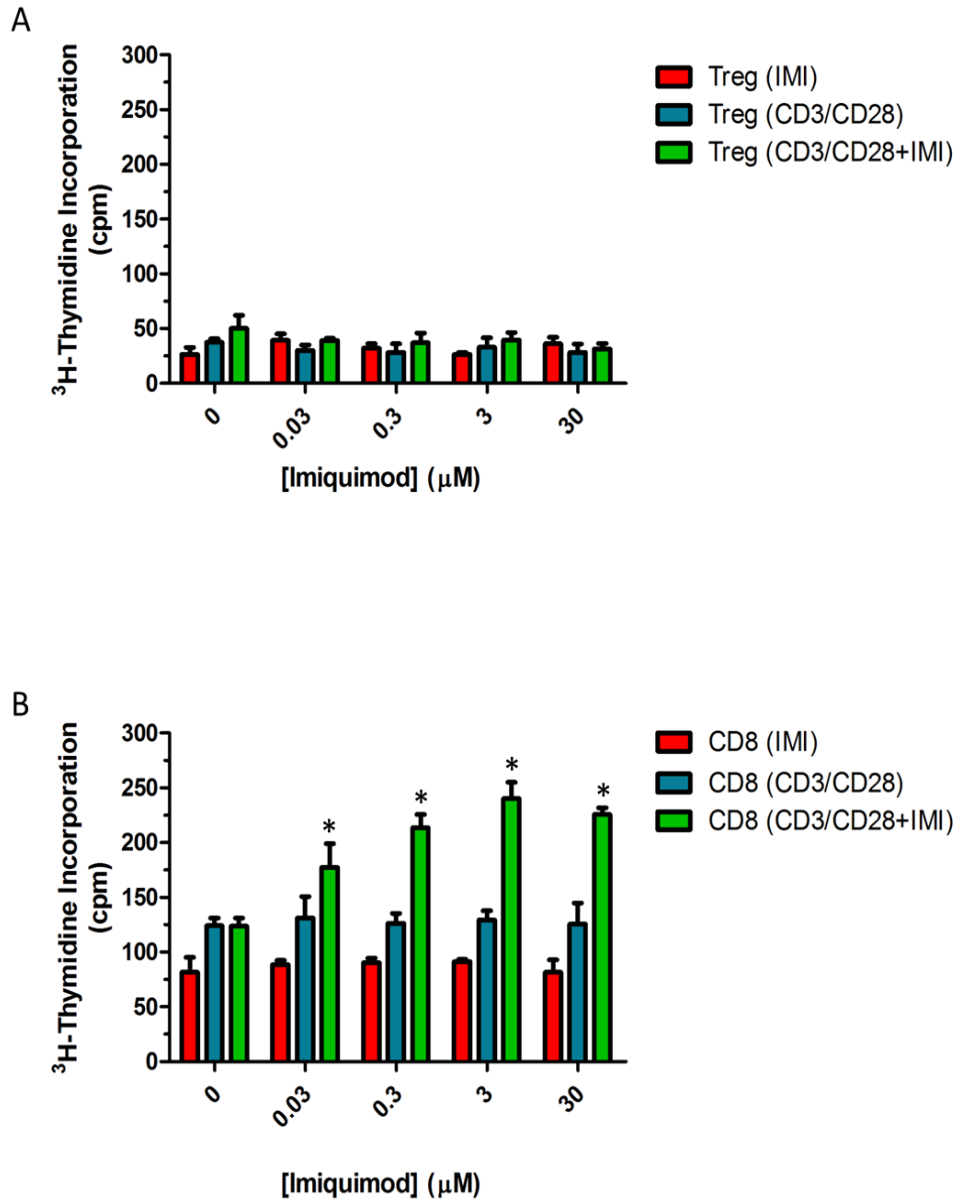


Figure 6.5 – Imiquimod Increases CD8+ T cells but not Treg Proliferation when co-stimulated with α CD3/ α CD28 for 24 hours. **A** - Treg. Co-stimulation of Treg with 10 μ g/ml α CD3 and 5 μ g/ml α CD28 and varying concentrations of Imiquimod (0 – 30 μ M) showed no change in proliferation compared to Imiquimod or α CD3/ α CD28 only. **B** – CD8⁺ T cell. Co-stimulation of CD8⁺ T cells with 10 μ g/ml α CD3 and 5 μ g/ml α CD28 and varying concentrations of Imiquimod (0 – 30 μ M) showed an increase in proliferation when compared to Imiquimod or α CD3/ α CD28 alone; $p < 0.05$ ($n = 3$). Figures representative of the means of three independent experiments from the different donors. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Error bars represent standard deviation.

6.3.3 Cell Activation and IFN- γ Expression

Figures 6.6 and 6.7 demonstrate the effect of Imiquimod on the activation status of CD8⁺ T cells. Firstly, in Figure 6.6, CD69 expression on CD8⁺ T cells stimulated with either Imiquimod or in conjunction with α CD3/ α CD28 was assessed using flow cytometric analysis. CD69 is an early membrane receptor transiently expressed on lymphocyte activation, not detected in resting lymphocytes, and selectively expressed in chronic inflammatory infiltrates and at the sites of active immune responses *in vivo* (95). It is widely used as a marker for T cell activation and as such was used to monitor the activation of CD8⁺ T cells in response to stimuli. There was no change in CD69 expression when treated with Imiquimod only (0 – 30 μ M, $n = 3$, $p > 0.05$). α CD3/ α CD28 alone caused an increase in CD69 expression (Figure 6.6; 0 μ M Imiquimod vs. α CD3/ α CD28 (%) – 7.70 vs. **9.65**, $n = 3$, $p < 0.05$). However, on titration of Imiquimod, a significant increase in CD69 expression was seen (0.03 – 30 μ M, $n = 3$, $p < 0.05$). The effect was dose dependent with a combination of 3 μ M Imiquimod and 10 μ g/ml α CD3 and 5 μ g/ml α CD28 stimulation providing the highest percentage of CD8⁺CD69⁺ cells (Figure 6.6; 0.03 vs. 0.3 vs. **3** vs. 30 μ M ; (CD69 %) – 11.42 vs. 12.33 vs. **14.26** vs. 14.04, $n = 3$; $p < 0.05$).

Another common technique used for assessing activated T cells is intracellular cytokine staining for IFN- γ (96). Intracellular cytokine staining allows the assessment of individual cells within a population and does not rely on the detection of secreted cytokines. It can provide an insight into the processes underway within a cell soon after stimulation/activation. Figure 6.7 shows the level of intracellular IFN- γ in CD8⁺ T cells after stimulation with either Imiquimod or in conjunction with α CD3/ α CD28. Treating CD8⁺ T cells with Imiquimod led to an increase in intracellular IFN- γ expression (0.03 – 30

μM , $n = 3$, $p < 0.05$). $\alpha\text{CD3}/\alpha\text{CD28}$ alone caused an increase in IFN- γ expression (Figure 6.7; 0 μM Imiquimod vs. $\alpha\text{CD3}/\alpha\text{CD28}$ (IFN- γ %) – 1.59 vs. **5.413**, $n = 3$, $p < 0.05$). As with the change in CD69 expression seen in Figure 6.6, titration of Imiquimod led to a significant increase in IFN- γ (0.03 – 30 μM , $n = 3$, $p < 0.05$). The effect was dose dependent with a combination of 30 μM Imiquimod and 10 $\mu\text{g}/\text{ml}$ αCD3 and 5 $\mu\text{g}/\text{ml}$ αCD28 stimulation providing the highest percentage of CD8⁺IFN- γ ⁺ cells (Figure 6.7; 0.03 vs. 0.3 vs. 3 vs. **30** μM ; (IFN- γ %) – 9.88 vs. 12.44 vs. 13.65 vs. **14.03**, $n = 3$; $p < 0.05$). Ideally, Treg would have also been assessed for CD69 and intracellular IFN- γ expression. However, restrictions on cell size, reagent availability and cost meant that the decision was made to forgo these experiments. Although in Chapter 3, a method for expanding Treg was optimised, it would be difficult extract any useful information from CD69/IFN- γ experiments. The rationale for this statement is due to the continuous stimuli provided to Treg whilst expanding. High IL-2 and αCD28 superagonist treatment lead to activation and subsequent proliferation of Treg. Therefore, upon treatment, CD69 would be artificially elevated in the expanded population of Treg (97-100) and so the effect of Imiquimod would be less inferable. Recent work by Daniel *et al.* (101) suggests that a subset of CD4⁺CD25⁺FOXP3⁺ Treg possess the ability to express IFN- γ . Again, this could skew the inference of Imiquimod-induced IFN- γ expression.

To confirm the intracellular cytokine results seen in Figure 6.7, supernatants from the CD8⁺ T cell stimulation assays were assessed by IFN- γ and IL-10 ELISA (Figure 6.8). Samples from Treg stimulation assays were also assessed. The data in Figure 6.8 validates those findings which were described earlier. Both Treg and CD8⁺ T cells demonstrate a dose-dependent increase in IFN- γ (Figure 6.8; Treg, 0.3 – 30 μM ; CD8, 0.3 – 30 μM , $n = 3$, $p <$

0.05) on Imiquimod treatment which was subsequently enhanced on the addition of α CD3/ α CD28 (Treg, 0.03 – 30 μ M; CD8, 0.03 – 30 μ M, $n = 3$, $p < 0.05$). 3 μ M Imiquimod, in combination with, 10 μ g/ml α CD3 and 5 μ g/ml α CD28 exhibited the highest concentration of IFN- γ secretion in both Treg and CD8⁺ T cells (Treg; 2.41 ng/ml, CD8; 4.68 ng/ml).

