Elucidating the Impact of $CD4^+$ T cells on Tumour Progression in Patients with Colorectal Cancer

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Summary

In recent years, substantial evidence has been generated demonstrating the importance of the immune system in preventing and controlling the growth of many cancers, including colorectal adenocarcinomas. In particular, populations of tumour-specific effector T cells appear to play a crucial role in restricting the generation and expansion of transformed neoplastic cells. However, the fact that tumours continue to grow in the presence of a seemingly intact immune system suggests that these responses are often inadequate.

$CD4^+Foxp3^+$ T regulatory cells (Tregs) have been shown to play a key role in modulating the immune system by keeping immune responses to self-antigens in check, thereby preventing autoimmunity. These cells also appear to be employed by tumours to protect against recognition and eradication, and have been demonstrated to impinge upon the anti-tumour immune response in humans. Furthermore, it appears that the tumour microenvironment facilitates the development of highly immunosuppressive T cells, which may also allow subsequent tumour progression.

In colorectal cancer, the relationship between Tregs and tumour progression is less clear – despite their well-documented ability to impinge on anti-tumour immune responses, increased tumour infiltrates have also been associated with prolonged survival. In this thesis, the phenotype and function of $CD4^+$ T cells derived from PBMC, colon and tumour samples were analysed for suppressive markers by FACS, and anti-tumour responses by IFN-γ ELISpot. CRC patients with more advanced tumours responded to fewer epitopes and generated a significantly weaker epitope-specific T cell response to the oncofoetal antigen, 5T4 than healthy donors. Human depletion experiments both *in vitro* and *in vivo* indicated suppression by $Foxp3^+$ regulatory $CD4^+$ T cells. These cells were found in abundance amongst tumour-infiltrating lymphocytes; however, another equally prominent population of IL-10 and TGF-β-producing $CD4^+Foxp3^-$ T cells were found to be >50-fold more suppressive. Thus, a major caveat to cancer immunotherapy is the suppressive tumour microenvironment, which contributes to the selective decline of measurable anti-tumour $CD4^+$ T cell responses as tumours progress. These responses were enhanced in metastatic CRC patients by depleting regulatory T cells.

It is hoped such findings will augment our understanding of how anti-tumour $CD4^+$ T cells are activated and conversely regulated, with the intention of designing better treatment for patients with colorectal cancer.

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List of Abbreviations

Ab	Antibody
APC	Allophycocyanin
APCs	Antigen presenting cells
BCL-2	B-cell lymphoma 2
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
Ci	Curie
CK	CellKine
CRC	Colorectal cancer
CTLA-4	Cytotoxic T-lymphocyte antigen 4
COX	Cyclooxygenase
DC	Dendritic cell
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme linked immunospot
FACS	Fluorescence activated cell sorting
FAP	Familial adenomatous polyposis
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
GARP	Glycoprotein A repetitions predominant
GITR	Glucocorticoid induced tumour necrosis factor receptor
HA	Haemagglutinin
HD	Healthy donor
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICOS	TCR-inducible costimulatory molecule
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IL	Interleukin
IMDM	Iscoves' modified Dulbecco's medium
IPEX	Immunodeficiency, polyendocrinopathy and enteropathy X-linked syndrome
LAG-3	Lymphocyte activation gene-3
LAP	Latency associated peptide
LN	Lymph node
MACS	Magnetic-activated cell sorting
MART-1	Melanoma antigen recognized by T cells-1
MCA	3-methylcholanthrene
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MVA	Modified vaccinia ankara
NK	Natural killer
NS	Not significant
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PD-1	Programmed death-1
PE	Phycoerythrin
PECy7	Phycoerythrin-Cy7
Pen/Strep	Penicillin / Streptomycin
PerCpCy5.5	Peridinin chlorophyll protein complex-Cy5.5
PHA	Phytohaemagglutinin
PMA	Phorbol myristate acetate
PPD	Tuberculin purified protein derivative
Rag	Recombination activating gene
RPMI	RPMI-1640 media
RT	Room temperature (18-23^{o}C)
SEM	Standard error of the mean
SFC	Spot forming cell
SPICE	Simplified presentation of incredibly complex evaluations
TaCTiCC	TroVax® and cyclophosphamide treatment in colorectal cancer
Tconv	$CD4^{+}Foxp3^{-}$ conventional T cells
TCR	T cell receptor
TDLN	Tumour draining lymph node
T_{FH}	Follicular-helper T cells
TGF-β	Transforming growth factor-beta
Th	T helper
TIL	Tumour infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin domain containing protein-3
TNFα	Tumour necrosis factor-alpha
TNM	Tumour / Nodes / Metastasis
Tr1	Type 1 regulatory T cell
Treg	Regulatory T cell
TT	Tetanus toxoid
VEGF	Vascular endothelial growth factor

Chapter 1 – Introduction

1.1 Tumourigenesis

1.1.1 The Initiation and Establishment of Cancer

The processes underlying the transformation of normal cells into malignant neoplasms involve epigenetic and mutational alterations, leading to unregulated cellular differentiation and proliferation. In the seminal review, 'The Hallmarks of Cancer', Hanahan and Weinberg describe six common principles that govern this transformation (Hanahan and Weinberg 2000). They comprise:

- The ability to sustain chronic proliferation by deregulating the production and release of growth promoting signals;
- Evading growth suppressors, either by circumventing the effect of tumour suppressor genes or by nullifying cell-cell contact inhibition;
- Resisting cell death and becoming resistant to multiple apoptotic mechanisms;
- Unlimited replicative potential by protecting against telomere erosion;
- Inducing angiogenesis via production of vascular endothelial growth factors, and;
- Activating invasion and metastasis by altering expression of cell-cell adhesion molecules.

Whilst these processes must occur for tumours to progress, the cancer cells actively interact with, and are heavily influenced by, their immediate environment comprising many different cell types. Thus, it is the totality of the tumour microenvironment that determines the outcome of tumourigenesis and much research has gone into analyzing the constituents of tumours.

1.1.2 Cancer Immunosurveillance

In 1909, Paul Ehrlich originally hypothesized that the growth of carcinomas could be constrained by cells of the host's immune system (reference in German*). Over the following 50 years, advances in the understanding of cancer immunobiology took a step forward with the development of inbred mouse strains and the successful immunisation of mice against syngeneic tumours (Old and Boyse 1964). This work led F. MacFarlane Burnet to propose the concept of "immunological surveillance". His experimental evidence suggested that "thymus-dependent cells of the body" constantly surveyed host tissues for transformed cells, given that neo-antigens would become upregulated on mouse tumours (Burnet 1964; Burnet 1970).

At the time, such work was considered controversial in the face of evidence that appeared to disprove the immunosurveillance hypothesis. Some of the most convincing research was based on findings in immunocompromised athymic nude mouse, which have far fewer T cells than wild type mice. When these mice were given chemically induced tumours (a fibrosarcoma after 3-methylcholanthrene injection) the latency period before tumour formation and the overall number of tumours were no different to those found in wild type mice (Stutman 1974). In addition, nude mice did not exhibit an increased incidence of spontaneous non-viral tumour formation (Outzen, Custer et al. 1975). This led some investigators to speculate that tumour cells were too similar to the normal cells from which they derived, thus the immune system could not recognize these cells or could become tolerized to them (Matzinger 1994).

Since then, technological advances in mouse genetics and monoclonal antibody production have helped substantiate the theory of cancer

* Ehrlich, P. Über den jetzigen stand der karzinomforschung. *Ned. Tijdschr. Geneeskd.* **5**, 273–290 (1909).

immunosurveillance. Some of the key findings involve the use of mouse strains deficient in recombination-activating gene (Rag-1 / -2), essential for the generation of mature B, T and NKT cells. These mice demonstrate an increased frequency and a broader spectrum of spontaneous tumours over wild type, age-matched littermates (Shankaran, Ikeda et al. 2001). Moreover, tumours that develop in Rag-2 deficient mice are rejected when transplanted into wild-type recipients; however, tumours from wild type mice are not rejected in other wild type or Rag-2 knockout mice. These experiments demonstrate a clear role for the adaptive immune system in recognising and eliminating cancerous cells (outlined in Figure 1.1); yet they also indicate how tumours developing in immune-competent hosts can lose immunogenicity, demonstrating how cancer immunosurveillance represents only one step of a broader immunological process.

1.1.3 Cancer Immunosurveillance in Humans

Whilst evidence for cancer immunosurveillance occurring in mice comes largely from data in immunocompromised animals, so too does evidence of cancer immunosurveillance occurring in humans. Patients receiving transplants are routinely given immunosuppressants to reduce the risk of organ rejection; these recipients have a 3-fold increased risk of cancer relative to the age and sex-matched general population (Vajdic and van Leeuwen 2009). A meta-analysis of the incidence of cancer in people with HIV/AIDS compared with immunosuppressed transplant recipients revealed that the similarity of the pattern of increased risk of cancer in the two populations was suggestive of immune deficiency, rather than other risk factors for cancer (Grulich, van Leeuwen et al. 2007). Whilst many of these cancers, including Kaposi's sarcoma and non-Hodgkin's lymphoma, have a known infectious

cause (usually viral, i.e. human papillomavirus or Epstein-Barr virus), some cancers such as non-melanoma skin cancer and lung cancer are markedly increased without a known cause. One potential explanation arises from the cancer immunosurveillance hypothesis, and the increase in rates of cancer in immunocompromised individuals is a result of reduced recognition or elimination of aberrant cells; certainly a reduced anti-viral immune capacity can result in increased rates of tumourigenesis. Given that there is not a general increase in all cancers, however, questions the cancer immunosurveillance hypothesis.

1.1.4 Cancer Immunoediting

The cancer immunoediting hypothesis describes how host immune responses can both protect against and promote tumour development (Dunn, Old et al. 2004; Schreiber, Old et al. 2011). The process of cancer immunoediting has been defined by three distinct phases: Elimination, representing the classical concept of cancer immunosurveillance; equilibrium, whereby tumour cells are not sufficiently removed and remain in a latent phase; and escape, when tumours have overcome the immunological restraints of the equilibrium phase and growth proceeds unabated. This hypothesis also describes how tumours can arise in seemingly immunocompetent hosts, despite the multitude of immune effector functions in place to protect against carcinogenesis.

During the elimination phase, it is likely that many tumour cells are effectively destroyed by immune cells, however new variants carrying more mutations may also arise. Thus, MCA tumours established in mice lacking either T cells, NKT cells, perforin or IFN-γ, are more immunogenic than those originating in an intact immune

system, since wild-type mice can easily reject tumours transplanted from such mice (Svane, Engel et al. 1996; Smyth, Thia et al. 2000; Street, Cretney et al. 2001). For tumour cells to evade immunosurveillance, certain resistance mechanisms must be acquired to disguise them. This can be achieved through a number of mechanisms. Firstly, tumour cells are known to be capable of altering and losing expression of immunogenic antigens. In one clinical trial, metastatic melanoma patients received adoptively transferred T cells specific for the tumour antigen MART-1; three out of five patients studied exhibited specific loss of MART-1 expression on the tumour cells after first infusion of T cells (Yee, Thompson et al. 2002). Secondly, immunologically sculpted tumour cell variants may overproduce certain cytokines, such as IL-6, IL-10, TGF-β and VEGF (vascular endothelial growth factor), which are capable of inactivating effector immune responses (Cabillic, Bouet-Toussaint et al. 2006), however, this may also represent a mere side-effect of the angiogenic and growth factor functions of these cytokines. In particular, TGF-β has been found to have a critical role both in suppressing immune cell effector functions and activating an epithelial-to-mesenchymal transition, which confers traits associated with high-grade malignancy on cancer cells, e.g. metastatic capability (Xu, Lamouille et al. 2009). Finally, tumour cells can also overcome the cytotoxic effects of immunity by inducing anti-apoptotic effector molecules such as the BCL-2 (B Cell Lymphoma-2) family of proteins (Placzek, Wei et al. 2010). Other mechanisms of tumour immune escape have been reviewed previously (Khong and Restifo 2002).

Transformed cells that do manage to evade elimination next enter a period of equilibrium. According to the immunoediting hypothesis, this is a period of dynamic interaction between the tumour cells and the host immune response, resulting in a dormant phase that may last up to several years in humans before the onset of

clinically apparent symptoms (Aguirre-Ghiso 2007). Evidence supporting this comes from the identification of minimal residual disease in the bone marrow and liver of patients with colorectal cancer, and the fact it can take a number of years for metastatic disease to become clinically apparent (Merrie, Yun et al. 1999). Further experimental evidence to corroborate these findings came from primary tumourigenesis experiments, whereby wild type mice were injected with low doses of MCA (Koebel, Vermi et al. 2007). These mice harboured occult cancer cells for prolonged periods but did not go on to develop palpable tumours, unless given antibodies to deplete T cells and IFN-γ; tumours would then appear at the injection site in half of these mice. Interestingly, both $CD4^+$ and $CD8^+$ T cells, but no innate immune cells, were crucial to the maintenance of the tumour cells in the equilibrium phase. These tumours are highly immunogenic, akin to the unedited MCA-induced sarcomas in $RAG^{-/-}$ mice, indicating that T cells could inhibit the creation of new, less immunogenic, variant tumour cells.

It should also be noted, that some experimental inconsistencies may inadvertently be implicating the adaptive immune response in controlling MCA-induced sarcomas. In the aforementioned studies, the immune-deficient mice were bred locally and control mice were purchased; when the same experiments were performed on knockout and wild-type mice obtained from the same colony, mice lacking IFN-γ expressing cell types or perforin do not show increased susceptibility to MCA. This argues against the idea of immune surveillance of MCA-induced carcinogenesis mediated by T cells (Qin and Blankenstein 2004).

Whilst the equilibrium phase of the immunoediting hypothesis may still provoke controversy, tumour cells must advance and evade immune responses in

order to become clinically identifiable. This process begins throughout the equilibrium phase, where tumour cell variants emerge with mutations providing them with increased resistance to immune attack. Ultimately, enough genetic or epigenetic changes occur in the tumour cells leading to variants that have acquired an ability to evade immune detection and elimination, utilising mechanisms described earlier. However, other factors appear to facilitate this transition, driven by the mobilization of immunosuppressive leukocytes in the tumour microenvironment. It has recently been shown that the transition to the escape phase from a state of equilibrium is mirrored by a phenotypic and functional shift in tumour-infiltrating dendritic cells (Scarlett, Rutkowski et al. 2012). This research utilised an inducible p53-dependent ovarian cancer mouse model to demonstrate that dendritic cells isolated from early stage carcinomas were capable of stimulating anti-tumour immune responses amongst T cells taken from the same tumour, yet late-stage derived dendritic cells heavily suppressed T cell activation and proliferation. Tumours may also promote the suppressive microenvironment by releasing certain cytokines and chemokines that result in the recruitment of immunosuppressive leukocyte populations, such as myeloid-derived suppressor cells and regulatory T cells (Tregs; discussed in more detail later) and help drive the suppression of anti-tumour effector immune responses both within the tumour and systemically (Curiel, Coukos et al. 2004; Frey and Monu 2006; Frey and Monu 2008).

Given such compelling evidence to show the role of host immunity in impeding tumour development, it was perhaps surprising that immune system evasion was omitted from the original 'Hallmarks of Cancer'. In 2011, in acknowledgement to the recent surge in interest, Hanahan and Weinberg updated their paper to include how cancer cells are able to evade recognition and consequent elimination by cells of

the host immune system. However, the authors highlight the dichotomous roles of immune cells, as subsets exist that can both antagonize and enhance tumour progression (Hanahan and Weinberg 2011). This is brought about by the infiltration of immune cells with tumour-promoting effects, and by the fact that tumour cells can induce marked inflammation.

Indeed, chronic inflammation has been shown to promote tumourigenesis and progression by enhancing angiogenesis and tissue invasion. This arises from the secretion of growth factors that sustain proliferative signaling, survival factors that limit cell death, proangiogenic factors and extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis (reviewed in (Grivennikov, Greten et al. 2010)). For example, proinflammatory cytokines such as IL-1β, IL-6 and TNF-α are present in many tumours and associated with tumour progression (Smith, Hobisch et al. 2001; Balkwill 2006; Lewis, Varghese et al. 2006). The exact mechanisms by which these cytokines promote tumour growth remain unclear, however, IL-1β has previously been shown to induce expression of VEGF in colorectal cancer, further facilitating angiogenesis and metastatic growth (Konishi, Miki et al. 2005). Ardent debates have arisen as to whether inflammation enhances or diminishes tumour progression, as a number of reports now indicate that pro-inflammatory tumour-associated macrophages can actually enhance beneficial anti-tumour T cell responses (Ong, Tan et al. 2012). Indeed, the problem seems to arise from the disparity in different tumour locations, since tumour-associated macrophages that infiltrate colorectal and skin carcinomas are associated with a good prognosis, whereas they are associated with a poor prognosis in most other cancers (Yuan, Chen et al. 2008). In addition, IFN-γ production by T cells and ultimate control of tumour burden can still exist within the tumour microenvironment even when tumours

containing high-levels of pro-inflammatory cytokines have become established (Teng, Andrews et al. 2010). Therefore, this work demonstrated that tumour-promoting inflammation and cancer immunosurveillance mechanisms could coexist within the same tumour model.

1.2 T cells

1.2.1 T cell Subsets

The immune system has evolved to protect humans from invading pathogens by recruiting cells of the adaptive immune system, namely B and T cells, that are capable of recognising non-self antigens, generating effector responses to eliminate specific pathogens or pathogen-infected cells, and develop memory to that specific pathogen. As a result, T cells with distinct functions exist to promote different types of immune responses and provide a homeostatic mechanism to regulate the response generated. T cells can therefore be grouped based on these effector functions, such as cytokine profile, and their corresponding molecular phenotype associated with those cells. For example, T cells expressing the TCR co-receptor CD8 are capable of producing cytolytic proteins to kill infected or transformed cells, whilst other T cells expressing CD4 can both help this cytotoxic response, by producing IL-2 and IFN-γ, and dampen excessive inflammation by producing IL-10 and TGF-β. Basic immunological research has focused on definitively identifying specific T cell subsets, since targeting individual subgroups of cells will likely be essential for the development of new treatments, particularly in the setting of cancer immunotherapy.

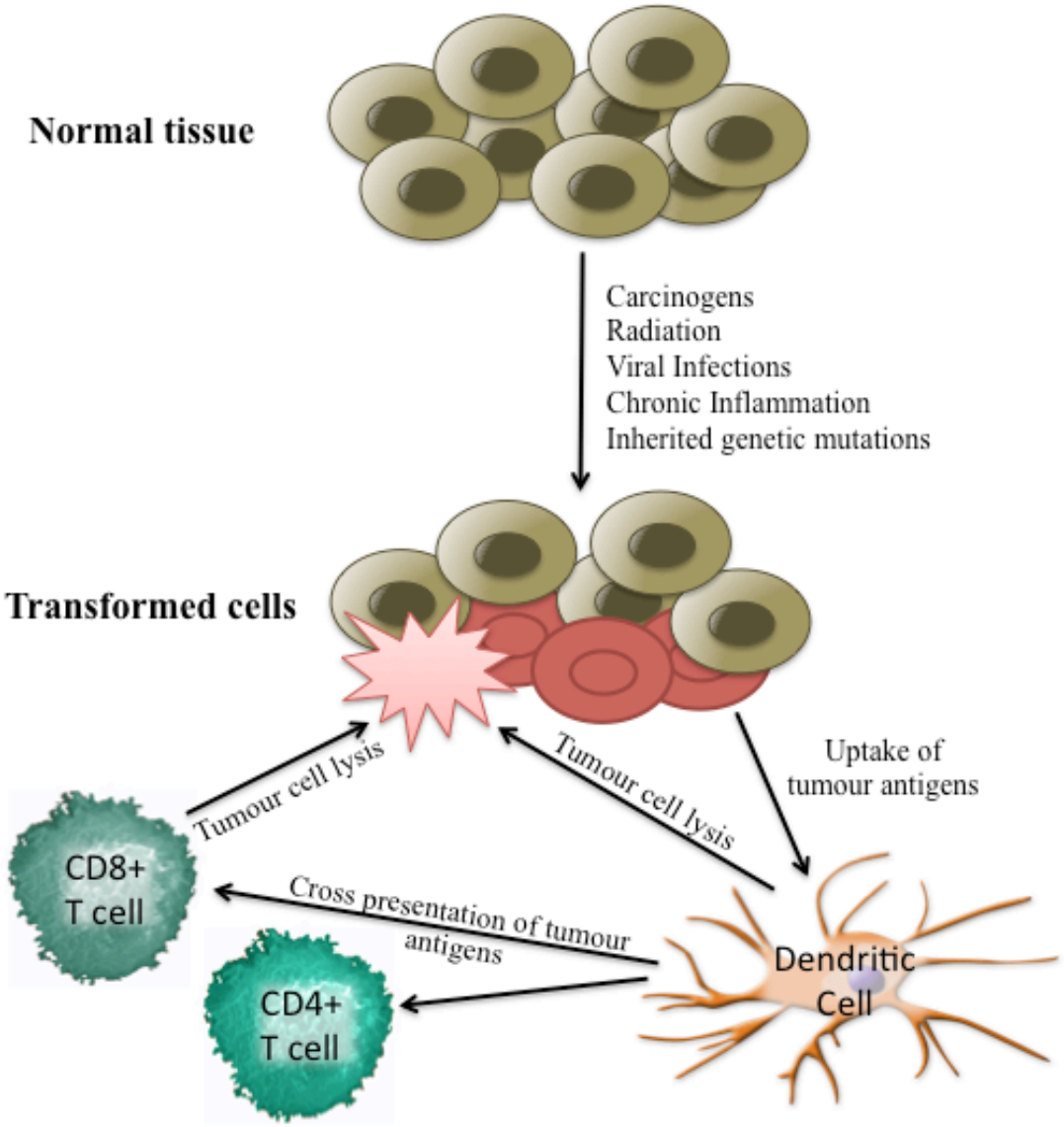


Figure 1.1 Cancer Immunosurveillance. Neoplastic cells, which become transformed by one of the methods indicated, can be recognised and killed by effector mechanisms of the immune system, mediated by the presentation of tumour antigens to T cells. However, cancer cells can become immunologically sculpted over time to avoid rejection, upregulate suppressive molecules that drive growth, and eventually proliferate to clinically identifiable tumours even in the presence of tumour infiltrating lymphocytes.

1.2.2 Regulatory T cells (Tregs)

Although evidence to show that some T cell subsets were capable of limiting immune responses has been around for over 40 years (Baker, Stashak et al. 1970), it is only in recent years that interest has intensified. Initial findings from Fiona Powrie's lab in the 1990's suggested that a subset of T cells expressing certain activation / memory cell markers could protect mice from autoimmune disease (Powrie and Mason 1990; Powrie, Leach et al. 1993). One major piece of evidence definitively identifying their existence was the discovery that the IL-2 receptor α-chain, CD25, is significantly upregulated on these suppressive $CD4^+$ T cells (Sakaguchi, Sakaguchi et al. 1995). This article demonstrated that the inoculation of athymic nude mice with spleen and lymph node cell suspensions depleted of $CD25^+$ cells caused severe multi-organ autoimmunity within 2 weeks and even death in 30% of the mice within 3 weeks. Research into these cells quickly accelerated with a number of reports showing that these $CD4^+CD25^+$ regulatory T cells could limit TCR induced expansion and activation of $CD4^+$ and $CD8^+$ T cells *in vitro*, both in mice (Thornton and Shevach 1998) and humans (Ng, Duggan et al. 2001; Stephens, Mottet et al. 2001). Numerous studies have since demonstrated the potent influence of Tregs in suppressing pathologic immune responses in autoimmune diseases, transplantation and more recently, cancer.

1.2.3 Phenotypic Markers of Tregs

For many years, the definitive identification of regulatory T cells was hampered by the fact that upregulation of CD25 also denoted activated T cells with a non-suppressive function (Kmieciak, Gowda et al. 2009). A key advancement was the

discovery that the forkhead / winged helix transcription factor, Foxp3, seemed to play a critical role in the development and function of Tregs (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003). $CD4^+CD25^+$ T cells in wild-type mice exclusively expressed Foxp3, whereas mice that lacked expression of the Foxp3 gene ('scurfy' mice) have a phenotype associated with defective T cell tolerance, including massive lymphoproliferation, diabetes, exfoliative dermatitis, thyroiditis and enteropathy. Such autoimmunity can be reversed by a transgene encoding the wild-type Foxp3 allele (Brunkow, Jeffery et al. 2001). A very similar phenotype is observed in humans suffering from a rare X-linked fatal autoimmune disease, known as IPEX (immune dysregulation, polyendocrinopathy, entereopathy, X-linked) syndrome (Wildin, Ramsdell et al. 2001). Such findings implicate an important role for Foxp3 in allowing T cells to control excessive autoimmunity and maintain immune homeostasis.

In mice, activation of non-Tregs, i.e. $CD4^+CD25^-$ T cells, does not induce Foxp3 expression, and ectopic expression of Foxp3 is sufficient to activate suppressive function in T cells (Fontenot, Gavin et al. 2003); hence Foxp3 appears to be a specific marker for murine Tregs. However, these findings are not completely replicable in human T cells. For example, TCR stimulation of $CD4^+CD25^-Foxp3^-$ effector T cells results in the induction of Foxp3 expression, but this is not sufficient to confer suppressive T cell activity (Wang, Ioan-Facsinay et al. 2007), thus arguing against a role for Foxp3 as a key regulator of suppression in human $CD4^+$ T cells (reviewed in (Roncarolo and Gregori 2008)).

Despite the controversy surrounding Foxp3 as a marker of suppressive T cells in humans, no other marker, either surface-bound or intracellular, can denote a larger proportion of T cells with immunoregulatory function and it is still currently

considered as the gold standard for Treg phenotyping in humans (Ziegler 2006; Feuerer, Hill et al. 2009). When T cells are isolated *ex vivo*, suppression of effector T cell cytokine production and proliferation correlates very strongly with the proportion of $Foxp3^+$ T cells present (Kryczek, Liu et al. 2009). However, the intracellular location of Foxp3 precludes its use as a phenotypic marker for Tregs in many experimental situations. Crucially, live cells cannot be sorted or depleted based on Foxp3 expression since antibody staining requires that the cell be permeabilised and fixed. Researchers wishing to isolate human Tregs from peripheral blood now do so using a panel of surface markers; most commonly this is done by sorting $CD4^+$ cells based high expression of CD25 and low expression of the IL-7 receptor α chain, CD127, which is downregulated specifically on suppressive T cells in the peripheral blood (Liu, Putnam et al. 2006).

However, many reports suggest that a significant number of regulatory T cells exist outside of this classical Treg phenotype. Of particular note is the demonstration that Foxp3 is expressed on a significant percentage (3-5%) of healthy human PBMC-derived $CD4^+CD25^-$ T cells. Thus, CD25-*negative* $CD4^+CD127^{lo}$ T cells also suppress effector T cell proliferation *in vitro* (Liu, Putnam et al. 2006). Further evidence compounding the phenotypic identification of Tregs came from work demonstrating that low CD127 expression is not an intrinsic characteristic of $Foxp3^+$ Tregs either, and that CD127 expression is dependent on T cell localization and activation status (Simonetta, Chiali et al. 2010). Interestingly, this study also identified other phenotypic markers that correlated with Foxp3 expression but not CD25 and CD127. These include the inducible costimulatory molecule, ICOS, and the αE integrin, CD103, which was originally described as a marker for T cells residing in the skin, lung and gut wall (Parker, Cepek et al. 1992) and is thought to

play a role in retention of T cells within epithelial compartments, since its ligand E-cadherin is predominantly expressed on epithelial cells but not the endothelium (Schon, Arya et al. 1999). Such markers have previously been attributed to activated Tregs (Herman, Freeman et al. 2004) and subsets of $CD25^+$ and $CD25^-$ $CD4^+$ T cells with suppressive characteristics *in vitro* (Lehmann, Huehn et al. 2002). Other phenotypic markers of Tregs are displayed in Table 1.1.

1.2.4 Naturally Occurring and Peripherally Induced Tregs

Clearly, a number of T cell subsets displaying disparate phenotypes are capable of suppressing antigen-specific immune responses. These cells can also be distinguished by the exact manner in which they enact immunosuppression (cell-contact mediated or regulatory cytokine production) and also by their origin. The majority of the classical, 'naturally occurring' regulatory T cells are generated in the thymus, where it is thought that T cells possessing a high affinity for self-antigens, in conjunction with various cytokine receptor signals, differentiate into the Foxp3-expressing Treg lineage (Liston, Nutsch et al. 2008). In the peripheral sites, these cells enforce self-tolerance by suppressing auto-antigen specific effector T cell responses. Hence, thymectomy in 3-day-old mice results in the development of autoimmunity, in particular destruction of the ovaries, since these mice lack naturally occurring Tregs (Fontenot, Dooley et al. 2005).

In contrast, many peripherally induced, 'adaptive' regulatory T cell subsets do not express Foxp3, demonstrating that Foxp3 expression alone is not sufficient for commitment to the Treg lineage. Even in patients with IPEX syndrome, a hypoproliferative and suppressive T cell subset producing IL-10, IFN-γ but not IL-4 is present (Passerini, Di Nunzio et al. 2011). These findings indicate that mutations in

the FOXP3 gene do not impair the function of T cells with a suppressive capacity, but that a peripherally induced Treg subset known as Tr1 cells, distinct from the naturally occurring Treg lineage, plays a crucial role in resolving inflammation and maintaining peripheral immune tolerance (Roncarolo, Gregori et al. 2006). In particular, Tr1 cells mediate their suppressive function via the secretion of large amounts of IL-10 and do not suppress through cell-contact mechanisms (Vieira, Christensen et al. 2004). It has recently been shown that these cells originate from the interaction of effector memory $CD4^{+}CD62L^{-}Foxp3^{-}$ T cells with their cognate antigen in the presence of the glycosaminoglycan, hyaluronan (Bollyky, Wu et al. 2011). This suggests that compromised cellular matrix integrity could promote the differentiation of T cells into Tr1 cells in order to maintain peripheral tolerance.

Another peripherally induced T cell subset with suppressive characteristics has also been observed, termed Th3 cells. These cells produce the regulatory cytokine TGF-β, but not IL-4 or IL-10, thus distinguishing them from Tr1-type cells (Weiner 2001). Importantly, these Th3 cells have been shown to confer tolerance in a mouse model of experimental autoimmune encephalomyelitis (EAE), and to suppress antigen-specific responses *in vitro*; in both cases the suppression is mediated specifically by TGF-β (Miller, Lider et al. 1992).

The distinction between adaptive Tregs and naturally occurring Tregs is the requirement of adaptive Tregs for further differentiation of a T cell subset post thymic development into a Treg phenotype as a consequence of exposure to antigen in a particular immunological context (Bluestone and Abbas 2003, Curotto de Lafaille and Lafaille 2009). Recent attempts to differentiate the two subsets by phenotype revealed the expression of a transcription factor called Helios, restricted to thymic-derived

Tregs in both mice and humans (Thornton, Korty et al. 2010). Data showed that 100% of $CD4^+CD8^-Foxp3^+$ thymocytes and around 70% of $CD4^+Foxp3^+$ Tregs in peripheral lymphoid tissues expressed Helios, thus raising the possibility that a significant number (~30%) of $CD4^+Foxp3^+$ Tregs are generated extra-thymically, i.e. through peripheral conversion of conventional T cells. However, recent controversies have arisen with some researchers suggesting that Helios could become upregulated by activated, proliferating Tregs induced to express Foxp3 *in vitro* by peptide stimulation in the presence of TGF-β (Verhagen and Wraith 2010; Akimova, Beier et al. 2011), despite original findings showing that neither mouse nor human T cells induced to express Foxp3 by TCR stimulation with TGF-β, expressed Helios (Thornton, Korty et al. 2010). More validation of this research is required in order to unequivocally denote Helios as a marker for thymic-derived Tregs, although a number of groups have since utilised this marker for this purpose, giving credence to the original research (Elkord, Sharma et al. 2011; McClymont, Putnam et al. 2011). Regardless of the controversy, recent findings from a number of groups identify $Helios^+$ Tregs as having greater suppressive properties than $Helios^-$ Tregs, and represent a distinct subset associated with CD103 and GITR expression and TGF-β production (Elkord 2012; Elkord and Al-Ramadi 2012; Zabransky, Nirschl et al. 2012).

1.2.5 Mechanisms of Suppression Mediated by Tregs

Extensive research into the mechanisms of T cell mediated suppression aims to unearth the control processes of peripheral tolerance with the potential of identifying novel therapeutic targets, given the importance of Tregs in a wide range of disease settings. Currently, it appears that Tregs are capable of inhibition at multiple levels, utilizing a plethora of inhibitory mechanisms depending on the nature, localization

Marker	Function and Expression in Human T cells
Foxp3	Transcription factor controlling many Treg functions and phenotypic markers, found to be a critical factor for maintaining immune homeostasis. Proposed to be a more specific marker of T regulatory cells than most cell surface markers.
Helios	A member of the Ikaros family of zinc finger transcription factors, Helios plays an important role in T cell development and homeostasis. It was originally proposed as a marker of thymus-derived Tregs, though expression also found on activated, proliferating Tregs.
CD25	The low affinity IL-2 receptor α chain may act as an IL-2 sink on Tregs, thus giving these cells regulatory potential by starving effector T cells of IL-2. CD25 is also markedly expressed on activated non-suppressive T cells.
CD39/CD73	Ectonucleotidases involved in the generation of extracellular adenosine, which may suppress effector T cell activation and effector function. CD73 has also mediates adhesion of lymphocytes to follicular dendritic cells and endothelial cells.
LAP (TGF-β)	The immunosuppressive cytokine TGF-β is synthesized as a large protein precursor and then secreted as a complex of TGF-β and LAP (latency-associated peptide), in which LAP associates with the mature TGF-β to prevent its activity. Cells expressing LAP appear to have suppressive function and it has been proposed as a marker for activated Tregs.
GARP	A receptor for latent TGF-β, GARP has been proposed to play a role in controlling various suppressor functions of Tregs. Tumour cells may also use GARP to express TGF-β or to capture TGF-β from their surroundings resulting in local suppression of anti-tumour immune responses or to induce Treg differentiation.
ICOS	The inducible costimulatory molecule is homologous to the CD28 / CTLA-4 proteins. It is expressed on T_{FH} cells, TCR-activated T cells and Tregs, particularly amongst TILs, and is known to induce secretion of IL-10 and TGF-β.
CD103	The αE integrin is primarily found on intestinal intraepithelial lymphocytes but is also expressed on a small subset on peripheral T cells and could denote T cells with a suppressive capacity. It is thought to play a role in T cell retention within mucosal tissues, such as the gut and skin.
CD127	The IL-7 receptor α chain has a role on regulating T cell development and proliferation, but appears to be downregulated specifically on peripheral $Foxp3^+$ Tregs.

Marker	Function and Expression in Human T cells
CTLA-4 (CD152)	Cytotoxic T lymphocyte antigen-4 is expressed predominantly on $Foxp3^+$ Tregs and negatively regulates T cell-mediated immune responses through interaction with CD80 and CD86 present on antigen presenting cells. It can therefore play a role in the induction and maintenance of immunological tolerance and can suppress dendritic cell maturation and function.
LAG-3 (CD223)	The CD4-homologue Lymphocyte Activation Gene-3 (CD223) is expressed on activated T cells and NK cells, as well as T regulatory cells. LAG-3 binds MHC class-II molecules, but with higher affinity than CD4. In association with the CD3 complex, LAG-3 can inhibit TCR-mediated signaling and regulate homeostatic T cell expansion.
GITR	Glucocorticoid-induced TNF receptor family-regulated gene is expressed on activated lymphocytes (upregulated by T cell receptor engagement) and by Tregs. GITR signaling has been shown to regulate T cell proliferation and TCR-mediated apoptosis. Once bound to the GITR-ligand, this serves to regulate the activation of Th1 cell subsets.

Table 1.1 Summary of common regulatory T cell markers and their function.
Tregs have been shown to express a number of different markers and molecules that contribute to their function and/or delineation, reflecting their heterogeneous nature. A summary of many of these markers/molecules is given in the table above, including the purported function and expression in human T cells.

and extent of the immune response. In particular, identifying which mechanisms are used and in what context will be paramount to our further understanding of this heterogeneous T cell subset.

A three-tiered model of Treg function has been proposed to differentiate the mechanisms of immunological suppression (Tang and Bluestone 2008). The first tier represents Tregs exerting homeostatic control over immune responses, initially in lymphoid organs to prevent the outgrowth of autoreactive T cells. When this steady-state is breached, for example in the presence of heightened frequencies of self-reactive effector T cells in autoimmune and transplantation settings, activated Tregs produce immunosuppressive cytokines and upregulate suppressive molecules, thereby engaging a second tier of 'damage control'. Finally, infectious tolerance is established through the induction of adaptive Tregs, since tissue destruction presents tissue antigens leading to more immune cell activation and TGF-β production. In the colon, TGF-β causes dendritic cells to express CD103, which in turn, are able to induce naïve T cells to differentiate into adaptive Treg cells via the production of retinoic acid (Coombes, Siddiqui et al. 2007). Throughout these tiers, many mechanisms exist to prevent and terminate immune responses; Vignali and colleagues have grouped these mechanisms into four 'basic modes of action' (Vignali, Collison et al. 2008). These incorporate the suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of dendritic cell maturation or function (summarized in Figure 1.2).

1.2.5.1 Inhibitory Cytokines

The inhibitory cytokines IL-10 and TGF-β have been associated with Treg activity in many studies, and are known to play a role in both preventing and causing

the pathogenesis of many diseases, including cancer. Initial support for the crucial role of TGF-β in regulating immune responses comes from studies in TGF-β knockout or TGF-β-receptor knockout mice. In both cases, mice develop a fatal autoimmune lymphoproliferative disease; further analysis of these mice reveals a normal phenotype and suppressive function of naturally occurring $CD4^+CD25^+$ Tregs, suggesting that TGF-β production by adaptive Tregs is responsible for the pathology (Lucas, Kim et al. 2000). Similarly, TGF-β deficient $Foxp3^+$ Tregs could not prevent the development of colitis, demonstrating an essential role for Treg derived TGF-β in dampening immune responses in the colon (Li, Wan and Flavell 2007).

A number of reports now demonstrate a role for membrane-bound TGF-β in mediating regulatory T cell function (Nakamura, Kitani et al. 2001; Ostroukhova, Qi et al. 2006). TGF-β is synthesized as a large protein precursor and then tethered to the cell membrane as a complex of TGF-β and LAP (latency-associated peptide) and LAP-expressing T cells are known to confer suppressive characteristics (Gandhi, Farez et al. 2010), consistent with the observation that Tregs inhibit via cell-to-cell contact. However, the phenotypic and functional relevance of LAP expression on T cells in human pathology is still to be fully researched, but current data suggests that soluble and/or membrane bound TGF-β is important for the function of Tregs and is implicated in tumour pathogenesis (Flavell, Sanjabi et al. 2010).

Likewise, IL-10 is known to play a crucial role in many immunoregulatory processes. A key study to analyse the role of IL-10 in mediating Treg suppression was performed by generating mice that lacked the ability to produce IL-10, specifically in $Foxp3^+$ Tregs (Rubtsov, Rasmussen et al. 2008). Although gut pathology was intensified in older mice, and heightened autoimmune responses in the lungs and skin

observed, no systemic autoimmunity was observed in these mice. This suggests that IL-10 derived from Foxp3^{+} Treg is necessary for immune homeostasis but may be restricted to the control of inflammatory responses at environmental and mucosal interfaces. In humans, a large number of putative, adaptive Treg subsets have been identified which express IL-10 in various settings (reviewed in (Fujio, Okamura et al. 2010)). These include the aforementioned Tr1 cells, CD46-stimulated T cells, and IL-10-secreting T cells induced by vitamin D3 and dexamethasone. However, assessing the detailed physiological function of these cells is difficult, because of the lack of specific markers that can reliably differentiate the population of IL-10-secreting Tregs from other T cells, although LAP, LAG-3 and NKG2D have been proposed as potential markers (Huang, Workman et al. 2004; Dai, Turtle et al. 2009; Gandhi, Farez et al. 2010). IL-10 can also be produced by activated, effector Th1 cells, demonstrating that many T cell subsets have the potential to limit an over exuberant immunological response (Anderson, Oukka et al. 2007).

Another immunosuppressive cytokine, IL-35, has been shown to be required for the maximal suppressive activity of Treg in mice (Collison, Workman et al. 2007). However, another report published soon after demonstrated that human Tregs do not express detectable amounts of IL-35, suggesting that it does not play a role in Treg mediated suppression in humans (Bardel, Larousserie et al. 2008). This has recently been called into question, with some research now suggesting that Treg production of IL-35 converts suppressed target conventional T cells into IL-35-induced Tregs, contributing another mechanism to infectious tolerance (Chaturvedi, Collison et al. 2011).

1.2.5.2 Cytolysis

A number of studies have associated regulatory function of $CD4^+CD25^+$ Tregs with their apparent cytotoxic activity via the secretion of granzymes A and B (Gondek, Lu et al. 2005; Zhao, Thornton et al. 2006). To date, this has only been demonstrated in mice, although these findings have been recapitulated by Cao *et al*., who showed that Tregs utilize granzyme B and perforin mediated killing to suppress the ability of NK cells and $CD8^+$ T cells to clear allogeneic and syngeneic tumours (Cao, Cai et al. 2007). Other cytolytic mechanisms associated with Treg suppressive activity include T cell apoptosis via galectin-1 production (Garin, Chu et al. 2007), lysis of antigen-presenting B cells by Fas-FasL interactions (Janssens, Carlier et al. 2003) and perforin-dependent dendritic cell killing in tumour draining lymph nodes (Boissonnas, Scholer-Dahirel et al. 2010), although more work is required to understand cytolysis as a suppressive function of Tregs in different settings.

1.2.5.3 Metabolic Disruption

As previously described, the expression of CD25 by Tregs provides them with a competitive advantage for IL-2 over conventional T cells which only express CD25 after TCR stimulation (Thornton and Shevach 1998). This ultimately results in cytokine deprivation-mediated apoptosis of conventional T cells, which require IL-2 for maintenance of the expression of genes involved in the regulation of cell growth and metabolism (Pandiyan, Zheng et al. 2007). Whilst the suppressive function of murine $Foxp3^+$ Tregs deficient of CD25 is not compromised *in vitro*, IL-2 signaling was found to be critical for maintaining the homeostasis and competitive fitness of Tregs *in vivo* (Fontenot, Rasmussen et al. 2005; Maloy and Powrie 2005).

Other mechanisms found to disrupt conventional T cell metabolism include prostaglandin E2, which can be produced by $Foxp3^+$ Tregs in humans. COX inhibitors and prostaglandin E2-receptor antagonists could reverse the suppression of effector T cell proliferation *in vitro*. (Yuan, Chen et al. 2010). Indeed, greater expression of Foxp3 in Tregs correlated with the expression of COX-2 and prostaglandin E2, thus it appears Foxp3 could control its production.

The ectonucleotidase CD39 is expressed upon $CD4^+Foxp3^+$ Tregs (both $CD25^+$ and $CD25^-$) and functions by hydrolyzing exogenous ATP to ADP and 5'AMP, which is hydrolyzed further to adenosine by another ectonucleotidase, CD73 (Borsellino, Kleinewietfeld et al. 2007). Adenosine nucleosides appear to inhibit the proliferation of conventional T cells *in vitro* and adenosine inhibitors are capable of reversing this suppression (Deaglio, Dwyer et al. 2007). In addition, intracellular cyclic adenosine monophosphate (cAMP) in naturally occurring Tregs play a key role in cell contact mediated suppression, whereby cAMP is transferred from Tregs into activated target $CD4^+$ T cells and/or antigen-presenting cells via gap junctions to suppress $CD4^+$ T cell function (Bopp, Becker et al. 2007; Bodor, Bopp et al. 2012).

1.2.5.4 Dendritic Cell Targeting

As well as targeting effector cells directly, Tregs can also modulate dendritic cell (DC) function, by suppressing antigen presentation and cytokine production required for the activation of effector T cells. Intravital microscopy has demonstrated that Tregs directly interact with DCs and are also able to reduce the period of interaction between effector T cells and DCs (Tadokoro, Shakhar et al. 2006; Tang, Adams et al. 2006). It was proposed that effector T cell function was attenuated due to

interactions between DCs and Tregs via the costimulatory molecule, cytotoxic T lymphocyte antigen-4 (CTLA-4). Indeed, CTLA-4 may be important for maximal regulatory T cell function but can also be upregulated by activated T cells to provide a negative signal thus limiting maximum activation potential (Greenwald, Boussiotis et al. 2001). Furthermore, CTLA-4-deficient Tregs are unable to suppress effector T cells since they cannot confer the necessary inhibitory signals to DCs in order to affect T cell activation (Oderup, Cederbom et al. 2006).

Another molecule capable of blocking DC maturation and function is the CD4 homologue and negative immunomodulator lymphocyte activation gene-3 (LAG-3 / CD223). LAG-3 binds MHC class-II molecules with higher affinity than CD4, has a negative intrinsic function and has been shown to be required for maximal Treg suppression (Huang, Workman et al. 2004; Workman 2004). Binding of LAG-3 to MHC class-II molecules expressed by immature DCs induces an immunoreceptor tyrosine-based activation motif (ITAM)-mediated inhibitory signaling pathway that suppresses DC maturation and their effector T cell stimulatory capacity (Liang, Workman et al. 2008). Interestingly, LAG-3 expression has been noted on a population of intratumoural T cells with direct suppressive effects on $CD8^+$ T cells (Gandhi, Lambley et al. 2006; Camisaschi, Casati et al. 2010). Exhausted and tolerised TILs also express LAG-3, further contributing to tumour-mediated immunosuppression (Woo, Turnis et al. 2012).

Neuropilin-1 (CD304) has been associated with increasing the interaction time between Tregs and DCs (Sarris, Andersen et al. 2008). Given that neuropilin-1 expression is confined mostly to Tregs, this is likely to give Tregs an advantage over conventional T cells, leading to a "default" suppression of immune responses in the absence of "danger signals" (Mizui and Kikutani 2008). Thus, it seems an

immunological balance exists between DCs and Tregs whereby Tregs can modulate DC phenotype and function, and DCs drive the differentiation of $Foxp3^+$ Tregs in order to control immune responses. Neuropilin-1 has also been proposed as a marker for thymic-derived Tregs (Yadav, Louvet et al. 2012), correlating with the expression of Helios amongst murine T cells. However, this finding is not recapitulated in humans (Milpied, Renand et al. 2009).

1.3 Colorectal Cancer

1.3.1 Incidence, Aetiology and Prevention

Colorectal cancer (CRC) is the second and third most commonly diagnosed malignancy in females and males, respectively, with 1.2 million individual diagnoses and over 600,000 deaths worldwide each year (Jemal, Bray et al. 2011). In the Cardiff and Vale NHS Trust (covering a population of ~500,000) there are over 250 newly diagnosed cases each year.

The incidence of CRC is far higher in economically developed countries than in developing countries, reflecting differences in environmental factors such as diet and lifestyle (Magalhaes, Peleteiro et al. 2012). In particular, a high intake of red and processed meat, most commonly associated with a western diet, significantly increases the risk of developing colon cancer, but not rectal cancer. How this occurs is still under scrutiny; a possible explanation comes from the formation of endogenous N-nitroso compounds under alkaline conditions in the gut. These compounds are typical breakdown products of haem found in red meats, but not white meat and fish, and likely contribute to DNA damage amongst epithelial cells (Kuhnle and Bingham 2007). No genetic predisposition is noted in the majority of CRC cases. However,

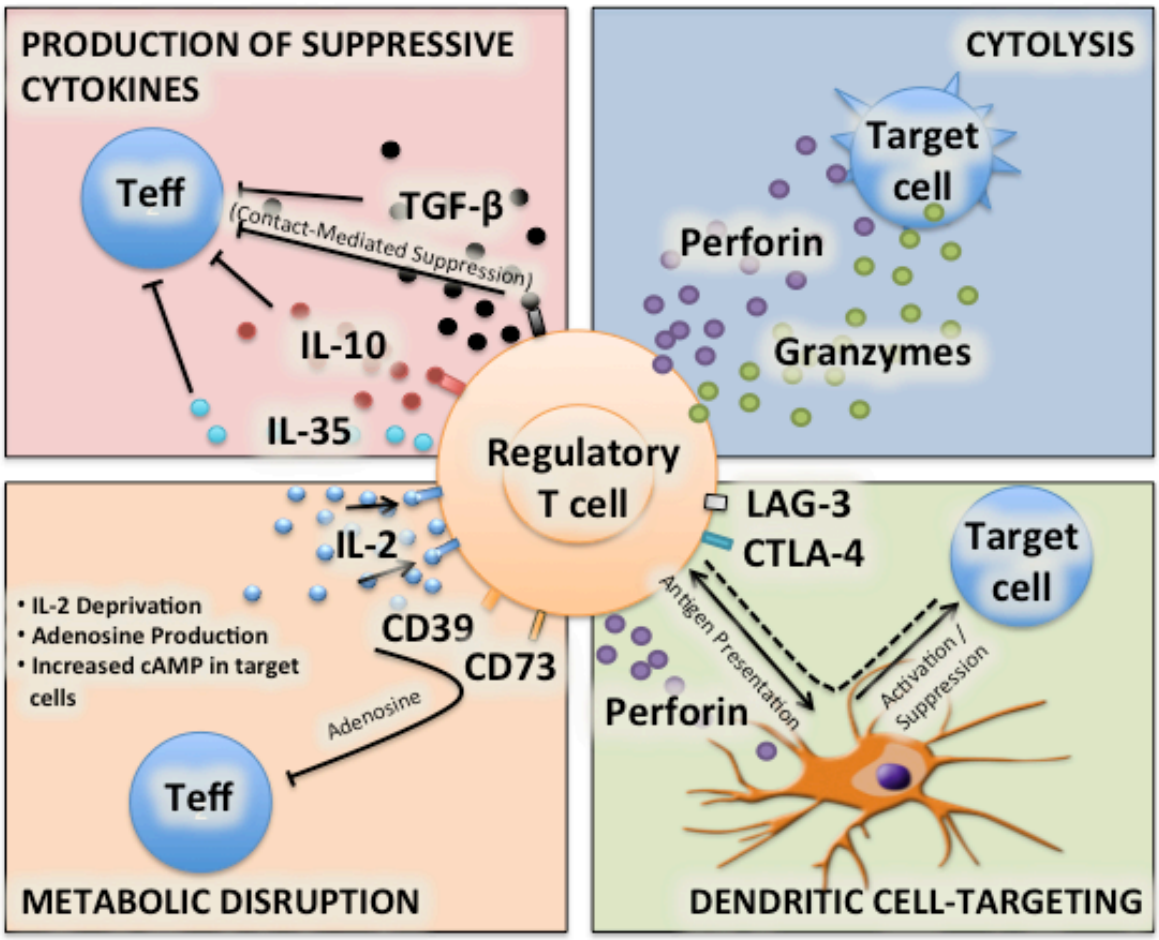


Figure 1.2 Mechanisms of suppression mediated by Treg. The mechanisms of suppression utilised by Tregs can be divided into four functional groups: suppression by inhibitory cytokines; cytolysis of target cells; suppression by metabolic disruption; and suppression by targeting dendritic cells.

most invasive colorectal tumours are thought to begin as polyps that grow on the inner lining of the colon or rectum. Regular colonoscopy screening tests can identify and remove polyps before they become cancerous. However, certain genetic abnormalities can lead some individuals to produce excessive numbers of colonic polyps; specific genetic syndromes, such as familial adenomatous polyposis (FAP), Lynch syndrome and Peutz-Jeghers syndrome, therefore increase the risk for developing CRC. In particular, mutations or deletion of the tumour suppressor gene APC (Adenomatous Polyposis coli) in humans leads to the development of FAP, but can also be responsible for other sporadic cases of CRC (Groden, Thliveris et al. 1991); virtually all individuals with FAP develop CRC by the age of 40. This gene has also been exploited in the APC^{min} mouse model to study the causes and development of adenomas.