A similar trend can be reported with regards to IL-10 secretion. Both Treg and CD8⁺ T cells demonstrate a dose-dependent increase in IL-10 (Treg, 0.03 – 30 μ M; CD8, 0.3 – 30 μ M, $n = 3$, $p < 0.05$) on Imiquimod treatment which was subsequently enhanced on the addition of α CD3/ α CD28 (Treg, 0.03 – 30 μ M; CD8, 0.3 – 30 μ M, $n = 3$, $p < 0.05$). 30 μ M Imiquimod, in combination with, 10 μ g/ml α CD3 and 5 μ g/ml α CD28 exhibited the highest concentration of IFN- γ secretion in both Treg and CD8⁺ T cells (Figure 6.8; Treg; 0.41 ng/ml, CD8; 0.68 ng/ml). An interesting observation is that on treatment with α CD3/ α CD28, Treg do not secrete IL-10. It is only on the addition of Imiquimod that a significant increase in IL-10 can be detected (Figure 6.8; 0 vs 0.03 vs. 0.3 vs. 3 vs. **30** μ M; (IL-10 ng/ml) – 0.04 vs. 0.07 vs. 0.07 vs. 0.150 vs. **0.413**, $n = 3$; $p < 0.05$). Another noticeable observation was that co-stimulation of Treg with α CD3/ α CD28 and 30 μ M Imiquimod leads to a further increase in IL-10 secretion (Figure 6.8; Imiquimod vs. **Imiquimod + α CD3/ α CD28** (ng/ml); 0.19 vs. **0.41**, $n = 3$, $p < 0.05$). This effect can be explained by work Baecher-Allan *et al.* (91) who suggested that TLR engagement and T cell stimulation provide strong activation signals which lead to the secretion of both IFN- γ and IL-10.

The data suggests that treatment with a TLR7 agonist leads to increased CD8⁺ T cell proliferation as well as increased CD69 and IFN- γ expression/secretion. There also appears to be a partial abrogation of the

suppressive activity of Treg against both CD4⁺CD25⁻ and CD8⁺ effector T cells when treated with Imiquimod. This enhancement of effector T cell function and reduction in the suppressive effects of Treg are suggestive of the role Imiquimod could have in enhancing CD8⁺ T cell function. The next step is to assess the effect of Imiquimod on plasmacytoid dendritic cells. As mentioned previously, there is an increased presence of pDC at the tumour site (35). The isolation and phenotypic characterisation of pDC when stimulated with Imiquimod are critical in understanding whether these antigen presenting cells will be of assistance in generating an anti-tumour immune response.

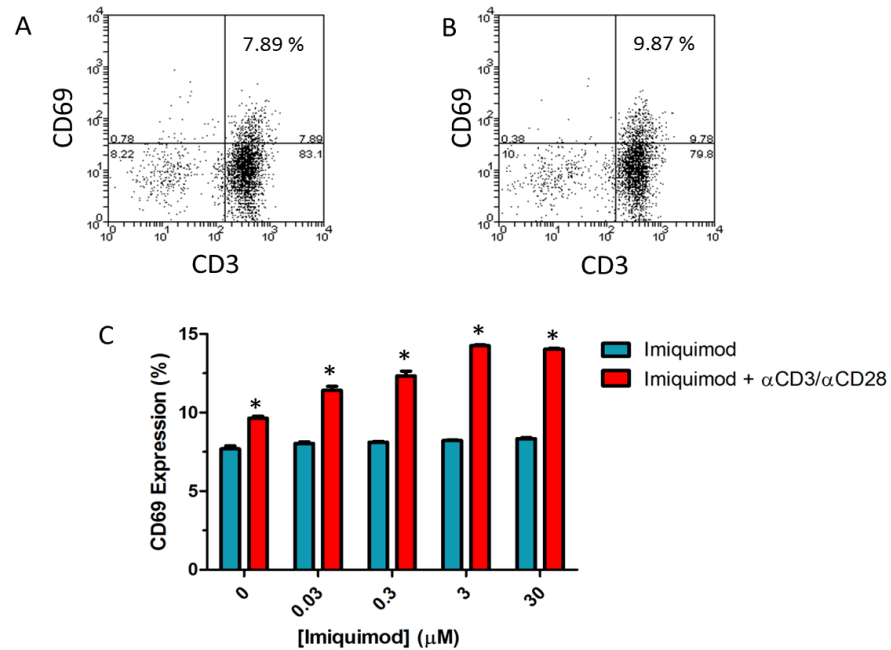


Figure 6.6 – Imiquimod Increases CD69 Expression on CD8⁺ T cells when Co-stimulated with αCD3/αCD28. **A** - FACS plot of CD3/CD69 expression on CD8⁺ T cells stimulated with Imiquimod for 24 hours. **B** - FACS plot of CD3/CD69 expression on CD8⁺ T cells stimulated with 10 μg/ml αCD3 and 5 μg/ml αCD28 for 24 hours. **C** – CD69 expression on CD8⁺ T cells stimulated with 10 μg/ml αCD3, 5 μg/ml αCD28 and varying concentrations of Imiquimod (0 – 30 μM). Peak CD69 expression was seen with 10 μg/ml αCD3 and 5 μg/ml αCD28 and 3 μM Imiquimod, *= $p < 0.05$; $n = 3$. CD69 expression determined in comparison to Mouse IgG-PE-Cy5 as isotype control. Figures representative of the means of three independent experiments from the different donors. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Error bars represent standard deviation.

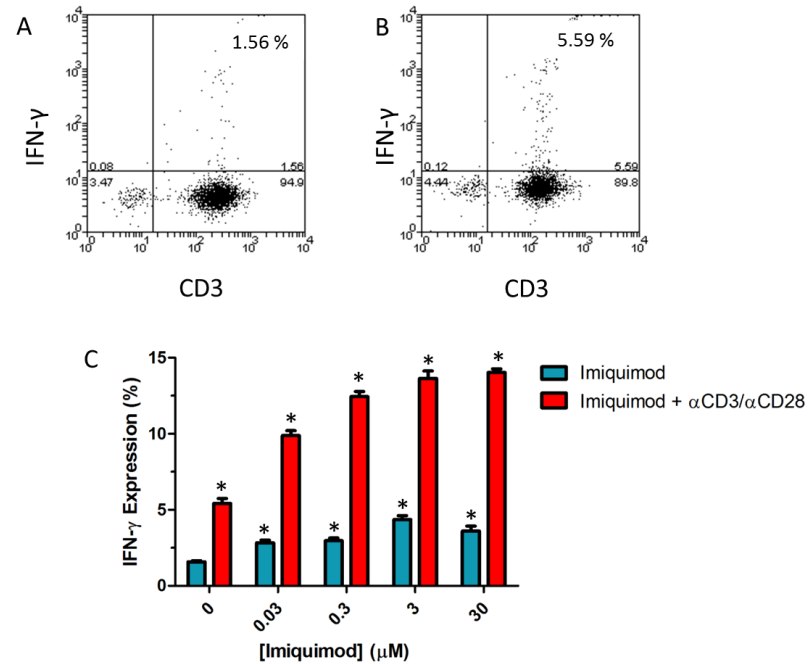


Figure 6.7 – Imiquimod Increases Intracellular IFN- γ Expression on CD8⁺ T cells when Co-stimulated with α CD3/ α CD28. **A** - FACS plot of CD3/IFN- γ expression on CD8⁺ T cells stimulated with Imiquimod for 24 hours. **B** - FACS plot of CD3/IFN- γ expression on CD8⁺ T cells stimulated with 10 μ g/ml α CD3 and 5 μ g/ml α CD28 for 24 hours. **C** – IFN- γ expression on CD8⁺ T cells stimulated with 10 μ g/ml α CD3, 5 μ g/ml α CD28 and varying concentrations of Imiquimod (0 – 30 μ M) for 24 hours. Peak IFN- γ expression was seen with 10 μ g/ml α CD3 and 5 μ g/ml α CD28 and 3 μ M Imiquimod, $\ast = p < 0.05$; $n = 3$. IFN- γ expression determined in comparison to Mouse IgG-FITC as isotype control. Figures representative of the means of three independent experiments from the different donors. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Error bars represent standard deviation.

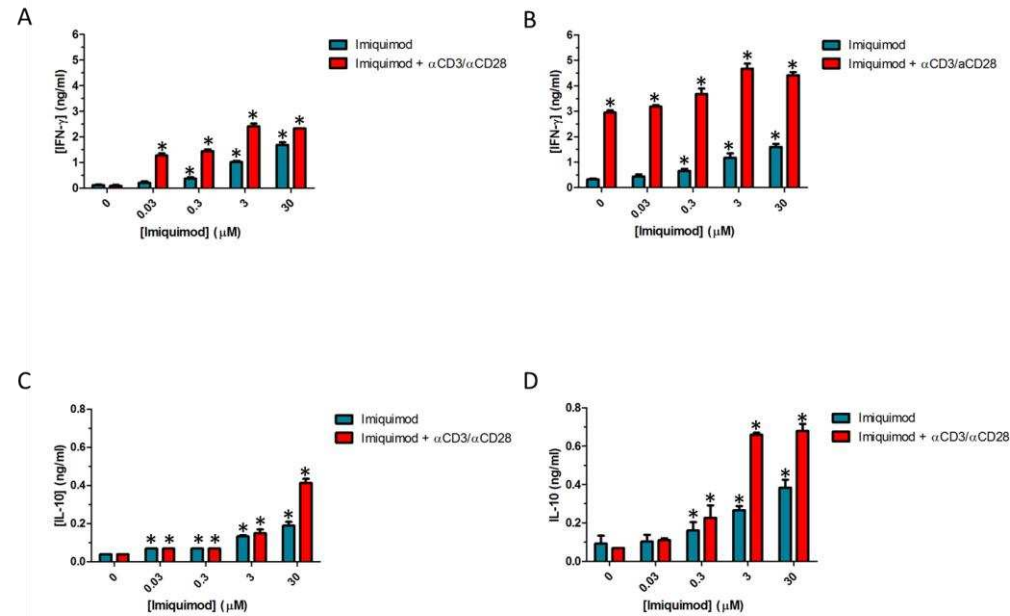


Figure 6.8 – Increased Production of IL-10 and IFN- γ from oth CD8⁺ T cells and Treg when Stimulated with α CD3/ α CD28 and Imiquimod. IFN- γ production is increased in both **A**- Treg and **B**- CD8⁺ T cells when stimulated with 10 μ g/ml α CD3, 5 μ g/ml α CD28 and varying concentrations of Imiquimod (0 – 30 μ M) for 24 hours. Peak IFN- γ secretion seen in both Treg and CD8⁺ T cells when stimulated with 10 μ g/ml α CD3, 5 μ g/ml α CD28 and 3 μ M Imiquimod. IL-10 production is also increased in **C** – Treg and **D** – CD8⁺ T cells when stimulated with 10 μ g/ml α CD3, 5 μ g/ml α CD28 and varying concentrations of Imiquimod (0 – 30 μ M) for 24 hours. Peak IL-10 secretion seen in both Treg and CD8⁺ T cells when stimulated with 10 μ g/ml α CD3, 5 μ g/ml α CD28 and 3 μ M Imiquimod. Error bars represent standard deviation. * p <0.05.. Detection limit = 40 pg/ml Figures representative of the means of three independent experiments from three different donors. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