CRC is also more common amongst individuals suffering from inflammatory bowel diseases such as ulcerative colitis and Crohn's (Triantafillidis, Nasioulas et al. 2009). A number of reports now provide strong evidence linking the use of chronic, low doses of non-steroidal anti-inflammatory drugs, such as aspirin, to prevent the growth of polyps and CRC (Thun, Jacobs et al. 2012). Such findings implicate a detrimental role for inflammation in colorectal tumourigenesis.

1.3.2 Initiation and Development of Colorectal Adenocarcinomas

A number of factors affect the development of dysplastic adenomas, generally arising from submucosal colonic polyps, into adenocarcinomas that invade through the muscularis mucosae. Genetic mutations in genes such as APC, KRAS and TP53 can accumulate amongst epithelial cells, resulting in chromosomal instability, microsatellite instability and DNA hypermethylation, which may further contribute to

tumourigenesis through transcriptional silencing of tumour-suppressor genes (Jones and Baylin 2002; Triantafillidis, Nasioulas et al. 2009). It has recently been proposed that such mutations also compromise junctional protein and mucus production by epithelial cells, allowing bacteria to translocate into the lamina propria. This triggers a local inflammatory response, activating myeloid cells, which in turn, causes a skewing in the cytokine profile of neighboring T cells (Grivennikov, Wang et al. 2012; Gallimore and Godkin 2013) (Figure 1.3). Production of interleukin-17 by these T cells is thought to drive proliferation, invasion and survival of the aberrant epithelial cells via STAT3 activation, leading to the onset of invasive carcinoma and further compromising barrier integrity. Thus, genetic mutations in epithelial cells, mucosal integrity, microbiota, and inflammation all play a role in the pathogenesis of CRC.

1.3.3 Colorectal Cancer Stem Cells

Following the initiation of colorectal carcinogenesis, the types of cells that propagate tumours remain unknown. Traditional stochastic models suggest that every cell within the tumour population is capable of tumour initiation and propagation. However, this has now been challenged by the recent identification of cancer stem cells, defined as the only cell type within tumours that can promote its growth and metastatic spread (reviewed in (Vaiopoulos, Kostakis et al. 2012)). Self-renewal, multipotency, limitless proliferation potential, angiogenic, and immune evasion features characterise cancer stem cells. In particular, colorectal stem cells have been defined by the expression of CD133, a transmembrane glycoprotein implicated in the organisation of the plasma membrane (Ricci-Vitiani, Lombardi et al. 2007). Only a small subset of $CD133^{+}$ cells was capable of initiating tumour growth in

immunodeficient mice, while negative cells were not. Since this work, CD133 expression has been independently correlated with stem cell potential, worse prognosis and low survival in colon cancer (Horst, Kriegl et al. 2008).

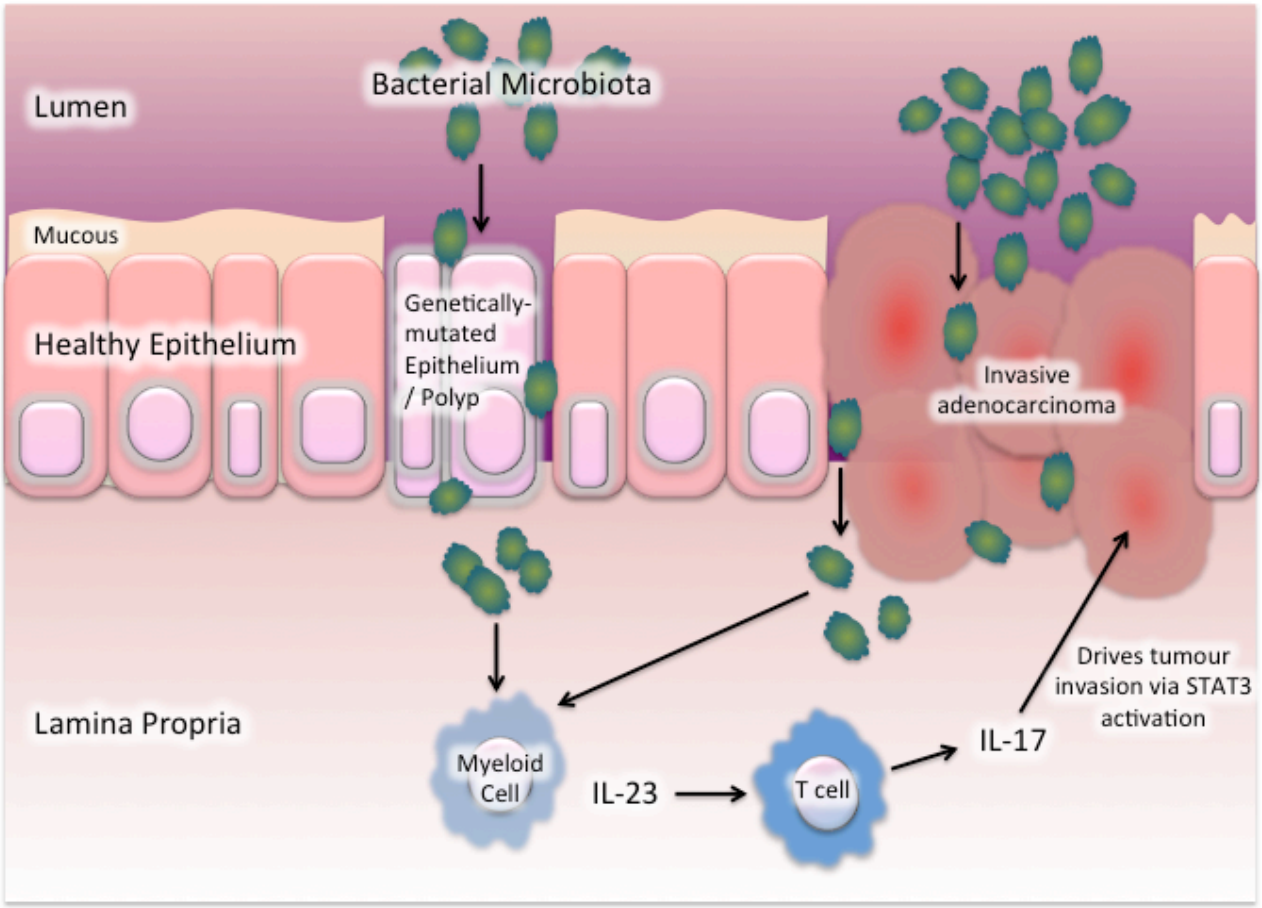
1.3.4 Metastasis and the Pre-Metastatic Niche

Whilst the epithelial-to-mesenchymal transition and the breakdown of cell-cell adhesion forms an integral part of the metastatic potential of colorectal cancer stem cells, it has been suggested that primary tumour cells orchestrate the genetic regulation and activation of specific chemokines and proteases in order to direct metastasis to a designated organ (Kaplan, Rafii et al. 2006). The increased expression of CXCL1 and matrix metalloproteinases-1 and-9 appear to precede metastasis, forming the foundations of creating a pre-metastatic niche (Minn, Gupta et al. 2005). Interestingly, compromised immunosurveillance via the increased infiltration of pro-inflammatory myeloid cells appears to be a novel mechanism through which primary tumours can also create favourable niches in secondary organs (Yan, Pickup et al. 2010).

1.3.5 Treatment of Colorectal Cancer

For individuals diagnosed with a primary localized tumour, a colectomy to remove the tumour mass and surrounding tissue is performed, typically via laparoscopic resection of the afflicted colon. In many cases, surgery is curative, however, 40-50% of these patients will relapse or die from metastatic disease. Treatment of advanced metastatic colorectal cancer has evolved considerably in the last decade; the introduction of tailored treatment (e.g. cetuximab, targeting the epidermal growth factor receptor expressed in ~80% metastatic colorectal tumours,

and bevacizumab which inhibits the action of vascular endothelial growth factor) and combination chemotherapy (e.g. FOLFOX, a combination of 5-Fluorouracil, oxaliplatin and leucovorin – drugs which block DNA synthesis and replication) has significantly enhanced patient survival (Segal and Saltz 2009). However, these regimes are not curative and are only recommended to reduce symptoms and prolong survival. For this reason, other treatments are sought. One of the most exciting avenues of research, with significant future potential, centers on utilizing the immune system to help treat cancer (see Discussion Chapter 5).



Adapted from (Gallimore and Godkin 2013).

Figure 1.3 Bacterial translocation can lead to gut inflammation and cancer progression. Genetically mutated epithelial cells, which may develop into colonic polyps and invasive adenocarcinomas, can become compromised in their ability to keep gut microbiota away from the lamina propria, since mucus production and junctional proteins are not formed sufficiently. This leads to a pro-inflammatory response whereby bacterial products stimulate toll-like receptors (TLRs). TLR activation causes myeloid cells to produce IL-23 in the mucosa, which has the notable downstream effect of causing T cells to produce IL-17. This skewed T cell cytokine profile is known to contribute to the progression of CRC and indeed, higher levels have been associated with a poorer prognosis (Tosolini, Kirilovsky et al. 2011).

1.4 T cells and Colorectal Cancer

1.4.1 Tumour Infiltrating Immune Cells

In 1931, American oncologist William MacCarty reported fifteen factors governing prognosis in the cancer patients he treated. The most compelling observation was that excised tumours with greater immune cell infiltrates would lead to an improved clinical outcome for that patient (MacCarty 1931). One of the first studies to formally identify the degree of tumour immune cell infiltration with patient prognosis was published in 1989 by Clark and colleagues. In their studies on melanoma tumour tissue, patients with a moderate-to-marked lymphocytic infiltrate within their primary melanoma had an 8-year survival rate of 88.5%, in comparison to 59.3% in patients with a sparse or absent lymphocytic infiltrate (Clark, Elder et al. 1989). Similar findings have since been obtained when analyzing ovarian (Zhang, Conejo-Garcia et al. 2003), head and neck (Shibuya, Nugyen et al. 2002), breast (Marrogi, Munshi et al. 1997), lung (Ito, Suzuki et al. 2005), prostate (Vesalainen, Lipponen et al. 1994) and colorectal (Ropponen, Eskelinen et al. 1997) carcinomas.

With the advent of monoclonal antibodies to accurately identify distinct cell types, it soon became clear that virtually all tumours contained immune cells to some degree, ranging from subtle infiltrations to gross inflammations (Pages, Galon et al. 2010). In particular, the identification of $CD3^+$ T cell infiltrates appeared to correlate most strikingly with disease outcome. In a seminal study analysing 490 colorectal tumour specimens for the type, density and location of immune cells, patient outcome could be significantly correlated to the degree of $CD3^+CD45RO^+$ T cell infiltration (Galon, Costes et al. 2006). Most striking was the observation that T cell infiltration was a better identifier of patient outcome than histopathological tumour grading.

Patients who presented with a more advanced TNM stage III tumour would actually have the same outcome as a TNM stage I patient if the tumour was readily infiltrated by T cells; conversely, a stage I patient with low levels of infiltrating T cells had an equally poor prognosis as a stage III patient. These results suggest that immune cells can actively control tumour recurrence and reduce metastatic potential, lending support to the hypothesis that the adaptive immune response can positively influence tumour control.

Given that the degree of immune cell infiltration into the colorectal tumour is a better prognostic indicator than histopathological staging, a taskforce has recently been established to include immunological biomarkers when classifying the excised tumour to facilitate stratification of patient treatment; this 'Immunoscore' involves harmonising immunohistochemical analysis of tumours to minimise variation (Galon, Franck et al. 2012).

1.4.2 Colorectal Tumour-Associated Antigens

One mechanism by which CRC patients may control their tumour is through direct recognition of epitopes derived specifically from tumour cells. 5T4 and CEA are oncofoetal glycoproteins that are markedly upregulated in the vast majority of colorectal adenocarcinomas but rarely on normal tissues, making them excellent targets for cancer immunotherapy and to study anti-tumour immune responses (Benchimol, Fuks et al. 1989; Starzynska, Rahi et al. 1992). 5T4 is not thought to be expressed on normal adult tissues, however, CEA is an autoantigen expressed at low levels in normal intestinal epithelium. Upregulation of both antigens forms an integrated component of the epithelial-to-mesenchymal transition, a process important

during embryonic development and metastatic spread of epithelial tumours, as discussed previously (Southgate, McGinn et al. 2010).

1.4.3 Anti-tumour T cell Responses in CRC patients

Several lines of research have now reported that both cancer patients and healthy individuals seem capable of producing readily detectable tumour-specific T cell responses to a number of tumour antigens, Our lab has previously characterised *ex vivo* effector Th1 responses to 5T4 and CEA in CRC patients, whereby IFN-γ is produced in response to tumour-antigen pulsed APCs (Clarke, Betts et al. 2006; Betts, Jones et al. 2012). In a five-year follow-up study analysing the effect of pre-operative CEA and 5T4-specific T cell responses, anti-CEA responses, but not anti-5T4 responses, were significantly associated with tumour *recurrence*, underlying the need to better characterise anti-tumour immune responses (submitted manuscript, see Appendix). Tumour-specific T cell responses are also significantly enhanced after the depletion of $CD25^+$ Tregs, as others have previously found (Danke, Koelle et al. 2004; Elkord, Burt et al. 2008). Thus, it seems Tregs have the potential to recognise tumour antigens, which are predominantly self-antigens, and suppress anti-tumour / anti-self responses. In one study, it appears that the repertoire of tumour antigens recognised by Tregs differs from those recognised by effector memory T cells in the majority of CRC patients (Bonertz, Weitz et al. 2009). In addition, depletion of Tregs *in vitro* only augmented effector T cell responses specific for the same epitope, thus detailed mapping of effector and regulatory T cell epitopes for a specific tumour antigen holds significant promise for future peptide based immunotherapies, and will be explored in more detail in this thesis.

An important question is whether tumour-specific T cells reside in, and are functional within the tumour. Currently, no studies have identified tumour-specific T cell responses from T cells isolated from colorectal tumours. Indeed the presence of highly restricted clonal T cell expansions in colorectal cancer is questionable given that a comparison of the TCR-Vβ repertoire restriction in the blood, colon and tumour tissue of CRC patients identified comparable restrictions, suggesting that colorectal tumours may not selectively recruit tumour-specific T cells (Ochsenreither, Fusi et al. 2010). This is in distinct contrast with melanoma, which is considered a more immunogenic cancer given the number of tumour-specific T cells and high degree of TCR repertoire restriction within the tumour (Thor Straten, Schrama et al. 2004). However, TCR repertoire restriction was higher in the blood of CRC patients than in healthy controls, suggesting there is a degree of clonal expansion of tumour-specific T cells in the periphery that may also reflect the constituent cells of colorectal tumours.

The clinical impact of different classes of colorectal tumour-infiltrating T cells has also been assessed through the identification of functional clusters of genes associated with Th1 (Tbet, IRF-1, IL-12Rb2, STAT-4), Th2 (IL-4, IL-5, IL-13), Th17 (RORC, IL-17A) and cytotoxicity (Granulysin, Granzyme B, Perforin) in 125 tumour specimens (Tosolini, Kirilovsky et al. 2011). The results of this study unequivocally identified patients with high expression of Th1 and cytotoxic cluster genes with prolonged disease-free survival. This data, coupled with the findings that a higher infiltrate of $CD3^+$ T cells correlates with disease-free survival (Galon, Costes et al. 2006), demonstrates how vital an effector T cell response can be in targeting cancerous tissue. However, the antigen specificity of these cells remains unknown.

1.4.4 Immunosuppressive Tumour Microenvironment

Although T cell responses can be effective at destroying tumour cells in certain cases, efficacy appears to be limited by the immunosuppressive influences of the tumour microenvironment. Enhanced expression of inhibitory molecules, e.g. PD-L1 and CD200R, has been reported on human malignancies and in multiple cases has been associated with enhanced metastatic potential and poor clinical outcome (Rygiel and Meyaard 2012; Topalian, Drake et al. 2012). This fits with the concept of adaptive resistance via immunoediting, where tumour cells may escape immune surveillance by expressing these ligands, survive, and grow out.

It is well established that colorectal cancer, like most human cancers, induces an increase in the frequency and proportion of regulatory T cells in peripheral blood and tumour infiltrating lymphocytes, as defined by Foxp3 expression amongst $CD4^+$ T cells (Ling, Pratap et al. 2007). This increased Treg frequency is associated with poor prognosis and progression of colorectal cancer due to Treg-mediated suppression of anti-tumour immunity (Betts, Jones et al. 2012). Increased intratumoural Treg frequencies have also been noted in latter stages of ovarian cancer (Curiel, Coukos et al. 2004), indicative of a role for Tregs in tumour progression, although results in colorectal cancer remain inconclusive and disputed. For example, accumulations of $Foxp3^+$ Treg in colorectal tumours, as evaluated by immunohistochemistry, are not always associated with a poor prognosis ((Loddenkemper, Schernus et al. 2006; Salama, Phillips et al. 2009) and unpublished findings from our own lab).

One plausible reason why Tregs may be associated with a good outcome comes from microarray analysis of tumour tissue, identifying that higher levels of Th17-related cytokines can be overtly detrimental to host survival. Th17 cells usually

arise in response to intestinal bacteria (where they exert a pro-inflammatory response) and reside in gut-associated lymphoid tissue. Within colorectal tumours, it has been hypothesized that $Foxp3^+$ Tregs regulate the production of IL-17 from Th17 cells, thus limiting inflammation and corresponding tumour progression, as discussed earlier (Whiteside 2012). However, since Tregs are also positively correlated with effector Th1 cell infiltrates, the positive impact of Tregs could just reflect a heightened, more potent anti-tumour immune response.

These findings remain controversial, however, since Tregs have commonly been associated with suppression of anti-tumour immune responses. A previously discussed mechanism of Treg suppression of immune responses is via the release of regulatory cytokines. Tumour infiltrating Tregs from numerous murine and human carcinomas are found to produce TGF-β and IL-10, and are known to inhibit anti-tumour immune responses by suppressing $CD4^+$ IFN-γ production and $CD8^+$ T cell cytotoxicity (Jarnicki, Lysaght et al. 2006; Strauss, Bergmann et al. 2007). Within colorectal tumours, very few functional studies have been carried out to determine Treg function, however, one paper has made an attempt to answer this and found $CD4^+CD25^+$ TILs to suppress effector T cells *in vitro* (Kryczek, Liu et al. 2009). Clearly more work is required to decipher the role of colorectal tumour infiltrating T cell subsets in the disease pathogenesis.

1.5 Summary

Anti-tumour T cell responses in CRC patients.

The existence and function of tumour-specific effector T cells is crucial to patient outcome. Currently, very little is known about the kinetics of an anti-tumour T cell response, and an in depth understanding is paramount to maximise the efficacy of

cancer vaccines utilising tumour-specific antigens to prime effector immune responses. In addition, evidence for cancer immunosurveillance occurring in humans is lacking, thus tumour antigen-specific Th1 and Treg responses were analysed in a cohort of healthy and age-matched donors in an effort to compare an individuals ability to mount an anti-tumour immune response. Finally, CRC patients were also examined for tumour-specific immunosuppression, and attempts made to reverse regulatory T cell involvement in cancer with concurrent tumour-specific T cell stimulation, utilising novel therapies in metastatic CRC patients.

Phenotypic and functional characterisation of colorectal tumour infiltrating T cells.
In order to design and direct anti-tumour therapies for maximal efficacy, it is of utmost importance to understand the network of biological interactions within the tumour microenvironment itself, with a particular focus on the suppressive mechanisms inhibiting anti-tumour immunity. A comprehensive overview of the phenotypic and functional characteristics of colorectal TILs will allow a greater understanding of the complex nature of T cell heterogeneity within the tumour, guiding and developing new drug treatment targets to diminish suppression and augment effective anti-tumour responses. The experiments described in this thesis aimed to establish the fundamental characteristics of T cells residing within the tumour, comparing these to corresponding cell subsets found in the colon and peripheral blood.

1.6 Research Questions

1) Can oncofoetal-antigen (5T4) specific $CD4^+$ T cell responses in colorectal cancer patients predict clinical outcome? Previous work has identified that 5T4-

specific T cells exist in CRC patients, but it is not known if cultured $CD4^+$ T cell IFN-γ / IL-10 responses correlates with tumour burden or disease outcome. These responses will be analysed in a cohort of CRC patients to determine whether the presence and magnitude of tumour-specific T cell responses could predict outcome and assess its potential as a disease biomarker.

2) Do oncofoetal-antigen (5T4) specific T cell responses exist in healthy individuals? Currently, there is paucity in our understanding as to the existence of T cells specific for oncofoetal antigens, and whether their presence in tumour-free individuals could represent a mechanism of immunosurveillance. In addition, the effect of age, gender and HLA-type on these responses is not known and will be analysed.

3) To what extent do regulatory T cells impinge on anti-tumour immune responses? Increased proportions of regulatory T cells have been reported in colorectal cancer patients, but their effect on suppressing anti-tumour immune responses remains largely unknown. The effect of Treg depletion on 5T4 T cell responses will be examined both *in vitro* and *in vivo,* using low-dose cyclophosphamide, to determine whether T cell-based anti-tumour immunity can be enhanced, leading to superior tumour control.

4) How do colorectal tumour-derived T cells differ in their phenotype and function to T cells located in the colonic mucosa or peripheral blood? A detailed phenotype and function of colorectal tumour infiltrating T cell subsets is currently lacking in the scientific literature. Such knowledge is fundamental to our understanding of how certain T cell subsets perform distinct functions and how these might be manipulated to enhance cancer immunotherapies.

Chapter 2 – Materials and Methods

2.1 Colorectal Cancer Patients

Patients presenting with an operable primary colorectal adenocarcinoma were seen by a consultant surgeon for resection of the affected colon. At pre-operative assessment, no more than seven days prior to surgery, the patient was consented in order to obtain specimens for use in this research (see Appendix section for copy of patient information sheet and consent form) and 30-40 mls of blood collected. Where possible, blood was sent for HLA typing (Welsh Transplantation and Immunogenetics Laboratory, Pontyclun, Cardiff). In some instances, patients were followed up at various time points after the operation to determine rates of tumour recurrence. In total, a cohort of 69 CRC patients were enrolled; cultured T cell responses were analysed in 27 of these patients (patient characteristics summarized in Table 2.1) and TIL analysis on tumour samples was performed from 33 patient samples (patient characteristics summarized in Table 2.2). A number of samples were analysed in both studies; some patient samples were used to set up and validate experimental methods and are not included in compiled results. The Bro Taf Local Research Ethics Committee granted ethical approval for this study.

2.1.1 Obtaining Patient Specimens

Following resection of the diseased bowel, specimens were taken to the pathology department for analysis. Here, a small section of the tumour was cut from the specimen, derived from the luminal aspect of the specimen so as not to interfere with the deep, invasive part of the tumour required for routine histopathological assessment.

Table 2.1 CRC patient characteristics where cultured 5T4 T cell responses have been analysed.

		Male	Female
n		16	11
Age (Range)		69 (38-86)	75 (49-90)
Tumour Location (%)	Ascending	2 (13)	6 (55)
	Transverse	0 (0)	1 (9)
	Descending	1 (6)	1 (9)
	Sigmoid	4 (25)	3 (27)
	Rectum	9 (56)	0 (0)
TNM Stage, 5th Edition (%)	T1	3 (19)	1 (9)
	T2	3 (19)	3 (27)
	T3	9 (56)	5 (45)
	T4	1 (6)	2 (18)
(Lymph Node Spread)	N0	9 (56)	5 (45)
	N1	5 (31)	2 (18)
	N2	2 (13)	4 (36)
Dukes' Stage (%)	A	6 (38)	2 (18)
	B	3 (19)	3 (27)
	C1	7 (44)	3 (27)
	C2	0 (0)	3 (27)
	D	0 (0)	0 (0)

Table 2.2 CRC patient characteristics where tumour-infiltrating lymphocytes have been analysed.

		Male	Female
n		22	11
Age (Range)		69 (38-86)	76 (49-88)
Tumour Location (%)	Ascending	2 (9)	5 (45)
	Transverse	0 (0)	1 (9)
	Descending	1 (5)	1 (9)
	Sigmoid	6 (27)	3 (27)
	Rectum	13 (59)	1 (9)
TNM Stage, 5th Edition (%)	T1	3 (14)	2 (18)
	T2	7 (32)	2 (18)
	T3	11 (50)	5 (45)
	T4	1 (5)	2 (18)
(Lymph Node Spread)	N0	15 (68)	5 (45)
	N1	5 (23)	2 (18)
	N2	2 (9)	4 (36)
Dukes' Stage (%)	A	9 (41)	2 (18)
	B	6 (27)	3 (27)
	C1	7 (32)	3 (27)
	C2	0 (0)	3 (27)
	D	0 (0)	0 (0)

Autologous colon samples were also cut from a macroscopically normal section of the excised tissue, at least 10 cm from the tumour. In some cases, easily identifiable lymph nodes present in the mesentery proximal to the colorectal tumour were also obtained. Samples of placenta were obtained from women undergoing elective Caesarian sections and small samples of inflamed colon from patients with Crohn's disease or ulcerative colitis were obtained from resected colon specimens. Informed consent was obtained from all participants. In most cases, samples were dissected and taken back to the lab within 30 minutes of the specimen being resected from the patient.

2.1.2 Histopathological Tumour Grading

Colorectal tumours were analysed for overall size, invasive status and lymph node involvement, confirmed by consultant pathologists at the University Hospital of Wales and University Hospital Llandough, Cardiff. Each specimen was then graded, depending on certain parameters, for Dukes' classification and TNM staging, as follows:

Dukes' A: Invasion into but not through the bowel wall.

Dukes' B: Invasion through the bowel wall; no lymph node involvement.

Dukes' C1: Extending into muscularis propria; local lymph nodes involved.

Dukes' C2: Penetrating through muscularis propria; local and Apical lymph nodes involved.

T(1-4): Size or direct extent of the primary tumour.

T1: Tumour no more than 2cm across (invades submucosa).

T2: Tumour more than 2cm but no more than 5cm across (invades muscularis propria).

T3: Tumour greater than 5cm across (invades into serosa).

T4: Tumour has grown into adjacent tissues.

N(0-2): Degree of lymph node involvement.

N0: No spread to regional lymph nodes.

N1: 1-3 regional lymph nodes involved.

N2: 4+ regional lymph nodes involved.

M(0/1): Presence of metastasis.

M0: No metastatic spread.

M1: Distant metastases.

2.1.3 TaCTiCC Clinical Trial

CRC patients with inoperable, metastatic, stable disease were enrolled on the phase II clinical trial, TaCTiCC. The aim of the trial was to assess the efficacy of using low-dose cyclophosphamide alongside vaccination with a modified vaccinia Ankara based vaccine containing the tumour antigen, 5T4, named TroVax® (Oxford BioMedica). Briefly, there were four arms to this trial: no treatment, TroVax® alone, low-dose cyclophosphamide alone and TroVax® plus low-dose cyclophosphamide. Patients were consented as per the trial protocol. Peripheral blood samples were assessed for effective Treg depletion and 5T4 T cell responsiveness, with the primary objective of determining whether such treatment results in an increased magnitude of anti-5T4 immune responses at week 7. A flowchart detailing the patient treatment regime by group is included in the Appendix section.

2.1.4 Healthy Donor and Age-Matched Controls

Blood was collected from healthy donors in the lab and also from age-matched controls with no history or clinical evidence of malignancy. All donors were consented before obtaining 20-50 ml blood samples. In all cases, samples were sent for HLA typing.

2.2 Lymphocyte Isolation

2.2.1 Preparation of Single Cell Suspensions from Tissues

Tumour and colon samples were minced with blades in a Petri dish and forced through 70 μm cell strainers to collect a single cell suspension. Cells were centrifuged twice in extraction media (IMDM + 2% AB serum, 20 μg/ml gentamicin, 2 μg/ml amphotericin B + 1X Pen/Strep, L-glutamine and sodium pyruvate) before plating for further use. In no instances were samples subjected to enzymatic dissociation, e.g. with collagenase / DNase.

2.2.2 Ficoll Separations

PBMCs were isolated by layering whole blood over Lymphoprep (Axis-Shield, Oslo, Norway) before centrifugation at 2000 rpm for 20 mins at RT^{o}C, with no brake. The PBMC layer was extracted using a Pasteur pipette and subsequently washed twice in R+ (RPMI 1640 + 1X Pen/Strep, L-glutamine and sodium pyruvate). Cells were enumerated using a haemocytometer under a light microscope by mixing 20 μl of cell suspension 1:1 with Trypan blue.

2.2.3 Colon / Tumour Infiltrating Lymphocyte Extraction

To isolate lymphocytes from epithelial or tumour cell fractions, single cell suspensions from each sample were resuspended in 5 mls extraction media. A discontinuous Ficoll density gradient was then created by layering 10 mls of 75% Ficoll (diluted in RPMI) over 10 mls 100% Ficoll in a falcon tube before slowly layering the cell suspension on top, as previously described (Whiteside, Miescher et al. 1986) and shown (Figure 2.1).

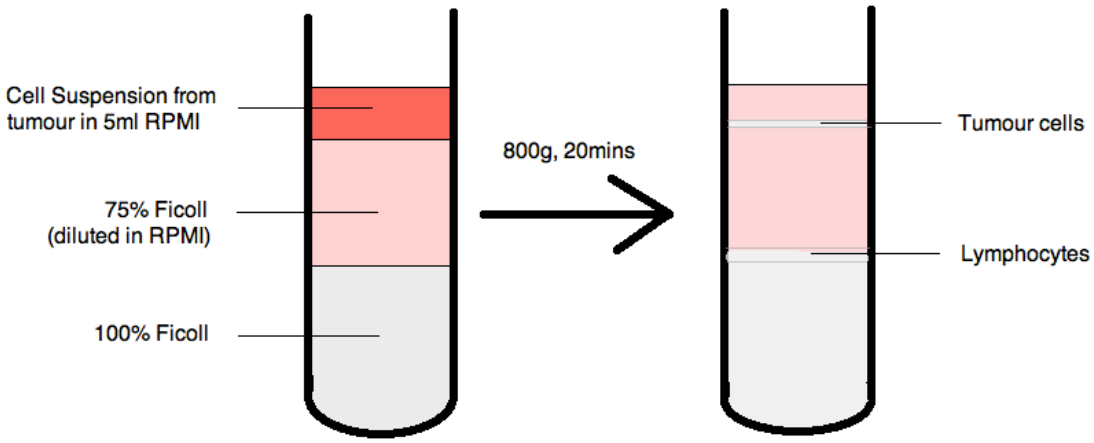


Figure 2.1 Isolation of lymphocytes from colorectal tumour specimens. CRC samples were mashed and forced through cell-strainers to obtain a single-cell suspension. This was washed and resuspended in 5ml extraction media, before being layered onto a discontinuous ficoll gradient, as shown above. After centrifugation, two distinct bands are formed where tumour cells and lymphocytes have separated based on their size and densities.

Samples were centrifuged at 2000 rpm for 20 mins at RT^{o}C, with no brake. The lymphocyte layer was extracted using a Pasteur pipette and subsequently washed twice in R+. Cells were enumerated using a haemocytometer by mixing 20 μl of cell suspension 1:1 with Trypan blue.

2.2.4 CD4^{+}CD25^{+} MACS Regulatory T cell Isolation

To isolate CD4^{+}CD25^{+} regulatory T cell fractions, PBMC were resuspended in MACS buffer and CD4^{+} cells were negatively selected by adding 10 μl biotin-antibody cocktail followed by 20 μl anti-biotin microbeads per 10^{7} PBMC in a total reaction volume of 100 μl MACS buffer (1x PBS, 0.5% BSA, 5 mM EDTA). Non-CD4^{+} cells were then depleted by running the cell suspension through a large depletion (LD) column, as per manufacturer's instructions (Miltenyi Biotec). To isolate the CD25^{+} and CD25^{-} fractions from the CD4^{+} cell fraction, cells were incubated with 10 μl of CD25 Microbeads II in 90 μl MACS buffer per $1x10^{7}$ cells and incubated for 15 minutes at 4^{o}C. A positive selection (MS) column was subsequently used and both the effluent (CD25$^{-/lo}$ cells) and the CD25^{hi} cells in the column were collected. Cells were washed twice with R+ before further use.

2.2.5 CD25 / CD45RO Depletion Using MACS Beads

CD25^{hi} cells were depleted from whole PBMC using CD25 Microbeads II by incubating $1x10^{7}$ PBMC with 10 μl of CD25 Microbeads II in 90 μl MACS buffer for 15 minutes at 4^{o}C. An LD column was used and the effluent (CD25$^{-/lo}$ PBMC fraction) collected. The effectiveness of depleting CD4+CD25+Foxp3+ Tregs using this method is demonstrated (Figure 2.2). For CD45RO^{+} T cell depletion, PBMC were

initially cultured with a PE-conjugated CD45RO antibody for 20 minutes at 4^{o}C. PBMCs were then washed twice and incubated with anti-PE microbeads for 15 minutes at 4^{o}C before running down an LD column. The collected effluent of CD45RO^{-} PBMC was washed, enumerated and checked for purity by FACS before use in assays.

2.2.6 Fluorescent Activated Cell Sorting

For functional studies, live PBMCs were sorted into effector ($CD4^{+}CD25^{-}$) and regulatory ($CD4^{+}CD25^{hi}CD127^{lo}$) T cell subsets using the monoclonal antibodies listed in Table 2.9.4. Various $CD4^{+}$ T cell subsets were sorted from colon and tumour specimens using a modified FACSAria II flow cytometer (BD Biosciences) or a Dako Cytomotion MoFlo (Beckman Coulter). Cell fractions were stained with fluorescent conjugated antibodies for 15 minutes at 4^{o}C in a total reaction volume of 40 μl of FACS buffer (1x PBS, 2% FCS, 5 mM EDTA) and filtered through 70 μm cell strainers before running samples. Gating strategies and cell purities are demonstrated (Chapter 4).

2.3 Freeze / Thaw PBMCs

Isolated PBMCs were counted then resuspended at a concentration of 5x10^{6}/ml in freezing media (90% FCS, 10% DMSO). 1ml aliquots of the sample were prepared and placed in Mr Frosty freezing containers (Nalgene), which provide a cooling rate of -1^{o}C /minute when placed in a -80^{o}C freezer. After 24-48 hours at -80^{o}C, vials were transferred to liquid nitrogen tanks for long-term storage.

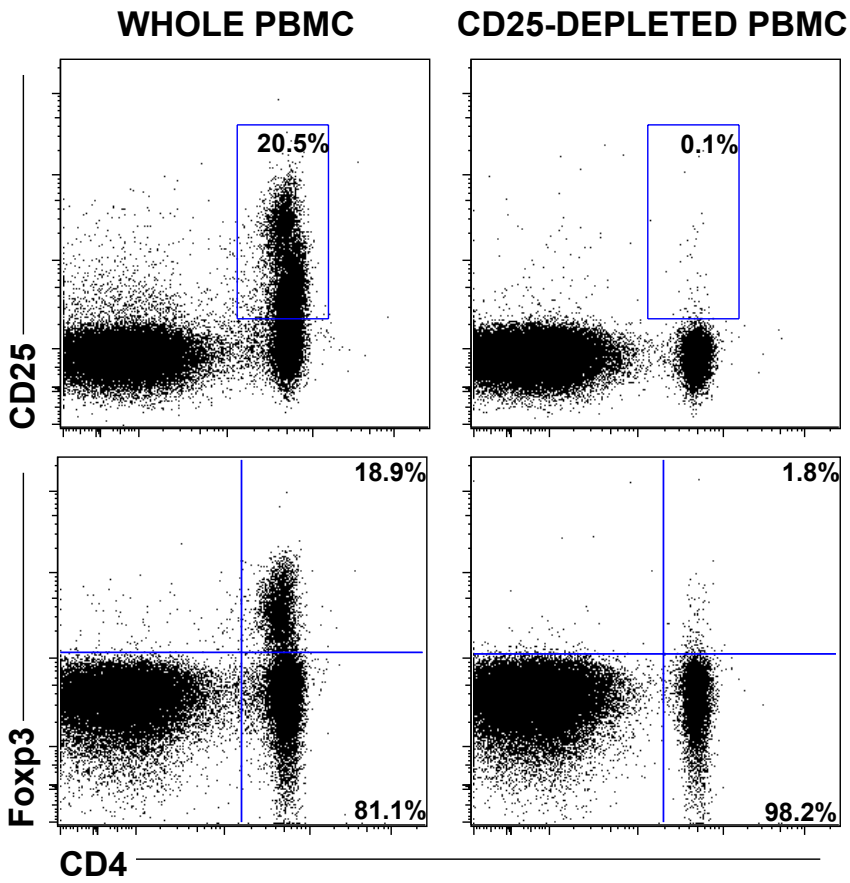


Figure 2.2 CD25 depletion using MACS beads and an LD column. The effectiveness of depleting $CD25^+$ T cells from $CD4^+$ was assessed by FACS. Corresponding levels of Foxp3 expression demonstrates the removal of the vast majority of $CD4^+CD25^+Foxp3^+$ Tregs prior to experimental use.

To thaw samples, vials were taken from liquid nitrogen and placed straight into a water bath set to 37^{o}C. Once the sample had almost completely thawed, a P1000 pipette was used to add the sample to pre-warmed R+ in a drop-wise fashion, to minimize oxidative stress on the cells. Cells were washed twice before use and counted. In general, a return of >70% viable cells was achieved.

2.4 Antigens

The common recall antigens tuberculin purified protein derivative (PPD) (Statens Serum Institut, Denmark), haemagglutinin protein (HA) (kindly provided by Dr John Skehel, NIMR) and Tetanus Toxoid (TT) (Statens Serum Institut, Denmark) were used to assess immune responses to a non-tumour antigen. All antigens were used at a final concentration of 10 μg/ml.

2.4.1 5T4 Peptide Pools and Matrix System

Forty-one separate 20 amino acid peptides, each overlapping by 10 amino acids, were designed and created to span the entire human 5T4 sequence (Table 2.3). The purity of each peptide was >95% (GLBiochem, Shanghai, China). In total, 13 peptide pools were created in a matrix system, each consisting of 5-7 peptides (as shown in Table 2.4). Peptides were added at a final concentration of 5 μg/ml/peptide. Final DMSO concentration in culture was never greater than 0.33%.

Table 2.3: 5T4 20mer peptide sequences

No.	Sequence	No.	Sequence	No.	Sequence
1	MPGGCSRGPAAGDGRLRLAR	15	LPSLRQLDLSHNPLADLSPF	29	LQGLPHIRVFLDNNPWVCDC
2	AGDGRLRLARLALVLLGWVS	16	HNPLADLSPFAFSGSNASVS	30	LDNNPWVCDCHMADMVTWLK
3	LALVLLGWVSSSSPTSSASS	17	AFSGSNASVSAPSPLVELIL	31	HMADMVTWLKETEVVQGKDR
4	SSSPTSSASSFSSSAPFLAS	18	APSPLVELILNHIVPPEDER	32	ETEVVQGKDRLTCAYPEKMR
5	FSSSAPFLASAVSAQPPLPD	19	NHIVPPEDERQNRSFEGMVV	33	LTCAYPEKMRNRVLLELNSA
6	AVSAQPPLPDQCPALCECSE	20	QNRSFEGMVVAALLAGRALQ	34	NRVLLELNSADLDCDPILPP
7	QCPALCECSEAARTVKCVNR	21	AALLAGRALQGLRRLELASN	35	DLDCDPILPPSLQTSYVFLG
8	AARTVKCVNRNLTEVPTDLP	22	GLRRLELASNHFLYLPRDVL	36	SLQTSYVFLGIVLALIGAIF
9	NLTEVPTDLPAYVRNLFLTG	23	HFLYLPRDVLAQLPSLRHLD	37	IVLALIGAIFLLVLYLNRKG
10	AYVRNLFLTGNQLAVLPAGA	24	AQLPSLRHLDLSNNSLVSLT	38	LLVLYLNRKGIKKWMHNIRD
11	NQLAVLPAGAFARRPPLAEL	25	LSNNSLVSLTYVSFRNLTHL	39	IKKWMHNIRDACRDHMEGYH
12	FARRPPLAELAALNLSGSRL	26	YVSFRNLTHLESLHLEDNAL	40	ACRDHMEGYHYRYEINADPR
13	AALNLSGSRLDEVRAGAFEH	27	ESLHLEDNALKVLHNGTLAE	41	YRYEINADPRLTNLSSNSDV
14	DEVRAGAFEHLPSLRQLDLS	28	KVLHNGTLAELQGLPHIRVF		

Table 2.4: 5T4 peptide pool (PP) matrix system

PP	1	2	3	4	5	6
7	1	2	3	4	5	6
8	7	8	9	10	11	12
9	13	14	15	16	17	18
10	19	20	21	22	23	24
11	25	26	27	28	29	30
12	31	32	33	34	35	36
13	37	38	39	40	41	

2.5 Primary T cell Culture

Isolated PBMC or purified T cell fractions were washed in R+ then resuspended at $2x10^6$/ml in OpTmizer CTS (Invitrogen) or advanced RPMI supplemented with 5% AB serum, before plating 100 μl / well in a 96 well plate (Nunc). Three PBMC lines were established to each of the 13 5T4 peptide pools and plates were kept in sterile culture conditions in an incubator (37^oC, 5% CO_2). In addition, cell lines were established to certain re-call antigens including tuberculin PPD, TT, HA and also to phytohaemagglutinin (PHA) as a positive control for T cell activation (IFN-γ production) and proliferation. Cells were supplemented with 10 μl CellKine media (Helvetica Healthcare) on day 3, 100 μl fresh media containing 40 IU/ml IL-2 (final concentration in culture = 20 IU/ml IL-2) on days 6 and 9, before analysing for antigen specificity using ELISpot on day 12.

2.6 IFN-γ / IL-10 ELISpot Assays

Polymer-backed 96-well filtration plates (MAIP-S-4510) (Millipore, Moslheim, France) were used for all ELISpot assays and all antibodies were obtained from Mabtech (Natka, Sweden). Initially, wells were coated with 50 μl of anti-IFN-γ capture antibody (1-D1K) / anti-IL-10 capture antibody (9D7) and left in the fridge at 4^oC overnight. Wells were washed five times with 150 μl PBS to remove excess coating antibody and blocked by coating wells with RPMI containing 10% human AB serum for 1 hour at 37^oC. Block was then discarded and cells were added to corresponding wells with and without stimulation. Plates were left overnight (18-24 hours) in the incubator (37^oC, 5% CO_2). Following five more washes with PBS, 50 μl of 1 μg/ml secondary biotinylated antibody (anti-IFN-γ: 7-B6-1, anti-IL-10: 12G8),

which recognise distinct epitopes from the coating antibodies, were added to each well and incubated for 1 hour in the incubator (37^{o}C, 5% CO_2). Wells were washed five more times with PBS before adding 50 μl streptavidin-alkaline phosphatase (Mabtech, Sweden), diluted 1:1000, for 30 mins – 1 hour at RT^{o}C. Following five more washes with PBS, the ELISpot wells were developed using the alkaline-phosphatase substrate kit from Bio-Rad (Hercules, California), comprising 4% AP colour development, 1% substrate A, 1% substrate B in dH_20. 100 μl of the development buffer was added to each well and the plate left for 5-15 mins, until spots were clearly visible. The reaction was stopped by washing the wells thoroughly with tap water. Cytokine-producing T cells were enumerated at the single-cell level by counting the number of spots per well using an automated ELISpot plate reader (Autoimmun Diagnostika GMBH, A.I.D., Strasberg, Germany) and analysed with the ELISpot 5.0 software package to ensure consistent analysis of spots between wells. Spots counts were verified manually.

2.6.1 *Ex vivo* ELISpot

Having isolated PBMC / T cell subsets, either fresh from whole blood or from frozen stocks, cells were counted and resuspended at 2-3x10^6/ml in OpTmizer CTS (Invitrogen) before adding 100-150 μl per well. Final cell counts are indicated where appropriate. Positive *ex vivo* responses were identified as having at least 10 spot-forming cells (SFC) per 2x10^5 PBMC, after subtraction of the background, and an increase of at least 50% above background.

2.6.2 Cultured ELISpot

PBMC lines that had been kept in culture were pooled from triplicate wells in identical culture conditions, washed, counted and resuspended at $2x10^6$/ml before being plating 100μl/well with or without the corresponding 5T4 peptide pool for direct comparison. Positive cultured responses were identified as having at least 20 spot-forming cells (SFC) per 10^5 PBMC, after subtraction of the background, and an increase of at least 50% above background.

2.7 Generation and Maintenance of T cell Clones

To generate 5T4-specific $CD4^+$ T cell clones, PBMC cultures were restimulated with the putative 5T4 epitope and cultured for a 12-day period. Strongly positive cell lines, as defined by a count of at least 100 SFC/10^5 cells minus background and double that of the negative control, were identified using the IFN-γ ELISpot. $CD4^+$ cells were negatively sorted using MACS CD4+ T cell isolation kit II (Miltenyi-Biotec, Germany) as per manufacturers instructions. Isolated cells were resuspended in T cell clone media comprising OpTmizer CTS (Invitrogen), 10% Human AB Serum, 20 U/ml IL-2, 2 μg/ml PHA and $2x10^5$ irradiated allogeneic PBMC, and cloned by limiting dilution in 96-well plates (Nunc). Plates were analysed for clones two weeks later and positive clones identified using the IFN-γ ELISpot, as defined by the formation of at least 20 SFC/10^4 and double that of the negative control. Samples of the 5T4 T cell clone were frozen for future use when cell numbers were sufficiently high enough. The rest were expanded using Dynabeads Human T-Activator CD3/CD28 (Invitrogen) and 100 U/ml IL-2 to stimulate for a 7-day period, before removing the beads and splitting the cells when the media began to turn yellow. Cells were then fed 40 U/ml IL-2 every 3-4 days until use.

2.8 Suppression Assays

2.8.1 ^{3}H-Thymidine Incorporation Assay

Having isolated subsets of T cells using a cell sorter, $CD4^{+}CD25^{-}CD127^{hi}$ conventional T cells were plated at $1x10^{5}$ cells per well in triplicate on a 96-well plate (Nunc) for studies on Tregs from peripheral blood and $2x10^{3}$ cells per well to study functional characteristics of isolated $CD4^{+}$ T cells from colon / tumour tissue. Cells were cultured in OpTmizer CTS media (Invitrogen) for 3 days at 37^{o}C, 5% CO_2, supplemented with Pen/Strep/L-Glut and stimulated with Dynabeads Human T-Activator CD3/CD28 (Invitrogen) at a bead to effector cell ratio of 1:2. Irradiated autologous PBMC were added to wells to make overall cell number in each assay consistent. Proliferation was measured by pulsing cells with 1 μCi/well of ^{3}H-thymidine for 6 hours, before harvesting onto filter mats using a TomTec Cell Harvester and analysing levels of tritiated hydrogen. The sensitivity of this assay is detailed (Figure 2.3).

2.8.2 CFSE-based Suppression Assay

The *in vitro* function of actively proliferating FACS-purified T cell subsets was analyzed by labeling autologous effector $CD4^{+}CD25^{-}CD127^{hi}$ T cells with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) and potential regulatory subsets with 2 μM PKH-26 (Sigma-Aldrich). To assess the impact of isolated TIL subsets on effector T cell proliferation, cells were co-incubated in 96 well plates at various ratios and stimulated with Dynabeads Human T-Activator CD3/CD28 (Invitrogen) at a bead to effector cell ratio of 1:2. After 3 days, cells were harvested and analysed using a FACSCanto II flow cytometer (BD Biosciences). Proliferation of

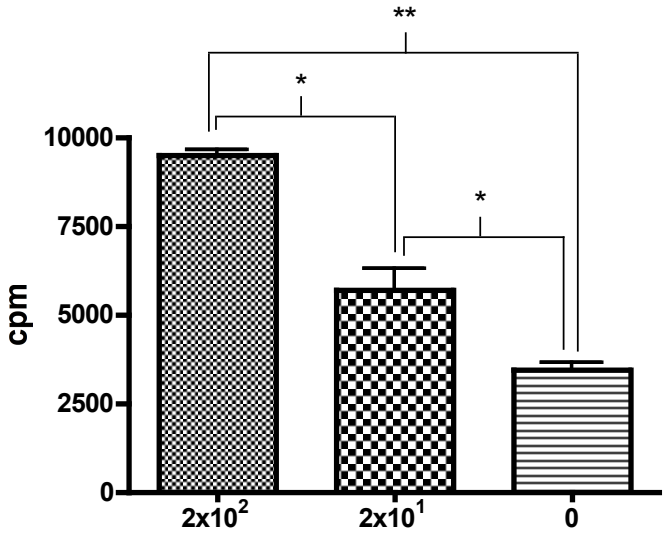


Figure 2.3 Sensitivity of ^{3}H-Thymidine proliferation assay. The indicated number of freshly isolated PBMC were stimulated with 2 µl Dynabeads Human T-Activator CD3/CD28 beads (equivalent to $8x10^4$ beads) and seeded with $2x10^4$ irradiated PBMC per well. ^{3}H-thymidine was added for the final 6 hours of a 72 hour assay before detecting radioactivity using a scintillation counter. cpm = counts per million. Significant differences are indicated; *$p < 0.05$, **$p < 0.01$.

T cell subsets was assessed by conventional gating and the division index was calculated using the proliferation platform in FlowJo software version 9.4 (TreeStar Inc.) as described previously (McMurchy and Levings 2012) and shown (Figure 4.18, Chapter 4).

2.9 Flow Cytometry

2.9.1 Antibody Staining

Cell samples were resuspended in PBS at a concentration of 2-5x10^6 cells/ml in 96-well plates (Nunc). Cells were initially stained with for viability with aqua amine-reactive viability dye (Invitrogen) for 15 minutes in the dark at RT^{o}C. Cells were then washed twice in FACS buffer and resuspended in 50 μl FACS buffer for surface marker staining. Anti-human conjugated antibodies were used in various combinations and allowed to incubate for 20 mins in the dark at 4^{o}C (see Table 2.5).