6.3.4 pDC Isolation and Stimulation with Imiquimod

pDC make up a very small percentage of the total PBMC population (102). Therefore their isolation and use in downstream applications makes their use *ex-vivo* particularly challenging. Using CD304 microbeads, isolation of pDC from PBMCs was conducted. CD304 (BDCA-4/Neuropilin-1) is specifically expressed by pDCs in human blood (102-106), bone marrow (103), and cord blood (107). Exclusive expression of CD304 (BDCA-4/Neuropilin-1) on pDCs allows their direct isolation. In blood and bone marrow, CD304 (BDCA-4/Neuropilin-1)⁺ pDCs are CD4⁺, CD45RA⁺, CD303 (BDCA-2)⁺, CD123⁺, CD141 (BDCA-3)^{dim}, CD1c (BDCA-1)⁻, and CD2⁻. They lack expression of lineage markers (CD3, CD14, CD16, CD19, CD20, CD56) and neither express myeloid markers such as CD13 and CD33, nor Fc receptors such as CD32, CD64, or FcεRI (103,104). Isolation of pDC was assessed via flow cytometry, using CD303 and CD123 as markers, an example of which is provided in Figure 6.9. The purity of pDC on isolation was 90.2% (85.3 – 94.6%).

From Figure 6.1, pDC express TLR7. Therefore, it was important to examine the effect of Imiquimod on these cells and thus gain evidence either for or against being a target in generating an anti-tumour immune response. Figure 6.10 illustrates the expression of the pDC maturation markers CD80, CD83 and CD86 when treated with Imiquimod. These markers are widely used for assessing the maturation of dendritic cells (40,62).

Treatment of pDC with Imiquimod causes the upregulation of CD80, CD83 and CD86 in a dose-dependent manner (Figure 6.10; 0.3 – 30 μM, $n = 3$, $p < 0.05$). Peak expression CD80 (Figure 6.10; 0.03 vs. 0.3 vs. **3** vs. 30 μM; (CD80%) – 37.0 vs. 42.2 vs. **61.87** vs. 53.53, $n = 3$), CD83 (Figure 6.10; 0.03

vs. 0.3 vs. **3** vs. 30 μM ; (CD83%) – 10.49 vs. 11.93 vs. **22.56** vs. 18.63, $n = 3$) and CD86 (Figure 6.10; 0.03 vs. 0.3 vs. **3** vs. 30 μM ; (CD86%) – 36.67 vs. 46.87 vs. **51.73** vs. 45.60, $n = 3$) was demonstrated when treated with 3 μM Imiquimod ($p < 0.05$). These results suggest that Imiquimod can aid pDC maturation.

CD83 is highly expressed on mature DC, but is not detectable on APCs that do not prime naive T cells such as immature DCs, resting B cells, and monocytes (108). Critically, prolonged CD83 expression leads to increased priming of human CD8⁺ T cells (108,109). Therefore, the dose-dependent increase seen on treating pDC with Imiquimod is of importance as, along with the increase in CD80 and CD86 (110). CD80 and CD86 are co-stimulatory molecules which play key role in the priming and activation of naïve T cells (111).

Increased co-stimulation of CD8⁺ T cells could potentiate an anti-tumour response. However, the increase in CD80 and CD86 could also potentiate Treg engagement and thus subsequent tolerisation through CTLA-4 (112,113). From the results above, there is evidence to suggest that Imiquimod has an effect on Treg. The expression of CTLA-4 has not been looked at in this thesis. Therefore, the effect on Imiquimod on CTLA-4 would be of great interest in understanding the effects of TLR7 engagement.

The supernatant of Imiquimod-stimulated pDC was analysed for their production of IFN- α , IL-6 and TNF- α via ELISA as illustrated in Figure 6.11. pDC treatment of Imiquimod led to an increase in IFN- α production in a dose dependent manner (Figure 6.11A; 0.03 – 30 μM , $n = 3$, $p < 0.05$). Peak secretion of IFN- α was observed when treated with 3 μM Imiquimod (IFN- α ; (ng/ml) - 0.03 vs. 0.3 vs. **3** vs. 30 μM ; – 0.10 vs. 0.25 vs. **0.67** vs. 0.42, $n = 3$,

$p < 0.05$). pDC are regarded as professional type I interferon (IFN- α and IFN- β) producing cells (114). Moreover, type I IFN modulates several aspects of the immune response, including pDC survival, mDC differentiation (115), modulation of Th1 and CD8⁺ T cell responses, cross-presentation and cross-priming independent of CD4⁺ T helper cells (116), upregulation of MHC and co-stimulatory molecules, activation of NK, and induction of primary antibody responses (117).

IL-6 is a pleiotropic cytokine involved in the physiology of virtually every organ system. pDC treatment of Imiquimod led to an increase in IL-6 production in a dose dependent manner (Figure 6.11B 0.03 – 30 μ M, $n = 3$, $p < 0.05$). Peak secretion of IL-6 was observed when treated with 30 μ M Imiquimod (Figure 6.11; IL-6; (ng/ml) - 0.03 vs. 0.3 vs. 3 vs. **30** μ M; – 0.76 vs. 3.66 vs. 8.20 vs. **13.31**, $n = 3$, $p < 0.05$). Recent studies have demonstrated that IL-6 has a very important role in regulating the balance between IL-17-producing Th17 cells and Treg. IL-6 induces the development of Th17 cells from naïve T cells together with TGF- β while inhibiting TGF- β -induced Treg differentiation (70). Given the critical role of IL-6 in altering the balance between Treg and Th17 cells, increasing the amount of IL-6 within the tumour microenvironment is, potentially, an effective approach to further limit the suppressive effect of Treg.

TNF- α is produced during the initiation of inflammatory responses and is critical for maintenance of chronic inflammation (118,119). pDC treatment of Imiquimod led to an increase in TNF- α production in a dose dependent manner (Figure 6.11C; 0.03 – 30 μ M, $n = 3$, $p < 0.05$). Peak secretion of TNF- α was observed when treated with 3 μ M Imiquimod (Figure 6.11C; TNF- α ; (ng/ml) - 0.03 vs. 0.3 vs. **3** vs. 30 μ M; – 0.43 vs. vs. 0.69 vs. **1.228** vs.

1.043, $n = 3$, $p < 0.05$). Given its established role in chronic inflammation, angiogenesis, tissue remodelling, tumour growth and metastasis, TNF- α is likely to be an important cytokine in a variety of cancers (120-122). There is also controversial evidence surrounding the effect of TNF- α on Treg suppression. Valencia *et al.* (123) suggests that TNF- α treatment of Treg leads to a decrease in Foxp3 expression in mice with a subsequent reduction in suppression activity. Whereas Chen *et al.* (124), amongst others, suggests that TNF- α can aide Treg expansion (125).

In addition to the above secreted cytokines pDC stimulated with viral-like ligands produce chemokines, such as CCL3, CCL4, CCL5, CXCL8 and CXCL10 which stimulate Th1, and NK cells migration to sites of inflammation (126,127). There is also evidence that pDC can induce significant IL-10 production by Treg (35,92). This indicates that pDC can be phenotypically and functionally modulated in the tumour microenvironment with either pro- or anti-inflammatory stimuli. On this basis, it would be advantageous to target pDC within the tumour microenvironment with Imiquimod.

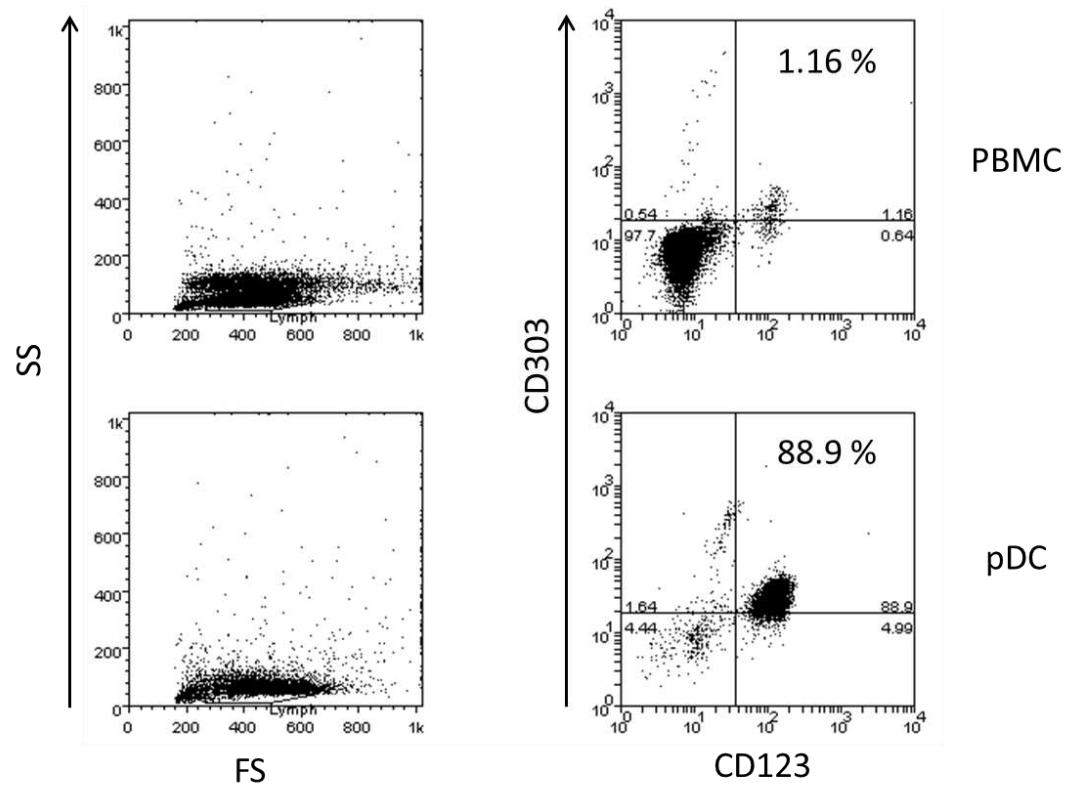


Figure 6.9 - Flow Cytometry Analysis of pDC cells Isolated using Magnetic Selection. Cells were stained for CD303 and CD123 expression. Lymphocytes were identified from forward/side scatter plots before populations of interest were gated upon. Analysis confirms CD303⁺CD123⁺ cells are approximately 1 % of the total PBMC populations which agrees with current population statistics.

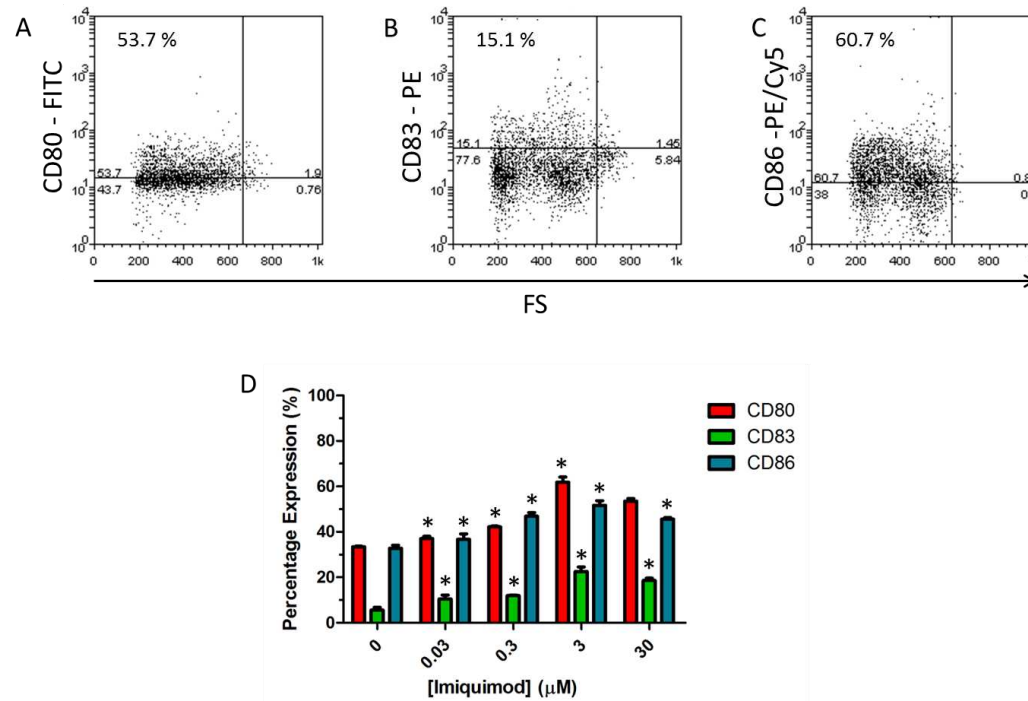


Figure 6.10 – Imiquimod Increases Expression of CD80, CD83 and CD86 on pDC. FACS plots of pDC maturation markers after 24 hours of Imiquimod treatment. **A** - CD80, **B**- CD83, **C** – CD86. **D** – Percentage expression of CD80, CD83 and CD86 on pDC stimulated with varying concentrations of Imiquimod (0 – 30 μM). Peak CD80, CD83 and CD86 expression was seen at 3 μM Imiquimod, *=p<0.05; n = 3. Percentage expression determined in comparison to Mouse IgG-FITC/PE/PE-Cy5 respectively as isotype controls. Figures representative of three independent experiments from the different donors. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Error bars represent standard deviation.

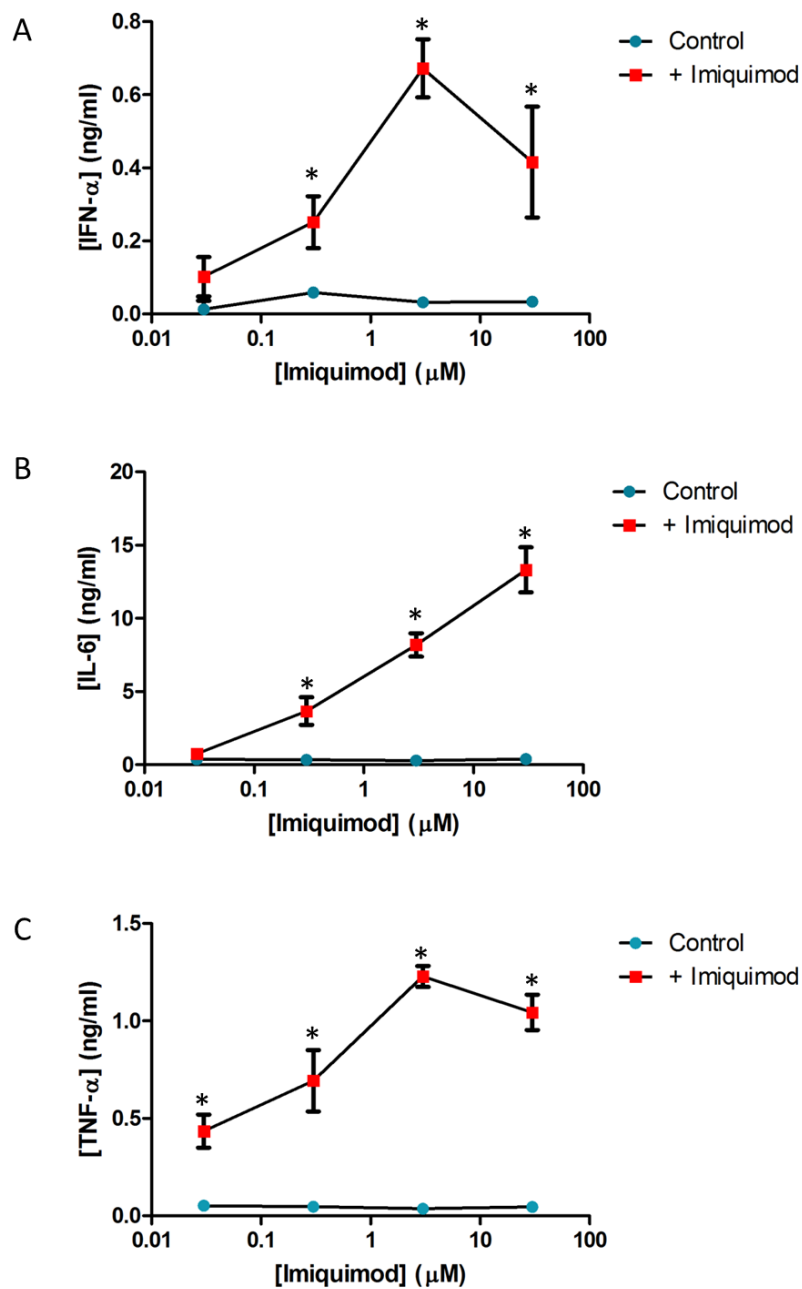


Figure 6.11 – Increased Cytokine Production from pDC Treated with Imiquimod.

Treatment with varying concentrations of Imiquimod (0 - 30 μM) for 24 hours leads to a dose dependent increase in IFN- α , IL-6 and TNF- α . **A** – IFN- α ; **B** – IL-6; **C**- TNF- α . Control cells treated with DMSO (0 – 10 % v/v). Peak concentrations of cytokines seen at the following concentrations of Imiquimod – IFN- α (3 μM), IL-6 (30 μM) and TNF- α (3 μM), $p < 0.05$; $n = 3$. Figure representative of the means of three independent experiments from three different donors. Error bars represent standard deviations. $*=p<0.05$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Detection limit = 40 pg/ml.

6.3.5 Stimulation of pDC on Administration of Imiquimod and Tumour-associated Antigen

Having seen an increase in cell maturation and the generation of a pro-inflammatory cytokine milieu, it is now important to assess the effects of Imiquimod, in conjunction with a tumour associated antigen, on pDC. In Chapter 5, there was a demonstration of the release of the tumour associated antigen MUC1 on treatment with Camptothecin. As mentioned previously, the increase in available MUC1 should increase the possibility for antigen presentation, both at the tumour site (23,24) and at distal draining lymph nodes (1,11,128).

With readily available antigen, the role of Imiquimod becomes critical in not only reducing the suppressive effect of Treg and potentiating CD8⁺ T cell proliferation and activation, but by inducing cross-presentation within pDC. In order to replicate this effect, pDC were treated with a combination of Imiquimod and an epitope derived from MUC1. The rationale for using an epitope, rather than the native protein was due to the cost involved in screening for multiple antigenic epitopes. The epitope used (LLLLTVLTV) was one of two sequences first discovered by Brossart *et al.* (129) who, using the PAP program, identified peptides from MUC1 which had a high probability of being presented by HLA-A2.