Following two wash steps with FACS buffer (PBS + 2% BSA), cells were permeabilized and fixed using a Fixation/Permeabilization kit (eBioscience) and incubated for 40 minutes at 4^{o}C. Following another wash step using 1X Permeabilization buffer, Fc receptors were blocked using rat serum for 15 minutes at 4^{o}C. The antibodies against intracellular markers were then added in various combinations and allowed to incubate for 30 minutes in the dark at 4^{o}C (see Table 2.6). The cells were then washed once with Perm buffer and fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich). Fixed cells were stored in the dark at 4°C until acquisition on a BD FACSCanto II. Representative flow cytometry plots and gating strategies for the analysis of comparable T cell subsets in blood, colon and tumour are

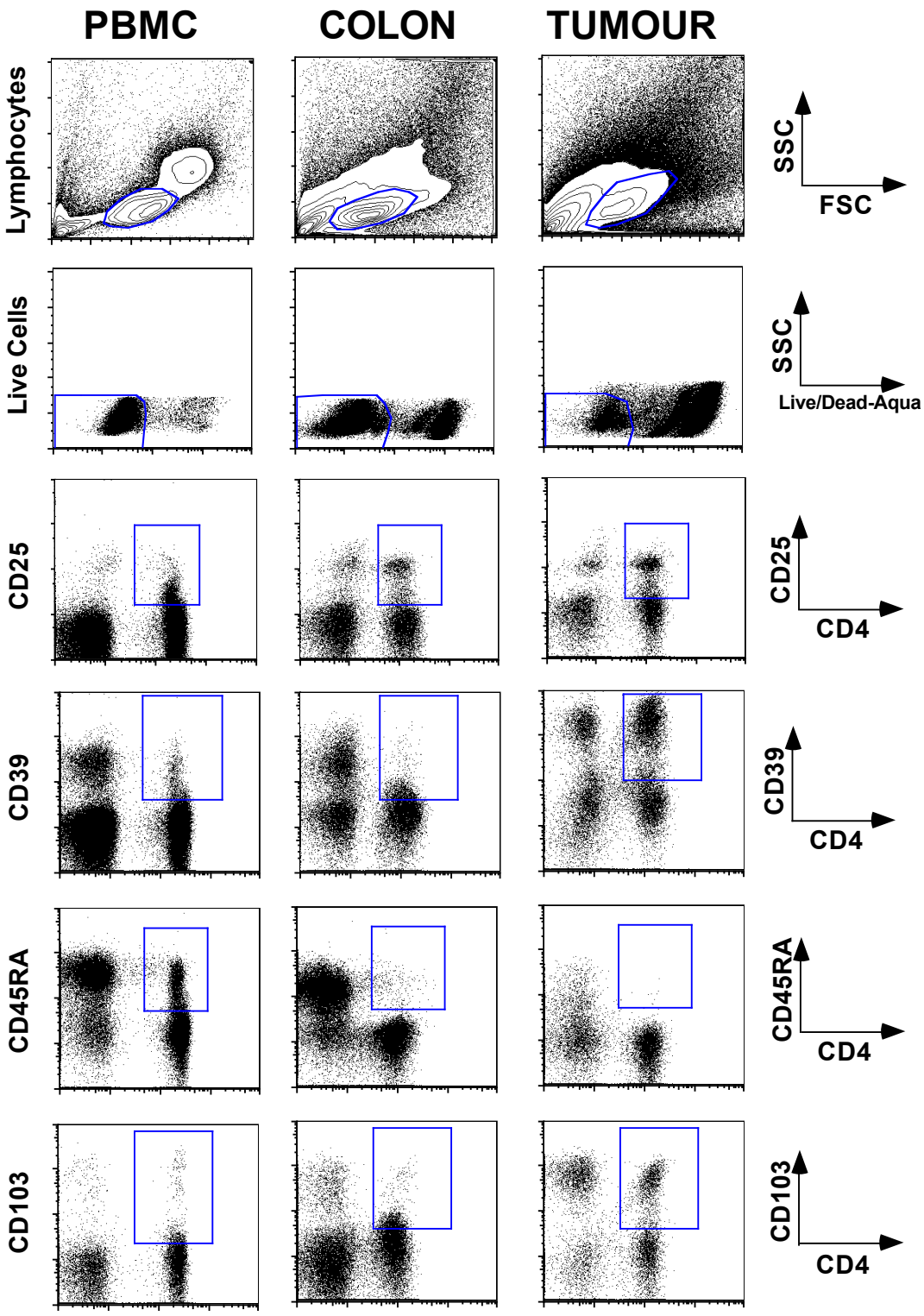


Figure 2.4 Representative flow cytometry plots showing expression of the indicated markers on $CD4^+$ T cells in blood (PBMC), unaffected colon and tumour samples.

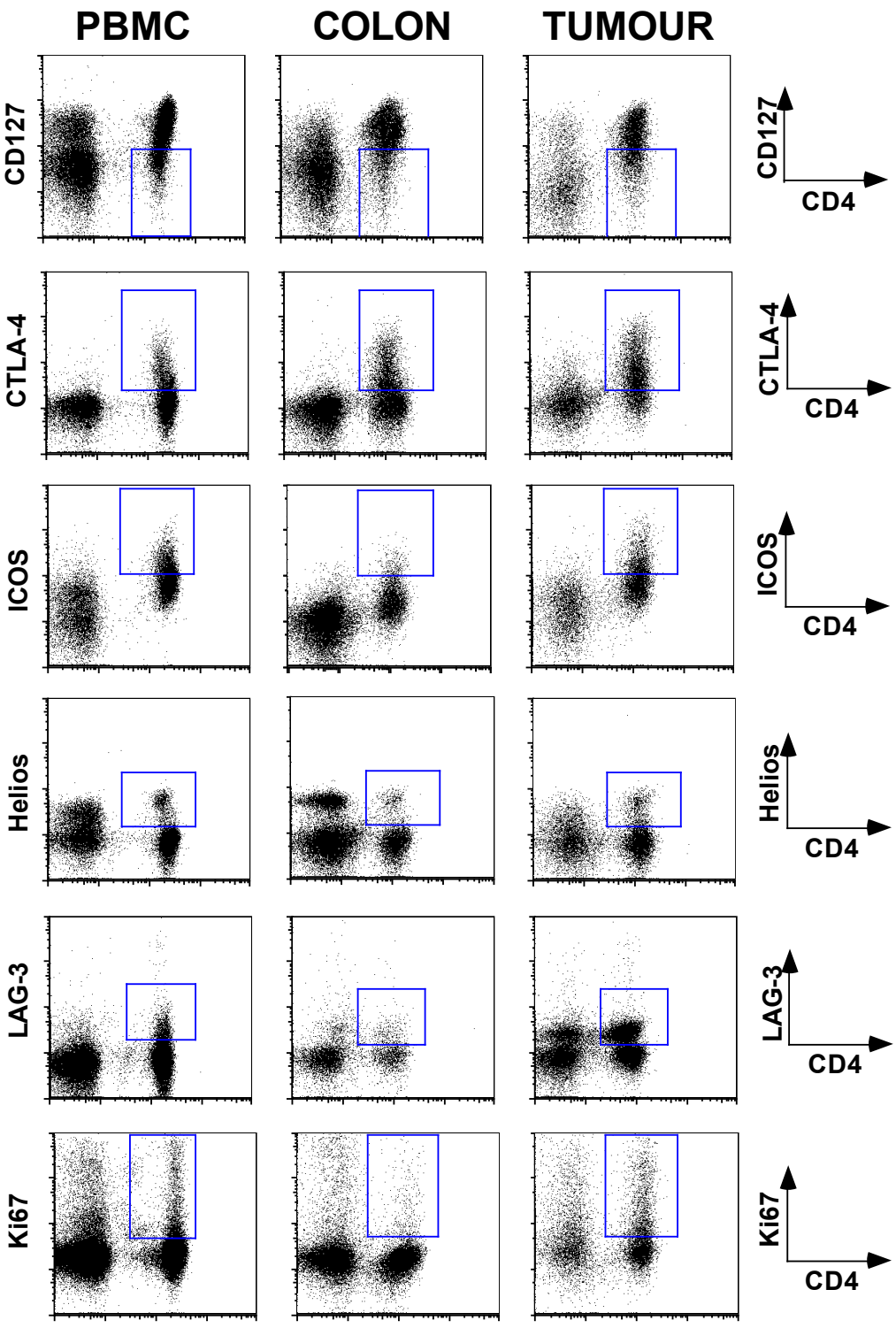


Figure 2.4 Cont'd. Representative flow cytometry plots showing expression of the indicated markers on $CD4^+$ T cells in blood (PBMC), unaffected colon and tumour samples.

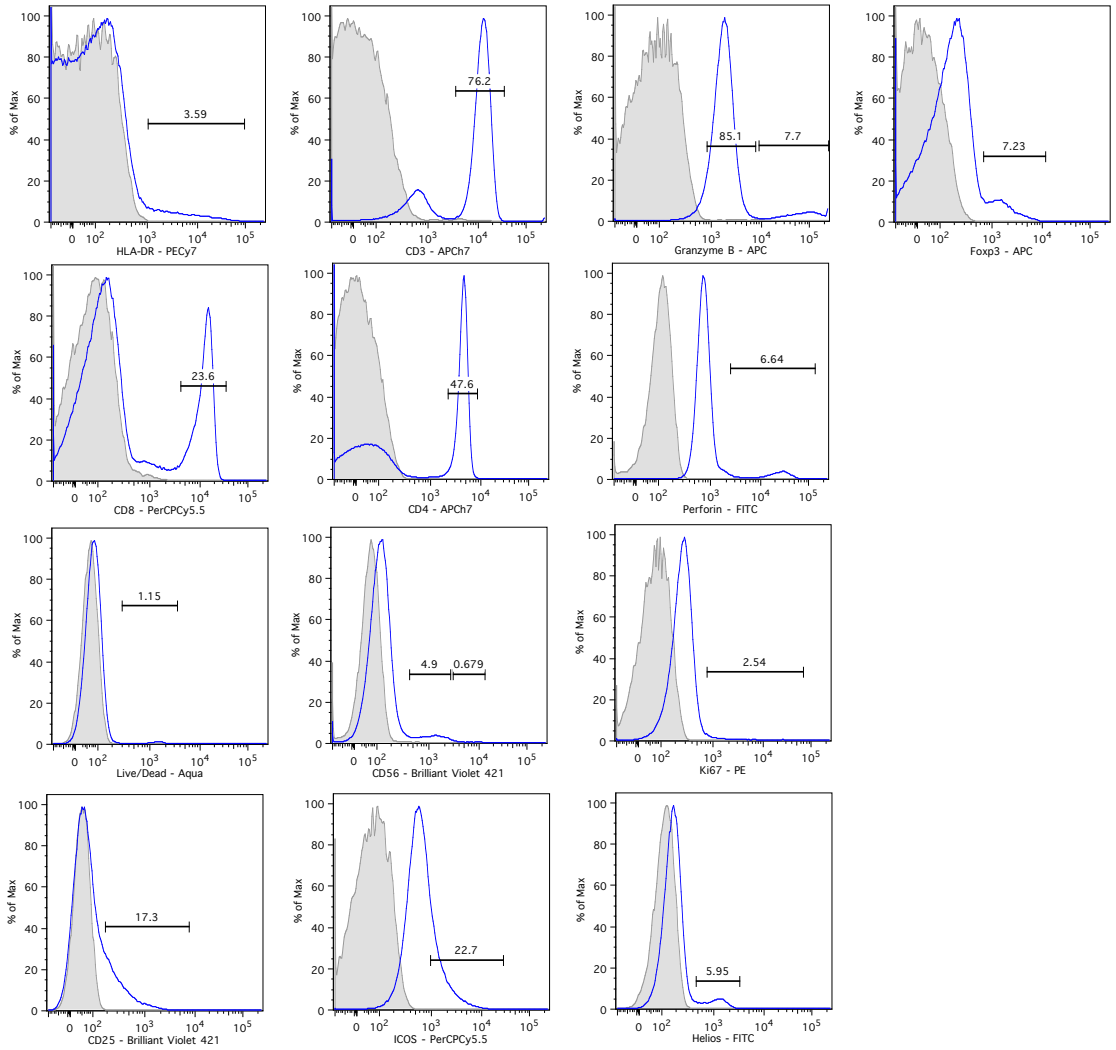


Figure 2.5 FMO Controls for commonly used fluorescently conjugated antibodies. Blue line indicates fluorescent staining of that particular antibody with concurrent use of all eight fluorescence channels on a BD FACSCanto II. Solid grey indicates the fluorescence minus the indicated antibody with concurrent use of the seven other fluorescence channels.

shown (Figure 2.4). Gates were drawn based on fluorescence-minus-one (FMO) controls (Figure 2.5).

2.9.2 MHC Class II Tetramer Staining

For MHC Class II tetramer staining of PBMC / T cell samples, cells were washed twice in ice-cold PBS. On the final wash step, cell pellets were resuspended in 0.1-0.5 μg tetramer / $1x10^6$ cells and incubated for 20 minutes at 4^oC in the dark. Cells were washed a further two times in ice-cold PBS. Live/dead viability and cell surface / intracellular stains were then carried out as described above, with the exception that the viability dye was incubated at 4^oC.

2.9.3 Activation for Intracellular Cytokine Analysis

In some instances, cells were stimulated with 20 nM PMA (Sigma-Aldrich) and 1 μg/ml Ionomycin (Sigma-Aldrich) for 4 hours at 37^oC. After 1 hour, 1 μl/ml GolgiStop (containing monensin; BD Pharmingen) was added and the cells then subjected to intracellular cytokine staining using the antibodies shown below (Table 2.6).

2.10 5T4 Immunohistochemistry

Fresh tissue samples obtained from surgery were immediately embedded in OCT compound, frozen in liquid nitrogen and stored in -80^oC freezers until use. 5μm thick sections were cut, placed on slides and fixed in acetone or 4% PFA. Slides were incubated with 1.5μg/ml primary anti-5T4 antibody (H8; Oxford Biomedica) overnight at 4^oC, alongside a mouse IgG1 negative control antibody (BD). Following

wash steps, DAB solution was added for 10 minutes, before counterstaining with haematoxylin. Slides were finally dehydrated through graded alcohols before viewing on a microscope.

2.11 Statistical and Graphical Analysis

GraphPad Prism Version 5 was used for all statistical analyses. All results are expressed as mean values together with the standard error of the mean where appropriate. Paired *t*-tests were used to compare data obtained from matched blood, colon and tumour tissue from the same patient. Unpaired *t*-tests were used for all other comparisons.

Initial analysis of flow cytometry data was performed using FlowJo version 9.3. Analyses and presentation of T cell subset distributions were performed by creating combinatorial Boolean gates to six parameters using Pestle software version 1.7. This data was imported into SPICE version 5.1, downloaded from http://exon.niaid.nih.gov (Roederer, Nozzi et al. 2011), to create graphical pie charts with arcs.

Table 2.5: Human Antibodies – Cell Surface Stains

Antigen	Conjugate	Clone	Company	Final Conc. (µg/ml)
CD3	PerCPCy5.5	UCHT1	BioLegend	2
CD3	APCh7	SK7	BD	2
CD4	Pacific Blue	RPA-T4	BD	3
CD4	PECy7	RPA-T4	BioLegend	2
CD4	PE	RPA-T4	BioLegend	2
CD4	APCh7	RPA-T4	BD	2
CD4	APC	RPA-T4	Miltenyi-Biotec	2
CD4	Brilliant-Violet 421	RPA-T4	BioLegend	2
CD8	FITC	SK1	BioLegend	2
CD8	PerCPCy5.5	RPA-T8	eBioscience	2
CD25	PE	M-A251	Miltenyi-Biotec	5
CD25	PECy7	BC-96	BioLegend	5
CD25	PECy7	M-A251	BD	6
CD25	APCh7	M-A251	BD	6
CD39	PECy7	eBioA1	eBioscience	3
CD45RA	PerCPCy5.5	HI100	eBioscience	3
CD45RO	PE	UCHL1	BD	3
CD49d	PE	9F10	BioLegend	4
CD56	Brilliant-Violet 421	HCD56	BioLegend	3
CD56	Alexa-Fluor 647	HCD56	BioLegend	3
CD103	PECy7	B-Ly7	eBioscience	2
CD107a	Pacific Blue	H4A3	BioLegend	4
CD127	PECy7	eBioRDR5	eBioscience	3
HLA-DR	PerCPCy5.5	L243	BioLegend	2
HLA-DR	PECy7	L243	BioLegend	2
LAP	PerCPCy5.5	BG/hLAP	BioLegend	4
LAP	Brilliant Violet 421	TW4-2F8	BioLegend	3
LAP	PECy7	TW4-2F8	BioLegend	3
ICOS	FITC	C398.4A	BioLegend	4
ICOS	PerCPCy5.5	C398.4A	BioLegend	4
PD-1	PerCPCy5.5	EH12.2H7	BioLegend	3
Tim-3	PE	F38-2E2	BioLegend	4
TGF-β	PE	9016	R&D Systems	5
LAG-3	PE	Polyclonal	R&D Systems	4

Table 2.6: Intracellular Stains

Antigen	Conjugate	Clone	Company	Final Conc. (μg/ml)
BCL-2	FITC	6C8	BD	2
Ki67	PE	20Raj1	eBioscience	1
CTLA-4	PE	BNI3	BD	2
Helios	FITC	22F6	BioLegend	2
Helios	Pacific Blue	22F6	BioLegend	2
Foxp3	APC	236A/E7	eBioscience	6
Foxp3	Alexa-Fluor 647	259D	BioLegend	6
TGF-β	PE	9016	R&D Systems	5
IFN-γ	FITC	4S.B3	BioLegend	5
IL-10	PECy7	JES3-9D7	BioLegend	6
IL-17	APCCy7	BL168	BioLegend	6

Chapter 3 – T cell responses to the oncofoetal antigen 5T4

3.1 Introduction

Tumour-associated antigens are common proteins that are markedly up-regulated in neoplastic cells, or proteins that are expressed mainly or solely in neoplastic cells i.e. tumour-specific antigens. The latter is an attractive group to target for therapy, as there should be limited cross reactivity to healthy tissue. The trophoblast cell surface glycoprotein 5T4 has a restricted expression to several human carcinomas including colorectal cancer (Starzynska, Rahi et al. 1992); this tumour-specific antigen lends itself as a candidate target to study tumour-directed immune responses for the purpose of improving tumour immunotherapy.

We have previously identified *ex vivo* $CD4^+$ T cell responses to 5T4 in approximately 30% of CRC patients awaiting surgical resection (Clarke, Betts et al. 2006; Betts, Jones et al. 2011). Despite this work, many questions remain over the role of $CD4^+$ T cell responses to tumour antigens. Are they present in healthy subjects and if so, does increasing age, or gender, affect these responses? Do they impede tumour growth, or does progressive tumour growth impinge on these responses, through mechanisms of tolerance, anergy, deletion or regulation?

To address some of these fundamental questions, this chapter describes the $CD4^+$ T cell responses from patients awaiting surgical resection of a primary colorectal adenocarcinoma. Peripheral blood-derived 5T4-specific T cell responses were analysed and compared to age-matched healthy donors for the breadth and magnitude of response to the tumour-associated antigen 5T4 using an overlapping peptide pool covering the entire protein. A particular emphasis was placed on determining how the range of epitopes recognized in HLA-typed populations, and the

magnitude of each epitope-specific T cell response, compared to colorectal tumour stage.

This chapter also explores data obtained from the phase II clinical trial, TaCTiCC (TroVax® and Cyclophosphamide Treatment in Colorectal Cancer). This trial utilizes the MVA-based vaccine TroVax®, which partly relies upon effective stimulation of 5T4-specific T cells to mediate anti-tumour immune responses (Harrop, Drury et al. 2007). A recent report has demonstrated the ability of 5T4-specific cytotoxic $CD8^+$ T cells to induce tumour cell death (Al-Taei, Salimu et al. 2012), outlining the potential for enhancing anti-5T4-specific responses in cancer patients. However, the clinical benefit of such an approach may be hampered by regulatory T cells, which are capable of suppressing effector 5T4-specific T cells when stimulated *in vitro* (Clarke, Betts et al. 2006). Indeed in mice challenged with 5T4-positive tumours followed by vaccination with a 5T4-encoding adenovirus, significant reduction in tumour burden was only noted alongside concurrent Treg depletion (Castro, Al-Muftah et al. 2011). In the trial presented here, low-dose cyclophosphamide is given to patients prior to TroVax® vaccination in an effort to reduce Treg numbers. The primary endpoint at week 7 is to achieve increased anti-5T4 immune responses after TroVax® and cyclophosphamide treatment, with a secondary endpoint of reduction in tumour burden. The preliminary experiments carried out here are designed to establish whether such immunotherapeutic strategies might be capable of enhancing anti-tumour immunity.

3.2 Results

3.2.1 Antigen-specific T cell expansion in culture

In order to analyse T cell specificity for 5T4 epitopes, short-term T cell cultures were established, as the low frequency of responses previously measured *ex vivo* renders detailed characterization of the response difficult. The effect of culturing PBMC with T cell epitopes was initially demonstrated using a known universal influenza haemagglutinin epitope (HA (305-320); "Flu 1"), which HLA-DR1^{+} (DRB1*01) individuals readily respond to. The C-terminal peptide-flanking region was also modified in an effort to enhance TCR binding affinity and T cell activation ("Flu 3") as previously described (Cole, Gallagher et al. 2012). Utilising IFN-γ ELISpot, very low-level Flu-specific IFN-γ T cell responses are found *ex vivo* (5-15 SFC/10^5 PBMC) (Figure 3.1A). This is mirrored by tetramer staining to the same epitope (Figure 3.1B). However, once the same PBMC fraction is cultured with the peptide for a 12-day period, flu-specific T cells undergo activation and clonal expansion *in vitro* to levels which become readily detectable by IFN-γ ELISpot and FACS (100-250 SFC/10^5 cultured PBMC). The same principles were applied to investigate 5T4-specific T cell responses in CRC patients.

3.2.2 5T4-specific T cell responses in CRC patients

PBMC isolated from HLA-typed CRC patients, awaiting surgical resection of a primary colorectal tumour, were stimulated with pools of 20mer 5T4 peptides spanning the entire protein (Tables 2.3 and 2.4). Initially, cells were subjected to *ex vivo* IFN-γ ELISpot to identify 5T4 epitopes, however, responses were rarely detected

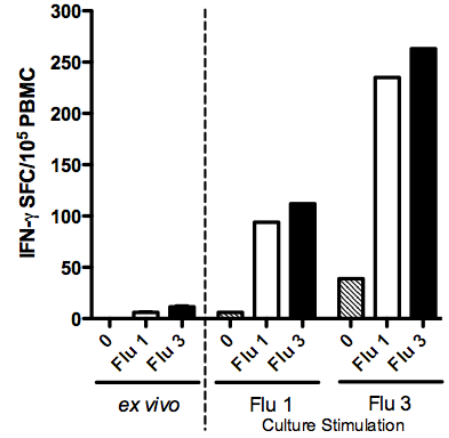
(2/16). Where responses were identified, these were of very low frequency (<10 SFC/10^5 PBMC), an example of which is shown in CRC patient 'MS16' (Figure 3.2).

5T4-specific T cells were detected more readily by IFN-γ ELISpot at day 12 of culture. Representative experiments from three CRC patients are shown (Figure 3.3). For patient 'MS209', peptide pools 1, 3, 7 and 13 were positive suggesting candidate peptides containing epitopes are peptides 1, 3, 37 and 39 (see matrix, Table 2.4). Some of these responses were robust, reaching frequencies of > 200 T cells / 10^5 cultured cells, enabling further analysis. However, some patients such as 'MS201' failed to mount any sizeable response and no definitive epitope could be detected (Figure 3.3A). Reasons for this will be explored later in the chapter. In total, 82% (22/27) of CRC patients tested produced an identifiable 5T4-specific T cell IFN-γ response to at least one 5T4 epitope.

3.2.3 5T4 responses by T cells isolated from different tissues

Although 5T4-specific T cells are identifiable amongst peripheral blood derived mononuclear cells, it is not known whether these cells can also be detected in other tissues. Lymphocytes were isolated from freshly resected colon specimens, which included, where possible, the colonic mucosa, tumour draining lymph nodes and the colorectal tumour. These lymphocytes were subjected to a 12-day culture period with 5T4 peptide pool-pulsed autologous irradiated PBMC (as APCs) and tested for antigen-specific IFN-γ responses. In most cases (6/7), no 5T4 responses were detected amongst tumour-infiltrating lymphocytes. In one case (CRC patient MS14), a 5T4 response to peptide pool 13 was detected in three compartments of blood, TDLN and tumour and an additional response to peptide pool 3 in blood

A:



B:

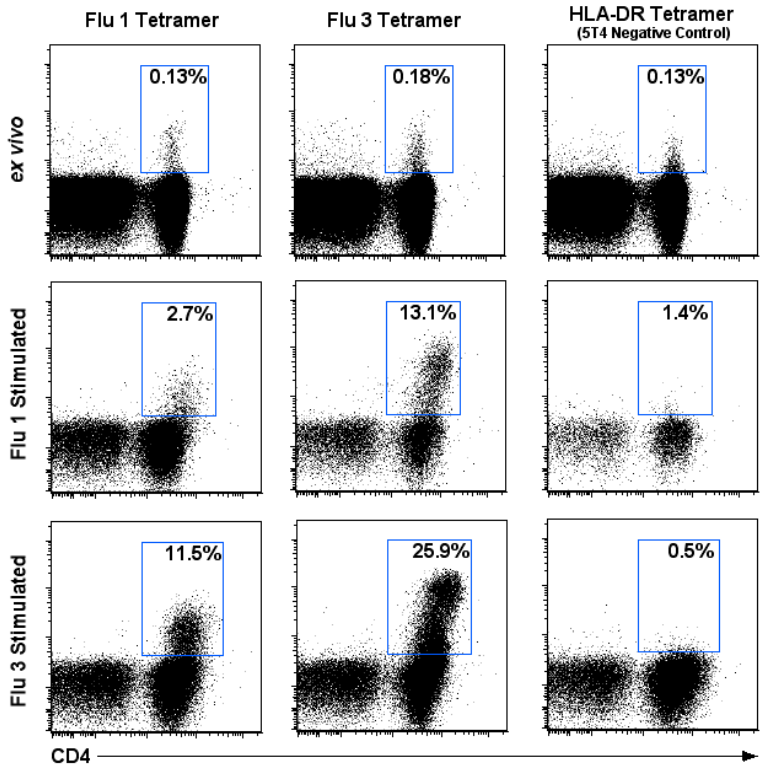


Figure 3.1 *In vitro* peptide stimulation of PBMC enriches for peptide specific CD4$^+$ T cells after 12 days in culture. Freshly isolated PBMC from an HLA-DR1$^+$ donor were stimulated with wild-type (Flu-1) or modified (Flu-3) HA(305-320) peptides and analysed by IFN-γ ELISpot for the number of IFN-γ$^+$ T cells (per 105 cells) both *ex vivo* (overnight stimulation) and following a culture period of 12 days *in vitro* (see Methods section 2.5, page 61 for details) (A). The same cells were also stained with class-II tetramers specific for the flu peptides *ex vivo*, or following the 12 day period of culture ('Flu 1 / Flu 3 Stimulated') (B). Data is representative of three DR1$^+$ donors.

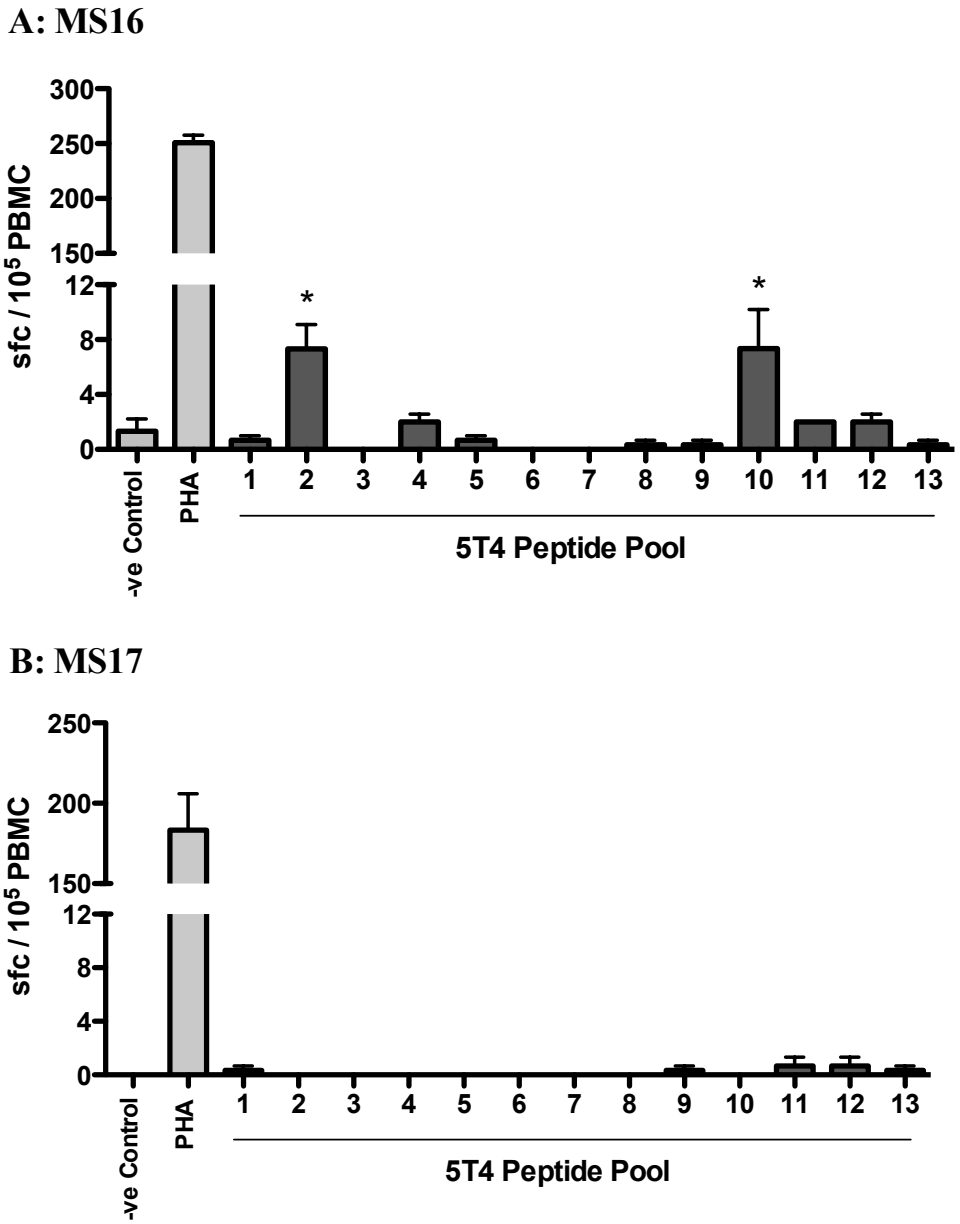


Figure 3.2 5T4-specific IFN-γ^+ T cell responses amongst PBMC are rarely detected *ex vivo*. Freshly isolated PBMC from 16 CRC patients have been assessed for the number of IFN-γ^+ T cells (per 10^5 PBMC) following overnight stimulation with the 5T4 peptide pools (see Table 2.4, page 60 for 5T4 peptide pools and matrix system). Examples shown represent a positively identified (A: CRC patient 'MS16') and negative (B: CRC patient 'MS17') response. (* Indicates positive response; sfc = spot forming cell).

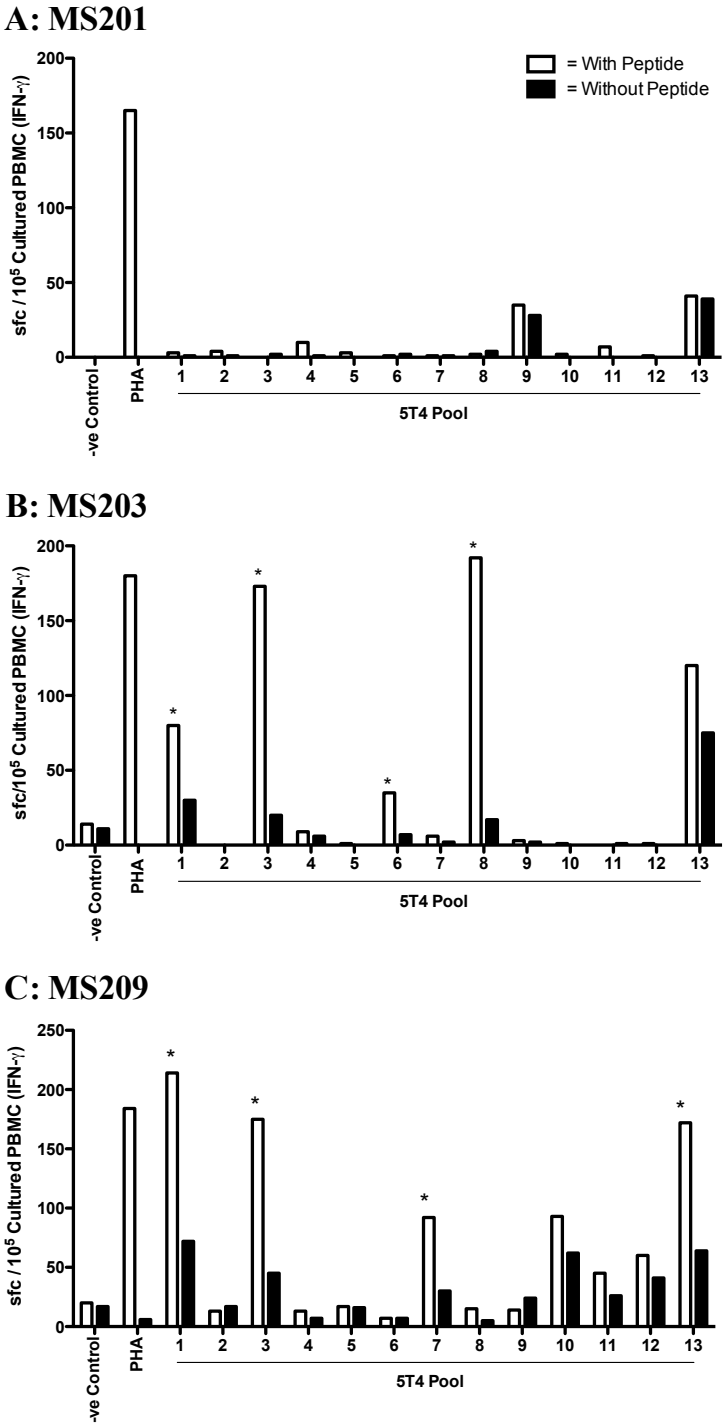


Figure 3.3 12-day *in vitro* culture of PBMC with 5T4 peptide pools enriches for measureable 5T4-specific T cell responses using IFN-γ ELISpot. Freshly isolated PBMC from 27 CRC patients were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured PBMC) specific for the 13 5T4 peptide pools, representative examples of which are shown (A: MS201, B: MS203, C: MS209; * indicates positive response; sfc = spot forming cell).

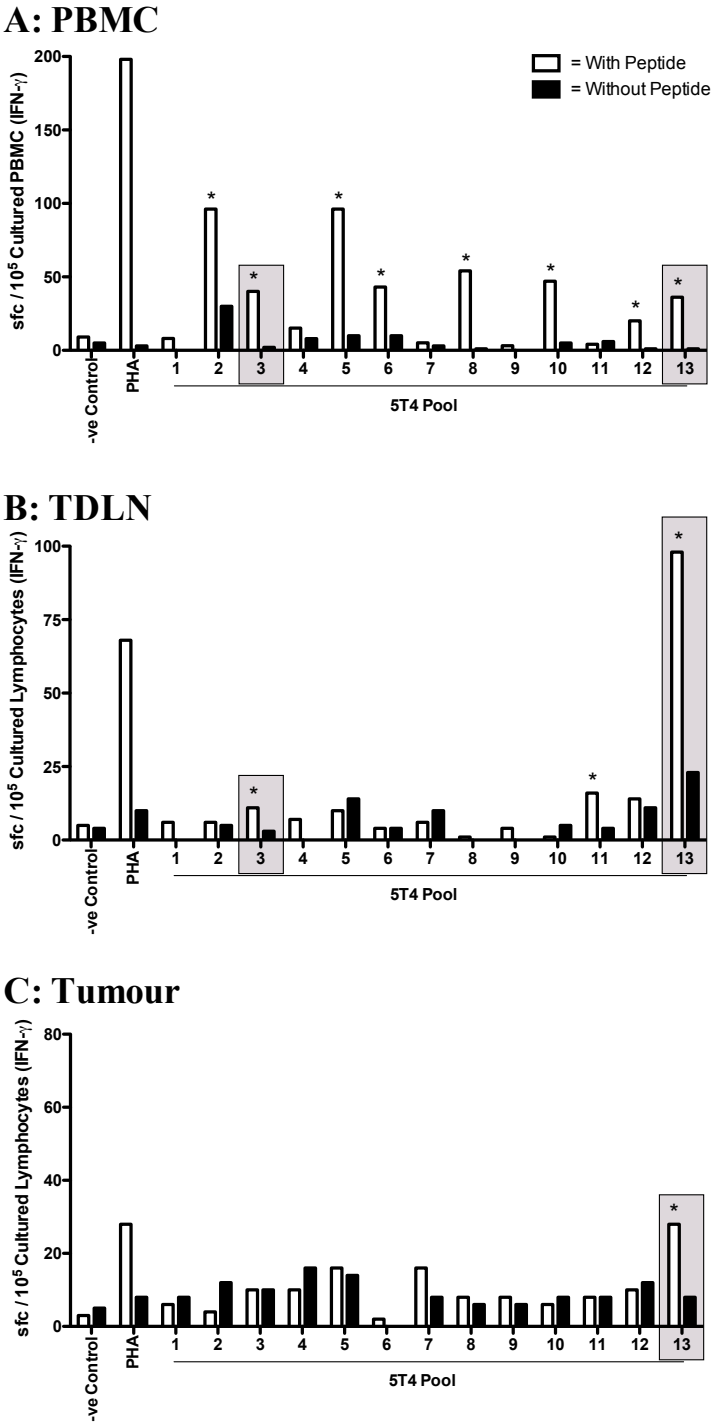


Figure 3.4 T cells from different compartments respond to similar 5T4 epitopes in a CRC patient. Freshly isolated lymphocytes isolated from peripheral blood (A), tumour-draining lymph node (B) and colorectal tumour (C) from CRC patient MS14 were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured lymphocytes were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Highlighted results indicate similar 5T4 epitope responses amongst T cells from different tissues. (* indicates positive response; sfc = spot forming cell)**.**

and TDLN, implicating peptide 39 as being an immunogenic epitope in this patient (Figure 3.4).

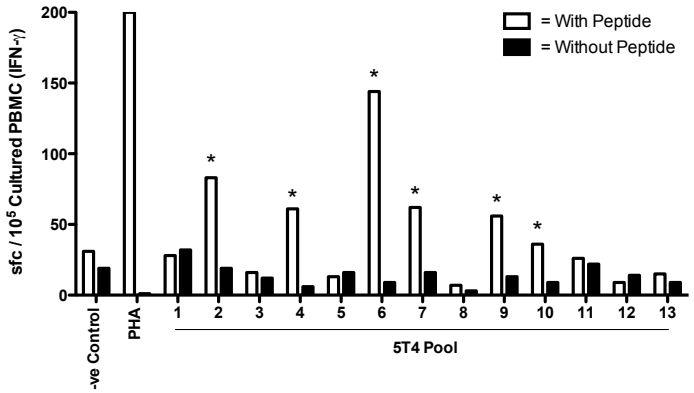
3.2.4 5T4 T cell responses in healthy donors

PBMC isolated from HLA-typed age-matched healthy donors were stimulated and cultured with the 5T4 peptide pools before analysis at day 12, as before. Representative experiments from three healthy donors are shown (Figure 3.5). These results were initially surprising, given the absence of *ex vivo* 5T4 responses in healthy donors found previously (Clarke, Betts et al. 2006); indeed healthy donors appeared to mount equal / superior cultured responses than CRC patients. In total, 100% (17/17) of healthy donors tested produced positive IFN-γ responses to ≥ 2 5T4 peptide epitopes, defined as two peptides that do not overlap and could individually contain putative epitopes.

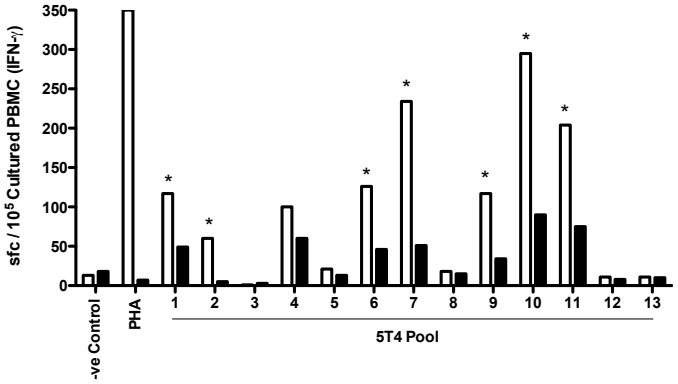
3.2.5 Identifying MHC Class-II restricted 5T4-derived epitopes

When two positive responses to 5T4 peptide pools implicate an epitope, restimulating cultured cells can test the immunogenicity of this single peptide (example shown Figure 3.6). Alongside this, it is possible to test for a CD4 / CD8 response by using blocking antibodies to HLA class-I (W6/32) or HLA class-II (HLA-DR; L243 or HLA-DQ; 1A3). The vast majority of IFN-γ responses were found to be restricted to $CD4^+$ T cells, probably reflecting the length of peptides used to stimulate the cells.

A: HD202



B: HD221



C: HD226

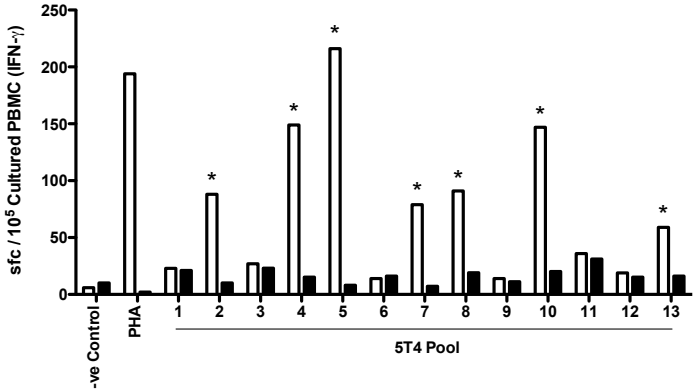


Figure 3.5 5T4-specific T cell responses are readily detectable in age-matched healthy donor controls following short-term culture. Freshly isolated PBMC from 17 healthy donors were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools, representative examples of which are shown (A: HD202, B: HD221, C: HD226; * indicates positive response; sfc = spot forming cell).

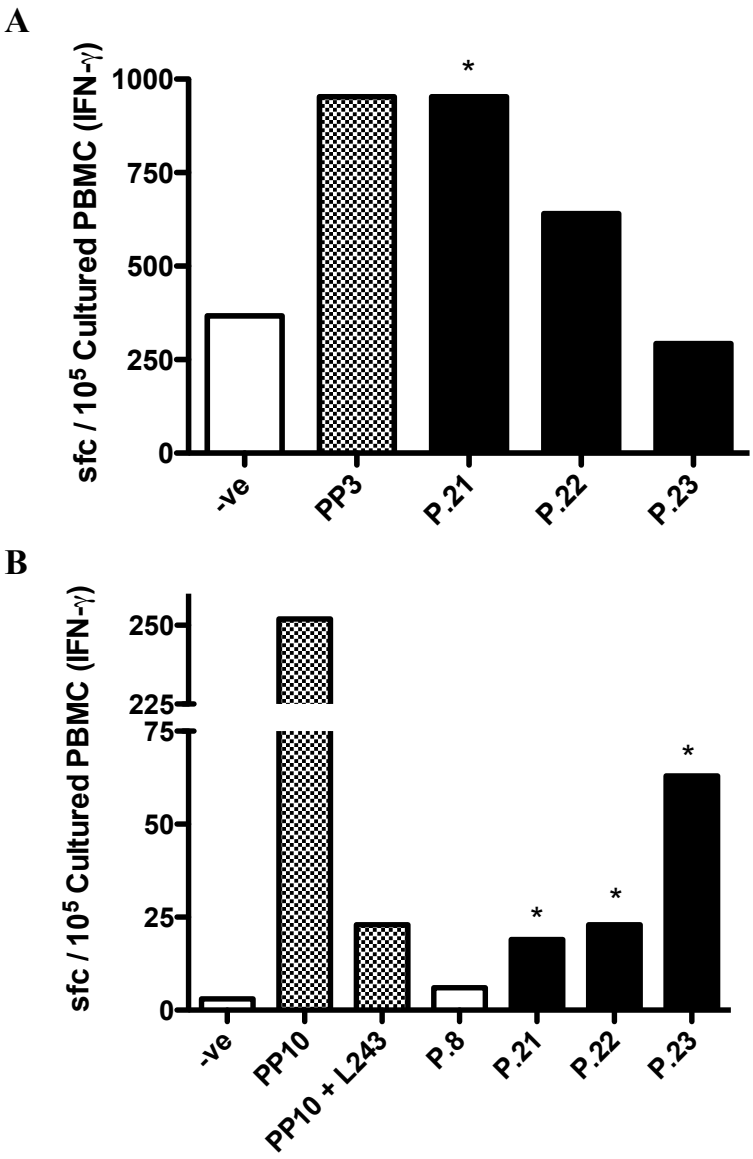


Figure 3.6 Peptide-specific CD4$^+$ T cell responses identified after culturing PBMC with 5T4 peptide pools. Freshly isolated PBMC from CRC patient MS24 were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Having identified positive 5T4 responses to peptide pools 3 (A) and 10 (B), cell lines were restimulated with corresponding peptides to identify specific epitopes. In this case, CD4$^+$ T cells produced IFN-γ in response to peptides 21-23. This epitope is HLA-DR restricted as addition of the HLA-DR blocking antibody, L243 resulted in a 90% decrease in response to peptide pool 10. **(*** Indicates positive response; sfc = spot forming cell).

3.2.5.1 Generation of $CD4^+$ 5T4-specific T cell clones

5T4-specific T cell clones were created from a number of healthy donors and CRC patients, as previously described (Methods section 2.7). IFN-γ ELISpot was utilized to identify T cell clones, which became activated upon recognition of their cognate antigen. Cloned T cells were also FACS stained to check T cell sub-type; as expected, given the length of peptide used to stimulate these cells, all clones were $CD4^+$ T cells (Figure 3.7). Where HLA-restriction was also definitively identified, this has been shown in red.

Two $CD4^+$ T cell clones, which produced the most robust anti-5T4 responses, were expanded and analysed in more detail. Examples derived from a CRC patient (Figure 3.8A) and a healthy donor (Figure 3.8B) are shown. T cell clones were $CD4^+$ and restricted by HLA-DR antigens (fine restriction mapping using matched/mismatched APCs showed HLA-DR1 (DRB1*01) in Figure 3.8A; HLA-DR4 (DRB1*04) in Figure 3.8B). Predicted epitope sequences were identified using epitope prediction software, which analysed the ligation strength for the sequence of amino acids to the specific MHC class-II structure.

Addition of whole 5T4 protein pulsed autologous irradiated APCs revealed the natural presentation of these 5T4 epitopes resulting in activation and IFN-γ production by these clones. As with other $CD4^+$ T cell clones/lines previously tested in the lab (Godkin, Jeanguet et al. 2001; Gallagher, Lauder et al. 2009), they were also able to produce IL-10 after peptide stimulation, a possible result of repeat TCR triggering of these cells, employed as a mechanism to control excessive immune responses (Saraiva, Christensen et al. 2009).

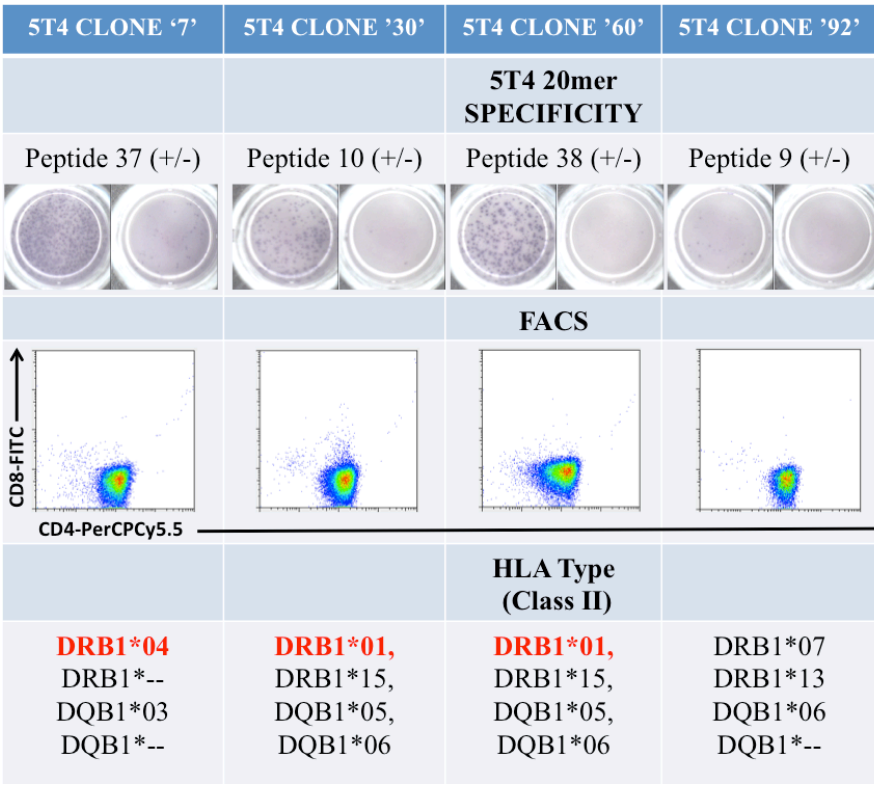


Figure 3.7 5T4-specific T cell clones. Clones were generated from a number of healthy donors and CRC patients, examples of which are shown. See Methods section 2.7, page 63 for information on the generation and cloning of 5T4-specific T cells). Clones were screened for 5T4 reactivity by IFN-γ ELISpot and analysed by FACS to identify T cell type. Where possible HLA-restriction was calculated by pulsing matched / mis-matched APCs with the specific 5T4 peptide before stimulating the T cell clone to test for reactivity (identified in red).

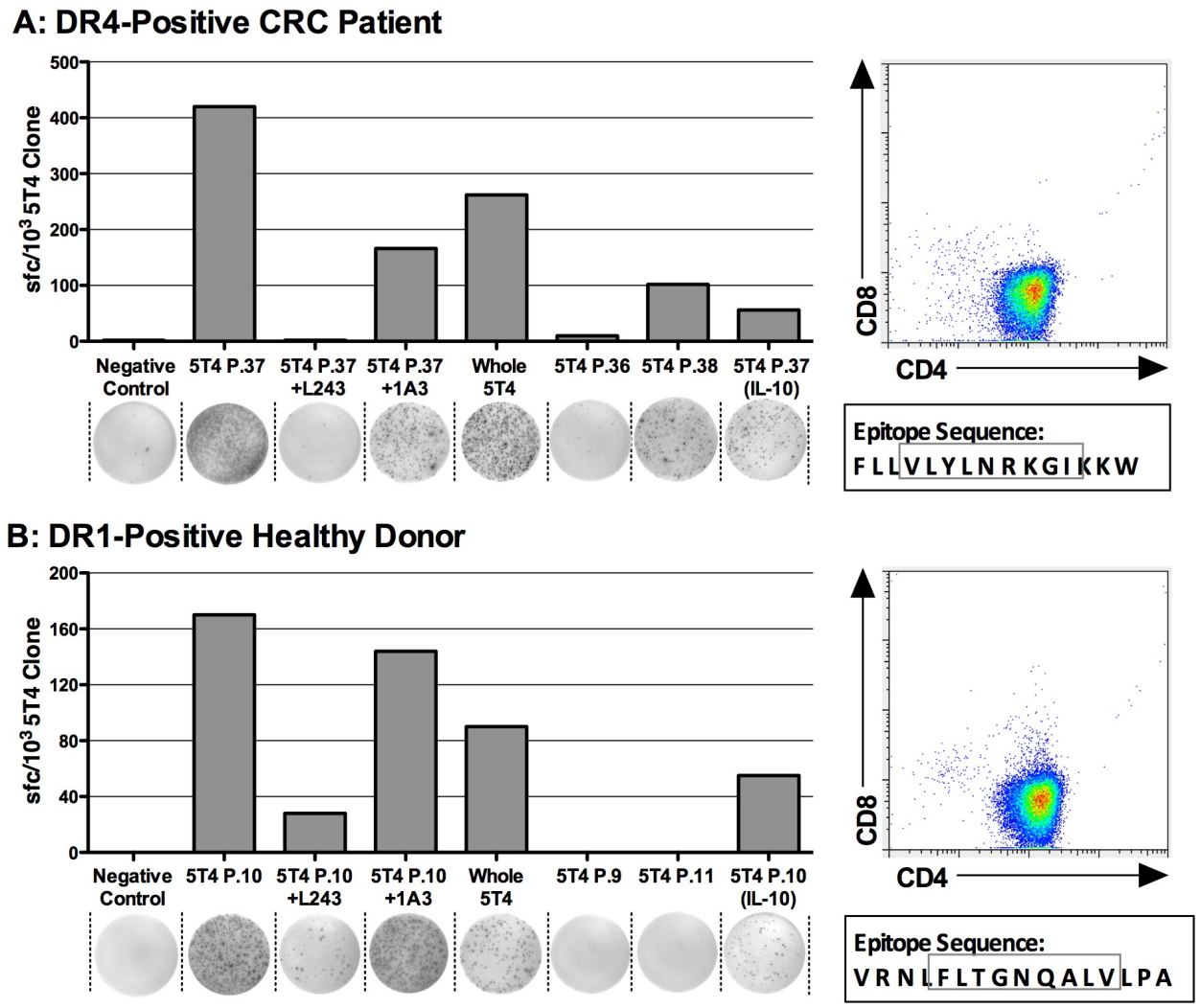


Figure 3.8. Cultured 5T4-specific CD4$^+$ T cells can be cloned from CRC patients (A) **and healthy donors** (B)**.** Graphs show IFN-γ / IL-10 production, detected using ELISpot, by 5T4-specific T cell clones co-cultured overnight with 5T4 peptide / protein-pulsed irradiated autologous PBMC in various indicated conditions. Results demonstrate the number of spot forming cells per 1000 5T4 clonal T cells plated. CD4 against CD8 FACS plots of the clone are shown (gated on live cells).

3.2.6 Comparison of 5T4-specific T cell responses in CRC patients and healthy donors

5T4 ELISpot data from all individuals (number of spot forming cells per 10^5 cultured PBMCs) were used to calculate the total summated response to all 5T4 peptides and the average response per 5T4 epitope. To distinguish between putative epitopes, distinct responses were defined as individual responses to non-overlapping peptides, i.e. responses to overlapping peptides were defined as containing one putative epitope. If doubt remained the lines were tested against individual peptides. The total response to the 5T4 peptide pools was significantly diminished in patients who were subsequently identified as having tumours which had penetrated the serosal surface of the bowel at operation and invaded local nymph nodes (i.e. Dukes' C or TNM stage 3+), as shown in Figure 3.9 (Dukes' A vs. Dukes' C CRC patients 548.1 ± 116.2 vs. 210.1 ± 72.73; $p = 0.017$) and concordantly between T1 and T3-graded CRC patients (607.0 ± 163.4 vs. 258.4 ± 70.5; $p = 0.041$).

Very similar findings were obtained when comparing 5T4 responses on a per epitope response basis (Figure 3.10). Healthy donors demonstrated superior responses to patients with increasingly advanced tumours (HD vs. Dukes' C: 127.4 ± 13.1 vs. 48.54 ± 15.8; $p = 0.0006$ and HD vs. T3: 127.4 ± 13.1 vs. 46.7 ± 13.4; $p = 0.0002$). Again, anti-5T4 T cell responses decreased with tumour progression, significantly between Dukes' A and Dukes' C patients (130.1 ± 26.94 vs. 48.5 ± 15.77; $p = 0.011$), T1 and T3-graded patients (120.8 ± 6.3 vs. 46.7 ± 13.4; $p = 0.011$) and even between T2 and T3-graded patients (124.7 ± 38.3 vs. 46.7 ± 13.4; $p = 0.024$).

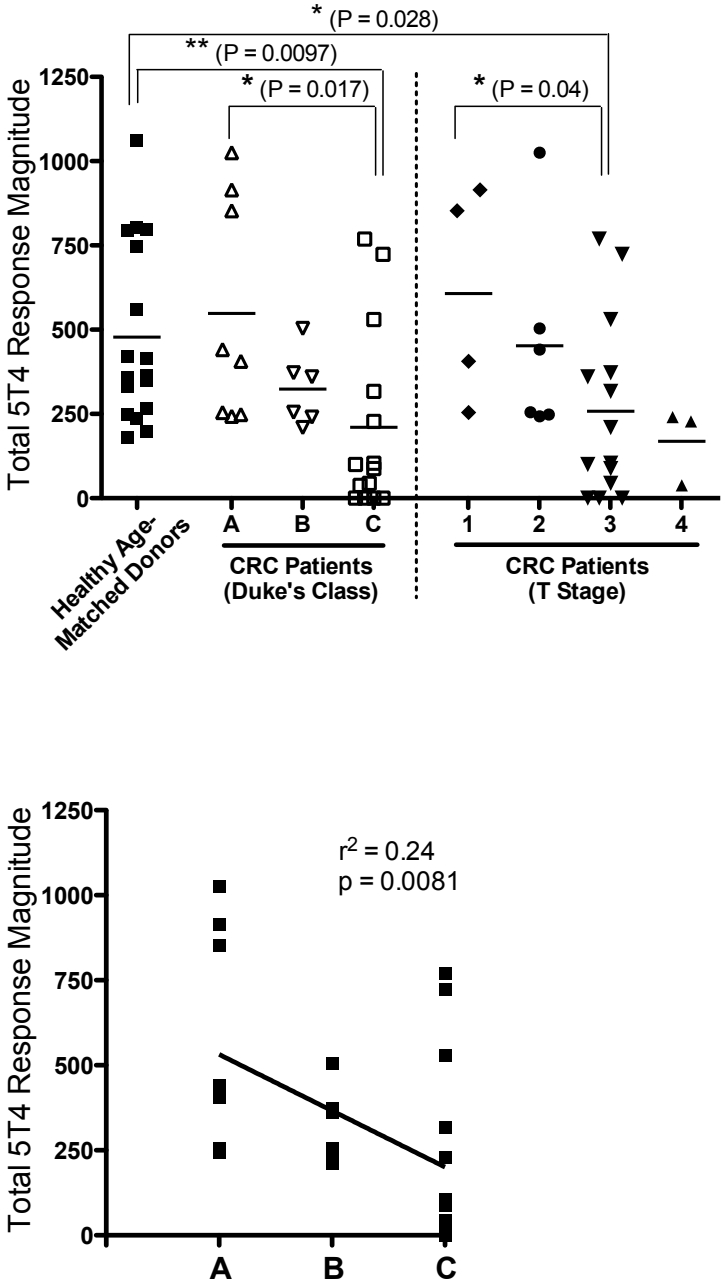


Figure 3.9 Total anti-5T4 T cell responses steadily decline in CRC patients with more advanced disease. Freshly isolated PBMC from 28 CRC patients and 16 healthy donors were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Positive responses were defined in terms of the overall number of IFN-γ producing 5T4-specific T cells to all peptide pools per 10^5 cultured PBMC (i.e. 'total 5T4 response magnitude'). Significant differences and regression analysis are indicated; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

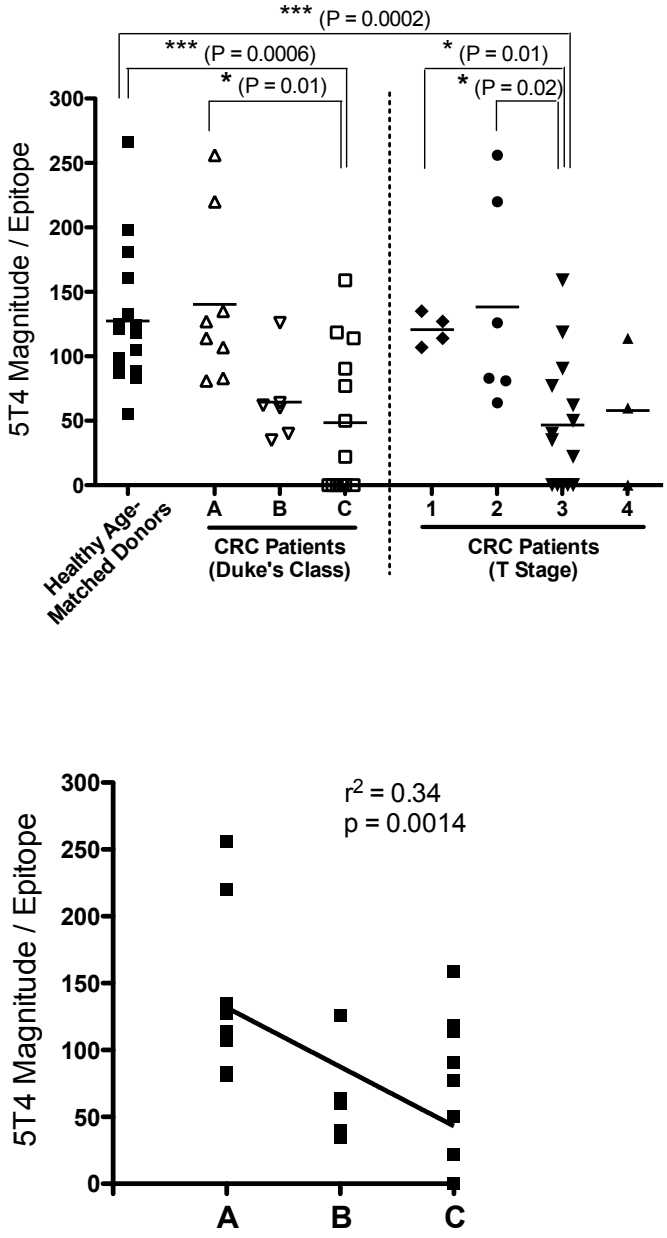


Figure 3.10 Epitope specific 5T4 T cell responses steadily decline in CRC patients with more advanced disease. Freshly isolated PBMC from 28 CRC patients and 16 healthy donors were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Positive responses were defined in terms of the average number of IFN-γ producing 5T4-specific T cells to each putative 5T4 epitope per 10^5 cultured PBMC (i.e. total magnitude divided by number of individual epitope responses ('5T4 Magnitude / Epitope')). Significant differences and regression analysis are indicated; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

These data demonstrate a steady reduction in the responsiveness of T cells to 5T4 measured by IFN-γ production in patients with increasingly advanced colorectal tumours. Despite this, T cell immunity to the recall antigen, PPD, appears unaffected (Figure 3.11). There does not appear to be non-specific immunosuppression, as we and others have previously noted (Betts, Jones et al. 2011) (Tassi, Gavazzi et al. 2008).

In the cohort of patients tested, 6 out of 13 patients with local lymph node spread produced no detectable 5T4 T cell response post-culture, whereas every patient whose tumour was contained to the bowel wall produced a 5T4 response (106.7 ± 19.1 vs. 48.5 ± 15.8; p = 0.027) (Figure 3.12A). However, no overall difference was noted between pathologically confirmed Dukes' C1 / C2 and TNM N1 / N2 graded CRC tumours (Figure 3.12B), indicating that tumour spread to the apical lymph node does not result in a further reduction in 5T4-specific T cell responsiveness.

The responses in all healthy controls (17/17) were robust, with a highly significant difference between healthy controls and patients with advanced cancer (HD vs. Dukes' C: 478.1 ± 64.3 vs. 210.1 ± 72.7; p = 0.0097 and HD vs. T3: 478.1 ± 64.3 vs. 258.4 ± 70.5; p = 0.028). At a cut off level of <200 SFCs/10^5, 0% (0/17) of healthy controls vs. 30% (8/27) of patients demonstrate such weak / absent responses (p = 0.031 Fishers' exact test; specificity 100%). Equally, with a cut off level of <75 SFCs/10^5/ 5T4 epitope, 6% (1/17) of healthy controls vs. 69% (11/27) of patients demonstrate poor responses (p = 0.0003 Fishers' exact test; specificity 94%) (Table 3.1). 12-month outcome data was available on 13 of 14 patients with Dukes' C tumours, within which these 8 patients with low level total

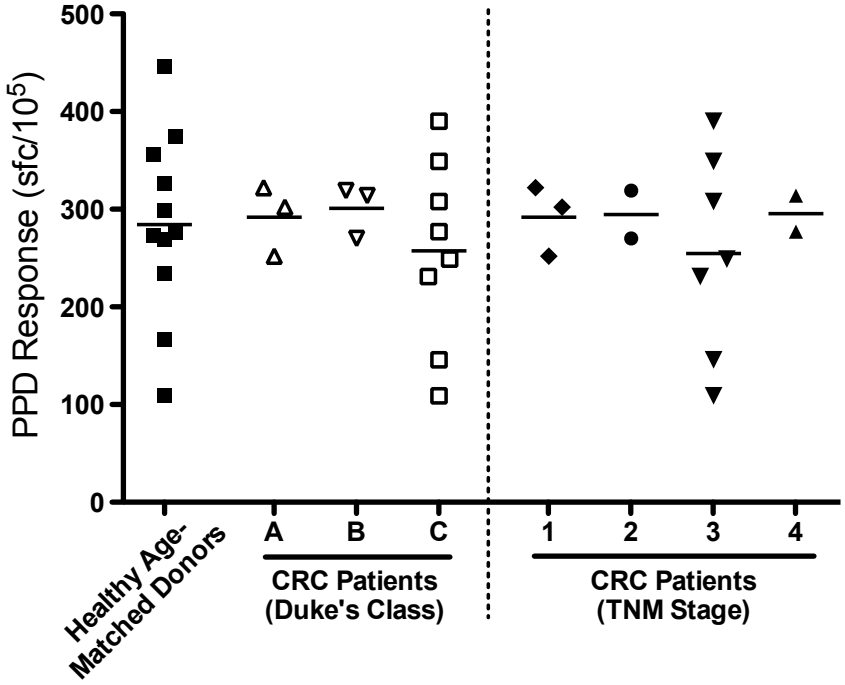


Figure 3.11 PPD-specific T cell responses remain unaffected in CRC patients. Freshly isolated PBMC from 14 CRC patients and 11 healthy donors were stimulated with a panel tuberculin PPD and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for PPD (i.e. spot forming cells – SFC).

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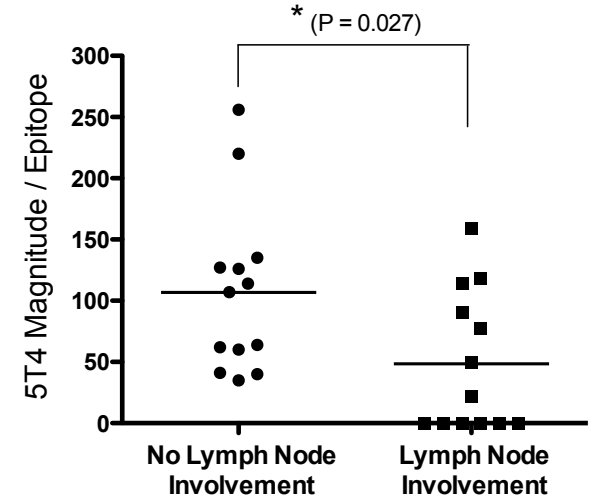

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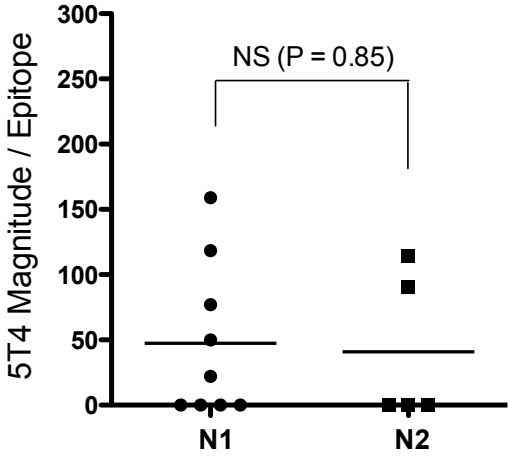

Figure 3.12 The effect of tumour spread to lymph nodes and anti-5T4 T cell responses. Freshly isolated PBMC from 26 CRC patients were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Positive responses were defined in terms of the average number of IFN-γ producing 5T4-specific T cells to each putative 5T4 epitope, per 10^5 cultured PBMC (i.e. total magnitude divided by number of individual epitope responses). This response was compared between CRC patients with and without histopathologically confirmed localized lymph node involvement (A) and between N1 and N2 graded patients (B). Significant differences are indicated; *$p < 0.05$.

Table 3.1 Statistical analysis of the difference in 5T4 T cell responses generated by healthy donor controls and colorectal cancer patients.

	TOTAL 5T4 RESPONSE	5T4 RESPONSE PER EPITOPE
Low-level response cut-off point	<200 SFCs/10^5	<75 SFCs/10^5/Epitope
Healthy Controls	0% (0/17)	6% (1/17)
CRC Patients	30% (8/27)	69% (11/27)
Fishers' Exact Test	p = 0.031; Specificity 100%	p = 0.0003; Specificity 94%

5T4 responses (<200 SFCs) reside. Five patients at 12 months had developed disease recurrence or metastatic disease, and of these patients, 80% (4/5) demonstrated low level (<200 SFCs) responses pre-operatively (Figure 3.13).

Five patients with low-level pre-operative 5T4 responses were assessed 6-18 months post-surgery. All 5 patients produced measureable increases in total IFN-γ production to all 5T4 peptides (pre-op responses; 107.6 ± 40.6 vs. post-op responses; 267.4 ± 71.4 SFCs / 10^5 cultured cells, p = 0.058) (Figure 3.14A), and 4/5 patients had increased anti-5T4 T cell responsiveness on a per-epitope basis (pre-op responses; 29.5 ± 13.0 vs. post-op responses; 95.6 ± 22.2, p = 0.038) (Figure 3.14B).

3.2.7 CRC patients have an elevated proportion of peripheral Tregs

3.2.7.1 Treg proportion and 5T4 T cell responses

Tregs have previously been shown to actively impinge on 5T4-specific anti-tumour T cell responses (Clarke, Betts et al. 2006; Elkord, Burt et al. 2008; Betts, Jones et al. 2011). It is also well documented that patients with cancer have increased frequencies of Tregs, as denoted by Foxp3 expression (Beyer and Schultze 2006). With the blood samples obtained from the same cohort of CRC patients used here for anti-5T4 T cell studies, flow cytometry was used to analyse the proportion of $CD4^+$ T cells that expressed Foxp3 to determine whether increased numbers of Tregs could correlate with the reduction in 5T4 responses. Indeed, the proportion of Tregs was most significantly increased in the PBMC of CRC patients with more advanced disease and concomitantly reduced T cell responses (Dukes' C vs. HD: 10.81% ± 0.79% vs. 7.76% ± 0.68%; p = 0.016 and T3 vs. HD: 11.42% ± 0.61% vs. 7.75% ± 0.68%; p = 0.0026) (Figure 3.15). Furthermore, the proportion of Tregs appeared to increase with

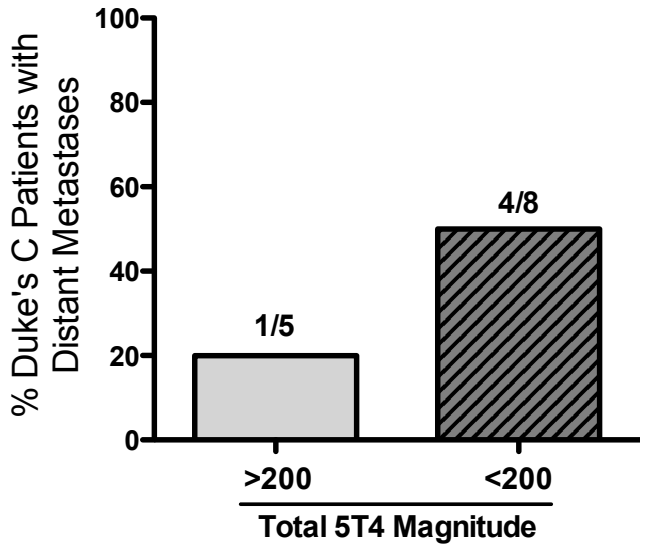

Figure 3.13 Dukes' C CRC patients with robust pre-operative 5T4 responses are less likely to develop metastatic disease. Freshly isolated PBMC, from CRC patients with histopathologically confirmed Dukes' C tumours at resection, were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Positive responses were defined in terms of the total number of IFN-γ producing 5T4-specific T cells to all positive 5T4 peptide pools, per 10^5 cultured PBMC (i.e. total magnitude). These responses were separated into two distinct groups based on whether they generated a good / poor total IFN-γ response to 5T4. A good response was considered to be a total 5T4 magnitude greater than 200 (which corresponded with a 5T4 magnitude / epitope of greater than 75). A poor response was considered to be a total 5T4 magnitude less than 120 (which corresponded with a 5T4 magnitude / epitope of less than 50). Patients were assessed at 12 months post surgery for metastatic spread and data was correlated with pre-operative anti-5T4 responses.

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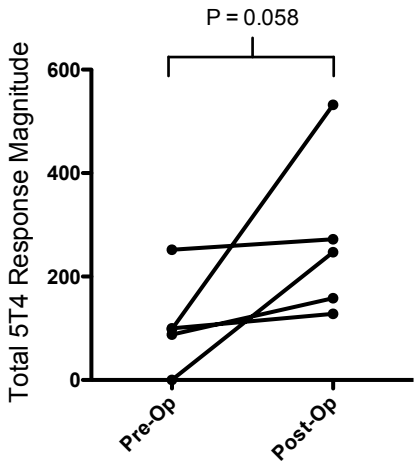

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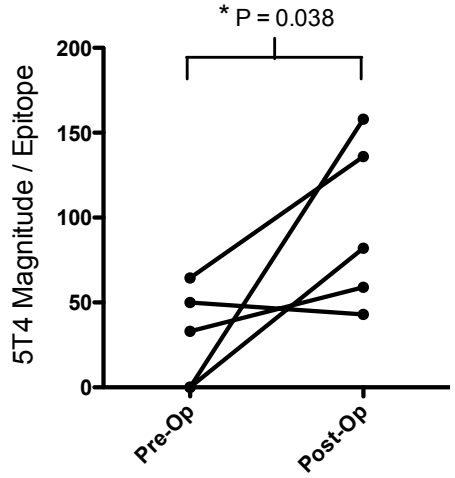

Figure 3.14 The effect of colorectal tumour resection on anti-5T4 T cell responses. Freshly isolated PBMC, from 5 CRC patients, were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Positive responses were defined in terms of the total number of IFN-γ producing 5T4-specific T cells to all positive 5T4 peptide pools, per 10^5 cultured PBMC (i.e. 'total 5T4 response magnitude') or the average number of IFN-γ producing 5T4-specific T cells to each putatively identified epitope (i.e. 'magnitude / epitope'). The five CRC patients studied here had relatively poor pre-operative 5T4 T cell responses (1 Dukes' B, 4 Dukes' C) and were subsequently analysed for total 5T4 responsiveness (A) and average 5T4 responsiveness per epitope (B) post-surgery. The responses were compared using a one-tailed paired *t*-test. Significant differences are indicated; *$p < 0.05$.

tumour progression in this cohort (T2 vs. T3: 8.48% ± 0.49% vs. 11.42% ± 0.61%; p = 0.0089).

A comparison of $Foxp3^+$ Treg proportion and 5T4 T cell responsiveness amongst individual donors was assessed (Figure 3.16). This identified a small, albeit not significant correlation between an increase in Treg proportion amongst peripheral T cells and the ability of these cells to mount an anti-5T4 response after short-term culture. Since peripheral Treg proportion increases with tumour advancement, Tregs could account for diminished 5T4 responses found in patients bearing more advanced tumours.

3.2.7.2 Cultured 5T4 T cell responses following depletion of Tregs from PBMC

The effect of removing Tregs prior to culture of PBMC with 5T4 peptide pools was studied in a number of healthy donors and CRC patients, an example of which is shown from a healthy donor 'HD203' (Figure 3.17). $CD4^+CD25^{hi}$ Tregs were removed using anti-CD25 microbead MACS kits as described in the methods. This resulted in the removal of between >95% of $Foxp3^+$ Tregs from the PBMC sample as determined by subsequent FACS analysis of sorted samples (see Appendix).

When comparing 5T4 responses generated before and after Treg depletion, the magnitude of responses increased but also *de novo* responses were observed in donor 'HD203'. IFN-γ responses to peptide pools 3, 5 and 10 (implicating 5T4 peptides 21 and 23) all increased after Treg depletion. In addition, responses were also generated to peptide pools 6, 8 and 12 (further implicating peptides 9, 11, 23, 32, 35 and 36). This raises the possibility that 5T4-specific Tregs exist to actively impinge upon the

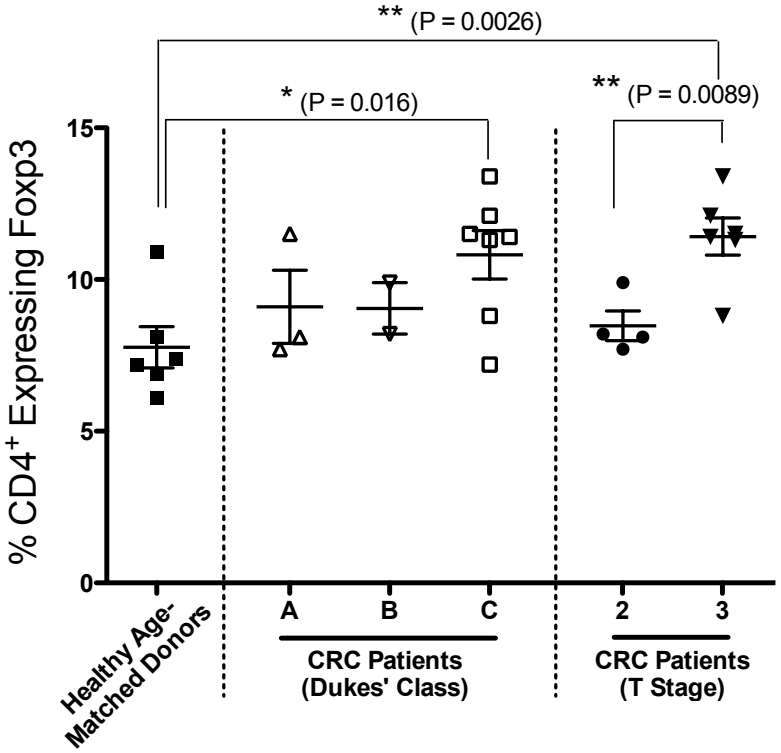

Figure 3.15 Foxp3$^+$ Regulatory T cell proportion increases in CRC patients with more advanced tumours. Freshly isolated PBMC from CRC patients and healthy age-matched controls were stained with fluorescently conjugated mAb to CD3, CD4 and Foxp3 and assessed for the proportion of live CD3$^+$CD4$^+$ T cells that expressed intracellular Foxp3 by FACS. Results from CRC patients were correlated to histopathological tumour score. Significant differences are indicated; *$p < 0.05$, **$p < 0.01$.

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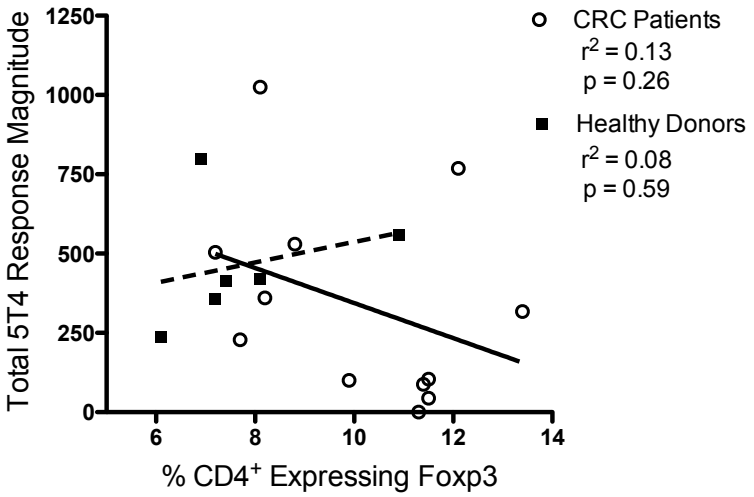

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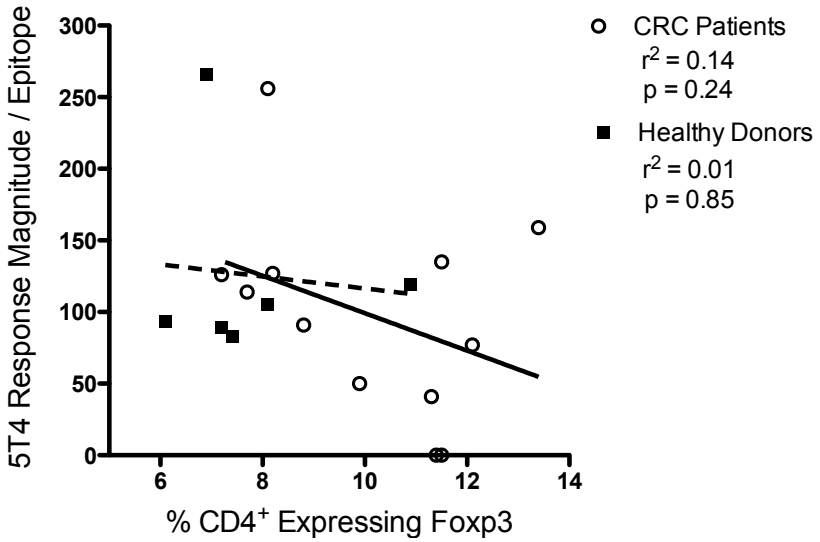

Figure 3.16 Treg proportion and 5T4 responsiveness. Freshly isolated PBMC were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Positive responses were defined in terms of the total number of IFN-γ producing 5T4-specific T cells to all positive 5T4 peptide pools, per 10^5 cultured PBMC (i.e. 'total magnitude') or the average number of IFN-γ producing 5T4-specific T cells to each putatively identified epitope (i.e. 'magnitude / epitope'). The percentage of PBMC-derived $CD4^+$ T cells expressing Foxp3 was correlated with the 5T4 response magnitude (A) and 5T4 magnitude per epitope (B) generated from the same PBMC sample amongst CRC patients and healthy age-matched donors. Lines of regression are shown (solid line = CRC patients; dashed line = healthy donors).

effector functions of 5T4-specific $CD4^+$ effector T cells. Furthermore, Tregs appear to recognise a broad range of 5T4 epitopes in this donor.

Further assessment of the response to 5T4 peptide 23 in donor HD203 revealed how removal of Tregs before PBMC stimulation with the individual peptide can result in a seven-fold increase in the magnitude of IFN-γ production generated at day 12. Such robust responses were confirmed to derive from a memory T cell population, since depletion of $CD45RO^+$ memory T cells before culture with peptide 23 resulted in no discernible response (Figure 3.17C).

3.2.8 Age-related effects on 5T4 T cell responses in CRC patients

Ageing has been associated with a decline in T cell function leading to the concept of immune senescence and increased susceptibility to infectious diseases and cancer (Raynor, Lages et al. 2012). Results in this chapter have demonstrated healthy age-matched donors produce better responses than CRC-patients. By correlating 5T4 responses with patient age, it was also noted that patients ≤ 60 years demonstrated better 5T4 responses on a per epitope basis than patients ≥ 80 years old (Figure 3.18A: 120.1 ± 21.7 vs. 42.5 ± 18.2; $p = 0.03$). However, this finding was not mirrored in the total 5T4 responses (Figure 3.18B).

Interestingly, in the cohort of patients tested here, there was a small but significant correlation between the age of patient at surgery and the tumour stage at resection (Figure 3.19A: Dukes' A vs. Dukes' B: 61.5 yrs ± 4.01 yrs vs. 74.83 yrs ± 3.82 yrs; $p = 0.038$ and T1 vs. T4: 56.0 yrs ± 4.89 yrs vs. 79.67 yrs ± 7.69 yrs; $p = 0.041$). This was not the case for two other cohorts of CRC patients (Figure 3.19B and C).

Overall, it seems that age of subject has little effect on measured $CD4^+$ anti-tumour responses.

3.2.9 HLA-DR type and 5T4 T cell responsiveness

If anti-5T4 $CD4^+$ T cell responses are protective, it is reasonable to hypothesize that there would be an advantage in maintaining a broad response to multiple epitopes. This might mean certain HLA types which are associated with fewer epitopes (or homozygosity of the HLA-DR region) may be seen more frequently in patients compared to background control population. The number of 5T4 peptides associated with an individuals' HLA-DR type is summarized in Figure 3.20. Three immunodominant regions of the 5T4 protein were responsible for 64% of 5T4 peptide responses generated, namely amino acids 70-100, 200-240 and 380-410. Each HLA type was associated with several peptides, the range being 4 peptides with HLA-DRB1*1301 to 9 with HLA-DRB1*0101. The HLA subtype frequencies for 52 CRC patients are summarized in Table 3.2 compared to the background population. These data did not demonstrate an effect of HLA subtype on the breadth or magnitude of $CD4^+$ T cell responses. Furthermore, no difference was found between homozygous and heterozygous donors for HLA-DR alleles and the number and magnitude of 5T4 responses generated (Figure 3.21).

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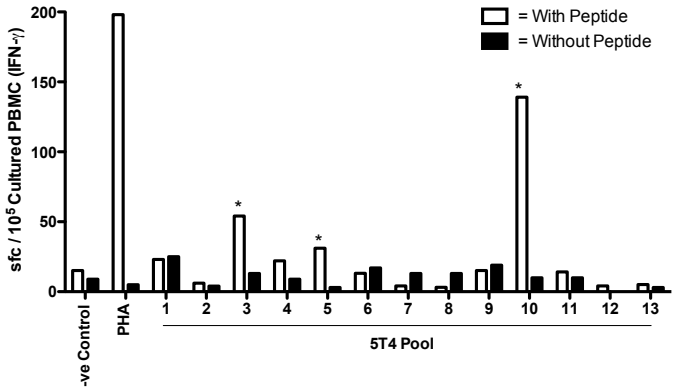

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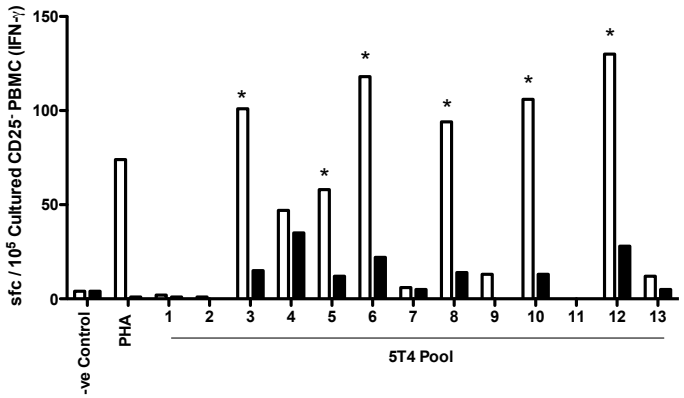
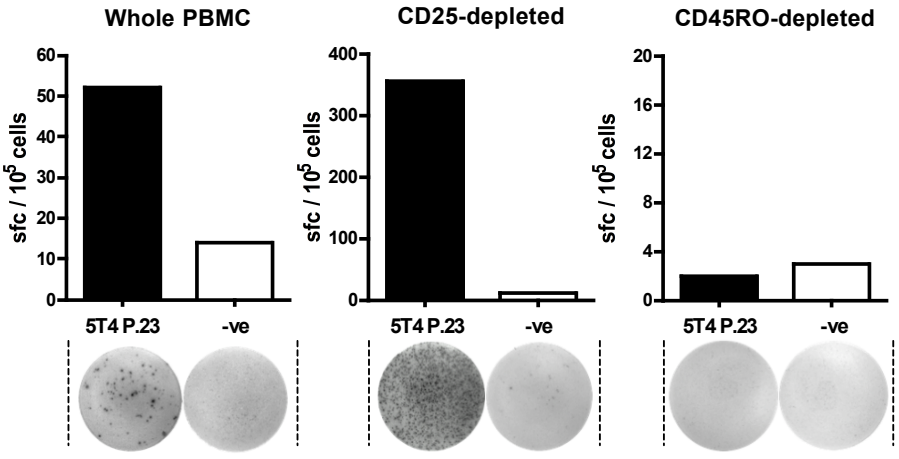


Figure 3.17 Treg depletion from PBMC prior to culture with 5T4 peptide pools. Freshly isolated PBMC (A) and CD25-depleted PBMC (B) from a healthy donor were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured cells were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Identified positive responses are shown (*). In the same donor, having identified 5T4 peptide 23 as being immunogenic, cell lines were established to this peptide in whole PBMC, CD25-depleted PBMC and CD45RO-depleted PBMC before subsequent analysis of peptide 23-specific IFN-γ production at day 12 by ELISpot (C).

C:



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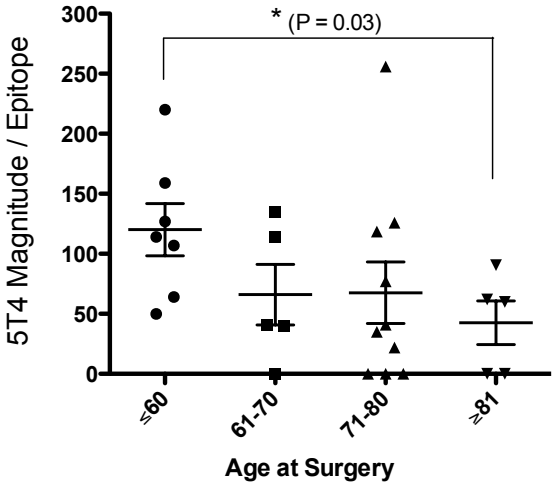

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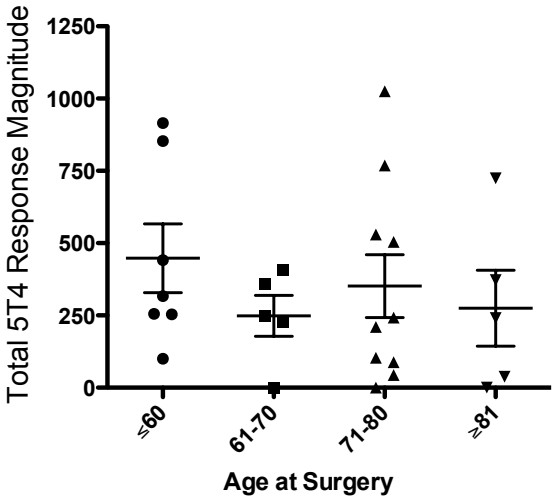


Figure 3.18 The effect of ageing on anti-5T4 responses in CRC patients. Freshly isolated PBMC from 27 CRC patients were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. Patient age at the time of going to surgery was correlated with the average number of IFN-γ producing 5T4-specific T cells to each putatively identified epitope (i.e. 'magnitude / epitope') (A) and to all positive 5T4 peptide pools, per 10^5 cultured PBMC (i.e. 'total magnitude') (B). Significant differences are indicated; *$p < 0.05$.

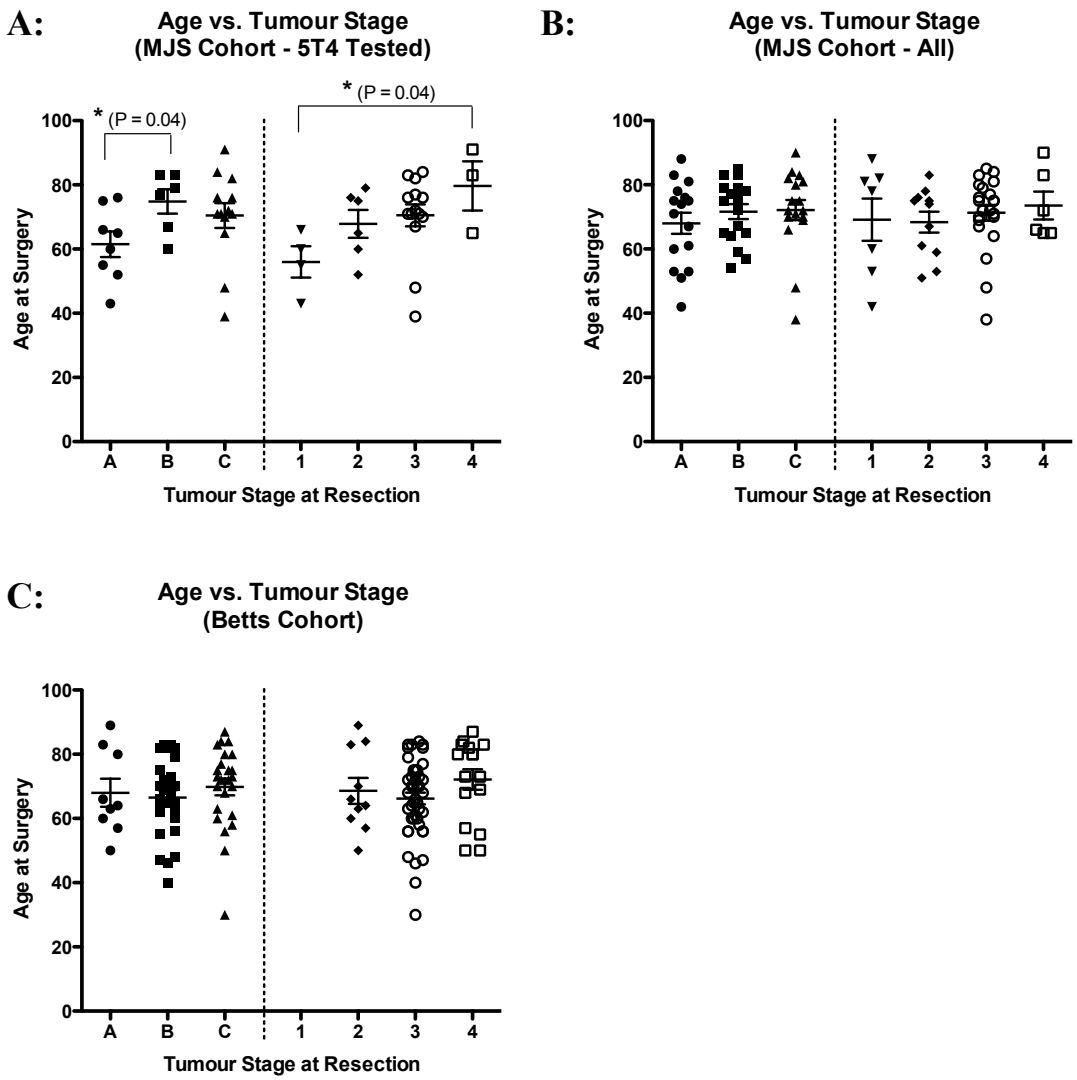


Figure 3.19 Analysis of patient age vs. tumour stage at resection. The age of the patient at time of surgery was correlated with tumour stage data taken from histopathological data of the excised tumour sample in three cohorts; a cohort of patients where 5T4 responses were measured (A), a cohort including patients from (A) but inclusive of all patients studied in this thesis (B) and an entirely distinct cohort taken from a previous PhD student in this lab (C: data from Gareth Betts).

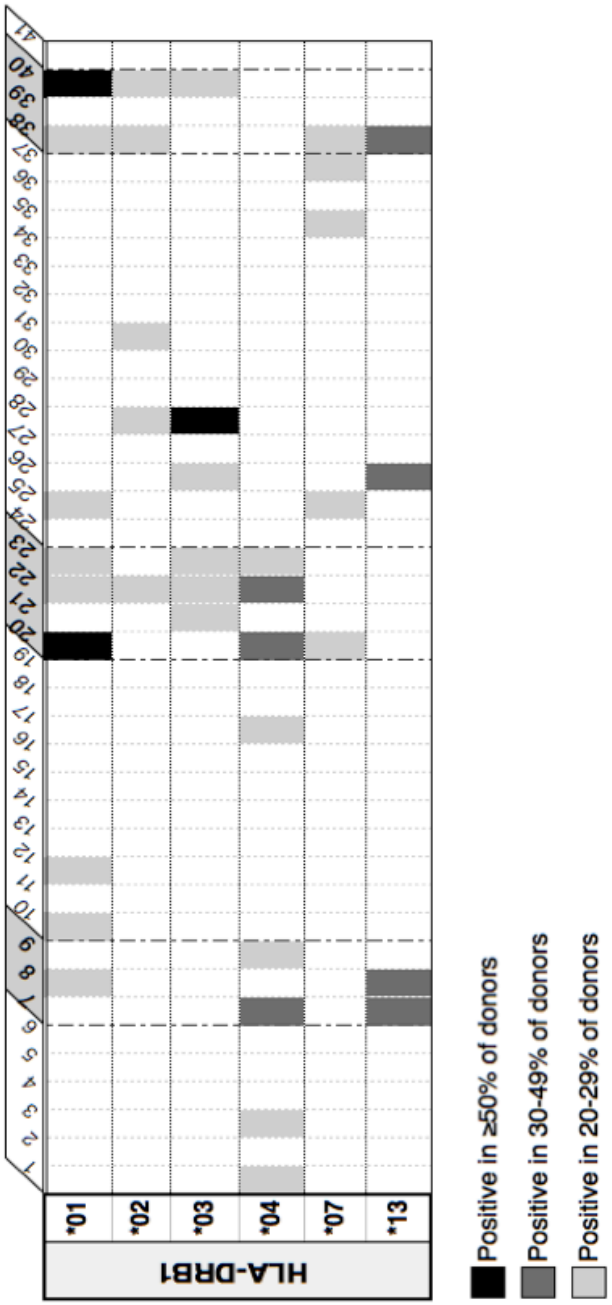


Figure 3.20 HLA-DR type and 5T4 T cell epitopes. Every positively identified 5T4 T cell response was correlated to donor HLA-DR type (see Methods section 2.6.2, page 63 for definition of positive response). Graph indicates 5T4 peptides that would regularly generate an immune response (Black = positive response identified in ≥50% of donors tested, dark grey = positive response identified in 30-49% of donors tested and light grey = positive response identified in 20-29% of donors tested).

Table 3.2: A comparison of HLA-DR allele frequencies between CRC patients and the population of Wales.

	Allele number CRC Patients (n = 52)	**Allele frequency CRC Patients** (%)	**Allele frequency Wales** (%)
HLA-DRB1*01	15	15.4	11
HLA-DRB1*15	12	13.2	14.3
HLA-DRB1*03	11	12.1	15.2
HLA-DRB1*04	26	28.6	20.4
HLA-DRB1*07	13	14.3	14.7
HLA-DRB1*08/09/10	6	6.6	3.3
HLA-DRB1*11/12	8	8.8	5.8
HLA-DRB1*13	10	9.9	9.7
HLA-DRB1*14	1	1.1	2.3

Allele frequency = total number of copies / 2n (Allele frequency Wales based on 39,979 subjects)

Phenotype frequency = number of individuals with gene / n

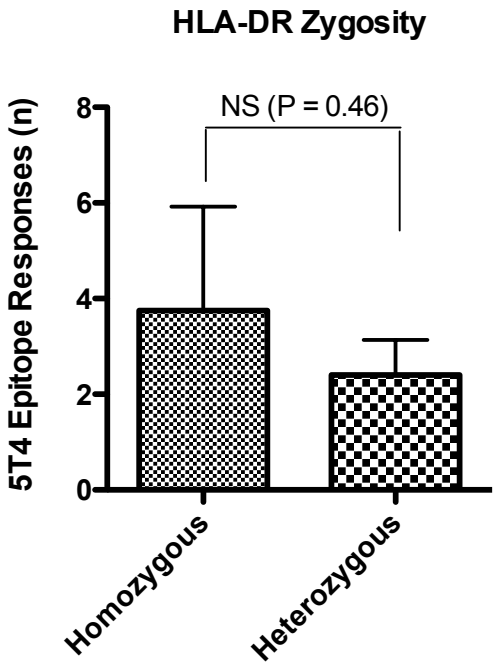


Figure 3.21 HLA-DR zygosity does not affect the number of positive 5T4 epitope responses generated. Freshly isolated PBMC from 12 CRC patients were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. The HLA-DR zygosity of the CRC patients was correlated with the number of putative 5T4 epitope responses that patient produced a positive IFN-γ response. Statistics show the results of an unpaired *t*-test.

3.2.10 Expression of 5T4 in cancerous / healthy / inflamed human tissue

The rationale for healthy donors producing sizeable anti-5T4 T cell responses remains unclear, although it is now well established that healthy donors do have tumour antigen-specific T cells present in the T cell pool (Danke, Koelle et al. 2004), (Campi, Crosti et al. 2003). In addition, 5T4-specific T cells have also previously been identified amongst healthy donors (Elkord, Burt et al. 2008), however reasons for this have not been explored until now. Although previous work analysing 5T4 expression on healthy tissue, including healthy colon samples, found minimal expression (Starzynska, Rahi et al. 1992), I hypothesised that inflamed tissue may upregulate such molecules to facilitate an inflammatory responses. The expression of 5T4 on inflamed tissue has not previously been determined.