Figure 6.12 illustrates the expression of the pDC maturation markers CD80, CD83 and CD86 when treated with 3 μ M Imiquimod and a concentration range of (0 – 100 μ g/ml) MUC1 peptide. The first observation to be made is that, on treatment with peptide only, there is a dose-dependent change in the expression of CD83 and CD86 but not CD80, suggesting there is uptake of exogenous antigen. Maximal expression of CD83 (Figure 6.12B; Peptide; 0

vs 20 vs. **40** vs 60 vs. 80 vs. 100 µg/ml; (CD83%) – 30.5 vs. 33.43 vs. **36.30** vs. 25.30 vs. 16.62 vs. 13.26, $n = 3$; $p < 0.05$) and CD86 (Figure 6.12C; Peptide; 0 vs 20 vs. **40** vs 60 vs. 80 vs. 100 µg/ml; (CD86%) – 31.17 vs. 31.40 vs. **46.33** vs. 38.10 vs. 36.23 vs. 34.30, $n = 3$; $p < 0.05$) was observed at a peptide concentration of 40 µg/ml. Interestingly, as peptide concentration increased, the expression of CD80, CD83 and CD86 decreased. This could possibly be due to the development of immunological tolerance (130). Too much antigen can lead to quiescent activation of antigen presenting cells, leading to the induction of tolerant mechanisms, such as the induction of Treg (31,131-133).

Co-administration of 3 µM Imiquimod saw an increase in the expression of CD83 and CD86. No change was seen in CD80 expression ($p > 0.05$). Treatment with Imiquimod did not alter the peak concentration of peptide required to provide maximal expression on CD83 (Figure 6.12B; Peptide + 3 µM Imiquimod; 0 vs 20 vs. **40** vs 60 vs. 80 vs. 100 µg/ml; (CD83 %) – 22.93 vs. 43.90 vs. **50.47** vs. 36.33 vs. 25.67 vs. 18.63, $n = 3$; $p < 0.05$) and CD86 (Figure 6.12C; Peptide+ 3 µM Imiquimod; 0 vs 20 vs. **40** vs 60 vs. 80 vs. 100 µg/ml; (CD86 %) – 33.10 vs. 51.22 vs. **60.47** vs. 52.80 vs. 47.13 vs. 46.47, $n = 3$; $p < 0.05$). Despite the significant elevated expression of these markers on peptide-pulsed pDC with Imiquimod, the antigen tolerance effect, mentioned above, still exists, albeit to a lesser extent. These results suggests that co-stimulation of pDC with Imiquimod, when pulsed with antigen, can increase cell maturity and activation markers. The lack of CD80 upregulation is not of major concern as work by Elloso and Scott (134), demonstrated that costimulation involving CD80 or CD86 can result in the production of either Th1 or Th2 cytokines, rather than a preferential induction of one type of response.

The supernatant of peptide-pulsed pDC, co-stimulated with Imiquimod was analysed for their production of IFN- α , IL-6 and TNF- α via ELISA as illustrated in Figure 6.13. In correlation with Figure 6.12, peptide-pulsed pDC, demonstrated a dose dependent increase in IFN- α , IL-6 and TNF- α (Figure 6.13; 20 – 100 $\mu\text{g/ml}$, $n = 3$, $p < 0.05$). Peak secretion of IFN- α (Figure 6.13A; 20 vs. **40** vs. 60 vs. 80 vs. 100 $\mu\text{g/ml}$; (IFN- α ng/ml) – 0.29 vs. **0.33** vs. 0.36 vs. 0.18 vs. 0.04, $n = 3$), IL-6 (Figure 6.13B; 20 vs. **40** vs. 60 vs. 80 vs. 100 $\mu\text{g/ml}$; (IL-6 ng/ml) – 2.6 vs. **3.03** vs. 1.72 vs. 1.27 vs. 1.17, $n = 3$) and TNF- α (Figure 6.13C; 20 vs. **40** vs. 60 vs. 80 vs. 100 $\mu\text{g/ml}$; (TNF- α ng/ml) – 0.55 vs. **0.77** vs. 0.43 vs. 0.34 vs. 0.25, $n = 3$) at 40 $\mu\text{g/ml}$ of peptide.

As before, 3 μM Imiquimod enhanced the production of these chemokines. Peak secretion of IFN- α (Figure 6.13A; 20 vs. **40** vs. 60 vs. 80 vs. 100 $\mu\text{g/ml}$; (IFN- α ng/ml) – 0.71 vs. **0.80** vs. 0.74 vs. 0.25 vs. 0.14, $n = 3$), IL-6 (Figure 6.13B; **20** vs. 40 vs. 60 vs. 80 vs. 100 $\mu\text{g/ml}$; (IL-6 ng/ml) – **9.27** vs. 8.99 vs. 7.06 vs. 6.12 vs. 3.39, $n = 3$) and TNF- α (Figure 6.13C; 20 vs. **40** vs. 60 vs. 80 vs. 100 $\mu\text{g/ml}$; (TNF- α ng/ml) – 1.25 vs. **1.43** vs. 0.89 vs. 0.64 vs. 0.41, $n = 3$) was demonstrated 20 $\mu\text{g/ml}$ for IL-6 and 40 $\mu\text{g/ml}$ for IFN- α and TNF- α .

These results are suggestive of pDC maturation and pro-inflammatory cytokine production when pulsed with antigen and Imiquimod. It could also be suggested that, contrary to some literature, the endocytic activity of pDC does exist (45-48), which improves upon co-stimulation with Imiquimod. Further work would need to be carried out on the degree of maturation that pDC undergo when treated with TLR7 agonists and to quantify their ability to endocytose antigen and present it efficiently.

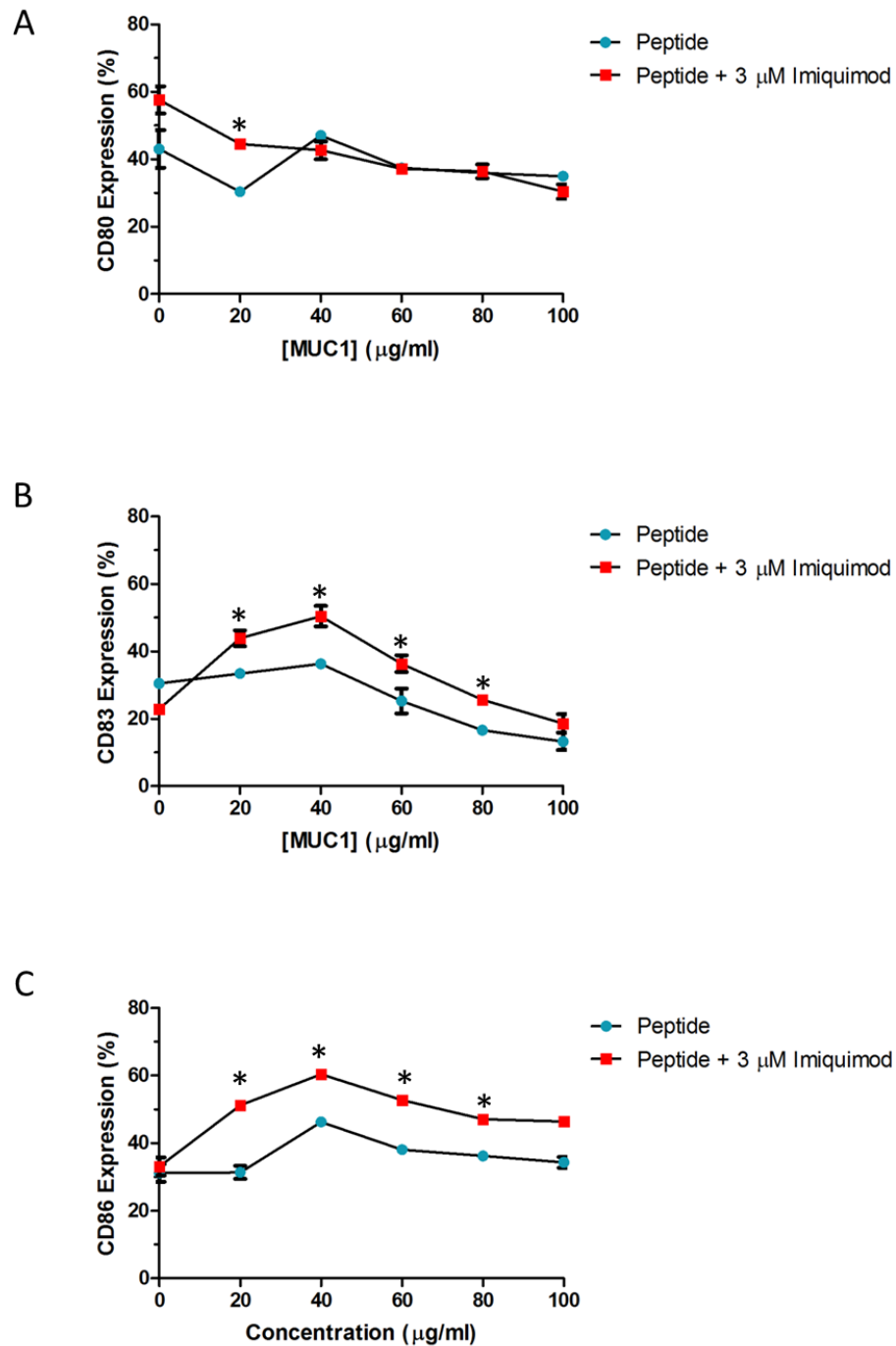


Figure 6.12 – Imiquimod Increases pDC Maturation Marker Expression when Co-administered with MUC1 Peptide. Expression of **A** - CD80, **B** - CD83 and **C** - CD86 is increased in pDC when stimulated with 3 μM Imiquimod and varying concentrations of MUC1 peptide (0 – 100 μg/ml) for 24 hours. Peak expression of CD80, CD83 and CD86 was seen at 40 μg/ml MUC1 peptide and 3 μM Imiquimod, $p < 0.05$; $n = 3$. Figure representative of the means of three independent experiments from three different donors. Error bars represent standard deviations. *= $p < 0.05$. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.

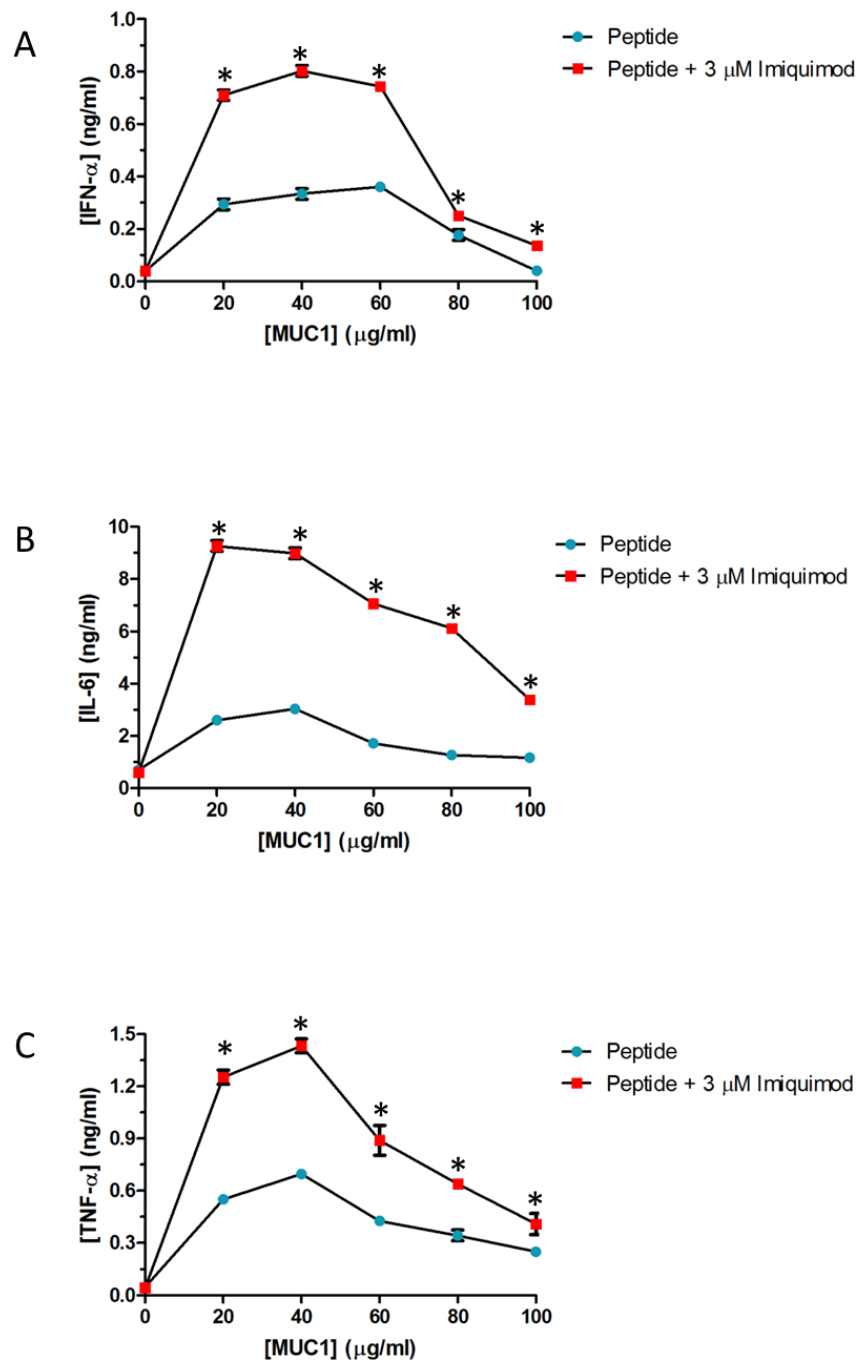


Figure 6.13 – Imiquimod Increases pDC Cytokine Production when Co-administered with MUC1 Peptide. Combined treatment of pDC with 3 μM Imiquimod and varying concentrations of MUC1 peptide (0 – 100 μg/ml) for 24 hours leads to a dose dependent increase in **A** - IFN-α, **B** - IL-6 and **C** - TNF-α. Peak concentrations of cytokines were seen at at 40 μg/ml MUC1 peptide and 3 μM Imiquimod. Figure representative of the means three independent experiments from the same donor. Error bars represent standard deviations. *=p<0.05. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test.. Detection limit = 40 pg/ml

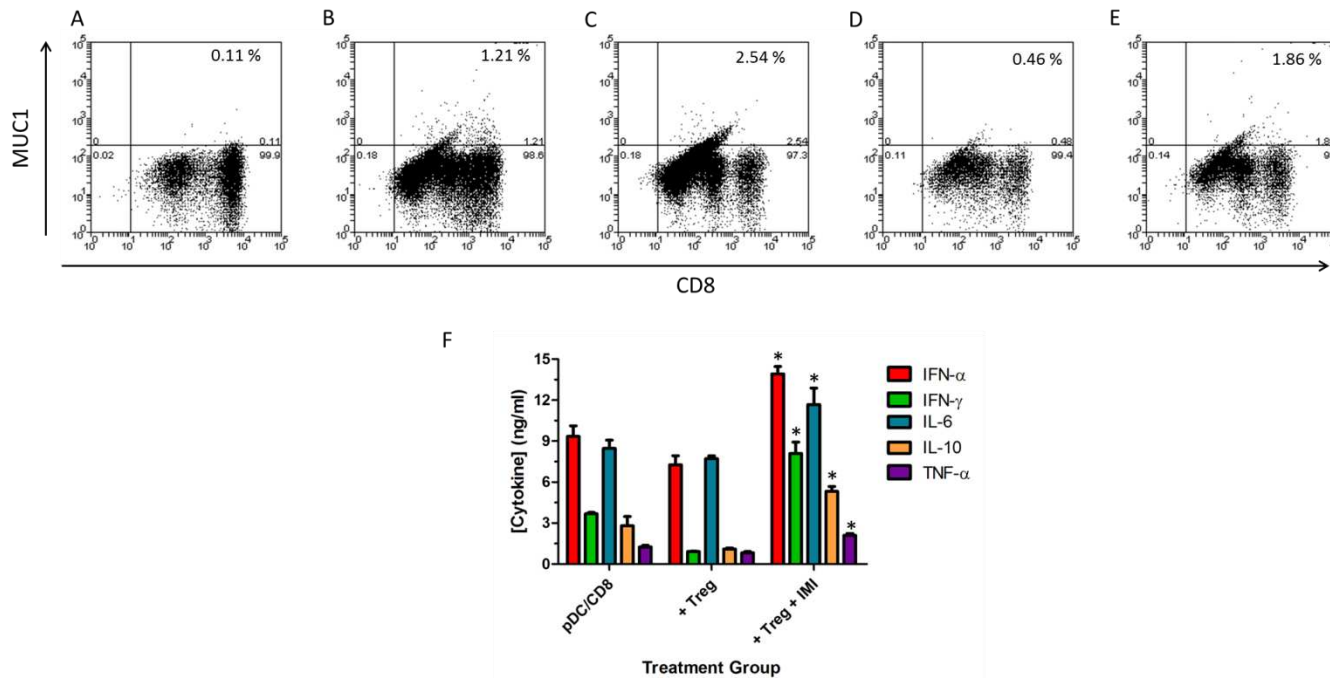


Figure 6.14 – Imiquimod Facilitates the Generation of MUC1⁺ CD8 T cells. pDC, CD8 and Treg were co-cultured for 7 days. FACS plots of CD8⁺MUC1⁺ cells, **A** – Negative control, 0 μ g/ml MUC1 peptide, **B** – 40 μ g/ml MUC1 peptide **C** – 40 μ g/ml MUC1 peptide plus 3 μ M Imiquimod, **D** - 40 μ g/ml MUC1 peptide plus Treg **E** - 40 μ g/ml MUC1 peptide plus Treg with 3 μ M Imiquimod. Addition of 3 μ M Imiquimod caused an increase in the number of CD8⁺MUC1⁺ T cells. **F** – Cytokine output of CD8⁺MUC1⁺ T cell generation. Increases in IFN- α , IFN- γ , IL-6, IL-10 and TNF- α were seen on addition of 3 μ M Imiquimod. Figure representative of the mean of one experiment run in triplicate. Error bars represent standard deviations. *= p <0.05. Statistical significance determined by a one-way ANOVA analysis with column comparisons using Tukey's Multiple Comparison Test. Detection limit = 40 pg/ml

6.3.6 Generation of CD8⁺MUC1⁺ T cells

With limited time and reagents, and having demonstrated the role Imiquimod could have in mediating CD8⁺ T cell proliferation as well as maturation of pDC, a small scale MUC1⁺ T cell generation assay was assembled. Figures 6.14A-E illustrates the percentage of CD8⁺MUC1⁺ T cells as identified using a MUC1-specific pentamer while Figure 6.14F depicts the corresponding cytokine output. The first observation to be made is from Figure 6.14B, where peptide-pulsed pDC and CD8⁺ T cells were co-cultured. 1.21 +/- 0.6 % of CD8⁺ T cells became positive for MUC1⁺ which suggests that pDC have some intrinsic cross-presentation activity. This effect was enhanced on the addition of 3 μM Imiquimod (Figure 6.14C), with 2.54 +/- 0.3 % of cells being CD8⁺MUC1⁺ T cells.

On addition of Treg to the co-culture system, the percentage of MUC1⁺ cells fell to 0.46 +/- 0.2 % (Figure 6.14D), indicating that Treg had inhibited CD8⁺ T cell priming (135). However, on the re-introduction of Imiquimod to the system, the fraction of antigen-specific CD8⁺ T cells increased to 1.86 %.