Patients undergoing colectomy for Crohn's disease or ulcerative colitis were consented and samples of inflamed colon were taken from surgery and analysed for expression of 5T4. Alongside this, a placenta was obtained from a woman undergoing an elective Caesarean section, for use as a positive control, since 5T4 is ubiquitously expressed and was originally identified on placental syncytiotrophoblasts, extravillous cytotrophoblast and the amniotic epithelium (Hole and Stern 1988). 5T4 expression was positively identified on placenta and colorectal tumour samples taken from this cohort of CRC patients (Figure 3.22). As expected healthy background colonic mucosa did not stain for 5T4. However, two colon sections from patients with inflammatory gut conditions (Crohn's disease and IBD) stained positive for 5T4. An example is shown of 5T4 expression within the setting of intestinal focal cryptitis. This finding requires further research to examine if other inflammatory conditions upregulate 5T4.

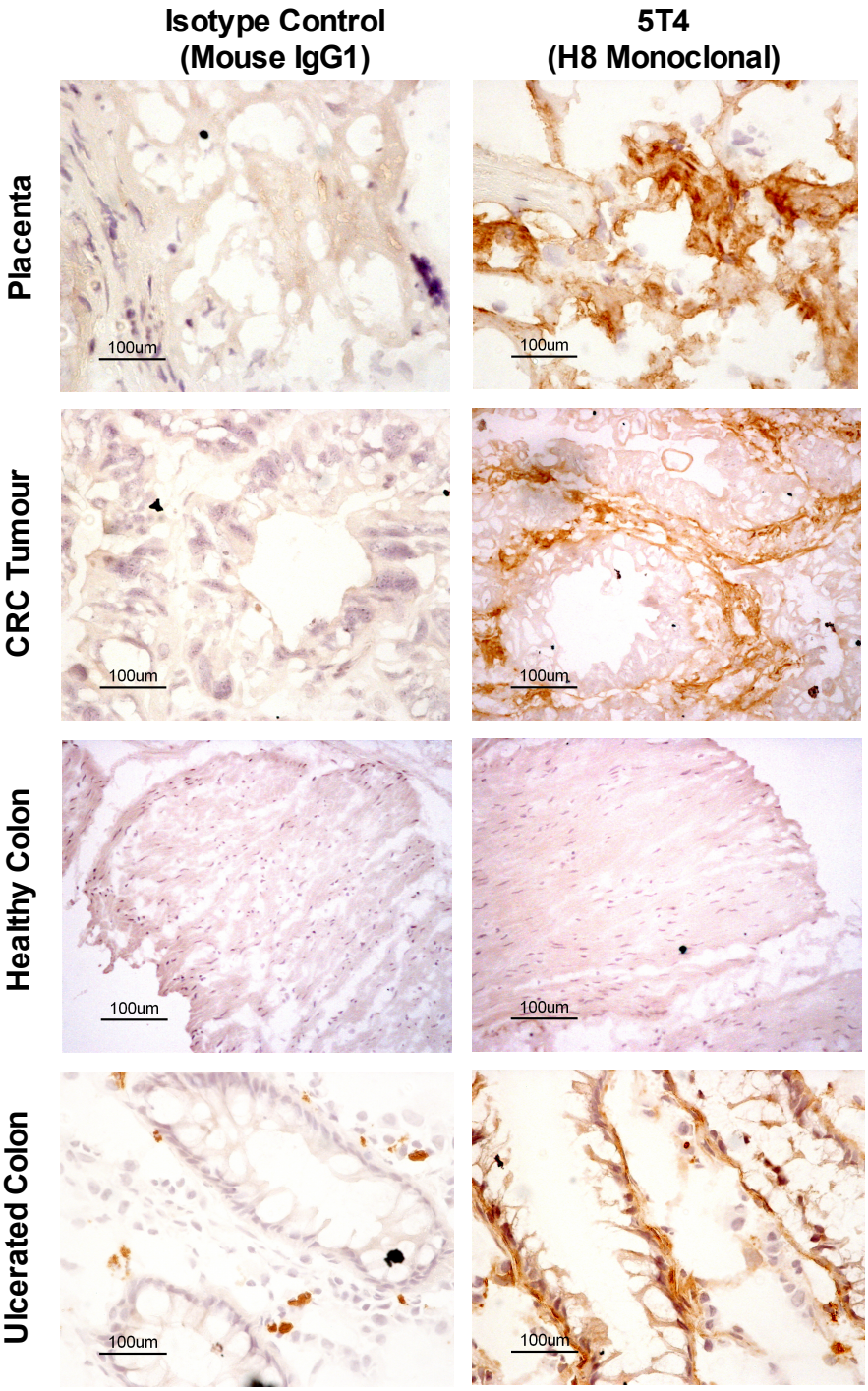


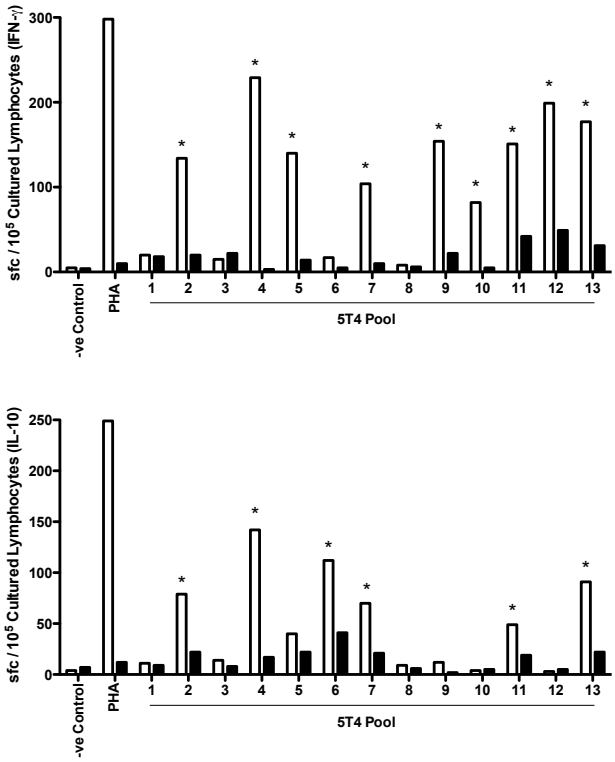
Figure 3.22 5T4 Immunohistochemistry staining of frozen sections. The expression of 5T4 was assessed on placenta (positive control), colorectal tumours, healthy colon and inflamed colonic tissue. Negative isotype controls (mouse IgG1) were performed alongside 5T4 staining on sections from the same samples.

3.2.11 5T4-specific IL-10 responses

So far in this chapter, the response to 5T4 epitopes have been defined in terms of IFN-γ production, since IFN-γ is produced upon activation of T cells with their cognate antigen. Another cytokine produced by subsets of $CD4^+$ T cells that has not been explored is interleukin-10 (IL-10), which, in contrast to IFN-γ, has potent suppressive effects upon many cell types, including other $CD4^+$ T cells (Fujio, Okamura et al. 2010). It was hypothesized that if 5T4-specific Tregs are indeed suppressing activation or effector function of 5T4-specific effector T cells, they themselves may be producing IL-10 to reduce the immune response. IL-10 ELISpots were therefore carried out with whole PBMC alongside CD25-depleted PBMC to identify whether $CD25^{hi}CD4^+$ T cells were responsible for inhibiting anti-5T4 immune responses via IL-10 production.

Results from one healthy donor show that where IFN-γ has been produced in response to a 5T4 epitope, IL-10 is also produced, i.e. positive responses were found to peptides 2, 4, 26, 28, 38 and 40 in both ELISpots (Figure 3.23A). Following depletion of $CD25^{hi}$ Tregs from the peripheral blood, some IFN-γ responses were diminished, e.g. peptides 2, 26 and 38, and new responses were identified, e.g. peptide 11. It is possible that removal of $CD25^{hi}$ CD4+ T cells could remove activated 5T4-specific T effector cells as well as 5T4-specific T regulatory cells prior to culture (Figure 3.23B). Interestingly, the IL-10 responses to certain peptides (e.g. peptide 28) remain unchanged, but other de novo responses were identified (i.e. peptide pools 9 and 12). This suggests that IL-10 production is not limited to classical $CD4^+CD25^{hi}Foxp3^+$ regulatory T cells, but is produced in sizeable quantities by other $Foxp3^-$ helper T cells.

A: HD212 PBMC



B: HD212 CD25-depleted PBMC

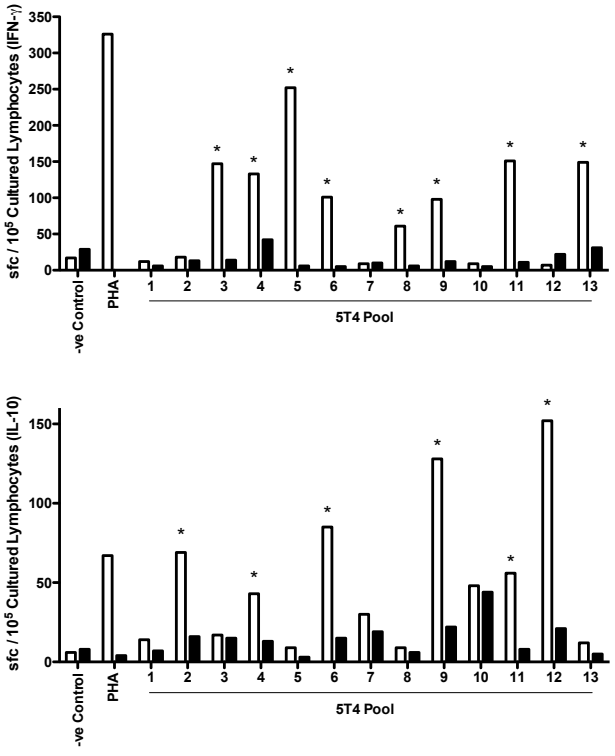


Figure 3.23 5T4-specific T cell production of IFN-γ and IL-10 after short-term culture. Freshly isolated PBMC (A) and CD25-depleted PBMC (B) from healthy donor 'HD212' were stimulated with a panel of 13 peptide pools covering the entire 5T4 sequence (see Table 2.4, page 60 for 5T4 peptide pools and matrix system) and cultured for a period of 12 days *in vitro*. Cultured PBMC were subsequently assessed for the number of IFN-γ^+ and IL-10^+ T cells (per 10^5 cultured lymphocytes) specific for the 13 5T4 peptide pools. (* indicates positive response).

3.2.11.1 5T4-specific IL-10 responses in pregnant women

Given the expression of 5T4 on trophoblasts, it is essential that pregnant women do not mount an immune response directed towards targeting 5T4-positive cells. Thus, regulation of 5T4-specific T cell responses must be robust to counteract this effect. To test this hypothesis, freshly isolated PBMC from three pregnant women (each in second trimester) were obtained and subjected to *ex vivo* IFN-γ and IL-10 ELISpots, testing for their reactivity to the 5T4 peptide pools. As expected, there were no IFN-γ responses to any 5T4 peptide (example shown in Figure 3.24A); however, a number of peptide pools produced sizeable IL-10 responses. In one donor in particular, peptide pools 4 and 8, implicating peptide 10 as being an immunogenic epitope, stimulated T cell IL-10 production (Figure 3.24B and C).

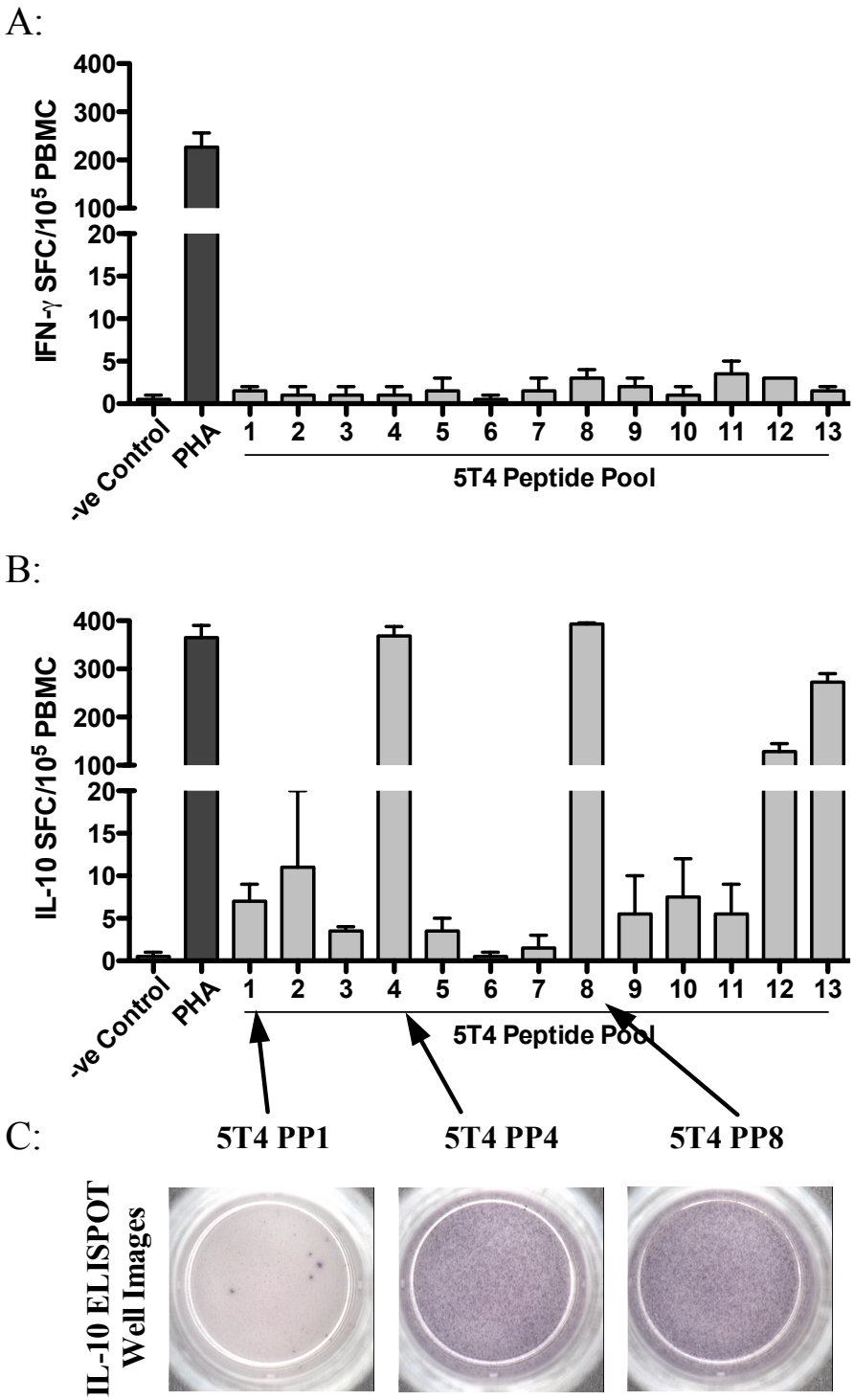


Figure 3.24 ***ex vivo*** **5T4-specific T cell responses in a pregnant woman.** Freshly isolated PBMC from a pregnant woman were assessed for the number of IFN-γ^+ (A) and IL-10^+ (B) T cells (per 10^5 PBMC) following overnight stimulation with the 5T4 peptide pools (see Table 2.4, page 60 for 5T4 peptide pools and matrix system). ELISpot well images of positive (5T4 PP4 and PP8) and negative (5T4 PP1) IL-10 responses are shown (C).

3.2.12 5T4 responses in CRC patients treated with TroVax® and cyclophosphamide

Following on from a large body of evidence in mice and humans demonstrating that regulatory T cells actively suppress 5T4-specific effector T cells, a phase II clinical trial was established to test the effect of Treg depletion alongside concurrent T cell stimulation with the 5T4-encoding, MVA-based vaccine TroVax®, in patients with metastatic colorectal cancer.

During this course of treatment, blood samples were frequently taken to assess for a number of immune parameters (see Appendix for trial flowchart). Cell counts from these samples indicate that in two patients recruited to the study, the number of $CD4^+$ and $CD8^+$ T cells per μl of whole blood increased significantly over a short period of time (Figure 3.25). In the space of four days, the number of $CD4^+$ T cells in Patient 101 tripled from 366/μl to 1094/μl and the number of $CD8^+$ T cells increased from 55/μl to 250/μl over eight days. Likewise, in Patient 102, $CD4^+$ T cells more than doubled in the space of 12 days, from 335/μl to 843/μl and $CD8^+$ T cells increased from 34/μl to 390/μl over the same period of time. A similar pattern was observed in NK and NKT cell numbers. Cell counts consistently reduced over the following weeks after the end of cyclophosphamide treatment.

$Foxp3^+$ regulatory T cell numbers in peripheral blood during treatment fluctuated greatly. In Patient 101, numbers actually doubled from 56/μl to 120/μl over the first four days. However, this coincided with a demonstrable increase in the $CD4^+$ effector T cell population; when analysing the overall proportion of $Foxp3^+$ Tregs amongst the peripheral $CD4^+$ T cell subset, this decreased by over 30% over the first

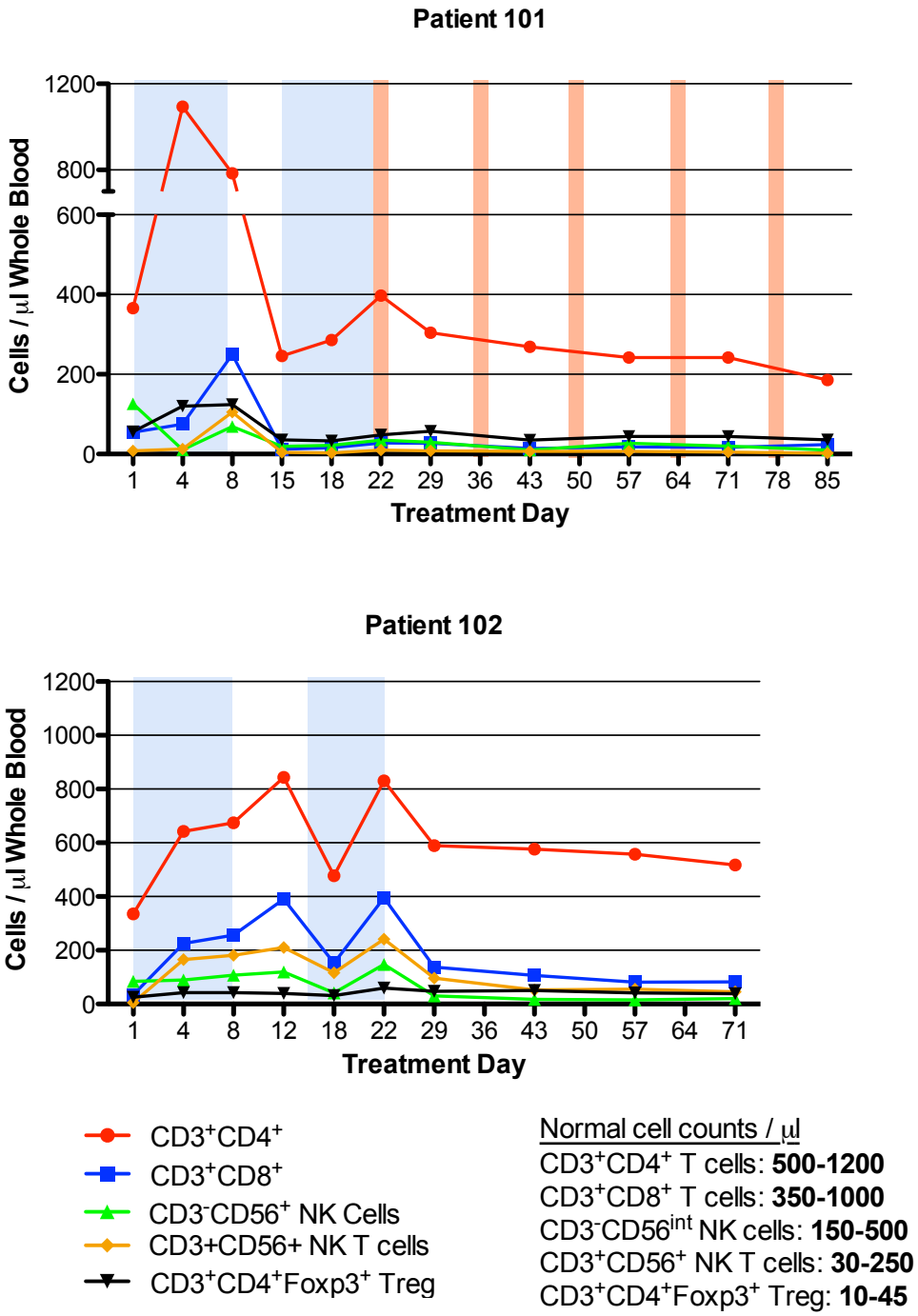


Figure 3.25 Whole blood cell counts from two patients taking low-dose cyclophosphamide. 50mg B.D. cyclophosphamide was given to patients for an initial 7-day period, followed by one-week off, then a further 6-days on (pale blue blocks). $CD4^+$, $CD8^+$ T cells, $Foxp3^+$ Tregs, NK and NKT cell counts per μl of whole blood were measured using flow cytometry-based methods. Patients were followed-up for a further 5-7 weeks to assess longer-term effects.

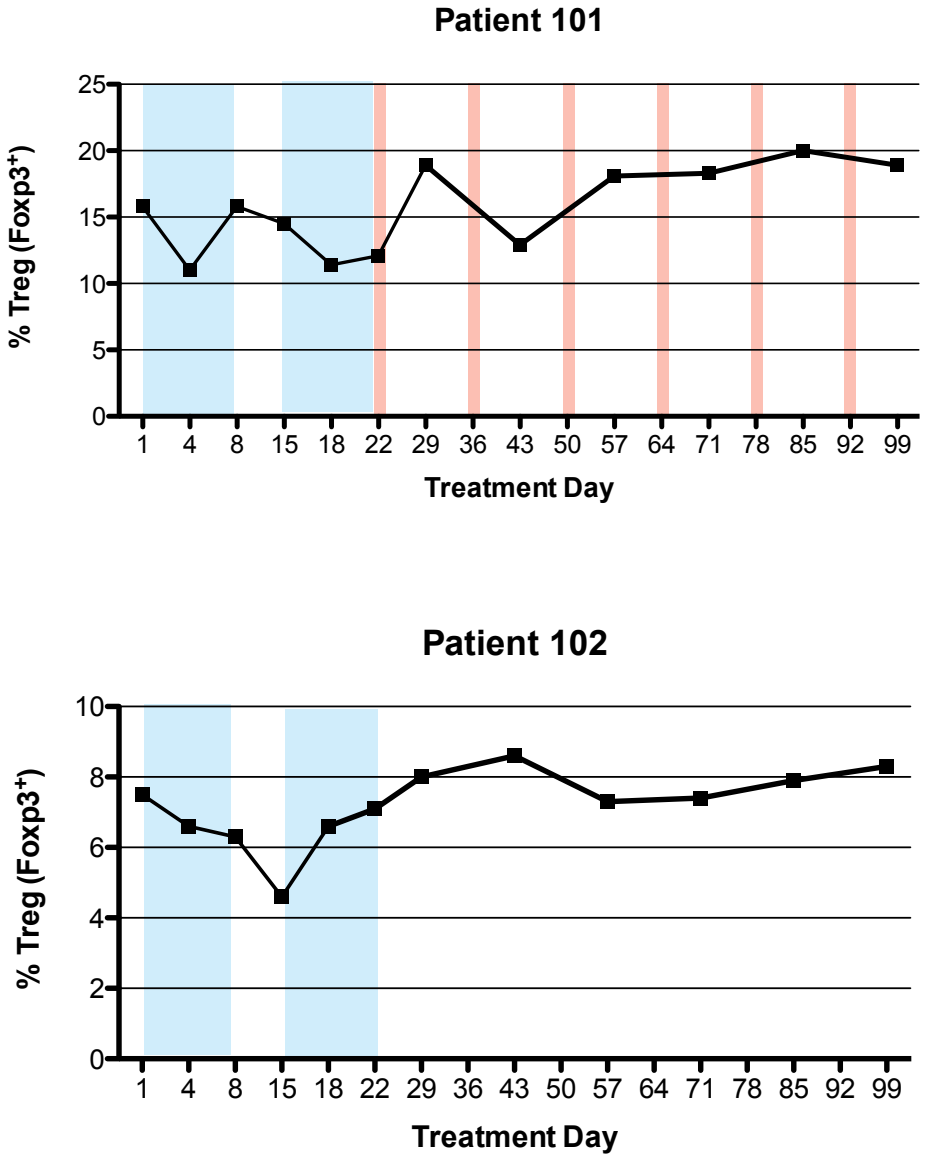


Figure 3.26 Proportion of CD4$^+$ T cells expressing Foxp3 in two patients taking low-dose cyclophosphamide. 50mg B.D. cyclophosphamide was given to patients for an initial 7-day period, followed by one-week off, then a further 6-days on (pale blue blocks). Over this time, freshly isolated PBMC were stained and analysed by FACS to assess for the proportion of Foxp3$^+$ regulatory T cells present amongst live peripheral CD4$^+$ T cells.

four days (Figure 3.26). By the end of metronomic cyclophosphamide treatment, the Treg proportion had reduced in Patient 101 by 24% and in Patient 102 by 5%. It is clear from the two patients studied here, however, that there is no long-term decrease in the proportion or number of $Foxp3^+$ regulatory T cells following cyclophosphamide as these quickly return to baseline levels. In addition, there was no great difference in the mean fluorescence intensity of Foxp3 expression on Tregs throughout treatment, despite initial reductions and consequent fluctuations (Figure 3.27). Neither patient relapsed throughout the course of the trial. More patients are currently under study to determine whether these results are reproducible.

The transcription factor Helios has previously been shown to denote a population of $Foxp3^+$ regulatory T cells derived from the thymus (Thornton, Korty et al. 2010), although this has recently been called into question (Gottschalk, Corse et al. 2012). Regardless, it is now established that this marker designates a more suppressive subset of $Foxp3^+$ Treg (Elkord and Al-Ramadi 2012). The expression of Helios was monitored in the $Foxp3^+$ Treg subset in these patients taking cyclophosphamide. In both patients, initial reductions in their proportions were found, indicating that cyclophosphamide was selectively depleting the more suppressive $Helios^+Foxp3^+$ Treg subset (Figure 3.28). However, the proportion of these cells recovered in both patients and no significant difference was found in either patient by the end of treatment; indeed in Patient 101, the proportion had actually increased.

The primary endpoint of this trial is to achieve an increase in anti-tumour immune responses at week 7 (day 43) in patients treated with both low-dose cyclophosphamide and TroVax® in comparison to controls. To do this, T cell

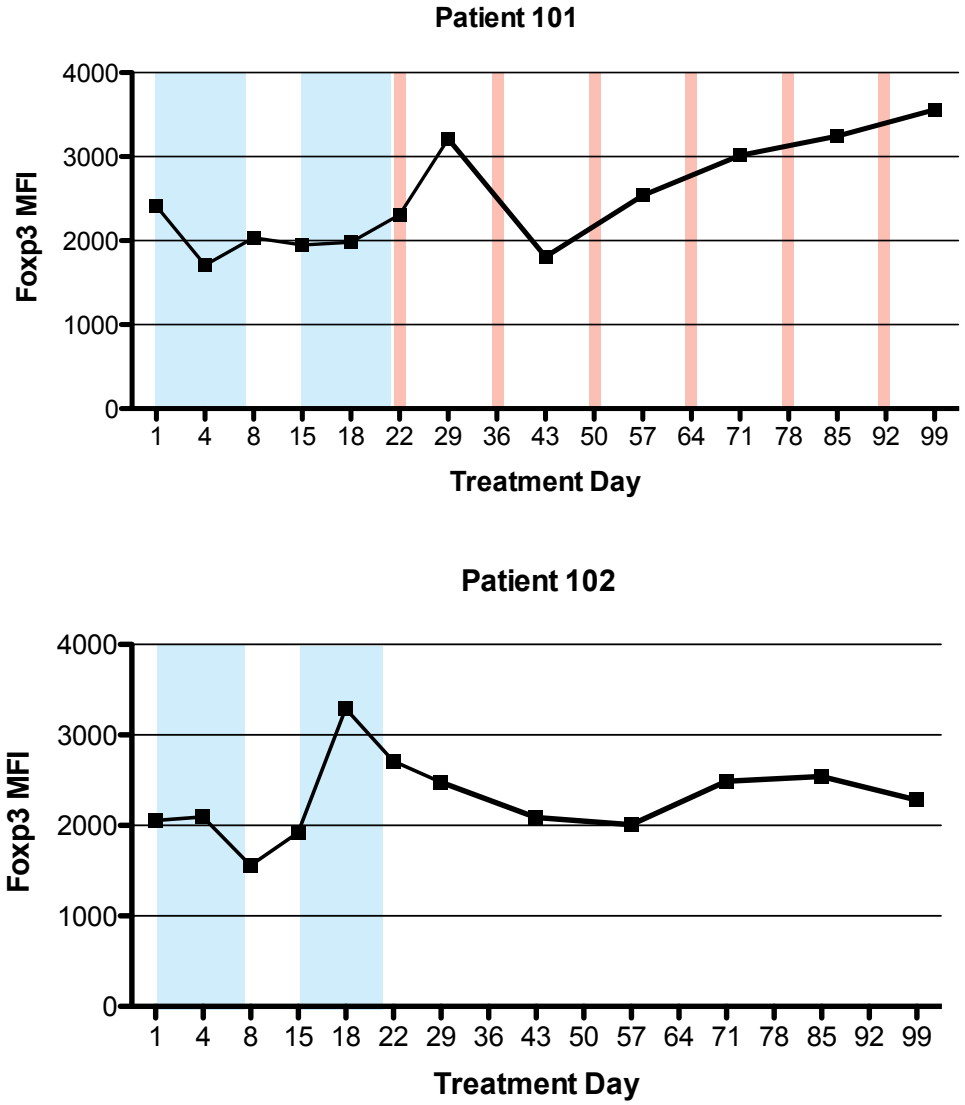


Figure 3.27 Mean fluorescence intensity of Foxp3 expression on $CD4^+Foxp3^+$ regulatory T cells in two patients taking low-dose cyclophosphamide. 50mg B.D. cyclophosphamide was given to patients for an initial 7-day period, followed by one-week off, then a further 6-days on (pale blue blocks). Over this time, freshly isolated PBMC were stained and analysed by FACS to assess for the mean fluorescence intensity of Foxp3 on live $CD4^+Foxp3^+$ peripheral Tregs.

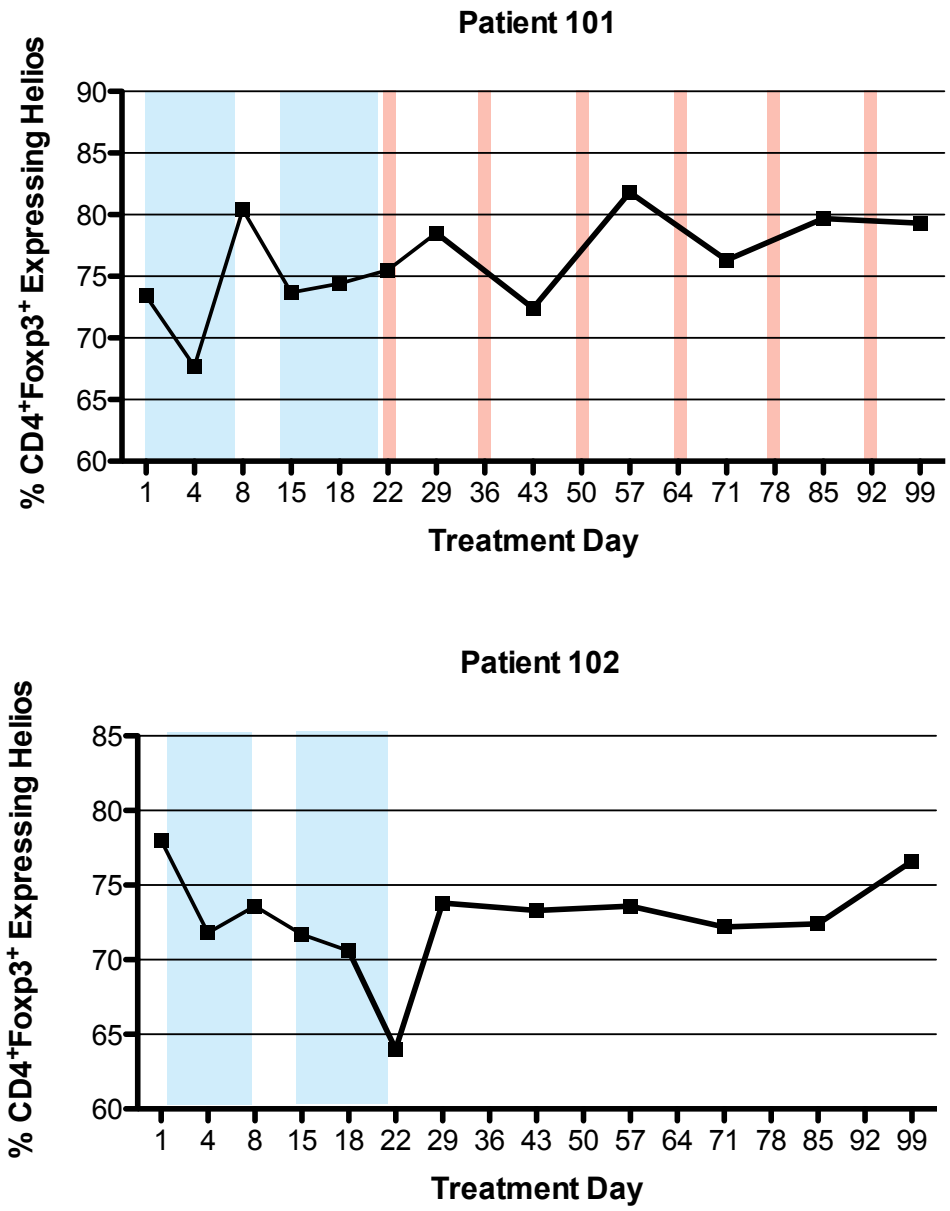


Figure 3.28 Proportion of $CD4^+Foxp3^+$ Tregs expressing Helios in two patients taking low-dose cyclophosphamide. 50mg B.D. cyclophosphamide was given to patients for an initial 7-day period, followed by one-week off, then a further 6-days on (pale blue blocks). Over this time, freshly isolated PBMC were stained and analysed by FACS to assess for the proportion of live $CD4^+Foxp3^+$ peripheral Tregs expressing Helios.

IFN-γ responses to 5T4 were measured using ELISpot at various time-points throughout the trial, again following a period of *in vitro* culture with 5T4 peptide pools, as described previously in this chapter. In Patient 102, who received low-dose cyclophosphamide alone, increases in anti-5T4 T cell responsiveness were found, both in terms of the cumulative response to all peptide pools, and the average response to each putative epitope, reaching a peak at day 15 (Figure 3.29). Responses soon dropped by day 29 and by day 43, these returned to baseline levels. This result indicates that administering metronomic low-dose cyclophosphamide alone can improve anti-tumour immune responses over a short period of time. However, T cell immune responses are also improved to the re-call antigens, tuberculin purified protein derivative and the influenza protein haemagglutinin (X-31) (Figure 3.31). Thus, the improvements in T cell IFN-γ production during cyclophosphamide treatment are not specific for 5T4-specific T cells.

Patient 101, who received low-dose cyclophosphamide followed by TroVax® vaccinations, responded in a similar fashion to the cyclophosphamide therapy, again reaching a peak at day 15 (Figure 3.30). However, instead of returning to baseline soon after stopping cyclophosphamide, the TroVax® vaccinations appear to sustain the existing anti-5T4 immune response up until week 7. After this, a reduction in 5T4 responses was noted, potentially due to the larger proportion of $Foxp3^+$ regulatory T cells observed at day 57 (see Figure 3.26). Indeed between day 43 and day 57, a 30% increase in the proportion of $CD4^+$ T cells expressing Foxp3 was found. It appears that TroVax® induces $Foxp3^+$ Tregs and it is highly probable that this subset of cells is responsible for suppressing anti-5T4 T cell responses in culture, given the data previously described in this chapter. In addition, T cell responses to the re-call antigens were high at day 71 (Figure 3.31), despite relatively poor 5T4 T cell

responses (Figure 3.30) and a very high proportion of peripheral $CD4^+$ T cells expressing Foxp3 (Figure 3.26).

Out of a total of ten patients currently enrolled on the trial, six have received low-dose cyclophosphamide. In agreement with the two patients studied in detail here, all patients demonstrated a transient depletion of peripheral blood-derived $Foxp3^+$ Tregs in the first 22 days of treatment (Figure 3.32A). This reduction in Treg proportion corresponds with a significant increase in the total magnitude of 5T4 responses generated (Figure 3.32B). This was not found in the four patients who did not receive cyclophosphamide.

In the same ten patients, seven received TroVax® vaccinations and their 5T4 T cell responses measured at day 0 and day 43 to assess the effectiveness of the vaccine to stimulate a T cell response. Overall, the data reveal a significant increase in the absolute difference in the magnitude of IFN-γ responses generated on a per epitope basis (Figure 3.33A). A similar trend was found in total 5T4 responses, albeit insignificant given the low numbers of patients at this point (Figure 3.33B). Thus, the 5T4 T cell responses generated by patient 101 appear to be replicable in the early stages of this trial.

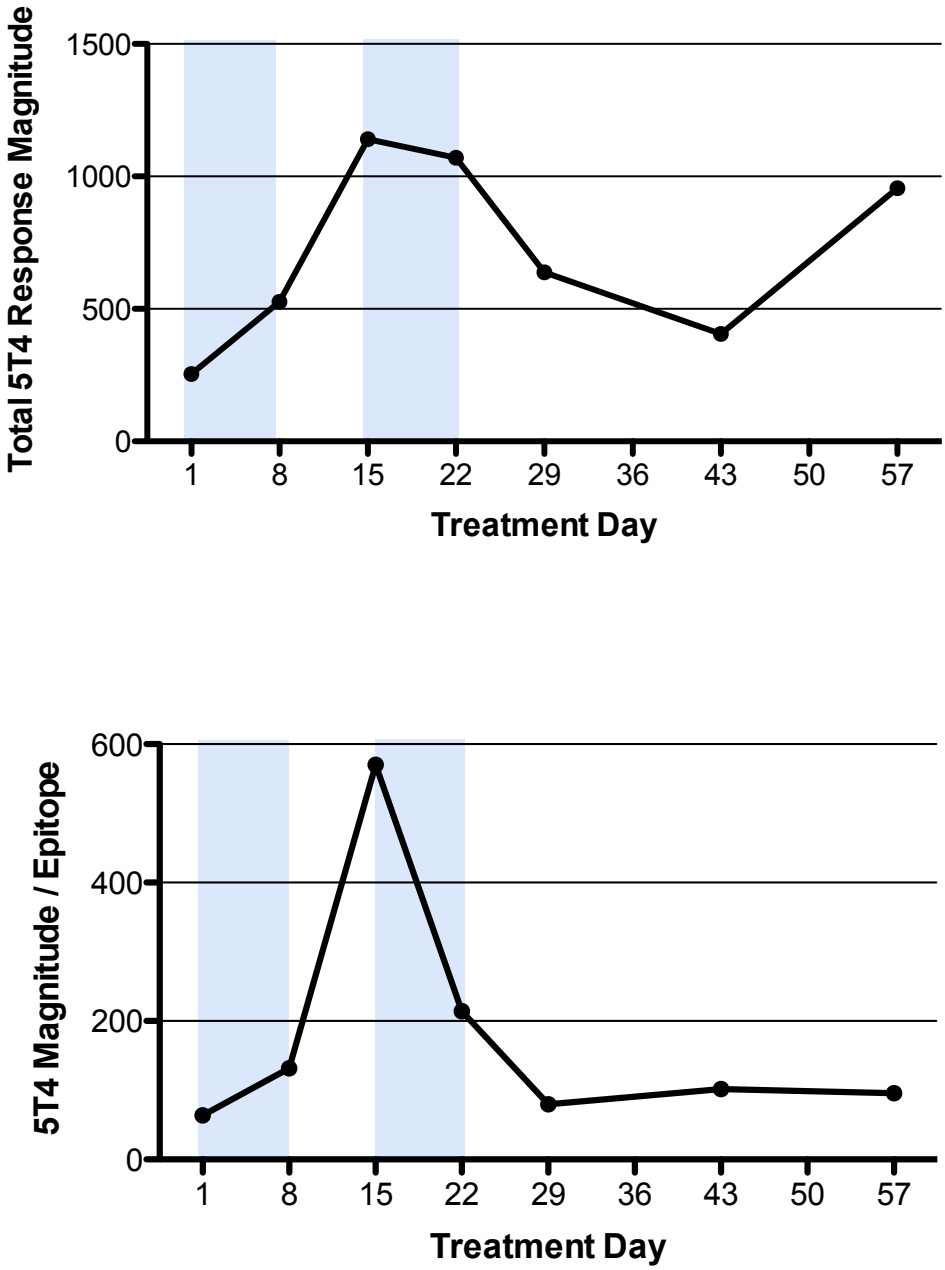


Figure 3.29 5T4-specific T cell responses in CRC patient '102' taking low-dose cyclophosphamide. 50mg B.D. cyclophosphamide was given to patient 102 for an initial 7-day period, followed by one-week off, then a further 6-days on (pale blue blocks). Over this time, freshly isolated PBMC were cultured with 5T4 peptide pools and analysed at day 12 by IFN-γ ELISpot for the total 5T4 response magnitude and 5T4 magnitude per epitope.

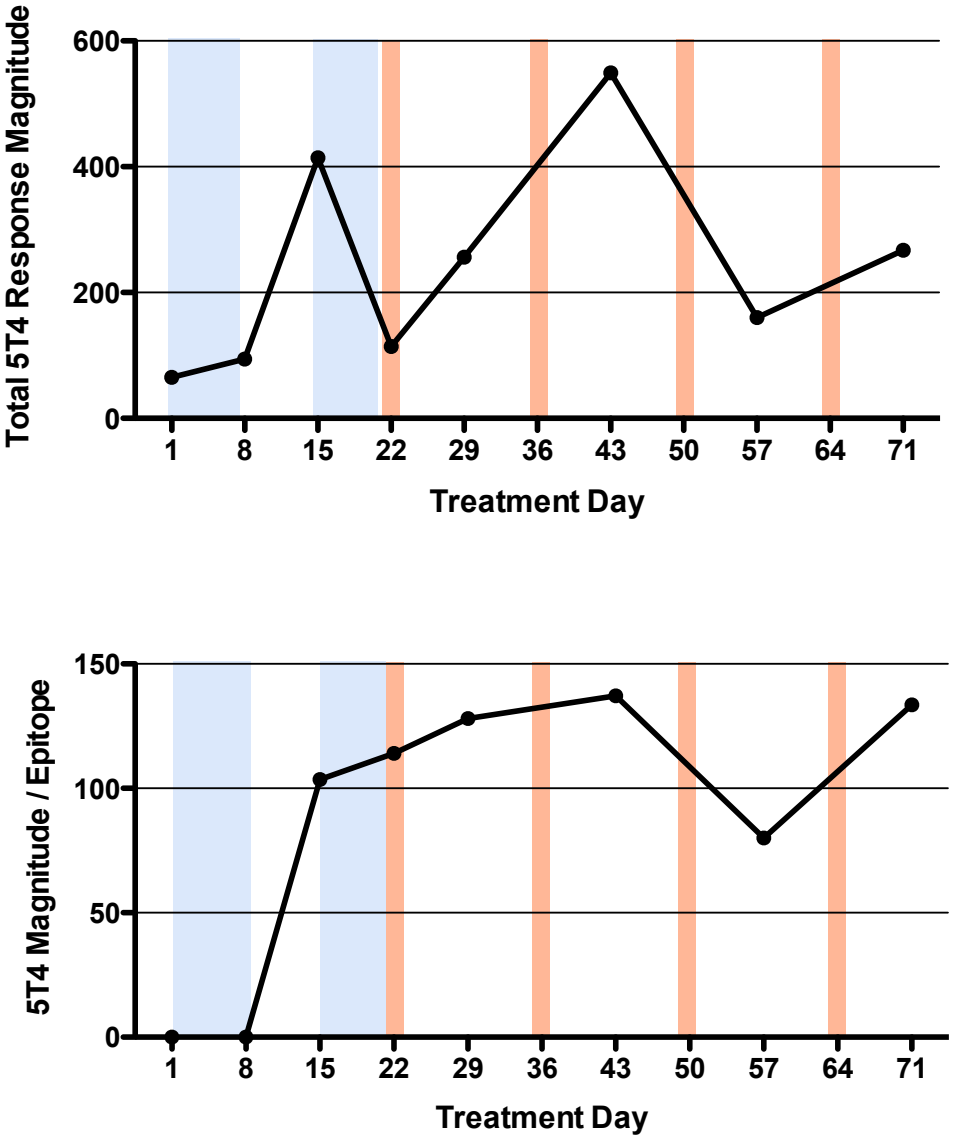


Figure 3.30 5T4-specific T cell responses in CRC patient '101' taking low-dose cyclophosphamide followed by four rounds of vaccination with TroVax®. 50mg B.D. cyclophosphamide was given to patient 101 for an initial 7-day period, followed by one-week off, then a further 6-days on (pale blue blocks). Following this, TroVax® vaccinations were administered (pale red blocks). Over this time, freshly isolated PBMC were cultured with 5T4 peptide pools and analysed at day 14 by IFN-γ ELISpot for the total 5T4 response magnitude and 5T4 magnitude per epitope.

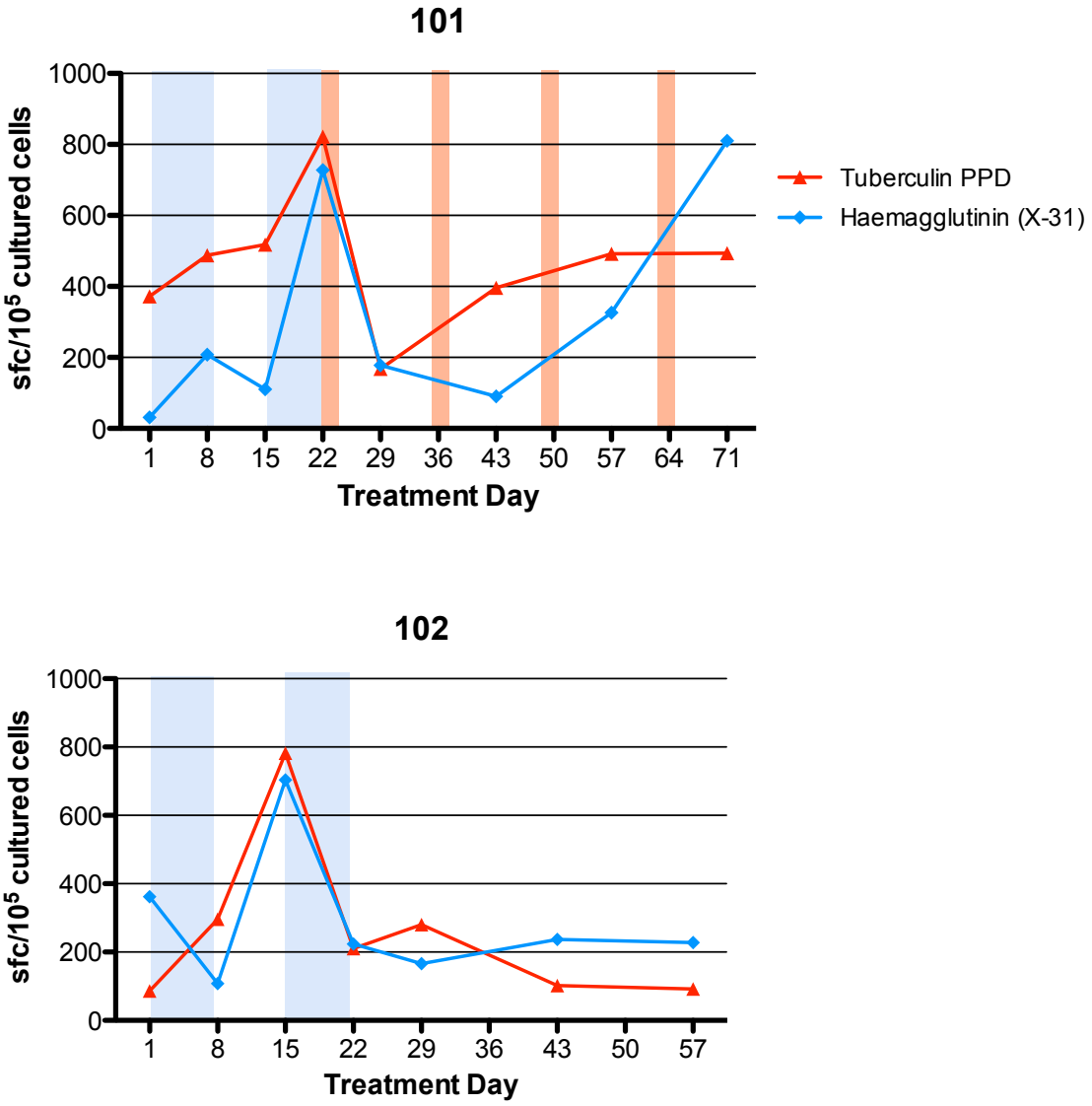


Figure 3.31 T cell responses to re-call antigens after low-dose cyclophosphamide ± TroVax® vaccination. Freshly isolated PBMC were cultured with the re-call antigens, tuberculin purified protein derivative (PPD) or haemagglutinin X-31 (HA) and specific T cell responses measured at day 14 by IFN-γ ELISpot.

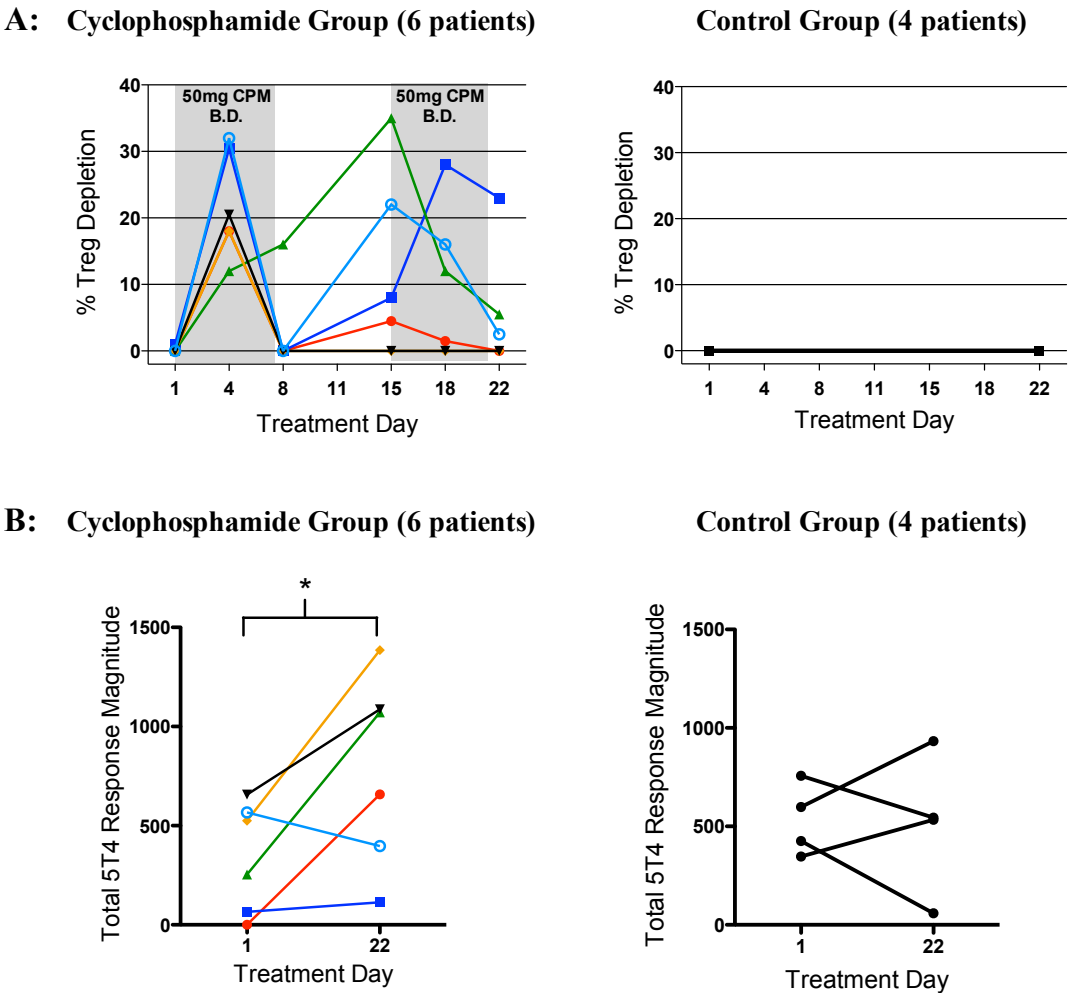


Figure 3.32 Low-dose cyclophosphamide reduces Treg proportion resulting in enhanced anti-5T4 T cell responses. Six metastatic (Stage IV) CRC patients were given 50mg B.D. cyclophosphamide at indicated time-points (grey bars) and a further 4 patients were assigned to a control group (no treatment). Treg proportion amongst peripheral blood was analysed throughout (A). Corresponding measurements of total 5T4 response (as defined in previous figures) were taken before the start (Day 1) and after (Day 22) treatment, resulting in a significant increase in the overall anti-5T4 response in the six patients taking cyclophosphamide; two-tailed *t*-test: *$p < 0.05$ (B).

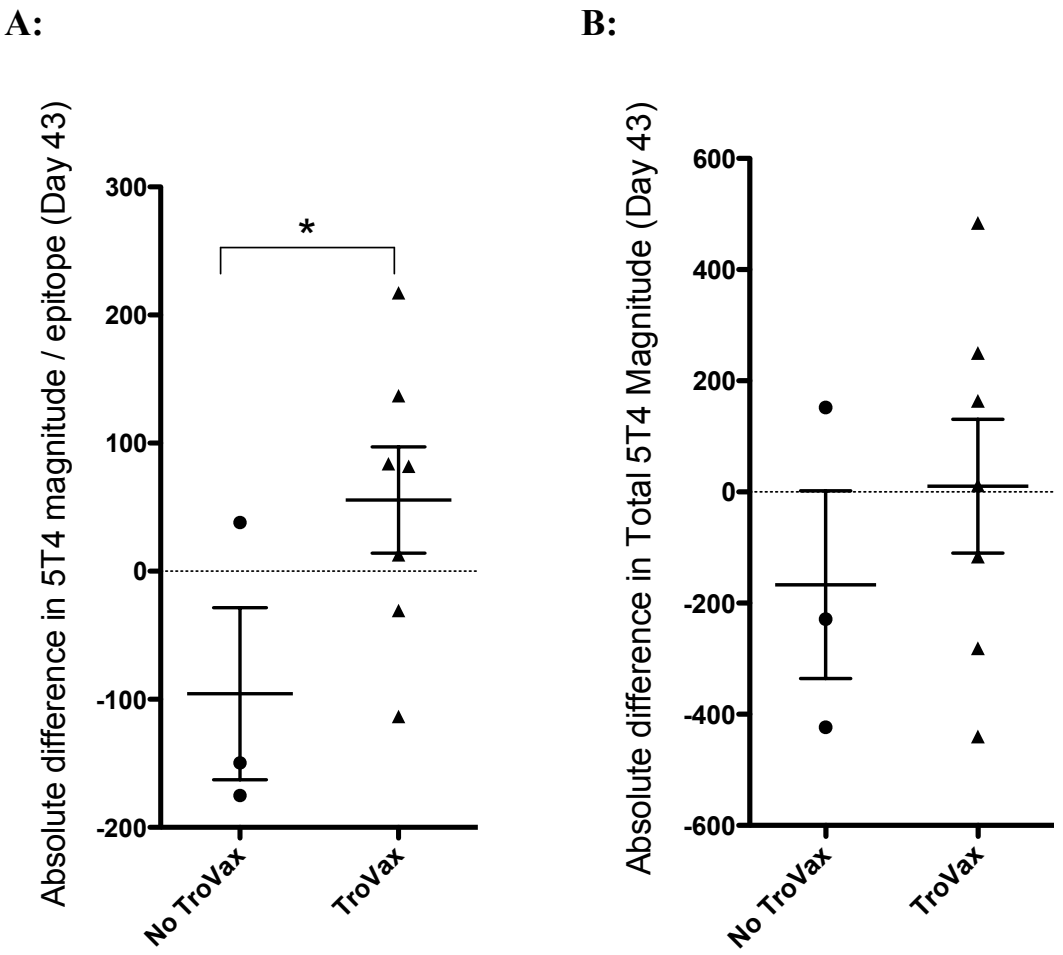


Figure 3.33 Absolute difference in 5T4 T cell response magnitude between day 0 and day 43 of trial. Measured IFN-γ responses (sfc/10^5) to the 5T4 peptide pools were measured at day 0 and day 43 and the absolute difference in the magnitude per epitope or total magnitude were calculated (as defined in previous figures). Significant differences are indicated; *$p < 0.05$.

3.3 Discussion

In this Chapter, an analysis of anti-tumour T cell responses to the oncofoetal antigen, 5T4, was performed. Specifically, experiments focused on investigating the range and magnitude of anti-5T4 T cell responses generated by cancer patients awaiting surgical resection of a colorectal tumour and comparing these to healthy age-matched donor controls. In this process, a number of 5T4-specific T cell clones were established, with the aim of producing class-II tetramers to help identify tumour-specific T cells present in other tissues by FACS. The effects of age, individual HLA-type, Treg proportion and progressive tumour growth on 5T4 T cell responses were also studied to decipher the mechanisms of tumour-mediated immunosuppression.

Successful anti-tumour adaptive immunity is dependent on activation of helper $CD4^+$ T cells by antigens upregulated in neoplastic cells. In CRC it appears that this process becomes restricted allowing for growth, and metastatic spread, of cancerous tissue (Clarke, Betts et al. 2006; Betts, Jones et al. 2011). The results in this chapter advance previous studies by demonstrating that the actual magnitude of anti-tumour $CD4^+$ T cell responses measured in peripheral blood can be correlated to the stage of the colorectal tumour, and indeed, can predict progression to metastatic spread.

Here, the oncofoetal antigen 5T4 was used as a candidate tumour-specific antigen. Utilizing 41 overlapping 20mer peptides allowed an unbiased approach to epitope mapping and negated the requirement for peptide binding algorithm software, as 5T4 peptides with high HLA binding affinities may not necessarily be those recognised *in vivo*. This is due to negative thymic deletion of T cells with high affinities for self-antigens, although other non-deleting mechanisms can also control the avidity with which T cells recognize self-antigens (Anderton and Wraith 2002).

5T4-specific T cell Responses in Healthy Donors

A range of 5T4-derived $CD4^+$ T cell epitopes was identified for individuals of each HLA-DR subtype using the 5T4 peptide pool matrix system. It was surprising that such robust responses to these epitopes were found in healthy controls, and raises the important question as to how these T cells are maintained at a high frequency in the $CD45RO^+$ memory pool. Although tumour-specific T cells have previously been encountered in healthy donors (Campi, Crosti et al. 2003; Danke, Koelle et al. 2004; Pickford, Watson et al. 2007; Elkord, Burt et al. 2008; Tassi, Gavazzi et al. 2008), no studies have been undertaken to ascertain why they exist. It was hypothesized here that 5T4 expression might not just be limited to colorectal tumours and placenta; one possibility is transient up-regulation of 5T4 in subjects with periods of inflammation of the colon. Patients suffering from the inflammatory bowel diseases ulcerative colitis or Crohn's disease, who underwent resection of the inflamed colon, were enrolled in this study to perform 5T4 immunohistochemistry analysis on obtained sections. We found widespread 5T4 expression in inflamed tissue of the gut, most obvious in areas of cryptitis, a common occurrence in IBD patients where an intestinal crypt has become inflamed. It is therefore plausible that 5T4-specific T cell responses may be produced to help resolve bouts of inflammation, perhaps gastroenteritis, encountered by an individual. 5T4-specific Tregs may also exist to regulate the anti-inflammatory response. It would be interesting to measure the magnitudes of 5T4 immune responses in such patients when inflammation is at its most severe. In addition, other inflamed tissues should be assessed for 5T4 expression, since this has not been explored previously.

A further possibility is that the existence of 5T4 immune responses in healthy donors reflects a continuing process of tumour immunosurveillance. This hypothesis requires T cells to recognize and target cancerous cells in order to prevent carcinogenesis and help maintain regular cellular homeostasis. Since 5T4 is expressed predominantly on well-differentiated cancerous tissue (Southall, Boxer et al. 1990), the existence of memory 5T4-specific T cell populations in healthy donors, which are capable of inducing cell death in 5T4-positive tumour cells (Al-Taei, Salimu et al. 2012) further supports this hypothesis.

Decreased 5T4 T cell Responses Correlates with Disease Progression

A major finding from the work presented in this chapter was the identification of decreased $CD4^+$ T cell responses to 5T4 significantly correlating with a steadily worse histopathological tumour grade (i.e.T1→T2→T3→T4). This indicates that patients with more advanced tumours, both in terms of invasiveness through the bowel wall and direct size / burden, have a reduced capacity for T cell-mediated anti-tumour immunity. This was further substantiated by follow-up data showing that those patients bearing advanced tumours with lymph node involvement, yet who had robust pre-operative 5T4 T cell responses, were 30% less likely to develop metastatic disease by 12-months post-surgery. Given the role of 5T4 in facilitating metastatic spread (Carsberg, Myers et al. 1996; Southgate, McGinn et al. 2010), it appears that 5T4 T cell reactivity is also related to the ability of an individual to control cancerous disease from metastasizing. The measured loss of anti-tumour $CD4^+$ T cell responses in blood also provide a basis for further studies to examine the usefulness of measuring T cell responses as a disease biomarker, since an average epitope-specific response of $\geq$75 SFC/10^5 cultured cells distinguished CRC patients and controls with

a specificity of 94%. No such blood test currently exists and it may prove very useful in determining the course of treatment given to cancer patients, as patients with poorer responses will require more aggressive therapy to eradicate the tumour.

Although an inadequate anti-5T4 immune response in CRC patients is found pre-surgery compared to responses in healthy controls, responses to the tuberculin recall antigen, PPD, remained unimpaired, confirming a tumour-antigen specific defect, as shown previously (Clarke, Betts et al. 2006). This is consistent with other reports identifying a reduction in CEA-specific T cell responses in pancreatic cancer patients, whilst anti-viral haemagglutinin-specific responses were analogous to healthy controls (Tassi, Gavazzi et al. 2008). A potential Th2 skew of anti-tumour T cells could be leading to the poor responses, since a Th1 response is required for maximal anti-tumour immunity (Hung, Hayashi et al. 1998). CEA-specific T cells isolated from cancer patients predominantly produce the Th2 cytokines IL-4 and IL-5, whereas the same cell subset from healthy donors produce IFN-γ. Crucially, this skew was limited to tumour-specific T cells and antiviral immunity remained unimpaired between cancer patients and healthy donors (Tassi, Gavazzi et al. 2008). Therefore, the resultant loss of 5T4-specific T cell responses found in more advanced disease may be the result of loss of Th1 function in these cells, rather than a reduction in numbers or responsiveness. It will be interesting to determine a possible immune deviation amongst 5T4-specific T cell responses as tumours progress, using IL-4 / IL-5 ELISpots.

A change of cytokine profile amongst tumour-specific T cells would most likely be caused by factors within the tumour microenvironment, where these cells may encounter antigen-loaded dendritic cells with Th2 polarizing capabilities (Moser

and Murphy 2000; Protti and De Monte 2012). It would be interesting to confirm the presence of 5T4-specific T cells amongst colorectal tumour infiltrating lymphocytes, since it is within this microenvironment that these cells must exert effector functions to eradicate cancerous cells (Bindea, Mlecnik et al. 2010). Although some evidence was provided for 5T4 T cell reactivity in the tumour and tumour draining lymph node in this chapter, this was not reproducible (1 CRC tumour out of 6 identified a positive 5T4 T cell line) and was limited to analysis of IFN-γ production. Th1 responses could either be impeded by large proportions of suppressive T cells present amongst lymphocyte subsets isolated from these compartments (see Chapter 4) or a Th2 skew of these cells would require analysis of Th2 / Th17 cytokines by ELISpot.

Tumour-specific Regulatory T cells

A large body of evidence also shows that regulatory T cells can suppress anti-tumour T cell responses (reviewed in (Nishikawa and Sakaguchi 2010)). Cancer-bearing individuals have increased proportions of Tregs in the periphery as defined by high levels of cell surface CD25 expression and, more recently, by intracellular Foxp3 expression (Betts, Clarke et al. 2006; Beyer and Schultze 2006; Miller, Lundberg et al. 2006; Ling, Pratap et al. 2007). Our lab recently reported that the presence of CRC drives a population of Tregs that inhibit anti-tumour immune responses to the tumour associated antigens 5T4 and CEA, and although excision of the tumour lead to normalization of Treg numbers, suppression of T cell responses prior to resection was still associated with tumour recurrence at 12 months (Betts, Jones et al. 2011). Although increased proportions of Tregs in the peripheral blood of cancer-bearing individuals has been widely reported, there are currently no studies

analysing changes in Treg proportion with tumour advancement. In the cohort of CRC patients examined here, a significant increase was noted in the proportion of peripheral blood-derived Tregs (i.e. $CD4^+$ T cells expressing the transcription factor Foxp3) as tumours progressed, defined by histopathological grading of tumour invasion through the bowel wall. The reason for elevated Treg proportions during malignancy is unclear. A possible explanation is that tumour antigens such as 5T4 and CEA are expressed to prevent immune recognition by stimulating regulatory T cells. Such antigens are expressed during foetal development as they are hypothesized to control maternal immune recognition of the partially allogeneic foetus. This is demonstrated by CEA-CEACAM-1 interactions directly suppressing T cell responses (Gray-Owen and Blumberg 2006). It is also possible that expression of such molecules drives proliferation, conversion or accumulation of regulatory T cells, owing to their large proportions within the tumour microenvironment (see Chapter 4).

Removal of Tregs *in vitro* resulted in greater 5T4 responses to certain epitopes, de novo epitope responses and no difference in response to other epitopes. Thus, it appears that Tregs are capable of suppressing only certain effector 5T4 T cell responses, meaning that Tregs don't recognize all 5T4 epitopes that stimulate effector T cell responses. This is important for future potential immunotherapy; by defining 5T4-derived MHC-class II restricted epitopes, superior peptide-based vaccines could be designed to incorporate $CD4^+$ T effector cell epitopes whilst minimising 5T4-specific Treg activation, with the potential to induce maximal anti-tumour effects. Work is on-going to definitively identify 5T4 Treg epitopes with this prospective treatment approach in mind.

A trend was also found between decreased 5T4 T cell responses after culture if the initial proportion of Foxp3$^+$ Tregs was relatively higher. It is tempting to speculate that Tregs may be responsible for inhibiting the establishment of 5T4-specific effector T cell activation and expansion over the 2-week period of culture, thus resulting in the diminished 5T4 responses identified in patients with more advanced tumours. If Tregs are stimulated by TAAs *in vivo*, this might inhibit effective cancer immunotherapy (Welters, Kenter et al. 2010). Furthermore, in Patient 101, a reduction in 5T4 T cell responsiveness was noted following the fourth TroVax® vaccination. This correlated with a stark increase in the proportion of peripherally derived Tregs and therefore the proportion of Tregs plated in the PBMC culture will be greater. If TroVax® has indeed induced a 5T4-specific Treg response, this may explain the decrease in responses found following repeat TroVax® vaccination in this patient.

Tolerance to tumour antigens could also be accomplished via "ignorance" of effector T cells to respond to such antigens in vivo, or further regulation by IL-10 secreting Tr1 cell subsets (Pickford, Watson et al. 2007). 5T4-specific IL-10 responses were readily detectable *in vitro*, even after Treg depletion, suggesting that Foxp3$^-$ Tr1 cells could be capable of inhibition of effector T cell responses. However, IFN-γ production was still mostly unaffected to the same epitope responses, although further experiments will be required to determine whether blocking IL-10 increases these responses further.

Depleting Tregs and Stimulating 5T4 Responses in CRC Patients

It has previously been proposed that a low 50mg B.D. iterative dosage of cyclophosphamide, referred to as "metronomic" therapy, was sufficient to selectively

deplete $CD4^+CD25^+$ regulatory T cells (Ghiringhelli, Menard et al. 2007). This therapy had the dual benefit of restoring T and NK cell effector functions and could therefore be considered as an effective immunotherapeutic adjunct. Here, two patients were recruited and given the same dose and regime previously identified to be effective; specifically, 50mg B.D. was taken for an initial 7-day period, followed by one week off, then a further 7-days back on treatment. TroVax® would then be administered to patients 24 hours following their last dose of cyclophosphamide. Preliminary findings from this trial reveal a striking increase in the $CD4^+$ and $CD8^+$ T cell number after cyclophosphamide treatment; despite small increases in $Foxp3^+$ Treg numbers, overall proportions of Tregs reduced in both patients. This response was also short-lived and TroVax® vaccination resulted in a further enlargement of the Treg proportion; this is in contrast to intravenous use of cyclophosphamide to deplete Tregs where an overall reduction in Treg numbers was still present 24 days after injection (Walter, Weinschenk et al. 2012).

One of the most compelling results of cyclophosphamide treatment in this trial was the induction of 5T4-specific T cell responses found in both patients at day 15 after the start of treatment. Very similar findings were obtained in a trial using identical cyclophosphamide therapy, whereby endogenous breast tumour–reactive T cells were detected in 27% of patients before cyclophosphamide treatment, which increased to 73% by day 14. Most strikingly, an increase in breast tumour–reactive T cells was associated with both stable disease and overall survival (Ge, Domschke et al. 2012). However, 5T4-specific T cell responses quickly diminished after completion of cyclophosphamide therapy; TroVax® vaccination was necessary and sufficient to sustain this 5T4 response. Although early findings from the trial indicate that these results appear reproducible, the results from all 54 enrolled patients will be analysed

throughout the trial and immunological responses correlated to clinical outcome to assess whether metronomic cyclophosphamide can enhance the effects of active immunotherapy.

Conclusion

In summary, an in depth analysis of oncofoetal antigen 5T4-specific T cell responses was performed, which revealed several interesting findings. Namely, these were:

- A significant and highly specific reduction of 5T4-specific T cell IFN-γ production in CRC patients with more advanced Dukes' C tumours, which was irrespective of HLA-type and age;
- Low pre-operative 5T4 T cell responses was indicative of an increased likelihood of metastatic disease 12 months after surgery;
- An increase in the proportion of peripheral Tregs as the colorectal tumour progresses, corresponding with poorer 5T4-specific T cell responses;
- The identification of robust anti-5T4 memory T cell responses in healthy donors and its expression on inflamed tissue in the gut;
- Metronomic low-dose cyclophosphamide therapy increases 5T4-specific T cell responses, re-call T cell responses, $CD4^+$ and $CD8^+$ T cell numbers and partially reduces Treg proportions;
- TroVax® appears to induce an increase in the Treg proportion in one CRC patient.

Collectively, the data presented in this chapter support the hypothesis that $Foxp3^+$ regulatory T cells actively impinge upon anti-tumour immune responses. In addition, they provide the basis for further studies to examine the usefulness of measuring anti-tumour T cell responses as a disease biomarker. Finally, preliminary data from

TaCTiCC, using Treg depletion strategies alongside immunotherapeutic vaccines, is providing useful insights into the effectiveness of this treatment regime in patients with metastatic colorectal cancer.

Chapter 4 – Phenotypic and Functional Analysis of Colorectal Tumour-Infiltrating Lymphocytes

4.1 Introduction

The adaptive immune system can be directed against neoplastic, transformed cells (Schreiber, Old et al. 2011). Although there is evidence that an increased $CD3^+$ T cell infiltrate improves prognosis in CRC (Galon, Costes et al. 2006), the fact that tumours still progress demonstrates a failure of anti-tumour immune responses to control the lesion effectively. Immunologically, there are several explanations that may work singularly or in conjunction to explain this observation. The tumour microenvironment seems to suppress immune responses as the tumour progresses, potentially reflecting a functional switch in tumour-infiltrating dendritic cells (DCs) towards an immunosuppressive phenotype (Scarlett, Rutkowski et al. 2012). This switch is encouraged by suppressive cytokines and growth factors within the tumour, including IL-10 (Fujio, Okamura et al. 2010), TGF-β (Wrzesinski, Wan et al. 2007; Flavell, Sanjabi et al. 2010), vascular endothelial growth factor (VEGF) (Ellis and Hicklin 2008) and the activity of indoleamine 2,3-dioxygenase (IDO) (Munn and Mellor 2007). Hence, anti-tumour T cells become less responsive in advanced tumours (Nagaraj, Gupta et al. 2007).

In addition to these factors, an antigen-specific suppression of effector T cell responses is mediated by a population of $CD4^+Foxp3^+$ regulatory T cells (Tregs) (Nishikawa and Sakaguchi 2010) (Gallimore and Godkin 2008), either directly or through indirect effects on DCs (Liang, Workman et al. 2008; Shevach 2011). These Tregs have an important role in preventing autoimmunity, but may also control immune responses to a range of tumours (Betts, Clarke et al. 2006). Work from this

laboratory has previously found that the presence of CRC is associated with an expanded and distinct population of Tregs in blood, which specifically inhibits anti-tumour immune responses (Clarke, Betts et al. 2006). Resection of the primary tumour led to a reduction in the magnitude of this peripheral Treg population, and pre-operative suppression of tumour-specific T cell function was associated with tumour recurrence one year later (Betts, Jones et al. 2011). However, a number of reports have demonstrated decreased $Foxp3^+$ Treg infiltrates in more advanced tumours that correlate with poor disease outcome (Salama, Phillips et al. 2009; Frey, Droeser et al. 2010). Differences in the proportions of peripheral and intra-tumoural $Foxp3^+$ Tregs could account for these seemingly disparate findings, but it is also possible that regulatory T cell populations other than $Foxp3^+$ Tregs mediate suppression of anti-tumour responses.

To understand the role of intra-tumoural Tregs, this chapter demonstrates work undertaken to perform a detailed phenotypic and functional analysis of various T cell subsets that infiltrate human colorectal tumours. Multi-parameter flow cytometry and functional assays of distinct populations isolated by fluorescence-activated cell sorting (FACS) were used to compare tumour-infiltrating lymphocytes (TILs) with the corresponding $CD4^+$ T cell subsets in healthy colon and peripheral blood. A marked difference in the phenotype of $Foxp3^+$ Tregs was observed, with intra-tumoural Tregs expressing far greater levels of markers associated with suppression. These data reveal the presence of a novel suppressive $CD4^+$ T cell population within colorectal tumours that is phenotypically and functionally distinct from $CD4^+Foxp3^+$ T cells and could be contributing to immunological suppression of anti-tumour T cell responses.

4.2 Results

4.2.1 Analysis of Foxp3 expression amongst T cell subsets in peripheral blood, colon and tumour.

The transcription factor Foxp3 is still widely regarded as the best marker for determining T cell subsets with suppressive capabilities and has been used extensively to identify regulatory T cells in patients with cancer (Kryczek, Liu et al. 2009; Nishikawa and Sakaguchi 2010). Initially, intracellular Foxp3 expression was analysed amongst $CD4^+$ T cells isolated from peripheral blood, colonic mucosa and colorectal tumour specimens, by flow cytometry. Examples of Treg identification and gating strategies are shown (Figure 4.1).

Compared to healthy age-matched controls (HD), the overall proportion of $CD4^+$ T cells expressing Foxp3 in the peripheral blood of CRC patients (n=13) was increased (patients 10.88% ± 0.77% vs. controls 7.77% ± 0.68%, p = 0.019; Figure 4.2A). The relative proportions of $Foxp3^+$ T cells remained remarkably consistent in CRC patients between peripheral blood and unaffected background colonic tissue (blood 10.88% ± 0.77% vs. colon 10.80% ± 0.87%); however, a significant increase was observed amongst colorectal tumour-infiltrating $CD4^+$ T cells (tumour 21.77% ± 2.46%; vs. colon, p = 0.0027 ; vs. blood, p = 0.0024). This is consistent with previous data obtained in our lab in a mouse model of cancer (Betts, Twohig et al. 2007) and other human tumours (Beyer and Schultze 2006; Whiteside 2012) and is indicative of a suppressive tumour microenvironment. However, the overall fluorescence intensity of Foxp3 expression did not significantly differ amongst $CD4^+Foxp3^+$ T cells in blood, colon or tumour (Figure 4.2B). Thus, whilst the overall proportion of $CD4^+$ T cells expressing Foxp3 is greatest in the tumour, they may be no more suppressive on an

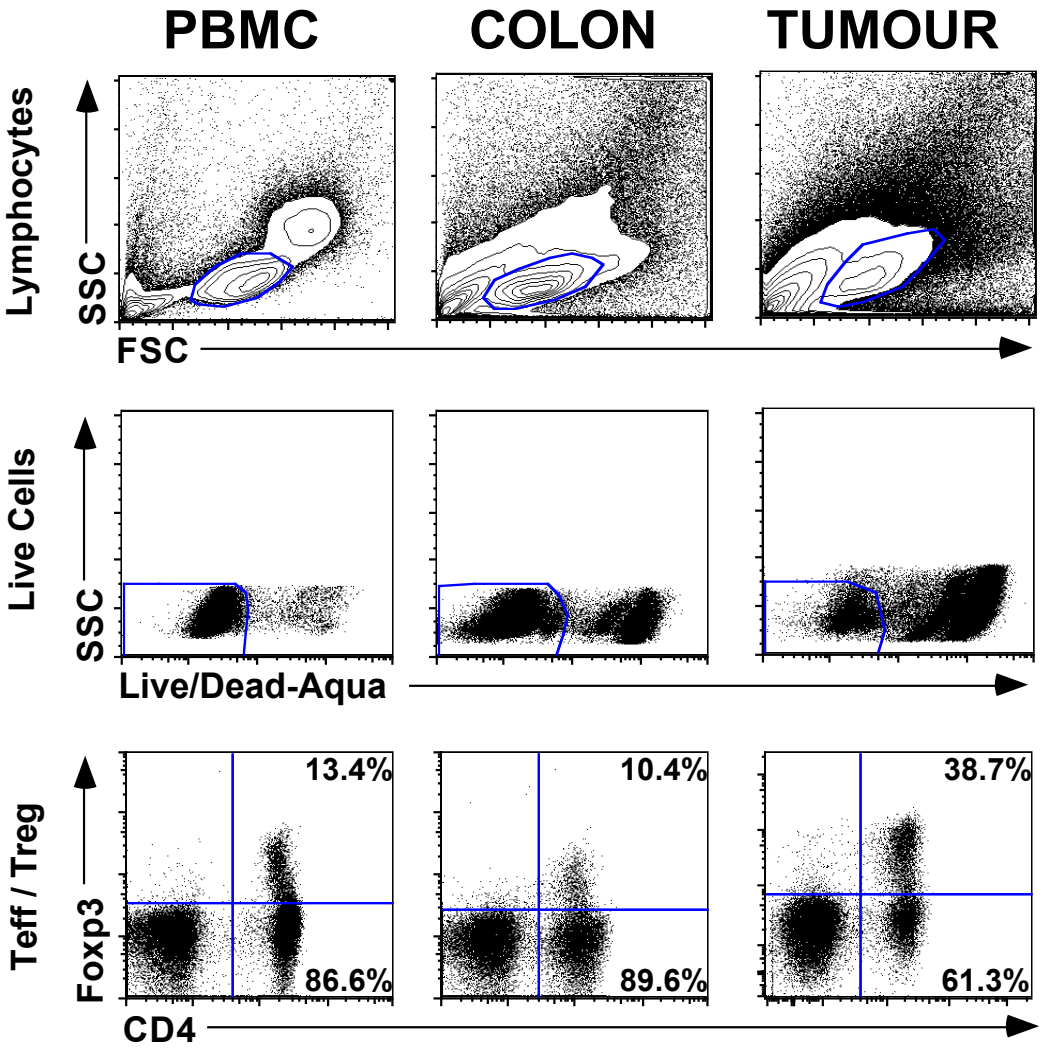


Figure 4.1 *Ex vivo* phenotypic analysis of regulatory CD4$^+$Foxp3$^+$ T cells in CRC patients. Representative bivariate flow cytometry plots showing Foxp3 expression on live CD4$^+$ T cells obtained from matched peripheral blood, unaffected colon and colorectal tumour samples. A lymphocyte gate was initially drawn based on forward scatter vs. side scatter profiles (top three plots). Next, a gate was placed around those lymphocytes that did not take up the live/dead-aqua stain (middle three plots). Finally, live lymphocytes were analysed for CD4 and Foxp3 expression, using the gating strategy shown above (bottom three plots).

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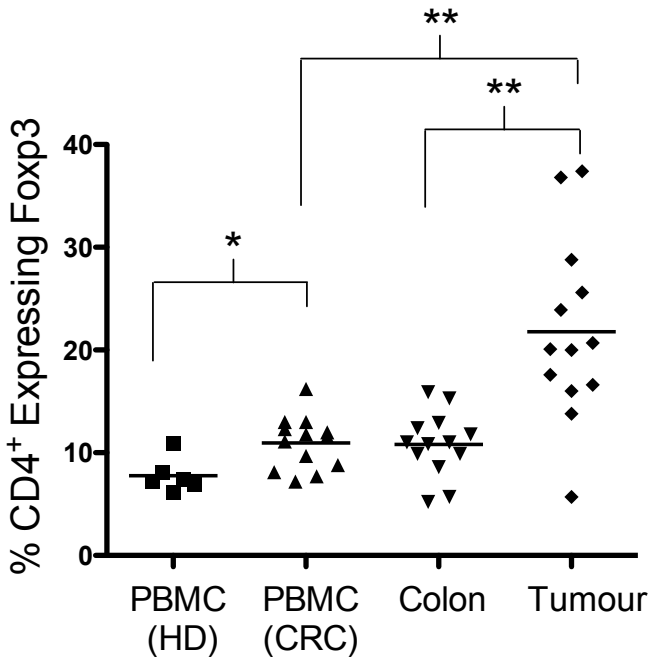

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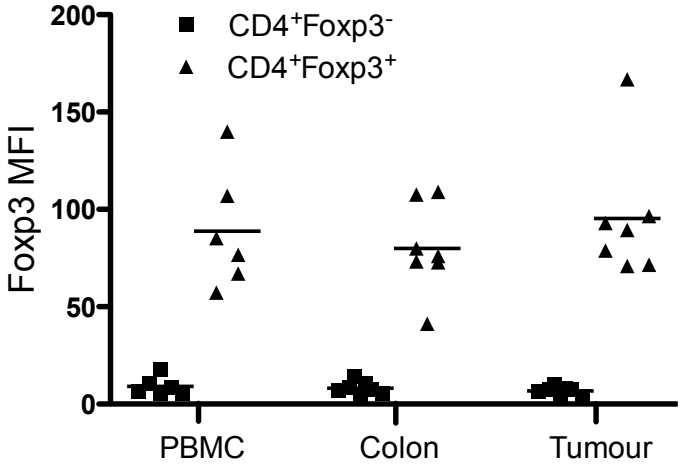

Figure 4.2 A comparison of $CD4^+$ T cells expressing Foxp3 in different compartments. (A) Percentage of live $CD4^+$ T cells expressing Foxp3 in PBMC samples from age-matched healthy donors (HD; n=6; mean age = 71 years), and PBMC, unaffected colon and tumour samples from CRC patients (n = 13; mean age, 72 years). Significant differences are indicated; *$p < 0.05$, **$p < 0.01$. (B) Mean fluorescence intensity of Foxp3 expression on live $CD4^+$ T cells.

individual cell basis than comparable cells found in peripheral blood or colon (Chauhan, Saban et al. 2009).

Low levels of Foxp3 expression amongst intratumoural single positive $CD8^+$ T cells were observed (Figure 4.3: 1.66% ± 0.46%; n = 8), in keeping with previous reports indicating that between 1-2% of $CD3^+CD8^+$ T cells express CD25 and Foxp3 in colorectal cancer tissue (Chaput, Louafi et al. 2009). This subset of cells, although only comprising a very small fraction of the total T cell pool, has been shown to bear suppressive characteristics *in vitro*. In the majority of tumour specimens, $CD4^+CD8^+$ double positive T cells were also identified and a relatively greater proportion of these cells expressed Foxp3 (Figure 4.3B; $CD4^+CD8^+$ T cells 22.95% ± 5.5% vs. $CD8^+$ T cells 1.66% ± 0.48%, p=0.0053). This is very similar to the proportion of single positive $CD4^+$ T cells that express Foxp3 ($CD4^+$ T cells 22.23% ± 3.22%). This double positive colorectal tumour infiltrating T cell fraction has recently been described to possess significant anti-tumour reactivity, via the production of TNF-α (Sarrabayrouse, Corvaisier et al. 2011); increased Foxp3 expression could be indicative of the activated status of these cells (Morgan, van Bilsen et al. 2005). Indeed, the vast majority (~90%) of $Foxp3^+$ T cells in the colorectal tumour samples analysed here were single-positive $CD4^+$ T cells (Figure 4.3C).

In results Chapter 3, increased proportions of $Foxp3^+$ Tregs amongst peripheral blood-derived $CD4^+$ T cells, were identified as the tumours progressed to more advanced stages. When analysing T cells that infiltrate colorectal adenocarcinomas, the relative proportions of Foxp3 expressing $CD4^+$ T cells significantly increases as tumours progress from Dukes' A to Dukes' C (Figure 4.4A: Dukes' A 17.03% ± 2.22% vs. Dukes' C 26.03% ± 2.49%, p=0.023), indicating that

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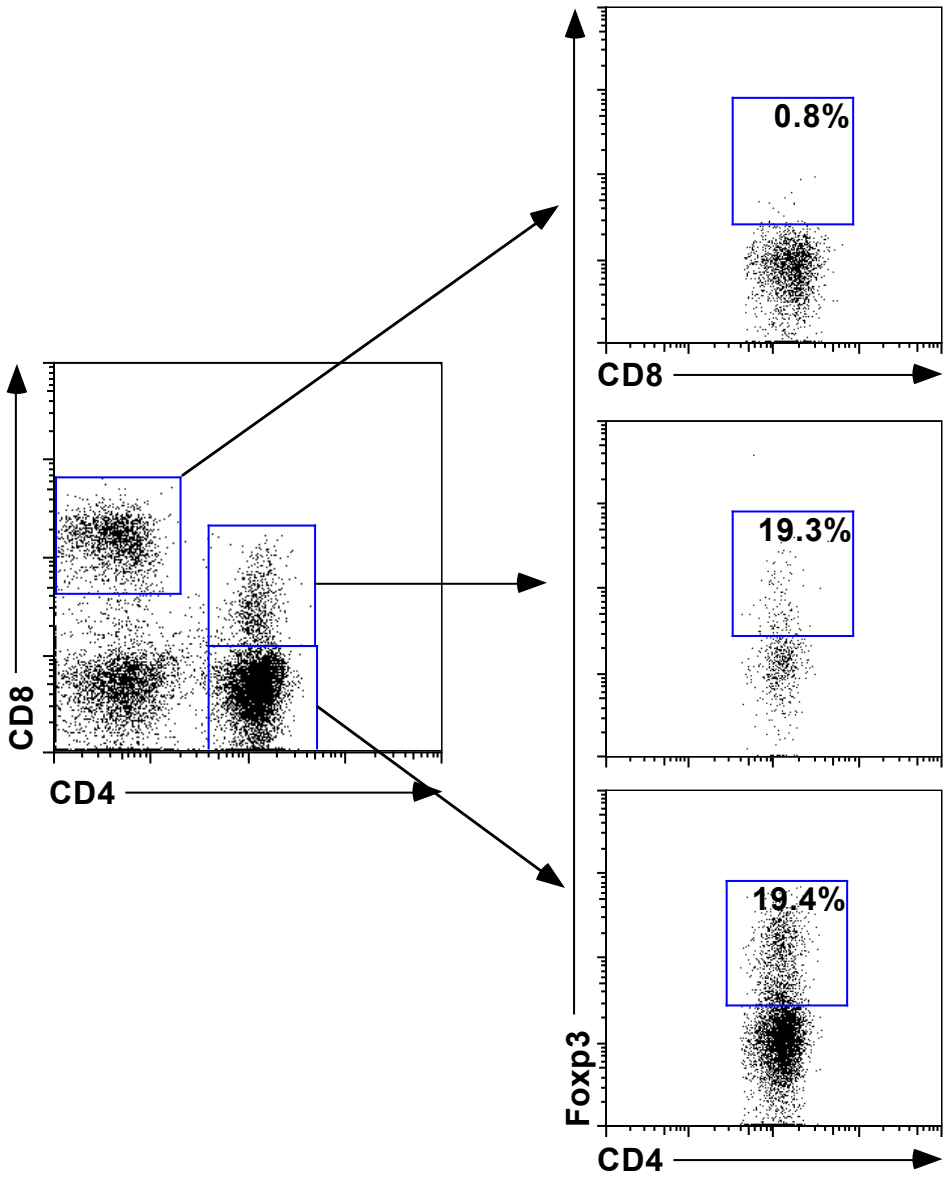

Figure 4.3 Colorectal tumour-derived single-positive CD8$^+$ T cells do not express Foxp3. Representative bivariate flow cytometry plots showing live CD4$^+$ and CD8$^+$ T cells obtained from colorectal tumour samples and the subsequent analysis of intracellular Foxp3 expression in the various subsets (A).

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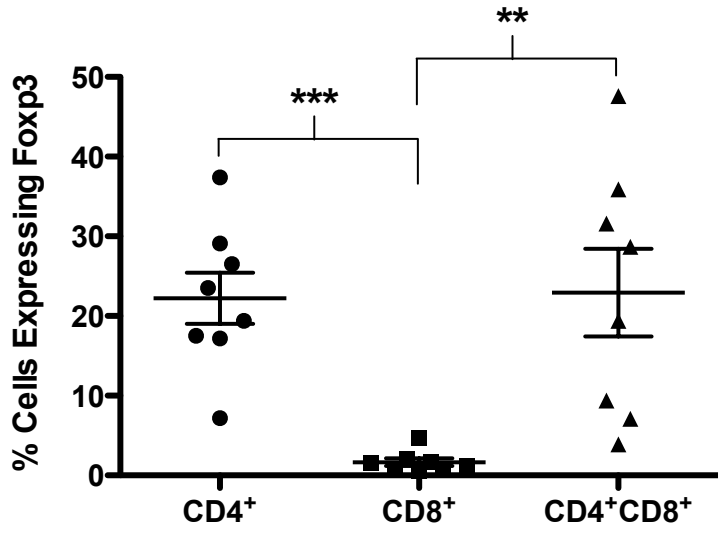

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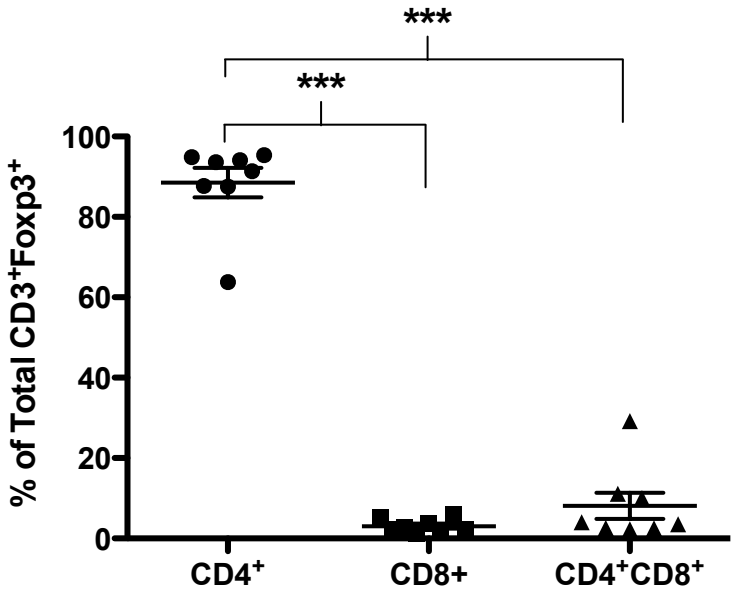

Figure 4.3 Cont'd. Colorectal tumour-derived single-positive $CD8^+$ T cells do not express Foxp3. Analysis of $CD3^+$ T cells from 9 colorectal tumour specimens for Foxp3 expression (B). Having gated on all live $CD3^+Foxp3^+$ TILs, the relative proportion of each subset (single positive $CD4^+$ / single positive $CD8^+$ / double positive $CD4^+CD8^+$) expressing Foxp3 reveals that the vast majority are single positive $CD4^+$ T cells. Significant differences are indicated; $^{**}p < 0.01$, $^{***}p < 0.001$.

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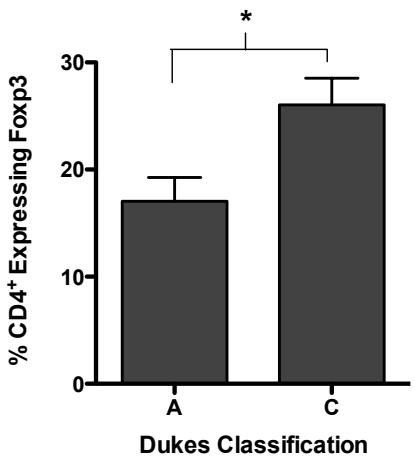

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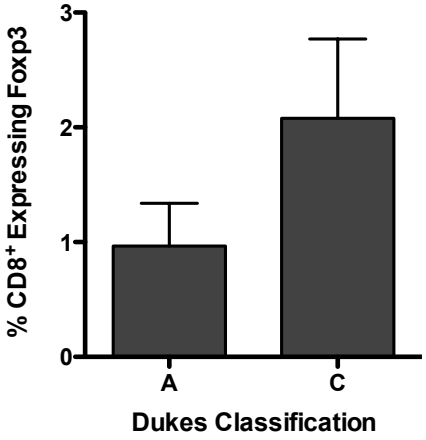

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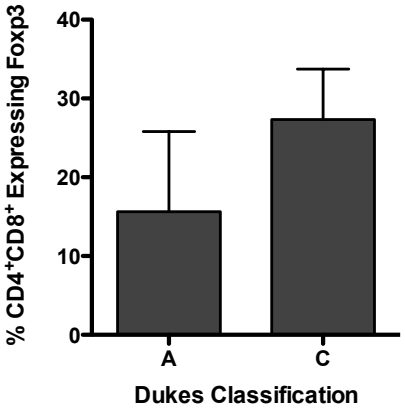

Figure 4.4 Increased Intracellular Foxp3 Expression in Intratumoural T cells as Tumours Progress. The proportion of live intratumoural $CD4^+$ (A), $CD8^+$ (B) and $CD4^+CD8^+$ double positive T cells (C) that express Foxp3 were correlated to Dukes' classification of the tumour at time of excision. (Dukes' A; n = 6, Dukes' C; n = 7). Significant differences are indicated; *$p < 0.05$.

these cells accumulate within tumours as they become more invasive. Similar, albeit non-significant increases were also found in single positive $CD8^+$ (Figure 4.4B) and double positive $CD4^+CD8^+$ T cells expressing Foxp3 (Figure 4.4C). Whilst this data corresponds favourably with previous findings from other laboratories, whereby CD4 and CD8 Treg infiltration correlates positively with tumour stage and microinvasive status (Chaput, Louafi et al. 2009), studies using immunohistochemistry to perform the same analysis in colorectal tumour tissue do not find this correlation (Loddenkemper, Schernus et al. 2006; Ling, Pratap et al. 2007).

4.2.2 Naturally-occurring and induced Treg infiltration of colorectal tumours

Our lab reported previously that conversion of effector T cells into Tregs does not account for the increased proportion of Tregs found in a murine tumour model (Hindley, Ferreira et al. 2011). Helios, a member of the Ikaros family of transcription factors, was recently identified as a marker of thymus-derived Tregs (Thornton, Korty et al. 2010). The majority of $Foxp3^+$ Tregs in CRC patients were $Helios^+$, indicating that these populations predominantly comprised naturally occurring Tregs (Figure 4.5A). However, the proportion of intra-tumoural $Foxp3^+$ Tregs that expressed Helios was slightly lower compared to the corresponding peripheral blood populations, suggesting that the degree of conversion may be slightly increased in the tumour (tumour 58.54% ± 1.56% vs. blood 67.61% ± 2.25%, $p = 0.012$). Nonetheless, this difference was small, indicating that conversion does not account for the substantial enrichment of $Foxp3^+$ Tregs in colorectal tumours. Based on the expression of Helios, approximately half of the Tregs in unaffected colon specimens appeared to be peripherally induced (colon 49.76% ± 2.77% vs. blood 67.61% ± 2.25%, $p = 0.001$). These findings are in line with previous reports demonstrating that the conversion of

Foxp3$^-$ T cells into Foxp3$^+$ T cells physiologically expands the Treg repertoire in the gut (Barnes and Powrie 2009). Thus, consistent with our previous findings in murine models, the enrichment of Tregs in tumours appears to be mainly constituted from a thymus-derived population (Hindley, Ferreira et al. 2011).

An interesting observation was that the proportion of PBMC-derived Tregs expressing Helios significantly increases with tumour advancement (Figure 4.5B; Dukes' A to D linear regression: $p = 0.0002$, $r^2 = 0.63$). Whilst healthy age-matched donors have significantly greater proportions of Helios$^+$ Tregs than Dukes' A CRC patients (HD 74.6% ± 1.94% vs. Dukes' A 62.73% ± 1.46%, $p = 0.0023$), this in turn is significantly lower than patients with more advanced Dukes' C / D patients (Dukes' A 62.73% ± 1.46% vs. Dukes' C 71.32% ± 2.54%, $p = 0.035$; vs. Dukes' D 81.34% ± 2.62%, $p = 0.0007$). This could reflect an induction of peripherally induced Tregs at early tumour stages or that Tregs in patients with more advanced tumours have a greater suppressive capacity. This finding was not replicated amongst intratumoural Foxp3$^+$ Tregs (Figure 4.5C) and Helios expression remained consistent with tumour advancement. Notably, a higher mean fluorescence intensity (MFI) of Foxp3 on intratumoural Helios$^+$ Tregs was also found (Figure 4.5D), indicative of the increased suppressive potential of this T cell subset (Chauhan, Saban et al. 2009).

In an attempt to define exhausted T cells (Sakuishi, Apetoh et al. 2010), the markers PD-1 and TIM-3 were included in the FACS staining panel. The T cell subset identified as having the greatest proportion of double positive PD-1$^+$TIM-3$^+$ T cells was intratumoural CD4$^+$Foxp3$^+$Helios$^+$ Tregs (Figure 4.6). Such an exhausted phenotype amongst TILs is thought to hinder productive anti-tumour immunity, but could also be upregulated on highly activated T cells.

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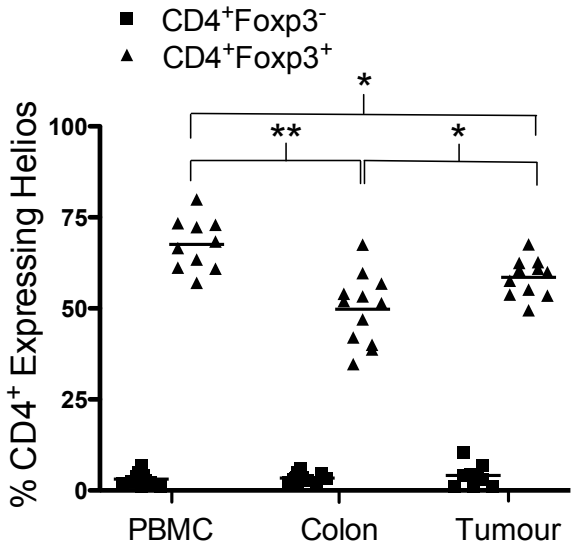


B: PBMC-derived Tregs

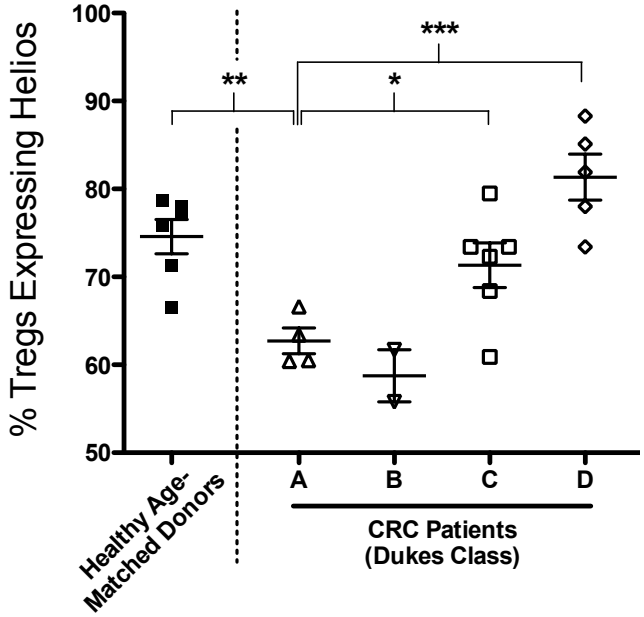
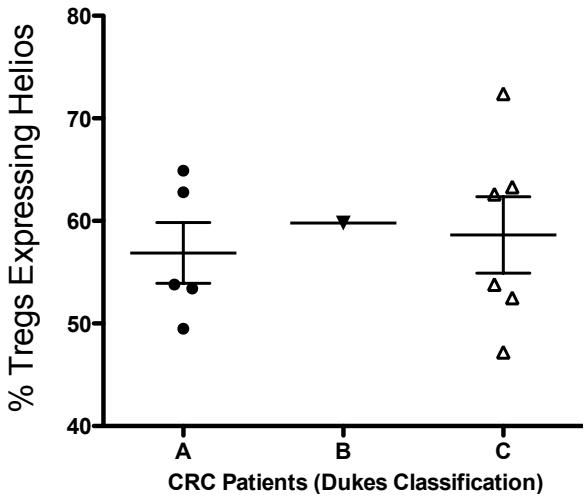


Figure 4.5 ***Ex vivo*** **Helios expression amongst CD4$^+$ T cells.** Intracellular expression of the Helios transcription factor was assessed on CD4$^+$Foxp3$^+$ Tregs (▲) and CD4$^+$Foxp3$^-$ T cells (■) isolated from blood (PBMC), unaffected colon and tumour samples from CRC patients (A). Peripheral blood-derived CD4$^+$Foxp3$^+$ Tregs were analysed for the expression of Helios and correlated to CRC patients Dukes' classification (B). Significant differences are indicated; *$p < 0.05$, **$p < 0.01$, ***$p<0.001$.