These CD8⁺MUC1⁺ T cells were sorted with the intention of using them in a cytotoxic T cell assay (136). However, when sorting, only 112 positive cells were collected. These were subsequently cultured under T cell expansion conditions as described in the methods section above. At the time of writing, these cells had now reached a total cell number of 4.8×10^4 , which was not enough for use in an assay.

The cytokine profiles of these assays demonstrated a similar trend. Treg significantly reduced the production of IFN-α (pDC/CD8 vs. **Treg** (ng/ml) – 9.35 vs. **7.26**, $n = 3$, $p < 0.05$), IFN-γ (pDC/CD8 vs. **Treg** (ng/ml) – 3.68 vs. **0.91**, $n = 3$, $p < 0.05$), IL-6 (pDC/CD8 vs. **Treg** (ng/ml) – 8.47 vs. **7.72**, $n = 3$,

$p < 0.05$), IL-10, (pDC/CD8 vs. **Treg** (ng/ml) – 2.81 vs. **1.11**, $n = 3$, $p < 0.05$) and TNF- α , (pDC/CD8 vs. **Treg** (ng/ml) – 1.26 vs. **0.83**, $n = 3$, $p < 0.05$).

Yet on addition of Imiquimod, there was a partial reversal of this effect IFN- α (Figure 6.14F; Treg vs. **Treg + 3 μ M Imiquimod** (ng/ml) – 7.26 vs. **13.92**, $n = 3$, $p < 0.05$), IFN- γ (Figure 6.14F; Treg vs. **Treg + 3 μ M Imiquimod** (ng/ml) – 0.91 vs. **8.09**, $n = 3$, $p < 0.05$), IL-6 (Figure 6.14F; Treg vs. **Treg + 3 μ M Imiquimod** (ng/ml) – 7.72 vs. **11.68**, $n = 3$, $p < 0.05$), IL-10, (Figure 6.14F; Treg vs. **Treg + 3 μ M Imiquimod** (ng/ml) – 1.11 vs. **5.31**, $n = 3$, $p < 0.05$) and TNF- α (Figure 6.14F; Treg vs. **Treg + 3 μ M Imiquimod** (ng/ml) – 0.83 vs. **2.10**, $n = 3$, $p < 0.05$). An interesting observation from Figure 6.14F was noted in that on treating the CD8/pDC/Treg co-culture there was a significant increase in all the cytokines measured. A possible explanation for this could be inferred from work carried out Sharma *et al.* (137) who described the use of a TLR9 ligand to reduce the suppressive effects of Treg. The key observation was that these newly ‘non-suppressive’ Treg acted like conventional T helper cells and improved the generation of antigen specific CD8⁺ T cells.

These effects could be attributed to the evidence provided earlier in this chapter. TLR7 agonism lead to a decrease in the suppressive effects of Treg and an increase in CD8⁺ T cell activation and proliferation, pDC maturation and pro-inflammatory cytokine secretion, there is an increased possibility of aiding antigen specific CD8⁺ T cell generation. However, as this experiment was only executed once it is difficult to make any meaningful conclusions from it.

6.4 Conclusions

This chapter sought to take advantage of MUC1 antigen release which would occur under the treatment of ovarian tumours with JPM137. Although successful release of antigen has been demonstrated, it is not adequate to assume that efficient MUC1-specific T cell priming will occur. Therefore, a method of potentiating the immune system such that antigen presentation, cytotoxic T cell proliferation and increasing the presence of pro-inflammatory cytokines was pursued.

The expression of TLR7 on a variety of immune cell populations; including pDC and CD8⁺ T cells meant that this became an attractive target for use as part of the immunotherapeutic strategy being developed in this thesis. Evidence regarding the expression of TLR7 on Treg was conflicting; however flow cytometry demonstrated a clear expression of the receptor. A TLR7 agonist, Imiquimod, was selected and a subsequent investigation into its effects on the aforementioned cell populations began. The suppressive effect of Treg on both CD4⁺CD25⁻ and CD8⁺ T cells were partially reversed with TLR7 engagement. Further investigation into the mechanism of how Imiquimod was acting suggested that an increase in CD8⁺ T cell proliferation combined with an altered cytokine profile; which had a greater pro-inflammatory element, were both major contributors. Additional work on the change in FOXP3 (88,138) and CTLA-4 (139) expression would add greater credence to the potential of TLR7-mediated abrogation of Treg function.

The effect of Imiquimod was not just restricted to T cells. pDC are a subset of antigen presenting cells that are known for the Type I interferon secretion and poor antigen endocytosis and cross-presentation. Their prevalence and contribution to the immune suppressive milieu within ovarian tumours has

been recently documented (32), making them an interesting target for therapeutic innervation. Treatment with Imiquimod induced pDC activation, maturation and the secretion of pro-inflammatory cytokines such as IFN- α , IL-6 and TNF- α . On the addition of Imiquimod, pDC pulsed with a MUC1 peptide, demonstrated a further increase in activation, maturation and cytokine release. Interestingly, an increase in pDC activation and cytokine release were seen on the addition of antigen only, suggesting that these cells do have the ability to present exogenous antigen, albeit in small amounts (140).

An attempt was then made to combine the above observations into an assay to generate MUC1⁺ cytotoxic T cells. Imiquimod slightly increased the fraction of MUC1⁺CD8⁺ T cells and was able to partially reverse the suppressive effect of Treg on cytotoxic T cell generation.

The above results suggest that Imiquimod would be of use in enhancing an anti-tumour response. The potential of TLR7 agonists to abrogate Treg function, as well as boost both pDC and CD8⁺ T cell activation would be advantageous in utilising the antigen which has become readily available on targeted tumour cell death.

6.5 References

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Chapter 7

General Conclusions and Future Work

The idea that the immune system can affect either cancer development or clearance has been the subject of debate for over a century. In the early 1900s, Paul Ehrlich was perhaps the first to reason that cancer would be quite common in long-lived organisms if not for the protective effects of immunity. It is now recognised that the immune system plays at least two distinct roles in cancer: elimination or escape.

In the elimination phase, innate and adaptive immunity work together to destroy developing tumours long before they become clinically apparent. Many of the immune molecules and cells that participate in the elimination phase, such as CD8⁺ T cells and Natural Killer cells have been identified. If this phase is successful, then the host remains free of cancer. However, a common occurrence is the avoidance of some cancer cells to immune-mediated cytotoxicity. These cells are no longer recognised by the immune system through i) loss of antigen expression and presentation, (ii) become insensitive to immune effector mechanisms (through the increased fraction of Treg or other immune suppressive cells in the tumour-immune infiltrate, or (iii) induce an immunosuppressive state within the tumour microenvironment (through upregulation of immune suppressive mechanisms such as Indoleamine-2,3-dioxygenase and Adenosine). This thesis explores, in principle, a strategy to propagate an anti-ovarian cancer immune response by targeting three different facets of ovarian cancer immunity; Regulatory T cell

(Treg) migration, poor release of the tumour-associated antigen, MUC1 and reduced cytotoxic T cell (CTL) proliferation.

Before embarking on the development of this strategy, studies were conducted to develop a suitable method by which to characterise Treg both functionally and phenotypically. Although there is still much debate surrounding a truly reliable human Treg phenotype it was decided that CD4⁺CD25⁺FOXP3⁺ cells were to be used in all experiments on the basis that these were commonly used markers within the literature. On isolation of Treg from whole blood using magnetic beads, it became clear that an expansion protocol needed to be devised in order to generate large populations of cells for downstream experiments. After comparing the cost/purity benefits of different stimuli used in expansion it was decided that an α CD28 superagonist would be used alongside high concentrations of IL-2. This allowed breakage of the anergic phenotype of Treg and thus provided suitably pure and large populations of cells. Expanded Treg maintained their phenotype and suppressive properties.

Treg demonstrated dose dependent suppression of both CD4⁺CD25⁻ and CD8⁺ T cells which is an indicator for the impairment of an anti-tumour immune response. Interestingly, the cytokine milieu generated by Treg suppression can be substantially altered, depending on the stimuli added. Mitogenic stimulation lead to the production of IFN- γ and IL-10 from Treg whereas TCR engagement with α CD3/ α CD28 does not. This could be attributed to the altered proliferative pathways that exist within Treg. It also indicated that induction of certain molecular pathways, in particular the NF- κ B pathway could offer a way to alter Treg function.

Dose-dependent Treg-mediated suppression suggested that their reduced presence led to greater proliferation of effector populations. This observation, together with those found in literature suggests that strategies to either manipulate Treg function or decrease the number of Treg within the tumour microenvironment will be of clinical benefit (1).

The evidence of increased Treg within tumour immune infiltrates being associated as a poor prognostic indicator gave credence to the theory that restricting Treg migration to the tumour site could help reduce the immune suppressive milieu. The clinical significance of increased Treg infiltration in progressively poorly prognostic ovarian cancer has been, in part, attributed to the chemokine-chemokine receptor axis CCL17/CCL22-CCR4. The expression of CCR4 and its specific chemokines has also been identified as a means for certain tumour types to undergo migration and metastatic seeding at distant sites. This evidence suggests that this method of cell 'recruitment' may be a target for therapeutic intervention.

Based on current literature and the data in this thesis, CCL17 and CCL22 are both implicated in Treg migration. The propensity of ovarian cancer cells to produce CCL17 and CCL22 in response to pro-inflammatory stimuli was also observed. This suggests that the immunological time course of events within the solid tumour microenvironment starts with a pro-inflammatory period, i.e. an anti-tumour response, followed by an ensuing regulatory, or anti-inflammatory, response. The release of CCL17 and CCL22 into the microenvironment could be considered part of that anti-inflammatory response; inducing the migration of Treg to dampen any local pro-inflammatory event.

The use of AZ1, a specific CCR4 antagonist demonstrated efficacy in reducing both Treg and ovarian cancer cell migration to both chemokines. With no change in cell viability or the suppressive function of Treg, CCR4 became the first target of the proposed treatment stratagem. The employment of AZ1 would be as an adjuvant. Evidence suggests that as the disease progresses there is an increase in Treg infiltration (2,3). This means that abrogating the chemotactic function of CCL17 and CCL22 would reduce Treg infiltrate over time as the disease progressed.