C: Tumour-derived $CD4^+Foxp3^+$ Tregs



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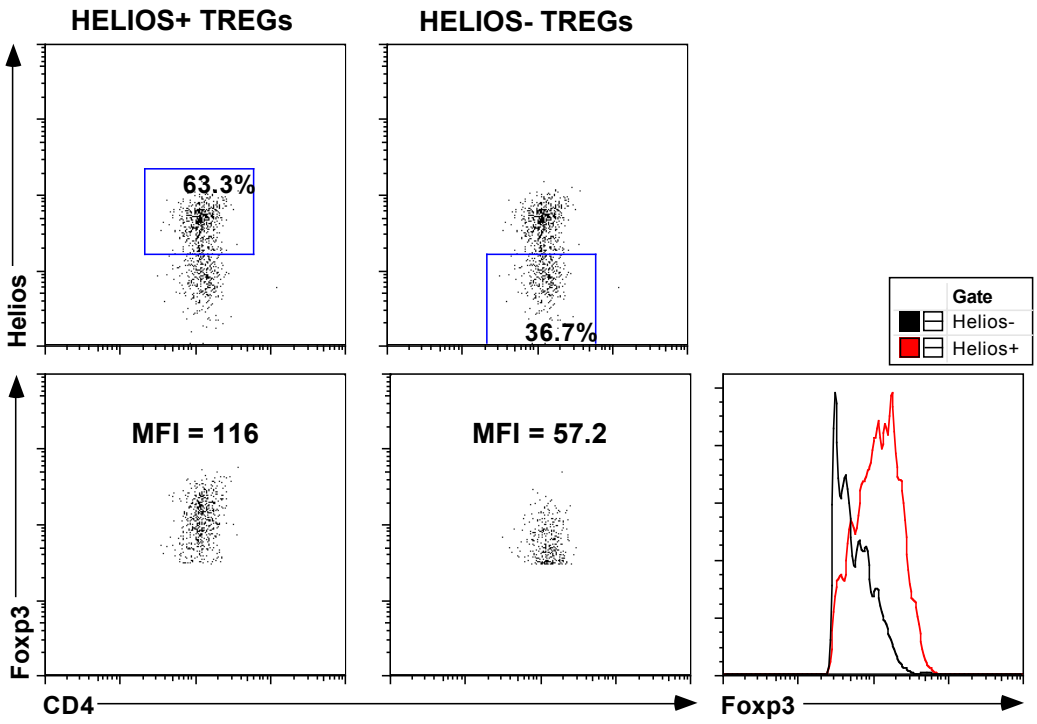

Figure 4.5 Cont'd. *Ex vivo* Helios expression amongst $CD4^+$ T cells. Tumour-derived $CD4^+Foxp3^+$ Tregs were analysed for the expression of Helios and correlated to CRC patients Dukes' classification (C). Significant differences are indicated; *p < 0.05, **p < 0.01, ***p<0.001. The mean fluorescence intensity of Foxp3 was subsequently examined in colorectal tumour-infiltrating $CD4^+Foxp3^+$ $Helios^+$ and $Helios^-$ Tregs, an example of which is shown with corresponding histogram (D).

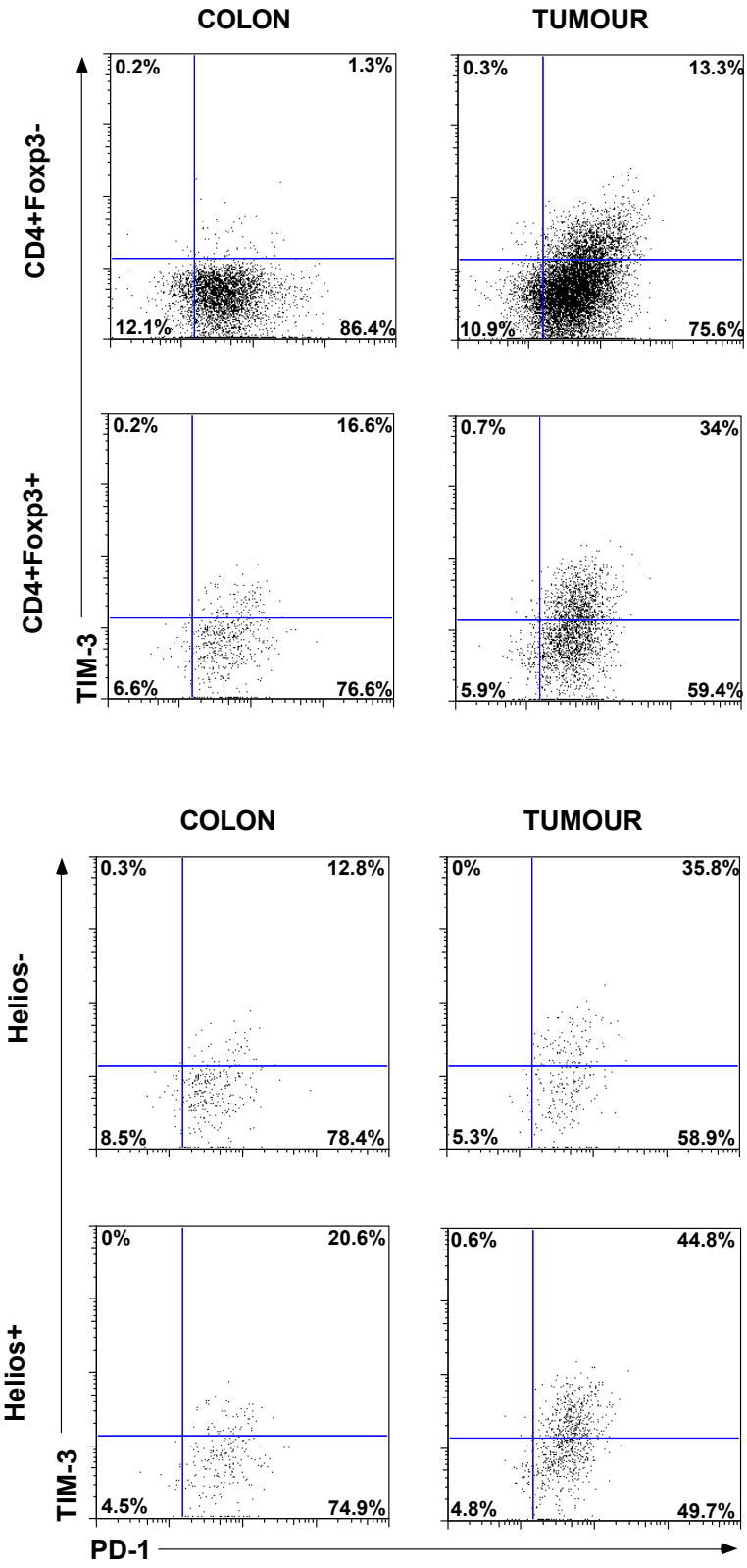

Figure 4.6 CD4^{+}Foxp3^{+} Helios^{+} TILs display the most exhausted phenotype. Live CD4^{+}Foxp3$^{+/-}$ (top four plots) and CD4^{+}Foxp3^{+}Helios$^{+/-}$ T cells (bottom four plots), found in the colon and tumour, were analysed for the co-expression of PD-1 and TIM-3 by FACS.

4.2.3 The majority of $CD4^+Foxp3^+$ regulatory T cells express ICOS

The inducible T cell co-stimulator (ICOS) molecule is expressed on T follicular helper cells (T_{FH}) and activated T cells; interactions with the ICOS-ligand enhance T cell proliferation, cytokine production and survival (Mahajan, Cervera et al. 2007). ICOS is also expressed abundantly on $CD4^+CD25^{high}Foxp3^+$ Tregs, particularly those found in tumours (Strauss, Bergmann et al. 2008). Here, a similar pattern was identified in this cohort of CRC patients; an example of ICOS expression amongst colorectal $CD4^+$ TILs is shown, alongside representative Foxp3 and Ki67 expression of $ICOS^+$ and $ICOS^-$ subsets (Figure 4.7A). ICOS expression appeared to define a large proportion of the $CD4^+Foxp3^+$ TILs (Figure 4.7B). In all patients studied, ICOS was expressed mainly on $CD4^+Foxp3^+$ T cells compared to $CD4^+Foxp3^-$ T cells in blood, colon and tumour (Figure 4.7C). The low levels of ICOS expression on $CD4^+Foxp3^-$ cells did not differ significantly by site (blood 13.91% ± 2.56% vs. colon 16.24% ± 4.07% vs. tumour 20.61% ± 3.49%, $p = 0.37$). However, in line with previous reports, $CD4^+Foxp3^+$ TILs expressed significantly higher levels of ICOS than peripheral blood $CD4^+Foxp3^+$ T cells (blood 45.46% ± 2.58% vs. colon 39.10% ± 6.81% vs. tumour 60.78% ± 4.42%, $p = 0.039$). Furthermore, there was a very strong correlation between Foxp3 expression and ICOS expression on $CD4^+$ TILs ($p = 0.004$, $R^2 = 0.71$; Figure 4.7D).

$CD4^+ICOS^{+/-}$ T cells were purified from colorectal tumour specimens to further assess their functional capacity. In a standard three day *in vitro* proliferation assay, $CD4^+ICOS^+$ TILs were significantly more proliferative in response to stimulation with αCD3/28 beads compared to the corresponding $CD4^+ICOS^-$ T cell subset (Two-way ANOVA of $CD4^+ICOS^+$ vs. $CD4^+ICOS^-$ at each cell count; $p<0.0001$; Figure 4.8A), a finding that is paralleled by *ex vivo* Ki67 staining (Figure

4.7A). In addition, isolated TILs were added back to FACS-sorted autologous PBMC-derived $CD4^+CD25^-$ T cells, stimulated with αCD3/28 beads, to assess for suppression over a 3-day assay. Varying degrees of suppression were found and due to excessive proliferation of isolated TILs in some experiments, this lead to a potential underestimation of the suppressive effect, as analysed by tritiated thymidine incorporation (Figure 4.8B). Where suppression could be analysed, what is striking from this data is that, although $CD4^+ICOS^+$ TILs were highly suppressive *in vitro* as expected, the $CD4^+ICOS^-$ TIL subset were in fact more suppressive at every regulatory to effector cell ratio. However, this should be interpreted with caution since the effector T cells present in the culture produce IL-2, which may cause the $CD4^+ICOS^+$ T cells to proliferate more than the $CD4^+ICOS^-$ T cells, thus masking their suppressive effect.

Taken together, the expression patterns of Foxp3, Helios and ICOS delineate a population of naturally occurring, highly proliferative Treg that infiltrate colorectal tumours.

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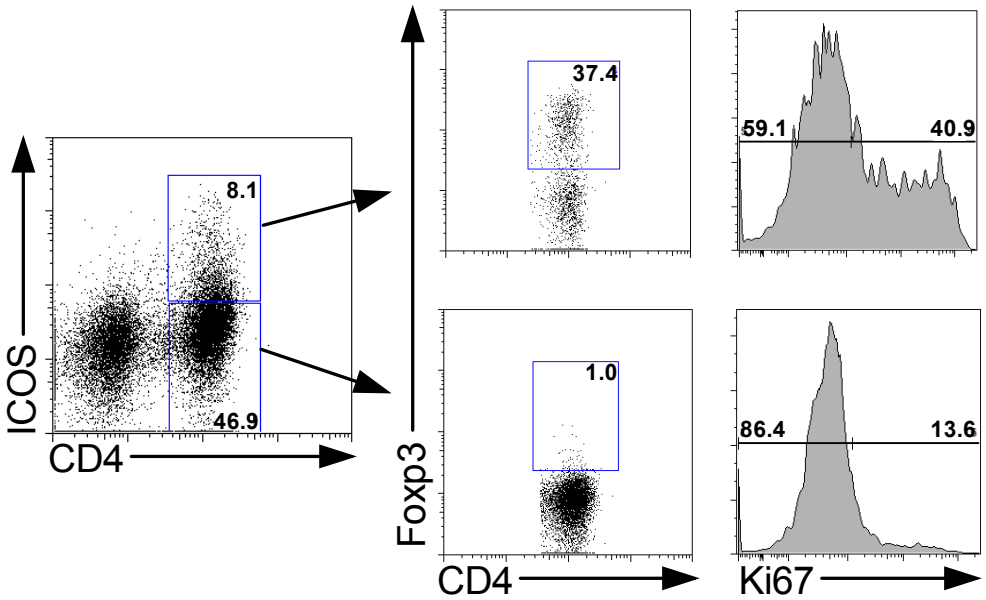
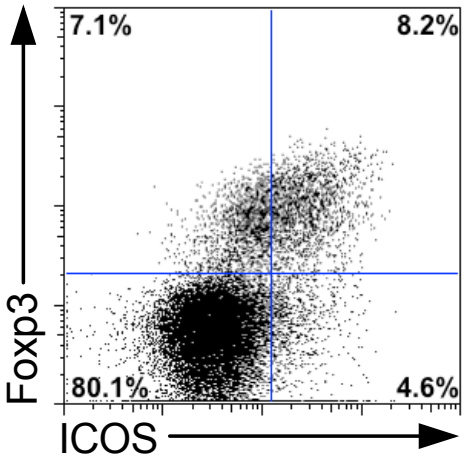
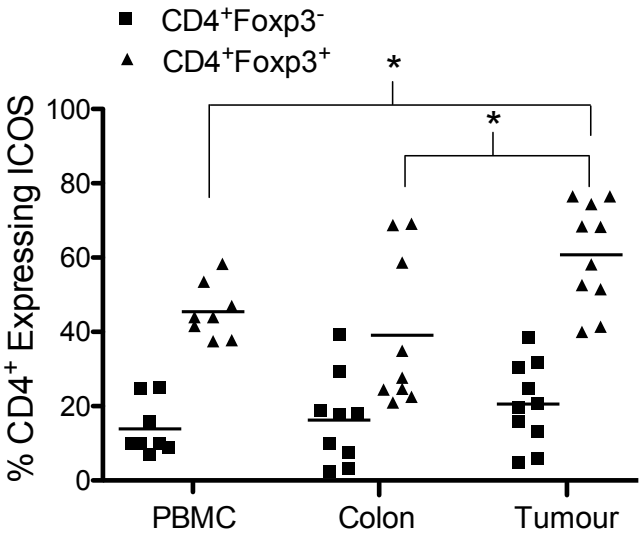

8.1
46.9
ICOS
CD4
37.4
1.0
Foxp3
CD4
59.1
40.9
86.4
13.6
Ki67

B:


7.1%
8.2%
80.1%
4.6%
Foxp3
ICOS

C:



D:

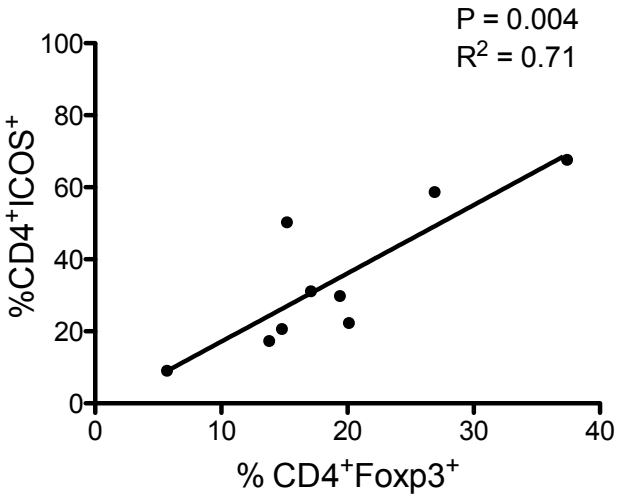
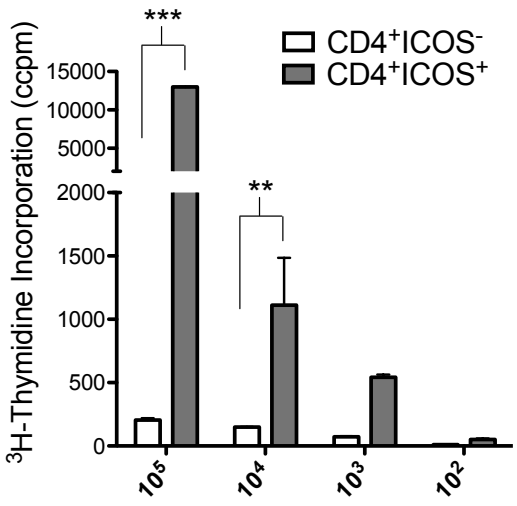


Figure 4.7 *Ex vivo* ICOS expression amongst $CD4^+$ T cells. Representative flow cytometry profiles showing immunofluorescence staining of live $CD4^+ICOS^+$ and $CD4^+ICOS^-$ TILs, and their corresponding expression of Foxp3 and Ki67 (A and B). Cell surface expression of ICOS was assessed on $CD4^+Foxp3^+$ Tregs (▲) and $CD4^+Foxp3^-$ T cells (■) isolated from blood (PBMC), unaffected colon and tumour samples from CRC patients. Significant differences are indicated; *$p < 0.05$. (C) Linear regression comparing the percentage of intra-tumoural $CD4^+$ T cells expressing ICOS with the percentage of intra-tumoural $CD4^+Foxp3^+$ Tregs (D).

A:



B:

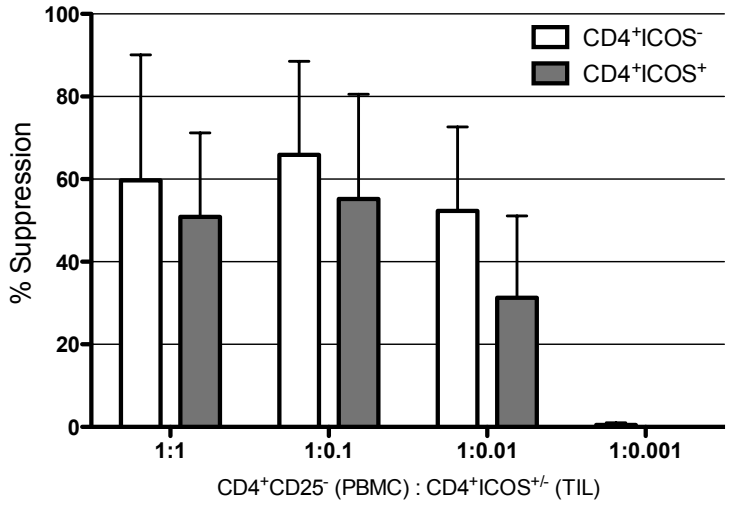


Figure 4.8 CD4$^+$ICOS$^+$ / ICOS$^-$ T cell function. CD4$^+$ICOS$^+$ and CD4$^+$ICOS$^-$ TILs were FACS sorted from a Dukes' A colorectal tumour and matched healthy colonic mucosa. (A) CD4$^+$ICOS$^{+/-}$ TILs were stimulated with αCD3/28 beads for 72 hours. Each cell dilution was carried out in duplicate. Two-way ANOVA with results of Bonferroni post-test analysis are indicated (***$p<0.001$, **$p < 0.01$). Sorted TILs were added back to autologous CD4$^+$CD25$^-$ effector T cells (FACS sorted from peripheral blood sample) at indicated ratios and cultured for a 72-hour period (B). Proliferation over the final 6 hours was measured by tritiated thymidine incorporation and % suppression calculated. Data is compiled from four independent experiments.

4.2.4 $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells derived from blood, healthy colon and colorectal tumours are phenotypically distinct

A detailed phenotypic analysis of $CD4^+$ T cells was conducted using a panel of antibodies specific for the markers CD25, CTLA-4, CD39, LAG-3, CD103, ICOS and Ki67, many of which are associated with natural Tregs (Miyara, Yoshioka et al. 2009). Representative flow cytometry plots are shown in Figure 2.4. Specifically, $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells from blood, healthy colon and colorectal tumour were compared (Figure 4.9 and Tables 4.1-4.3). $CD4^+$ T cells derived from each compartment expressed distinct patterns of these phenotypic markers. Expression levels of several markers, such as CTLA-4 and CD39, increased steadily on both $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells when comparing blood to healthy colon to tumour (Figure 4.9). Many markers associated with Tregs, such as the IL-2 receptor α-chain CD25 and CTLA-4, are also known to be present on activated T cells (Kmieciak, Gowda et al. 2009). Accordingly, this upregulation could reflect an altered state of activation in these distinct colonic and tumoural niches. This is illustrated by CD25 expression (Figure 4.9A), which remained relatively stable on $Foxp3^+$ T cells but was significantly increased amongst $Foxp3^-$ T cells (blood 9.69% ± 1.08% vs. colon 28.93% ± 3.51%, $p = 0.0009$; blood vs. tumour 41.23% ± 3.05%, $p < 0.0001$). Likewise, a greater proportion of intra-tumoural $CD4^+Foxp3^-$ T cells proliferate in comparison to blood and colon, as denoted by Ki67 expression (blood 5.39% ± 0.46% vs. colon 7.37% ± 1.43%, $p = 0.27$; blood vs. tumour 26.07% ± 3.96%, $p = 0.0015$; colon vs. tumour, $p = 0.0039$) (Figure 4.9D). Reduced levels of the IL-7 receptor CD127 and the anti-apoptotic protein BCL-2 were also observed amongst the $CD4^+Foxp3^-$ TILs (Figure 4.9G and H, respectively). CTLA-4 (Figure 4.9B), which

is an important negative immunomodulator that decreases cellular cytokine production and proliferation (Schneider, Downey et al. 2006), followed a similar pattern of expression to CD25 (blood 5.54% ± 1.77% vs. colon 28.03% ± 7.66%, $p = 0.024$; blood vs. tumour 43.61% ± 5.51%, $p = 0.0004$). There was also a significant increase in CTLA-4 expression on intra-tumoural $Foxp3^+$ Tregs (blood 51.34% ± 6.91% vs. colon 77.08% ± 6.83%, $p = 0.0052$; blood vs. tumour 89.60% ± 3.72%, $p = 0.0002$).

CD39 is an ectonucleotidase, which together with CD73 drives catabolism of extracellular ATP resulting in the generation of adenosine, an immunosuppressive molecule often elevated in cancer tissue (Deaglio, Dwyer et al. 2007). Both molecules are often expressed on $Foxp3^+$ T cells, and generation of adenosine by these cells is one of the means by which intra-tumoural Tregs may enact their suppressive function (Deaglio, Dwyer et al. 2007). We found that $Foxp3^+$ Tregs displayed markedly increased expression of CD39 in healthy colon and tumour (blood 44.10% ± 4.94% vs. colon 75.42% ± 8.16%, $p = 0.0003$; blood vs. tumour 90.83% ± 4.53%, $p < 0.0001$) (Figure 4.9C). However, as with CTLA-4, a marked and significant increase in CD39 expression was also observed amongst intra-tumoural $Foxp3^-$ T cells (blood 5.24% ± 0.99% vs. colon 55.58 ± 9.85%, $p = 0.0011$; blood vs. tumour 74.8% ± 6.79, $p < 0.0001$).

The marked increase in CD39, together with CD25 and CTLA-4, in the $Foxp3^-$ population suggests a possible regulatory role for these cells within the tumour environment. Accordingly, we examined other markers associated with regulatory function, including the CD4 homologue, lymphocyte activation gene-3 (LAG-3) (Huang, Workman et al. 2004; Woo, Turnis et al. 2012). Although expression of

LAG-3 amongst $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells was comparable (Figure 4.9E), intra-tumoural levels were markedly increased on both $CD4^+Foxp3^-$ TILs (blood 10.0% ± 3.1% and colon 11.44% ± 3.74% vs. tumour 34.92% ± 8.18%, p = 0.023) and $CD4^+Foxp3^+$ Tregs (blood 7.24% ± 1.56% and colon 12.69% ± 2.49% vs. tumour 28.18% ± 5.68%, p = 0.02).

The αE integrin, CD103, binds to the receptor E-cadherin and is thought to play a role in T cell retention within the gut (Schon, Arya et al. 1999); it has also been shown to denote populations of highly suppressive $CD25^+$ and $CD25^-$ T cells (Lehmann, Huehn et al. 2002). In the cohort of patients studied here, CD103 expression was elevated in colorectal tumours, particularly within the $CD4^+Foxp3^-$ population (blood 2.08% ± 0.22% vs. colon 9.54% ± 2.49%, p = 0.022; blood and colon vs. tumour 34.83% ± 8.06%, p = 0.01; Figure 4.9F). Recent reports claim that CD103 expression is significantly increased on CRC-resident Tregs compared to unaffected colon (Svensson, Olofsson et al. 2012); however, despite a trend, this was not found to be the case in this cohort (colon 5.56% ± 1.50% vs. tumour 10.19% ± 2.66%, p = 0.13).

In summary, a significant proportion of intra-tumoural $CD4^+Foxp3^-$ cells demonstrated a unique phenotype characterized by high expression levels of molecules predominantly associated with Tregs, including CD25, CD39, CTLA-4, LAG-3, and CD103 (summarized in Figure 4.9 and Tables 4.1-4.2). This finding warranted further investigation into the function of these $CD4^+Foxp3^-$ cells and, specifically, to determine whether they could contribute to intra-tumoural immunosuppression.

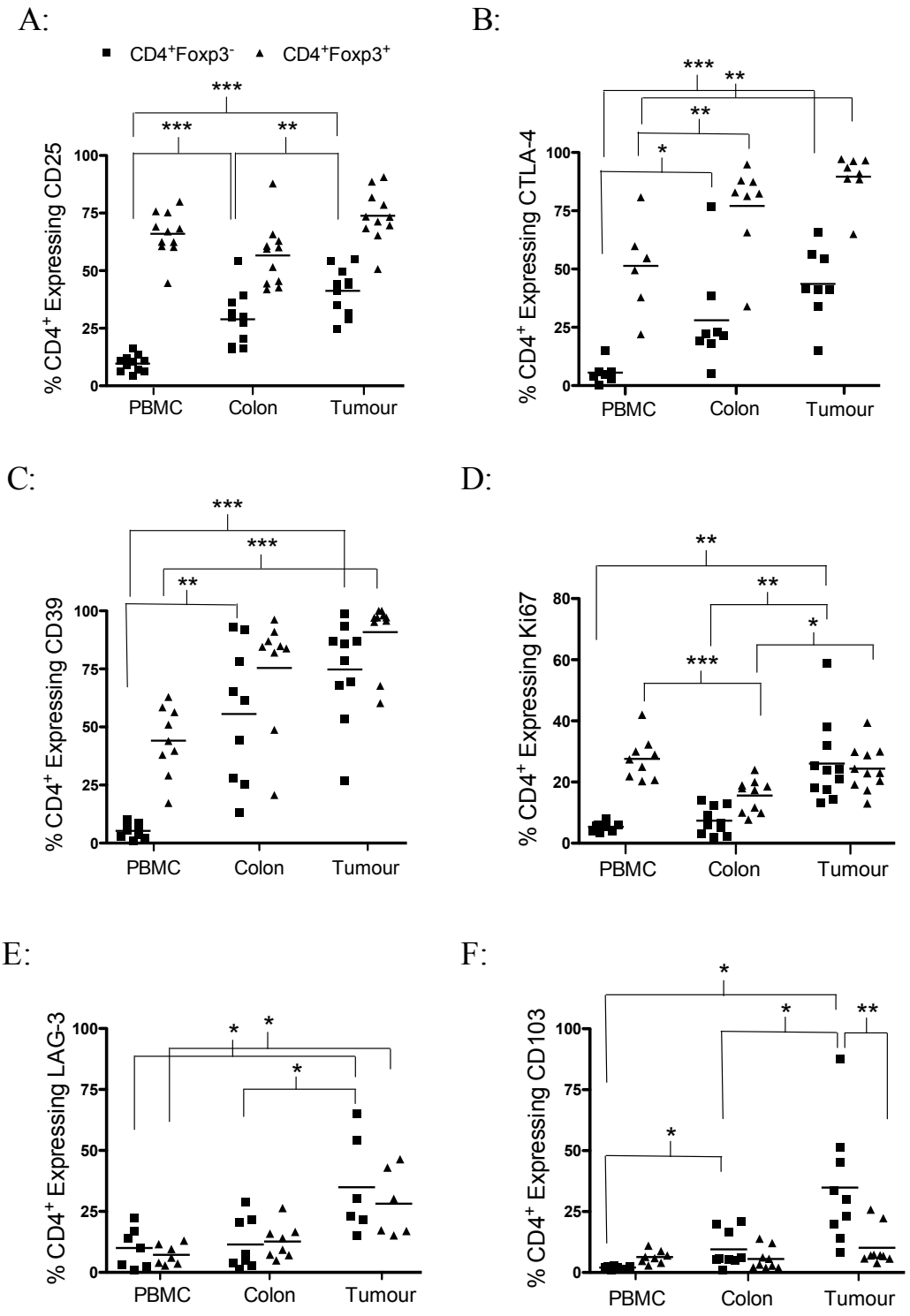


Figure 4.9 *Ex vivo* phenotypic analysis of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells in different compartments. Compiled flow cytometry data are shown for expression of CD25 (A), CTLA-4 (B), CD39 (C), Ki67 (D), LAG-3 (E) and CD103 (F) amongst $CD4^+Foxp3^+$ Tregs (▲) and $CD4^+Foxp3^-$ T cells (■) in matched blood (PBMC), unaffected colon and tumour samples. Significant differences are indicated; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

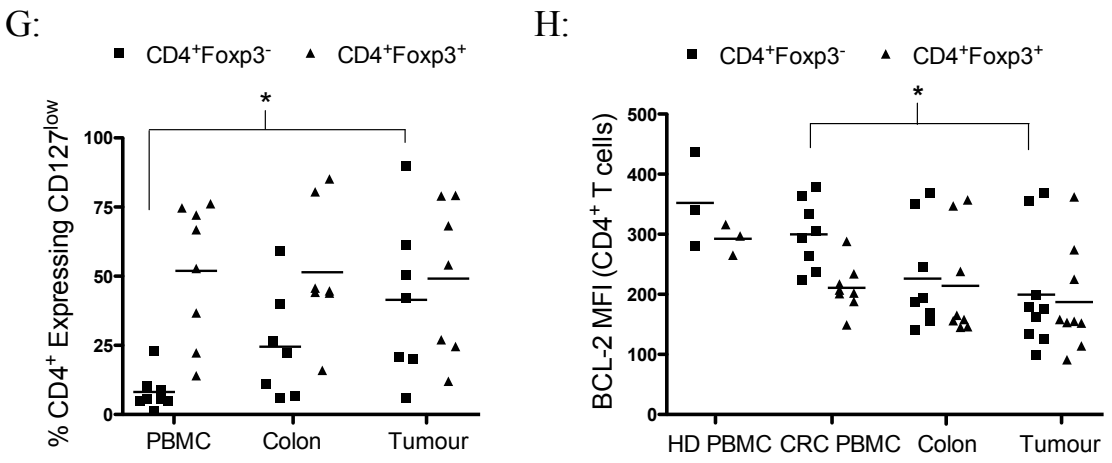


Figure 4.9 Cont'd. *Ex vivo* phenotypic analysis of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells in different compartments. Compiled flow cytometry data are shown for expression of $CD127^{low}$ (G) and BCL-2 (H) amongst $CD4^+Foxp3^+$ Tregs (▲) and $CD4^+Foxp3^-$ T cells (■) in matched blood (PBMC), unaffected colon and tumour samples. For BCL-2, data are shown as mean fluorescence intensity (MFI). Significant differences are indicated; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 4.1: Phenotypic overview of $CD4^{+}Foxp3^{+/-}$ T cells.

		CTLA-4	LAP	LAG-3	CD39	CD103	Ki67	CD25	Helios	ICOS
Tumour	$CD4^{+}FoxP3^{-}$	++	++	++	+++	++	++	++	-	+
	$CD4^{+}FoxP3^{+}$	+++	+	+	+++	+	++	+++	+++	+++
Colon	$CD4^{+}FoxP3^{-}$	++	+	-	+++	+	-	+	-	+
	$CD4^{+}FoxP3^{+}$	+++	+	-	+++	-	+	++	++	++
PBMC	$CD4^{+}FoxP3^{-}$	-	-	-	-	-	-	-	-	+
	$CD4^{+}FoxP3^{+}$	++	-	-	++	-	++	++	+++	++

Table 4.2: Phenotypic overview of $CD4^{+}ICOS^{+/-}$ T cells.

		Foxp3	LAG-3	CD39	CD103	Ki67	CD25	Helios	LAP
Tumour	$CD4^{+}ICOS^{-}$	-	++	+++	++	++	++	-	++
	$CD4^{+}ICOS^{+}$	++	+	+++	+	++	++	++	+
Colon	$CD4^{+}ICOS^{-}$	-	+	++	-	-	+	-	+
	$CD4^{+}ICOS^{+}$	++	+	+++	-	+	+	+	+
PBMC	$CD4^{+}ICOS^{-}$	-	-	-	-	-	-	-	-
	$CD4^{+}ICOS^{+}$	++	-	++	-	+	++	++	-

- = Average Expression < 10% of cells

+ = Average Expression 10% - 24.9% of cells

++ = Average Expression 25% - 49.9% of cells

+++ = Average Expression > 50% of cells

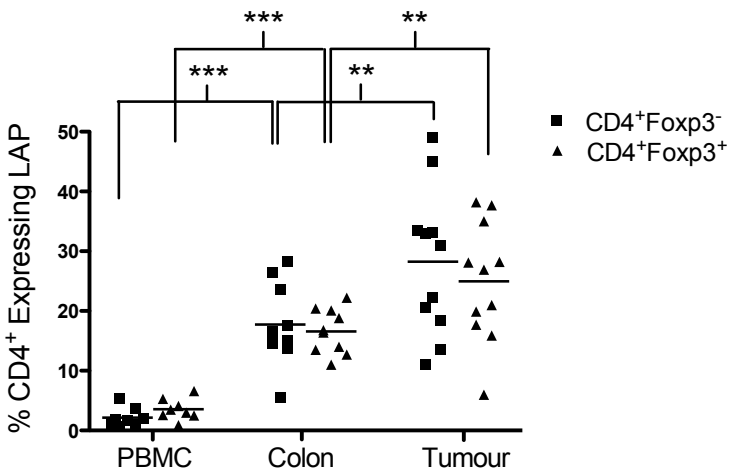
4.2.5 $CD4^+LAP^+Foxp3^-$ TILs act as a major regulatory T cell subset

The latency-associated peptide (LAP) has previously been described as a marker of regulatory T cells in human peripheral blood that are distinct from conventional $Foxp3^+$ Tregs (Gandhi, Farez et al. 2010). Here, LAP expression was analysed on $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells from blood, healthy colon and tumour samples. Although comparable levels were observed in peripheral blood, LAP expression was markedly increased on both $Foxp3^+$ T cells (blood 3.56% ± 0.63% vs. colon 16.59% ± 1.18%, $p < 0.0001$; blood vs. tumour 24.96% ± 3.01%, $p = 0.0007$) and $Foxp3^-$ T cells (blood 2.15% ± 0.56% vs. colon 17.72% ± 2.13%, $p = 0.0001$; blood vs. tumour 28.24% ± 3.69%, $p = 0.0007$) in healthy colon and tumour samples (Figure 4.10A). This marked enrichment of $CD4^+LAP^+$ T cells was most striking in early stage Dukes' A tumours (Figure 4.10B). It has previously been shown that $CD4^+Foxp3^-LAP^+$ T cells are found at significantly greater proportions amongst peripheral blood-derived T cells in CRC patients with metastatic disease (Mahalingam, Lin et al. 2012); whilst a positive trend was found here between early (Dukes' A/B) and later stage (Dukes' C) CRC patients, this was not significant ($p = 0.18$; Figure 4.10C).

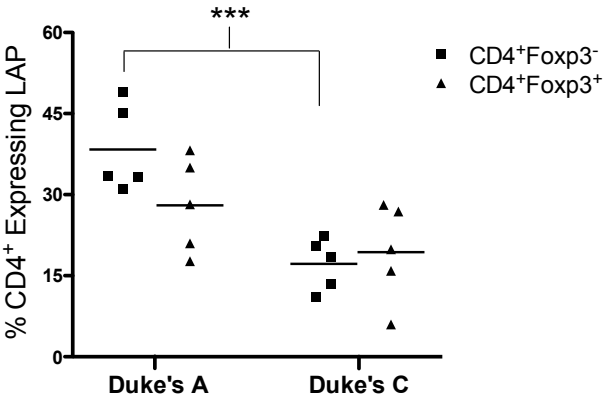
The majority of $CD4^+LAP^+$ TILs co-expressed LAG-3, CD25 and PD-1 (Figure 4.10D and Table 4.3); in contrast, <10% of the corresponding $CD4^+LAP^-$ cells expressed LAG-3 and CD25. Of note, however, most $CD4^+LAP^+$ TILs (> 85%) did not express Foxp3. Although a greater proportion of $CD4^+LAP^+$ T cells express both PD-1 and TIM-3 in the tumour than the colon, this is no greater than $CD4^+LAP^-$ TILs (Figure 4.10E).

Collectively, these observations demonstrate that the $CD4^+LAP^+$ T cell subset is largely distinct from conventional $Foxp3^+$ Tregs and significantly enriched in tumours compared to blood (Figure 4.11). The co-expression of the suppressive markers CD25, CD39, CTLA-4, Helios and LAP were visually collated by importing representative FACS data, from live $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells present in peripheral blood, background colonic mucosa and colorectal tumours, into SPICE charts (Figure 4.12). Such analysis reveals the marked co-expression of regulatory markers, with a profound accumulation around $CD4^+LAP^+Foxp3^-$ T cells that infiltrate colorectal tumours. In addition, whilst these markers associate with Foxp3 expression in $CD4^+$ T cells present in peripheral blood and normal colon, this is not the case for T cells in the tumour and as such, Foxp3 may not necessarily be a good marker to distinguish suppressive from non-suppressive T cells in colorectal cancer.

A:



B: Tumour-derived $CD4^+LAP^+$ T cells



C: PBMC-derived $CD4^+Foxp3^-LAP^+$ T cells

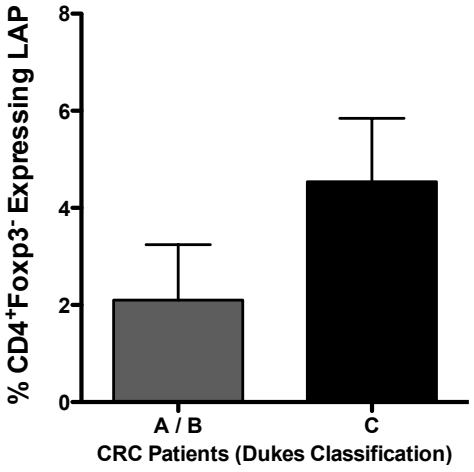
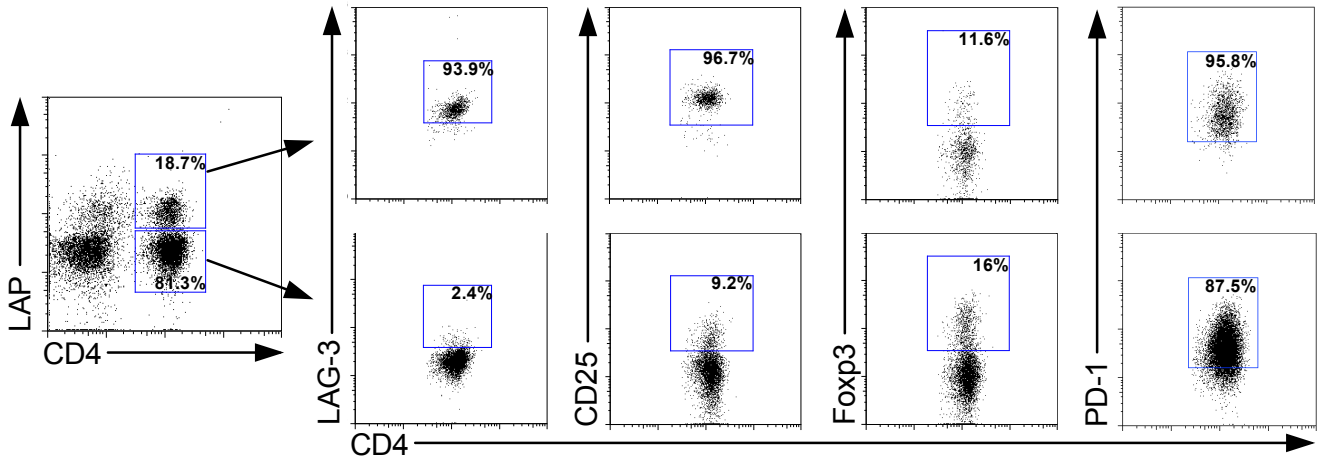


Figure 4.10 $CD4^+$ T cells expressing LAP and LAG-3, but not Foxp3, are enriched in colorectal tumours. Expression of LAP on $CD4^+Foxp3^+$ Tregs (▲) and $CD4^+Foxp3^-$ T cells (■) from matched blood, unaffected colon and tumour samples (A), with the latter stratified as originating from histo-pathologically confirmed Dukes' grade A (early) and Dukes' grade C (advanced) tumours (B). The proportion of peripheral blood-derived $CD4^+Foxp3^-$ T cells expressing LAP was correlated to early (Dukes' A/B) or advanced (Dukes' C) tumours (C). Significant differences are indicated; $^{**}p < 0.01$, $^{***}p < 0.001$.

D:



E:

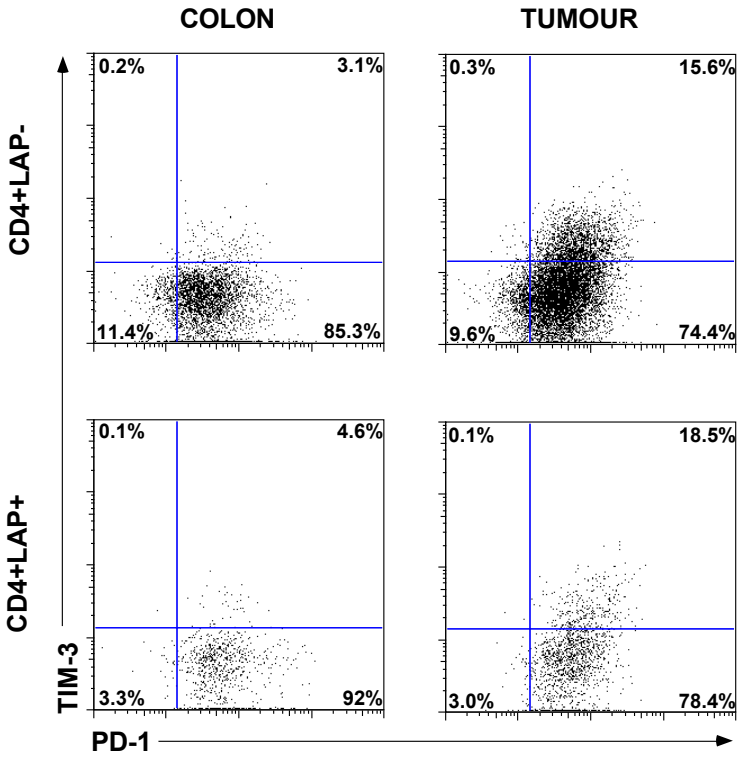


Figure 4.10 Cont'd. CD4$^+$ T cells expressing LAP and LAG-3, but not Foxp3, are enriched in colorectal tumours. Representative phenotypic analysis of CD4$^+$LAP$^+$ and CD4$^+$LAP$^-$ TILs, showing expression profiles of LAG-3, CD25, PD-1 and Foxp3 (D), and dual expression of PD-1 and TIM-3 (E).

Table 4.3: Phenotypic overview of $CD4^+LAP^{+/-}$ T cells.

		CTLA-4	Foxp3	LAG-3	CD39	CD103	Ki67	CD25	ICOS
Tumour	$CD4^+LAP^-$	+++	++	-	+++	+	++	-	+
	$CD4^+LAP^+$	+++	+	+++	+++	++	++	+++	+
Colon	$CD4^+LAP^-$	++	+	-	++	-	+	-	-
	$CD4^+LAP^+$	++	+	-	+++	-	+	+++	+
PBMC	$CD4^+LAP^-$	+	-	-	-	-	-	-	+
	$CD4^+LAP^+$	+	-	-	-	-	+	+++	++

- = Average Expression < 10% of cells

+ = Average Expression 10% - 24.9% of cells

++ = Average Expression 25% - 49.9% of cells

+++ = Average Expression > 50% of cells

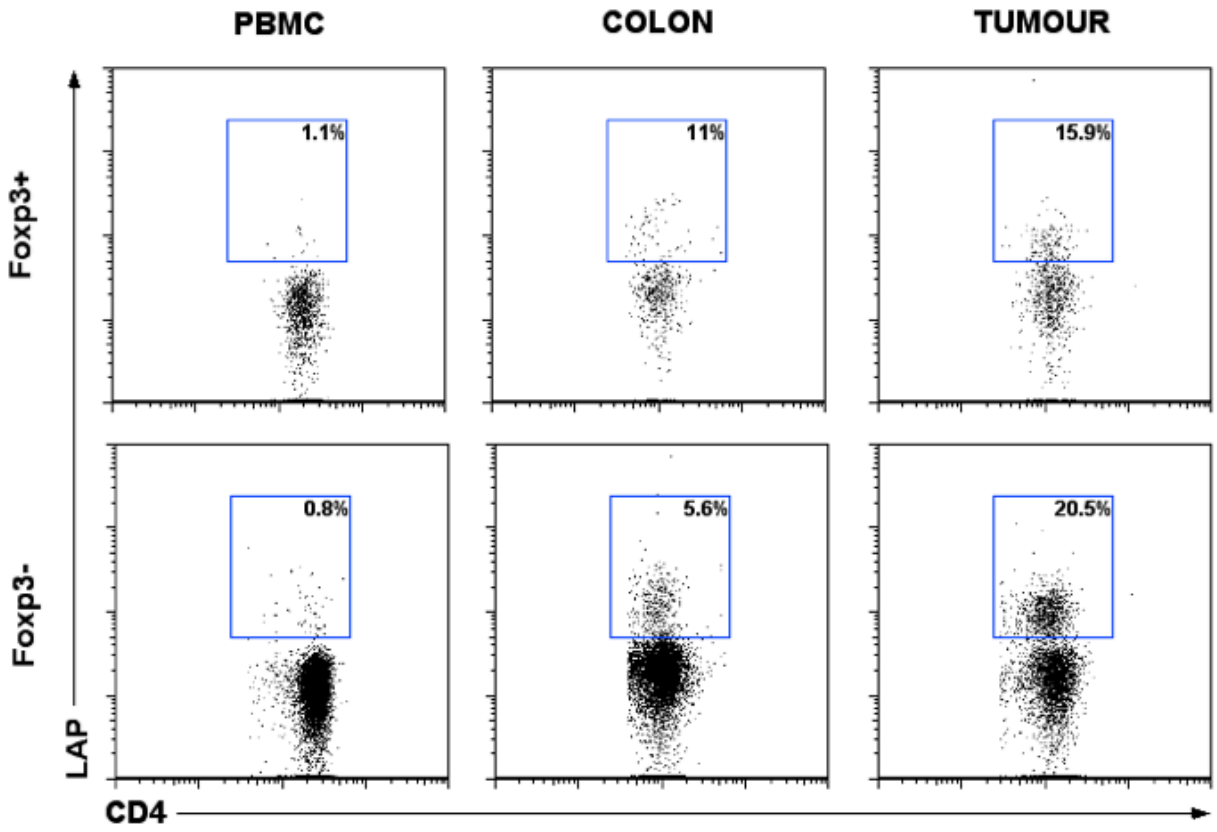


Figure 4.11 Expression of membrane bound TGF-β1 (LAP) on live Foxp3$^{+/-}$ CD4^{+} T cells. Representative bivariate FACS plots showing the expression of LAP on CD4^{+} T cells isolated from peripheral blood, colon and tumour samples. The highest proportion of LAP expression is amongst CD4^{+}Foxp3^{-} T cells found infiltrating colorectal tumours.