The reduction in the infiltrating capacity of Treg would help in the propagation of an anti-tumour immune response. However, in order to potentiate this effect, it was theorised that an alteration in the suppressive capacity of Treg as well as an improvement the release and presentation of tumour-associated antigen would be necessary to gain real clinical benefit.

To explore the facilitation of adequate antigen expression, the cytotoxic Camptothecin was used. *In vitro*, the treatment of ovarian cancer cells with this drug led to the release of the tumour-associated antigen MUC1. This antigen has been shown to be highly expressed in ovarian cancer and thus its release into the extracellular environment could be advantageous in attempting to generate an anti-ovarian tumour immune response. However, it must be noted that a saturation of immune populations with antigen can lead to antigen-specific tolerance. Clinically, despite a cytoreduction in tumour burden and the increase in MUC1 availability, the lymphodepletive effect of Camptothecin would mean that generating a potent anti-tumour immune response would be difficult. This was highlighted by the significant lack in PBMC proliferation but also in terms of a reduction in CD8⁺ T cell.

Interestingly, and in concordance with current literature, low doses of Camptothecin reduced the number of Treg from PBMC.

In order to improve tumour targeting and thus reduce peripheral lymphodepletion, JPM137 was created. This novel drug delivery system, encapsulated Camptothecin within a hydrophilic polymer to improve its solubility and pharmacokinetic profile. The addition of the Leuteinizing-hormone releasing hormone ligand (LHRH) gave the construct ovarian tumour specific targeting properties. JPM137 demonstrated similar but not superior cytotoxic performance compared to Camptothecin while maintaining CD8⁺ T cell populations and reducing Treg. Critically, JPM137 would have an effect at the tumour site, depleting intra-tumoural Treg while maintaining CD8⁺ T cells whereas Camptothecin would cause peripheral, as well as localised immune suppression which would not be as advantageous or clinically beneficial.

The release of MUC1 and subsequent maintenance of immune cell viability meant that there is a significant opportunity to induce an antigen specific anti-tumour immune response. Due to a variety of immune suppressive mechanisms it is not adequate to assume that efficient MUC1-specific T cell priming will occur. Therefore a method of potentiating the immune system such that antigen presentation, cytotoxic T cell proliferation and the presence of pro-inflammatory cytokines increased was explained.

The expression of Toll-like receptor 7 on a variety of immune cell populations; including pDC and CD8⁺ T cells meant that this became an attractive target for use as part of the immunotherapeutic strategy being developed in this thesis. Evidence regarding the expression of TLR7 on Treg was conflictive, at best; however flow cytometry demonstrated a clear expression of the

receptor. By using a TLR7 agonist, Imiquimod, the suppressive effect of Treg on both CD4⁺CD25⁻ and CD8⁺ T cells was partially reversed with TLR7 engagement. Further investigation into where and how Imiquimod was acting suggested that an increase in CD8⁺ T cell proliferation combined with an altered cytokine profile; which had a greater pro-inflammatory element, were both major contributors.

The effect of Imiquimod was not just restricted to T cells. Plasmacytoid dendritic cells are a subset of antigen presenting cells that are known for their Type I interferon secretion and poor antigen endocytosis and cross-presentation. Their prevalence and contribution to the immune suppressive milieu within ovarian tumours has been recently documented (9), making them an interesting target for therapeutic innervation. Treatment with Imiquimod induced pDC activation, maturation and the secretion of pro-inflammatory cytokines such as IFN- α , IL-6 and TNF- α . On the addition of Imiquimod, pDC pulsed with a MUC1 peptide, demonstrated a further increase in activation, maturation and cytokine release. Interestingly, an increase in pDC activation and cytokine release were seen on the addition of antigen only, suggesting that these cells do have the ability to present exogenous antigen, albeit in small amounts (10).

An attempt was then made to combine the above observations into a model assay with which to generate MUC1⁺ cytotoxic T cells. Imiquimod slightly increased the fraction of cytotoxic T cells and was able to partially reverse the suppressive effect of Treg on cytotoxic T cell generation, although the cytotoxic effect of these MUC1⁺T cells was not evaluated.

To improve the potential of this treatment strategy, key alterations can be made. The TLR7 agonist, Imiquimod could be replaced with its daughter

compound, Resiquimod. Resiquimod has been shown to have activity against both TLR7 and TLR8 and is reportedly fifty times more potent than Imiquimod (11). This should further reduce the suppressive effect of Treg as well increase antigen processing and CD8⁺ T cell proliferation. As mentioned in Chapter 5, the performance of JPM137 could be improved by modifying the presence of LHRH on the constructs surface. This would allow for greater accumulation within the ovarian tumour site and thus improve cytotoxicity. The choice of cytotoxic could also be altered. Camptothecin was picked due to itself relative inexpensive cost and for the evidence regarding drug encapsulation into hydrophilic polymers. Current treatment regimens for ovarian cancer include Paclitaxel and Cisplatin (12) and it is not unreasonable to suggest that a similar approach could be taken with these drugs.

In order to evaluate the proposed strategy more effectively, *in vivo* work is essential. In recent years, the development of syngeneic mouse models have provided a greater testing bed for chemo- and immuno-therapeutic strategies (13). However, one of the major issues with assessing the use of these models would be the effect of TLR7 agonists on Treg. As mentioned previously TLR7 engagement in mice leads to an increase in Treg suppressive activity (14). Standard human tumour xenografts such as those used by Dharap *et al.*(15) would be suitable for evaluating the cytotoxic effect of JPM137 as well determining the extent to which MUC1 is released peripherally on tumour cell death. The most suitable model would be an engraftment of HLA matched immune cells and tumour cells in an nude-mouse (16), however their reliability and cost would make this difficult to use.

Overall, this thesis has highlighted the potential importance of polypharmacy in treating ovarian cancer. The treatment stratagem employed herewith, has

demonstrated a reduction in the immune suppressive behaviour of Treg as well as its chemotaxis and reduction in tumour cell migration. The combination of chemo- and immunotherapy aides tumour debulking, release of the tumour-associated antigen MUC1 and the subsequent presentation and potential durable priming of antigen specific-cytotoxic T cells (Figure 7.1).

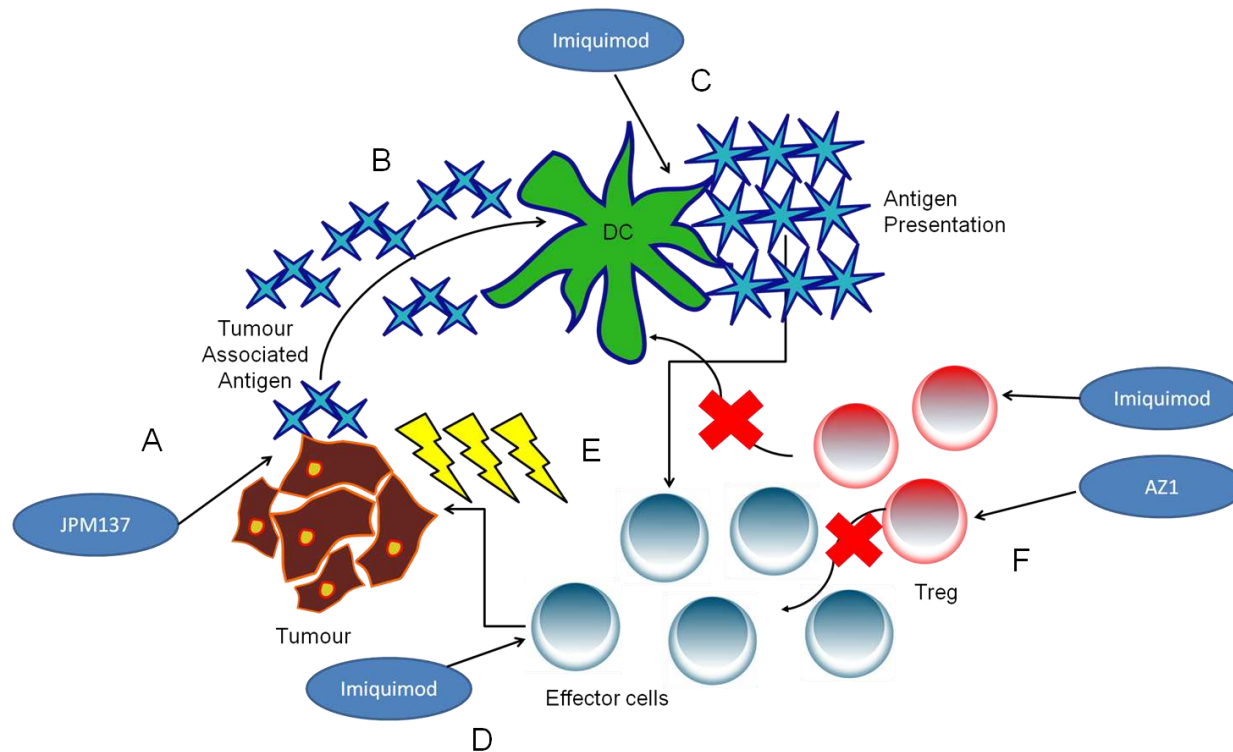


Figure 7.1 – The Effect of JPM137, Imiquimod and AZ1 on Tumour Immunity. **A** – JPM137 localises at the tumour site and cause cytolysis of the tumour. **B**- Tumour cell death leads to the release of tumour associated antigen. **C** – Imiquimod activates dendritic cells at tumour draining- and peripheral- lymph nodes causing antigen uptake, improved antigen presentation and generation of antigen specific cytotoxic T cells. **D** – Imiquimod induces the proliferation of CD8⁺ T cells and improves the production of pro-inflammatory cytokines (**E**). **F** – Imiquimod reduces the suppressive effects of Treg while AZ1 blocks their migration to the tumour site (denoted by red crosses). The net result is reduced tumour burden and a durable anti-tumour immune response.

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