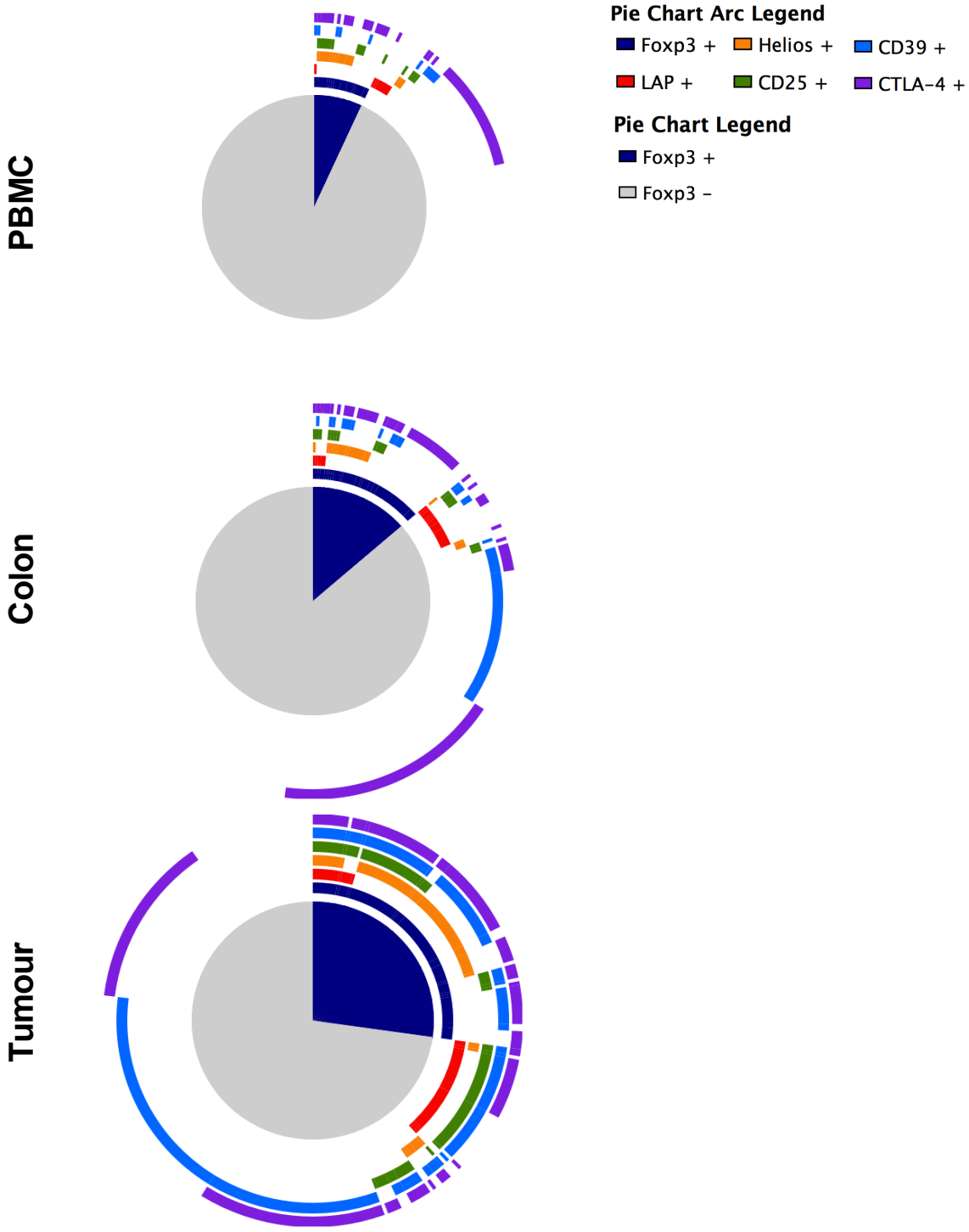


Figure 4.12 Concatenated *ex vivo* phenotypic analysis of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells in different compartments originating from one representative CRC patient. Pie charts shows the relative proportion of live $Foxp3^+$ (blue) and $Foxp3^-$ (grey) $CD4^+$ T cells in each compartment; pie chart arcs show expression of the indicated markers in these subsets.

Next, PBMC, colon and tumour infiltrating lymphocytes were stimulated polyclonally with PMA and ionomycin to determine cytokine production, namely IFN-γ, IL-10 and IL-17A, by $CD4^+LAP^+$ T cells present in these compartments. Upon stimulation, around 15-20% of $CD4^+LAP^+$ T cells in peripheral blood produce the potent, multifunctional Th1 cytokine, IFN-γ (Figure 4.13A), consistent with previous observations from other groups (Gandhi, Farez et al. 2010; Mahalingam, Lin et al. 2012). However, this cell subset produces far less IFN-γ in the colon (Figure 4.13B) and almost no IFN-γ in the tumour (Figure 4.13C), where $CD4^+LAP^-$ T cells significantly predominate the IFN-γ production ($CD4^+LAP^-$ 28.3% ± 2.8% vs. $CD4^+LAP^+$ 2.2% ± 1.1%, $p = 0.013$).

IL-17A-secreting $CD4^+$ 'Th17 cells' have been implicated in a number of autoimmune disorders but play important roles in anti-microbial immunity (Weaver and Hatton 2009; Maloy and Powrie 2011) and have been shown to enhance (Martin-Orozco, Muranski et al. 2009) and suppress anti-tumour immune responses (Ma and Dong 2011). Here, very little IL-17A production was detected amongst T cells derived from the peripheral blood of CRC patients (Figure 4.13A). The small population of Th17 cells detected (~0.3% of CD4's) were $CD4^+LAP^-$ T cells. A greater proportion of $CD4^+$ T cells produced IL-17A in the colon (5-10% of CD4's; Figure 4.13B), unsurprising given their aforementioned role in anti-microbial immunity. Here, little difference was observed between the proportion of $CD4^+LAP^+$ and $CD4^+LAP^-$ T cells producing this cytokine ($CD4^+LAP^-$ 7.2% ± 0.3% vs. $CD4^+LAP^+$ 5.7% ± 3.9%, $p = 0.73$). In the colorectal tumour specimens analysed, over 10% of the $CD4^+LAP^-$ T cell population produced IL-17A, whilst $CD4^+LAP^+$ T cells produced relatively little ($CD4^+LAP^-$ 13.8% ± 2.6% vs. $CD4^+LAP^+$ 4.5% ± 1.9%,

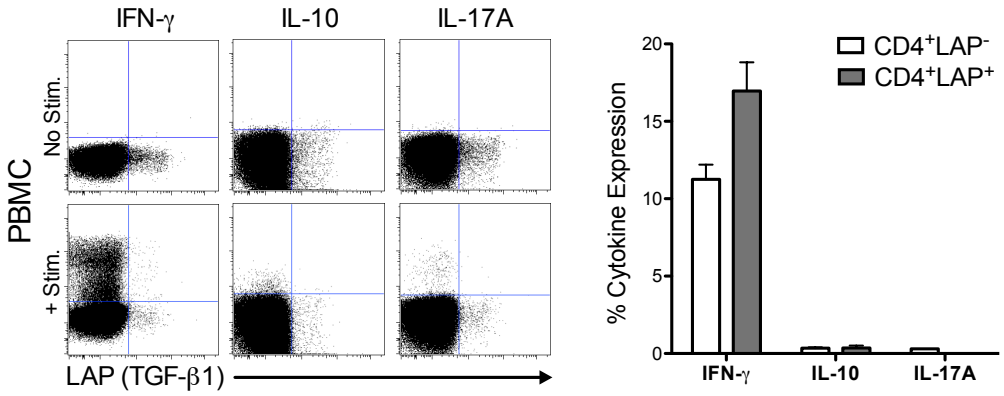
p = 0.0503; Figure 4.13C). It has been shown that high expression of IL-17 in colorectal tumours is detrimental to patient survival (Tosolini, Kirilovsky et al. 2011).

Very little production of the immunosuppressive cytokine IL-10 was detected amongst peripheral blood-derived $CD4^+$ T cells (Figure 4.13A). This is in stark contrast to the colon and tumour-infiltrating lymphocytes, as $CD4^+LAP^+$ T cells produced large amounts of IL-10 (Figure 4.13B and C). Indeed, these cells represented the predominant $CD4^+$ T cell fraction responsible for IL-10 production in these compartments of these CRC patients (Colon; $CD4^+LAP^-$ 0.9% ± 0.05% vs. $CD4^+LAP^+$ 9.4% ± 0.6%, p = 0.0042 / Tumour; $CD4^+LAP^-$ 1.1% ± 0.4% vs. $CD4^+LAP^+$ 33.5% ± 5.4%, p = 0.013)

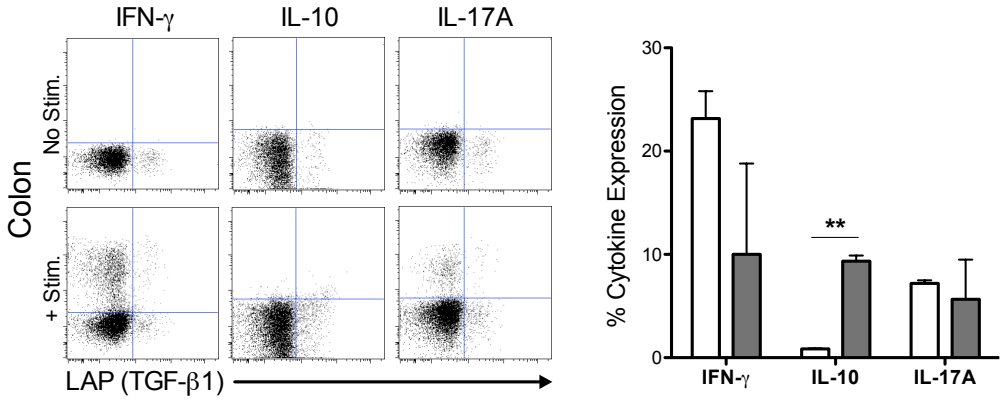
$CD4^+LAP^+$ T cells also stained for membrane-bound active TGF-β without prior activation (Figure 4.14), indicating that LAP expression denotes populations of cells that produce TGF-β, consistent with previous reports (Saito, Kinoshita et al. 2001). This was to be expected, since TGF-β is secreted in a complex with latent TGF-β binding protein and LAP (Taylor 2009). The immunosuppressive effects of $CD4^+CD25^+$ T cells are largely mediated by cell-contact dependent mechanisms, with membrane-bound TGF-β shown to contribute to such regulation (Nakamura, Kitani et al. 2001).

Together, these data illustrate how phenotypically comparable T cell subsets can take on divergent functions in different compartments, and support the premise that LAP expression denotes a population of intra-tumoural $CD4^+Foxp3^-$ cells with considerable immunosuppressive potential.

A: PBMC



B: Colon



C: Tumour

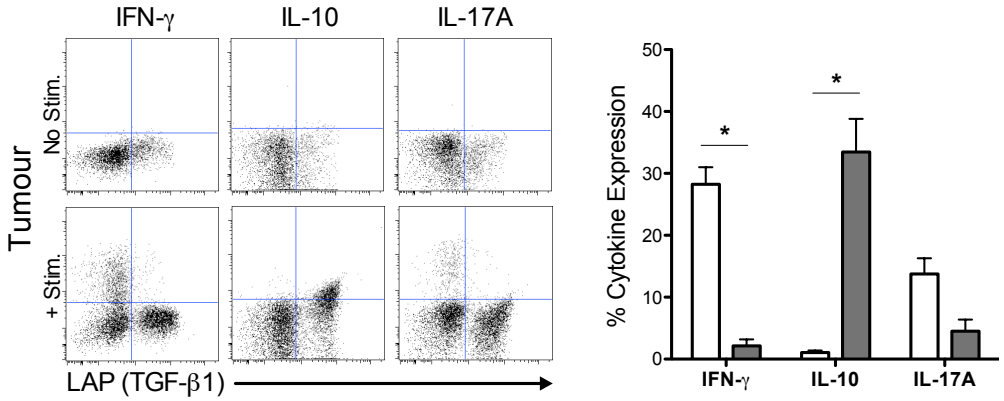


Figure 4.13 CD4$^+$LAP$^+$ TILs produce IL-10. Intracellular cytokine staining of stimulated CD4$^+$LAP$^+$ and corresponding CD4$^+$LAP$^-$ live T cells from matched peripheral blood (A), unaffected colon (B) and tumour samples (C). Representative profiles for IL-17A, IFN-γ and IL-10 are shown from a CRC patient with a Dukes' B, T3-staged tumour.

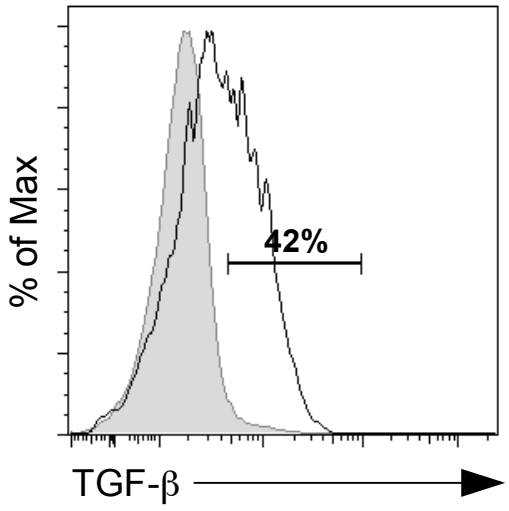


Figure 4.14 $CD4^+LAP^+$ TILs stain positive for active TGF-β1. Representative example of TGF-β1 expression on resting, live $CD4^+LAP^+$ TILs (A: Solid black line = TGF-β1, grey shading = isotype control).

To assess the functional significance of LAP expression, $CD4^+LAP^+$ and $CD4^+LAP^-$ T cells were purified from colorectal tumour samples by FACS (Figure 4.16B). Stimulation of the $CD4^+LAP^+$ TIL population *in vitro* with αCD3/CD28 beads revealed that nearly 90% of these cells were capable of cell division over a 3-day proliferation assay (Figure 4.15); this stands in marked contrast to the anergic phenotype described for conventional Tregs *in vitro* (Min, Thornton et al. 2007), despite the high degree of turnover seen *in vivo* (Vukmanovic-Stejic, Zhang et al. 2006). Proliferation of potentially suppressive T cell populations negates the use of tritiated thymidine incorporation to analyse for suppression, since the turnover of the suppressive population can potentially mask the inhibition of responder T cells, as previously noted. Instead, a CFSE / PKH-26-based suppression assay was utilized, so that the division index of suppressor and responder T cell subsets could be analysed independently over the course of the suppression assay.

The ability of $CD4^+LAP^+$ and $CD4^+LAP^-$ T cells to inhibit effector $CD4^+$ T cell proliferation *in vitro* was assessed using standard suppressor assays and compared to conventional Tregs, which were sorted as $CD4^+CD25^{hi}CD127^{lo}$ T cells (Foxp3 expression > 90%; Figure 4.16A). Autologous PBMC-derived $CD4^+CD25^-$ T cells (Foxp3 expression < 5%) labeled with CFSE were used as targets in these suppression assays, co-cultured with isolated TIL subsets, labelled with PKH-26, at ratios of 1:1, 1:0.5, 1:0.1 and 1:0.01. A direct comparison of $CD4^+LAP^+$ T cells, $CD4^+LAP^-$ T cells and conventional Tregs demonstrated that LAP^+ TILs were able to suppress target cell proliferation ~50-fold more potently than either of the other two subsets (data from four independent experiments; Figure 4.17A). Interestingly, the $CD4^+LAP^-$ population could still suppress at higher ratios, possibly due to the presence of $Foxp3^+$ Tregs. The suppressive effects of $CD4^+LAP^+$ TILs were largely mediated by TGF-β, since

addition of the TGF-β blocking antibody 1D11 partially restored effector T cell proliferation levels, reducing the suppressive effects of these cells by over 50% (Figure 4.17B).

Thus, tumour-derived $CD4^+$ T cells expressing LAP are highly immunosuppressive, despite low levels of Foxp3 expression, and represent a major regulatory T cell population amongst colorectal TILs.

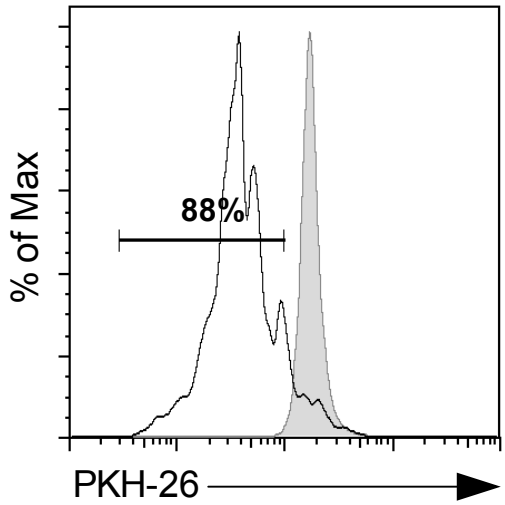
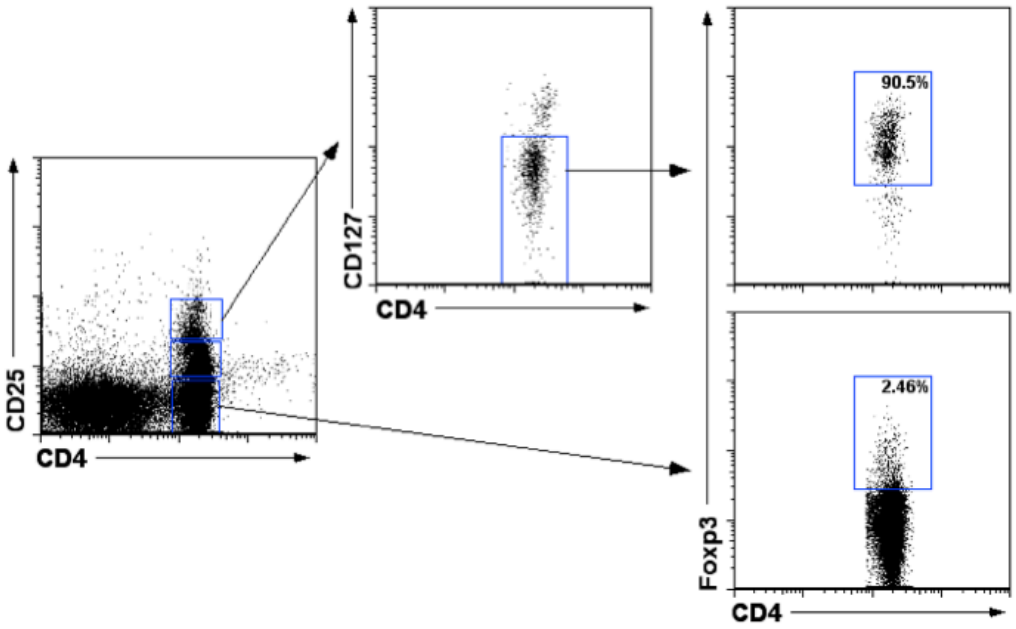


Figure 4.15 *In vitro* proliferative capacity of $CD4^+LAP^+$ TILs. Upon stimulation with CD3/CD28 beads, $CD4^+LAP^+$ TILs are highly proliferative *in vitro* over the course of a three day culture, as denoted by dilution of the cell tracking dye, PKH-26 (solid black line = live $CD4^+LAP^+$ T cells labelled with PKH-26, grey shading = PKH-26-labelled unstimulated $CD4^+$ T cell control).

A:



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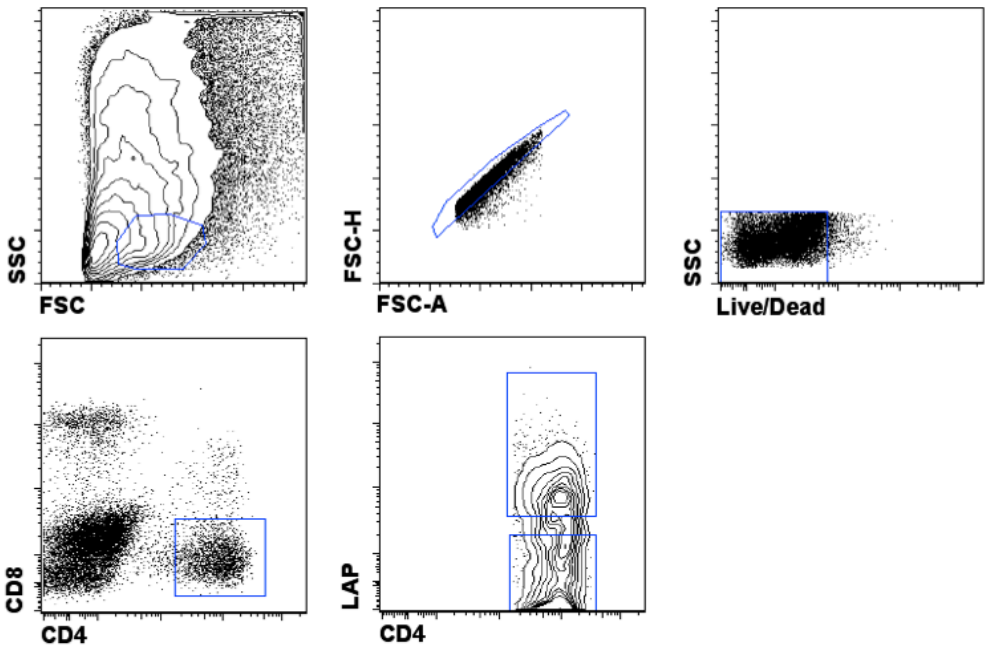
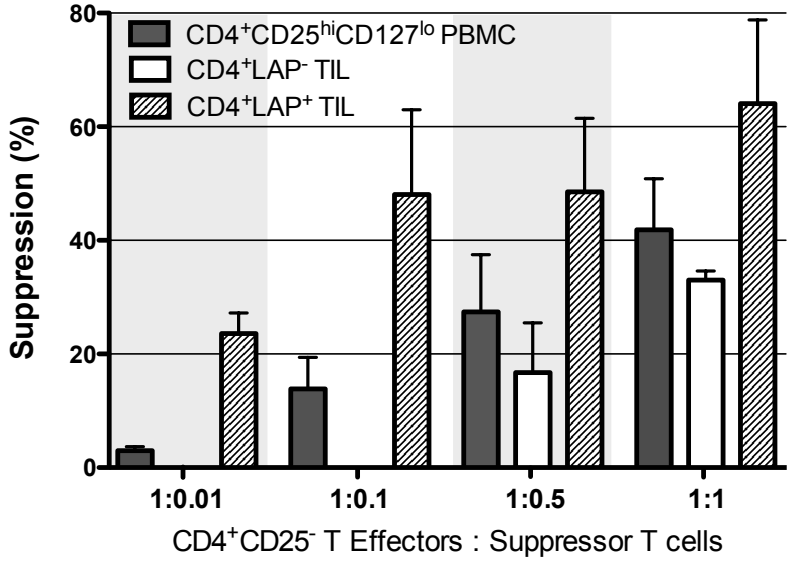


Figure 4.16 Fluorescent Activated Cell Sorting of T cell Populations. Representative flow cytometry plots showing the gating strategy used to sort live $CD4^{+}CD25^{hi}CD127^{lo}$ Tregs and live $CD4^{+}CD25^{-}$ T effector cells from PBMCs, and Foxp3 expression profiles in these subsets (A). Representative flow cytometry plots showing the gating strategy used to sort doublet-excluded, live ($CD8^{-}$) $CD4^{+}LAP^{+}$ and $CD4^{+}LAP^{-}$ TILs (B).

A:



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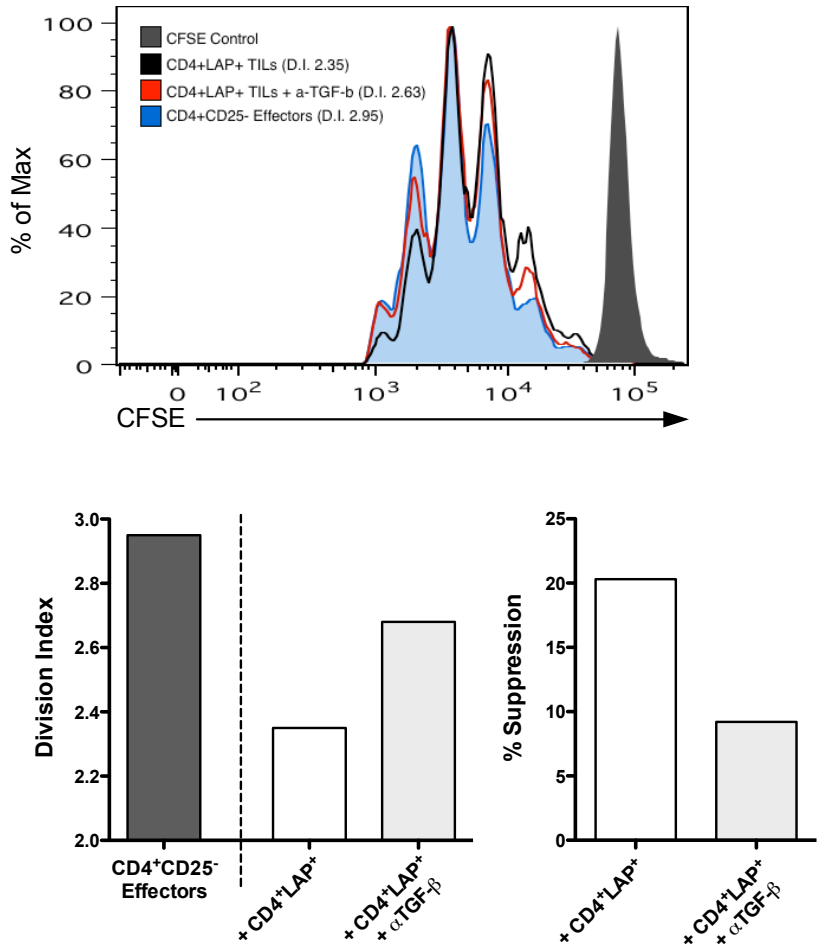

Figure 4.17 $CD4^+LAP^+$ TILs are >100 fold more suppressive than conventional Tregs *in vitro*. (A) $CD4^+CD25^{hi}$ $CD127^{lo}$ T cells were FACS-purified from PBMCs, together with $CD4^+LAP^+$ and $CD4^+LAP^-$ T cells from the colorectal tumour of the same patient and co-incubated with autologous $CD4^+CD25^-$ T effector cells at the indicated ratios (1 = 4 x 10^4 cells). Suppression indicates the percent reduction in proliferation of activated autologous effector T cells over a period of 60 hours. Data is inclusive of four independent experiments, showing degree of variability at each ratio (mean ± SEM). (B) Addition of the αTGF-β blocking antibody 1D11 (10 μg/ml final concentration) restored proliferation of effector T cells cultured at an E:T ratio of 25:1. Histogram shows raw CFSE dilution of each indicated condition, plus graphical data analysing the corresponding division index and % suppression.

4.3 Conclusion

Adaptive immune responses can control tumours, as illustrated by successful vaccination and adoptive immunotherapy studies (Restifo, Dudley et al. 2012). However, many immunotherapy trials have shown poor response rates, potentially due to the inhibition of tumour-specific responses by immune regulatory networks. A large body of data now associate increased frequencies of $CD4^+Foxp3^+$ T cells (Tregs) with a range of tumours (Betts, Clarke et al. 2006). Furthermore, Treg accumulation is linked with poor outcome in several malignancies, including ovarian cancer (Curiel, Coukos et al. 2004) and CRC (Betts, Jones et al. 2011). Previous work from this laboratory showed that anti-tumour $CD4^+$ T cell responses were reduced in patients with CRC prior to resection of the tumour; this phenomenon was related to Treg frequencies in peripheral blood, defined initially by high levels of CD25 expression on $CD4^+$ T cells (Clarke, Betts et al. 2006), and more recently by expression of the transcription factor Foxp3 ((Betts, Jones et al. 2011) and Chapter 3).

Despite substantial evidence indicating that regulatory T cells impinge upon anti-tumour immune responses in patients with cancer (Nishikawa and Sakaguchi 2010), many studies in humans have focused largely on blood-derived Tregs. This raises the question of whether intra-tumoural Tregs carry out the same function as those in blood. Indeed, the role of colorectal tumour-infiltrating Tregs in particular has been widely debated (Ladoire, Martin et al. 2011). Aberrant intra-tumoural expression of Foxp3, which may not denote a homogenous population of suppressive T cells, has been mooted as a reason why Treg numbers seem to be high in early tumours with a better prognosis (Morgan, van Bilsen et al. 2005; Ziegler 2007; Kmieciak, Gowda et al. 2009). Here, $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$ T cell infiltration

were actually found to increase in proportion with advancing tumour stage, in keeping with previous analysis of colorectal tumours by FACS (Chaput, Louafi et al. 2009). Studies using immunohistochemistry to perform the same analysis in colorectal tumour tissue do not find this correlation (Loddenkemper, Schernus et al. 2006; Ling, Pratap et al. 2007). This has led some groups to question the usefulness of immunohistochemical analysis of Foxp3 in human tumours (Badoual, Hans et al. 2009), since other non-suppressive T cells can upregulate Foxp3 (Walker, Kasprowicz et al. 2003; Roncador, Brown et al. 2005) in addition to significant cytoplasmic expression within colorectal tumour cells themselves (Karanikas, Speletas et al. 2008). This may explain why some groups have found an association between improved survival and a higher density of intratumoural $Foxp3^+$ T cells, despite their well-documented suppressive effects on anti-tumour immunity (Nishikawa and Sakaguchi 2010; Betts, Jones et al. 2012). This apparent contradiction may be resolved by clinical trials that deplete $Foxp3^+$ Tregs in CRC patients as a potential adjuvant to anti-tumour T cell stimulation (see Chapter 3). Nonetheless, detailed insights into the complexity and function of colorectal tumour-infiltrating T cell subsets are essential to guide the rational manipulation of T cell responses for the purpose of optimizing anti-tumour immunity.

Access to matched blood, healthy colon and colorectal tumour samples from multiple CRC patients enabled us to conduct a detailed phenotypic comparison of $CD4^+$ T cells derived from these different compartments. The relative proportion of $CD4^+$ T cells that expressed Foxp3, conventionally classified as Tregs, was significantly higher within tumours compared to healthy colon. Expression of Helios was confined to this $Foxp3^+$ population, an indication that these cells are potentially more suppressive than the corresponding $CD4^+Foxp3^+Helios^-$ subset, given their

higher levels of Foxp3 expression ((Figure 4.5D) and (Chauhan, Saban et al. 2009; Elkord and Al-Ramadi 2012)). Furthermore, an intriguing and highly significant increase in the proportion of peripheral Tregs expressing Helios was noted, as tumours advanced from Dukes' A to Dukes' D. This could reflect an increase in the output of thymic-derived Tregs in response to prolonged or heightened tumour-specific T cell activation, or it could represent an increase in the suppressive capacity of the peripheral Tregs in an attempt to mediate self-tolerance. It would be of interest to define the specificity of these T cells to see if the increased proportions of Tregs found in CRC patients over healthy donors is due to chronic stimulation by tumour-associated antigens. Within the colorectal tumour, whilst the majority of $Foxp3^+$ Tregs were $Helios^+$, this proportion was significantly lower than found in blood. This suggests that there is an additional element of Treg induction occurring in the tumour microenvironment. In the colon, there appears to be an even greater induction of Tregs, a probable result of the necessity to maintain intestinal homeostasis in the presence of immunogenic gut microflora (Maloy and Powrie 2011).

$Foxp3^+$ Tregs were found to be highly enriched for ICOS-expressing cells. ICOS has previously been demonstrated to provide a critical signal for the development of Th17 cells and follicular-helper T cells (T_{FH}), since ICOS-deficient mice have diminished numbers of these cell types (Bauquet, Jin et al. 2009). ICOS also appears to be critical to regulatory T cell maintenance within the tumour microenvironment; the expression of ICOS-L by tumour cells can promote the activation and expansion of $CD4^+Foxp3^+$ T cells, a large proportion of which express ICOS, thus maintaining high expression of Foxp3 amongst TILs (Martin-Orozco, Li et al. 2010). Within a melanoma microenvironment, $ICOS^{hi}$ Tregs have been shown to mediate stronger suppression than $ICOS^-$ Tregs (Strauss, Bergmann et al. 2008).

Analysis of the function of colorectal TILs based on ICOS expression in this chapter was restricted by the use of tritiated thymidine incorporation to analyse for suppression. However, both $ICOS^+$ and $ICOS^-$ $CD4^+$ TILs appeared capable of mounting a regulatory effect upon stimulated effector $CD4^+CD25^-$ T cells, suggesting that both isolated cell fractions contain suppressive T cell subsets and highlighting the heterogeneity of regulatory T cells within the tumour microenvironment.

An extensive comparison of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ populations revealed that the expression of other markers classically associated with Tregs in peripheral blood, including CD25, CTLA-4, CD39 and CD127, were less specific for intra-tumoural $Foxp3^+$ cells (Figure 4.12). This raised the possibility that cells other than $Foxp3^+$ T cells were capable of mediating immunosuppression within the tumour environment and further questions its usefulness as a marker for defining regulatory T cells in this compartment. Such suppressive molecules are upregulated on effector memory T cells during antigen-driven activation; strikingly, however, intra-tumoural $CD4^+Foxp3^-$ cells did not express the activation marker ICOS, thereby suggesting that their unique phenotypic profile was not solely the result of T cell activation.

Expression of the ectonucleotidase CD39 suggested that intra-tumoural $CD4^+Foxp3^-$ T cells might exert a regulatory role. This was further borne out by analyses of LAP and LAG-3 expression. Suppressive T cells that express LAP but not Foxp3 have previously been identified in humans (Gandhi, Farez et al. 2010) and their presence amongst colorectal TILs has recently been verified (Mahalingam, Lin et al. 2012). In addition, T cell expression of the CD4 homologue and negative regulator, LAG-3, has been shown to maintain tolerance to self and tumour antigens by directly regulating $CD8^+$ T cell effector function (Grosso, Kelleher et al. 2007; Matsuzaki, Gnjatic et al. 2010); synergy between LAG-3 with PD-1 on TILs has also been shown

to promote tumoural immune escape in a number of models (Woo, Turnis et al. 2012). In the work presented here, a large proportion of $CD4^+$ T cells that expressed LAP, part of the membrane-bound latent TGF-β complex, were detected. LAP requires cleavage by certain integrins and proteases before active TGF-β can be released (Tran 2012), but its expression is associated with cells capable of suppression, since membrane bound TGF-β is a major mechanism of cell contact-dependent immunosuppression (Nakamura, Kitani et al. 2001). Increased proportions of $CD4^+LAP^+$ T cells amongst peripheral blood and tumour-infiltrating lymphocytes have recently been described, however functional analysis of these cells was limited to blood-derived samples (Mahalingam, Lin et al. 2012). In agreement with this work, we found LAP was only expressed on very small populations of T cells in the peripheral blood; indeed, LAP expression appeared to be specifically associated with the tumour microenvironment. These $CD4^+LAP^+$ cells co-expressed LAG-3 in the tumour, but only a minority expressed Foxp3, consistent with descriptions of LAG-3^+ T cells that infiltrate Hodgkin lymphomas and bear suppressive activity without Foxp3 expression (Gandhi, Lambley et al. 2006). Functional analysis revealed that these cells produced IL-10 and TGF-β, and exerted suppressive activities *in vitro* that were around 50-fold more potent than "conventional" PBMC-derived $Foxp3^+$ Tregs. These cells appeared incapable of producing IFN-γ within the tumour, and therefore represent a functionally distinct $CD4^+LAP^+$ T cell subset from the corresponding cells derived from blood ((Figure 4.13) and (Mahalingam, Lin et al. 2012)). In addition, $CD4^+LAP^+$ T cells isolated from peripheral blood have previously been described as hypoproliferative *in vitro* (Gandhi, Farez et al. 2010); although upon further analysis of this data, ~10% are $Ki67^+$ *ex vivo* (similar data was found here; Table 4.3) and they do proliferate *in vitro*, albeit only to half the extent of $CD4^+LAP^-$ T cells.

Overall, these data reveal the presence of a potent suppressive $CD4^+Foxp3^-$ T cell population within the colorectal tumour regulatory landscape. These $CD4^+LAP^+$ T cells, which co-express significant levels of CTLA-4, LAG-3, PD-1, CD39, CD25, CD103 and Ki67, appear to be highly activated, divide easily both *in vivo* and *in vitro*, and are distinct from the regulatory populations found in healthy colon and peripheral blood. Recent studies by Donkor and colleagues demonstrated that oncogene-induced prostate tumour growth and metastasis could be blocked by increased cytotoxic T cell activity, but that the failure of immune protection involved TGF-β-mediated suppression of tumour antigen-specific T cell responses. Most compellingly, the major source of TGF-β was the T cells themselves, rather than either the tumour cells or $CD4^+Foxp3^+$ regulatory T cells (Donkor, Sarkar et al. 2011; Sarkar, Donkor et al. 2011). This is in close agreement to the work presented in this chapter, whereby a major regulatory subset of TIL that does not require Foxp3 for suppression, but instead expresses TGF-β and IL-10, has been identified. Indeed, TGF-β appears to play a substantial role in the suppressive capabilities of these cells, although the role of IL-10 needs to be elucidated further. This $CD4^+LAP^+$ T cell population likely controls anti-tumour immune responses in the local environment, even in the face of systemic anti-tumour responses present in the early stages of colorectal tumour development.

Therapeutic strategies that aim to overcome regulatory T cell activity as a means of enhancing anti-tumour immune responses need to take into account this novel intra-tumoural subset of highly suppressive $CD4^+Foxp3^-$ T cells identified in this chapter. This may include selective blockade of TGF-β signalling, specifically mediated by T cells, which adversely polarize the tumour environment (Flavell, Sanjabi et al. 2010). Future studies will also analyse whether blocking LAG-3

signalling can enhance anti-tumour responses and improve the responsiveness of effector Th1 responses (as previously shown (Grosso, Kelleher et al. 2007)), crucial to the host for effective tumour eradication (Bos and Sherman 2010; Tosolini, Kirilovsky et al. 2011). LAG-3 blocking antibodies are currently in the pre-clinical stage of development, but it is hoped future immunotherapeutic regimes could incorporate this to enhance anti-tumour immunity.

Chapter 5 – Final Discussion

This thesis has examined the role of effector and regulatory T cell subsets on the progression of colorectal carcinoma in humans. The major focus of this work centred around two key areas crucial to immunological tumour control: analysing the existence and functional capabilities of oncofoetal antigen-specific effector and regulatory T cells; and in depth phenotypic and functional evaluation of T cells that infiltrate colorectal tumours, comparing these to corresponding subsets found in healthy colonic mucosa and peripheral blood. The results of these experiments provide further evidence of increased immunological regulation of anti-tumour T cell immune responses in patients with colorectal cancer. Initially, this was shown by an increase in the proportion of peripheral blood derived $Foxp3^+$ Tregs as tumours became more advanced, which corresponded with a significant reduction in the magnitude of 5T4-specific Th1 responses generated by these patients. Furthermore, tumour infiltrating $Foxp3^+$ T cells displayed a phenotype associated with enhanced immunosuppression over $Foxp3^+$ cells in the colon and peripheral blood; however, a significant proportion of $Foxp3^-$ TILs expressed regulatory markers such as LAG-3 and LAP, and displayed the most prominent suppressive characteristics *in vitro*. Together, these findings support the rationale for inhibiting T cells capable of anti-tumour immunosuppression with concurrent stimulation of anti-tumour effector responses in the treatment of colorectal cancer.

It is now well established that some T cells do possess the capability of tumour control, following the observation that higher densities of intratumoural $CD3^+CD45RO^+$ T cells resulted in a longer median patient survival, regardless of histopathological tumour stage (Galon, Costes et al. 2006). This is a striking finding

as previously the staging of the tumour had been the gold standard for prognosis, and is still being used today to direct treatment. These results suggest that T cells can mediate effective deletion of cancerous cells in the colon, or at least can positively contribute to the control of tumour burden. However, T cell sub-populations vary enormously in function and a number of subsets exist with the capacity to actively suppress antigen-specific T cell responses, including the thymus-derived $Foxp3^+$ regulatory T cells (Tregs) which, as demonstrated in this thesis, are particularly abundant in colorectal cancer patients and are also enriched within the colorectal tumour microenvironment (Chapter 4 and (Ling, Pratap et al. 2007)). Whilst there seems to be a logical rationale to selectively deplete $Foxp3^+$ Tregs to enhance anti-tumour immunity (Betts, Clarke et al. 2006), the observation that early stage CRC is enriched for $Foxp3^+$ Tregs, and therefore is associated with improved patient outcome after tumour resection, raises a cautionary note (Salama, Phillips et al. 2009).

5.1 5T4-specific T cell responses in CRC patients and healthy donors

T cells are capable of attacking neoplastic cells through recognition of tumour-associated antigens (TAA) presented to them by antigen-presenting cells (APCs). Numerous TAAs for human carcinomas have been identified and current strategies for prospective immunotherapy include the identification of TAA-derived epitopes presented on MHC class I and class II molecules to $CD8^+$ and $CD4^+$ T cells, respectively, since both subsets of T cell are important for effective anti-tumour immunity (Campi, Crosti et al. 2003; Shingler, Chikoti et al. 2008; Bos and Sherman 2010). The oncofoetal antigens CEA and 5T4 represent excellent targets for immunotherapy as they are abundantly expressed on colorectal cancer cells but less so

on healthy tissue (Figure 3.22 and (Starzynska, Rahi et al. 1992; Hammarstrom 1999)).

T cells specific for such antigens, identified using the highly sensitive IFN-γ ELISpot assay, are found in CRC patients (Clarke, Betts et al. 2006; Smyth, Elkord et al. 2006; Elkord, Burt et al. 2008; Betts, Jones et al. 2011) and interestingly, in healthy donors (Chapter 3 and (Elkord, Burt et al. 2008)), suggesting a role in tumour immunosurveillance. The presence of autoreactive T cells in the peripheral blood of healthy individuals, with medium to high avidity for their cognate antigen, has previously been documented (Danke, Koelle et al. 2004). In this study, 5/7 healthy donors had detectable NY-ESO-1-specific $CD4^+$ T cells *ex vivo*, analysed by MHC class-II specific tetramers. The conclusions from this work were that autoreactive T cells are present in all healthy donors since thymic deletion is not 100% efficient. Given that, for the work presented here, PBMCs were cultured *in vitro*, it is perhaps not surprising that healthy individuals produced such robust responses to the oncofoetal antigen, since culturing PBMCs magnifies antigen-specific T cells (Ebert, MacRaild et al. 2012). In fact, healthy donors demonstrated a greater magnitude of 5T4-specific T cell responses than CRC patients (see Chapter 3.3 and Figure 5.1).

Subsequent analysis of the diminished T cell responses measured in CRC patients revealed that there was a gradual and significant loss in response correlating with the steady progressive worsening of the cancer stage. Hence, the size of the 5T4 response, measured in patients pre-operatively, may identify those with more advanced (or aggressive?) tumours, and also identify subjects most likely to develop early metastatic disease. The loss of responses did not seem to be influenced by the

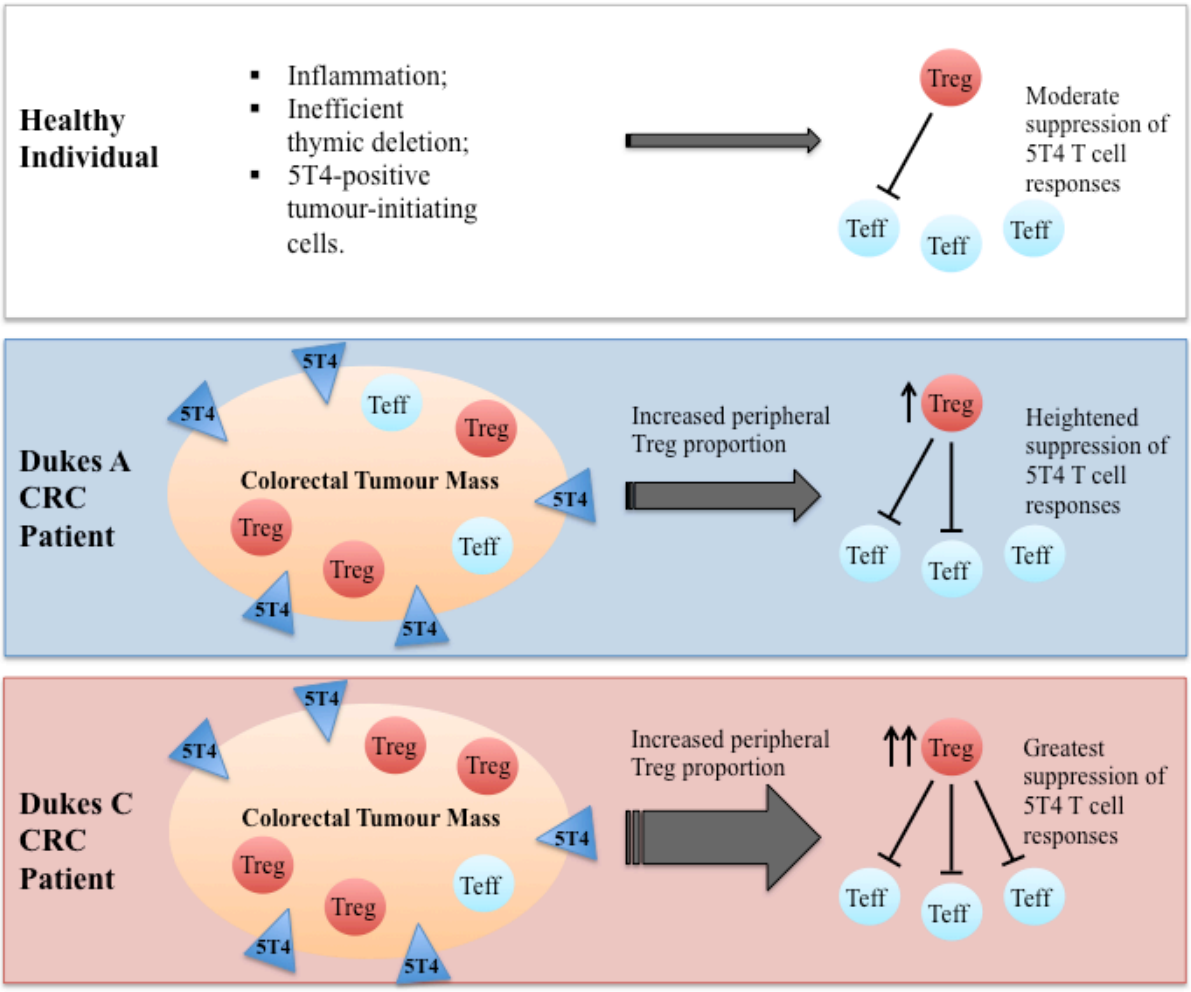

Figure 5.1 Overview of 5T4 T cell Origin and Consequent Suppression by Tregs. In healthy individuals, 5T4 is not expressed or is expressed at very low levels by normal tissue. 5T4 is upregulated on inflamed tissue in the gut, and found on tumour-initiating cells (cancer stem cells), thus 5T4 T cell responses may arise from mechanisms of inflammatory resolution, immunosurveillance or incomplete thymic deletion, where they are regulated by 5T4-specific Tregs in the periphery. In the presence of a primary colorectal adenocarcinoma, decreased 5T4-specfic T cell responses are found in the blood of these patients. However, removal of Tregs both *in vitro* and *in vivo* can restore some of these responses. Thus, the tumour drives a population of regulatory T cells, which increase in proportion with tumour advancement and lead to further suppression of 5T4-specific Th1 responses.

subjects HLA type, or range of epitopes recognised, but most strikingly corresponded with a tumour-specific population of regulatory $Foxp3^+$ T cells. This predictable decline from healthy age-matched controls to CRC patients is such that a cut off of in the frequency of responses (200 cognate $CD4^+$ T cells / 10^5 cultured cells) is 100% specific for the presence of CRC in the subjects studied so far. Results from this thesis open up the potential to extend this study to other tumour antigens and larger cohorts (for 5T4), and use blood from patients undergoing bowel cancer screening to assess its applicability i.e. loss of 5T4 response as a biomarker of disease.

In this thesis, 5T4-specific T cell responses were largely limited to the analysis of the effector cytokine IFN-γ, produced by T cells activated in the presence of their cognate antigen. The expression of IFN-γ by helper and cytotoxic T cells has previously been shown to be most beneficial for maximal anti-tumour effects; in addition to CD28, granzyme B and perforin, the expression of these effector molecules within colorectal tumours resulted in prolonged disease-free survival for that patient (Tosolini, Kirilovsky et al. 2011). However, even in the presence of large numbers of tumour-specific T cells, tumours may continue to progress due to T cell dysfunction via a skew in the cytokine profile of these cells (Boon, Coulie et al. 2006; Tassi, Gavazzi et al. 2008). Indeed for CRC, a skewed Th17 profile appears to be detrimental: patients with high levels of IL-17 in tumours, produced by Th17 T cells and macrophages, have a significantly worse disease-free survival than patients with low IL-17 levels (Tosolini, Kirilovsky et al. 2011). This may be a result of IL-17 encouraging local production of VEGF, facilitating angiogenesis and tumour growth (Liu, Duan et al. 2011). An extensive cytokine profiling of 5T4-specific T cells was not studied here; however, it will be interesting to determine whether the reduction in

IFN-γ producing 5T4-specific T cell responses with tumour progression could also be due to a change in cytokine production or a decrease in overall numbers of these cells.

5.2 Regulatory T cells and Cancer Progression

Since many tumour-associated antigens are also self-antigens, mechanisms of immunological tolerance can restrict the effectiveness of tumour-specific effector T cells. In order to maintain tolerance, Tregs are capable of suppressing such responses (Nishikawa and Sakaguchi 2010) and it is apparent that tumours employ Tregs as a defence mechanism against host immune surveillance (Curiel 2007). Intratumoural Tregs appear to play a role in cancer progression, since their selective depletion results in tumour rejection in many mouse models of cancer (Gallimore and Godkin 2008). In CRC, patients have increased proportions of Tregs in peripheral blood and amongst tumour-infiltrating lymphocytes (TILs), and mounting evidence suggests that these cells impinge upon the beneficial TAA-specific responses (Vence, Palucka et al. 2007; Bonertz, Weitz et al. 2009). This is further substantiated by experiments presented in this thesis, since using low-dose cyclophosphamide to reduce the proportion of peripheral Tregs in patients with metastatic colorectal cancer resulted in enhanced anti-5T4 T cell responses. Previous work from this lab also identified that surgical removal of the primary tumour is effective in normalizing peripheral Treg levels in CRC patients and in unmasking previously suppressed anti-tumour T cell responses (Betts, Jones et al. 2012). Furthermore, Treg-mediated suppression of tumour-specific $CD4^+$ T cells prior to surgery was associated with tumour recurrence at 12 months and 5 years (unpublished data). These findings imply that depletion of Tregs in CRC patients before the onset of metastatic disease (i.e. post-surgical

removal of a primary colorectal adenocarcinoma) might enhance overall patient survival.

Yet the role of Tregs that infiltrate colorectal tumours is under debate (Ladoire, Martin et al. 2011), as some reports demonstrate that a favourable prognosis i.e. early tumour stage is associated with a higher density of tumour-infiltrating $Foxp3^+$ cells (Salama, Phillips et al. 2009; Frey, Droeser et al. 2010), in contrast to other groups that did not find this (Loddenkemper, Schernus et al. 2006). This paradox could be explained by divergent expression of Foxp3 that may not be denoting a homogenous population of suppressive T cells, and potential inaccuracies encountered when measuring Foxp3 expression by immunohistochemistry, as discussed in Chapter 4.3. Indeed, Foxp3 can become upregulated on activated effector T cells with apparently no regulatory function (Ziegler 2007; Hoffmann, Boeld et al. 2009), so caution must be taken to denote $Foxp3^+$ T cells as 'regulatory'. Other theories have been proffered for this counter-intuitive observation. Matera et al. speculated that $Foxp3^+$ Tregs do not possess immunosuppressive function in the context of CRC and instead their presence might just be a homeostatic mechanism to control a robust immune response (Matera, Sandrucci et al. 2010). Others have hypothesized that Tregs can attenuate a Th17-mediated pro-inflammatory and tumour-enhancing response (Ladoire, Martin et al. 2011). Whilst this may be plausible, there is currently no evidence to suggest that $Foxp3^+$ T cells isolated *ex vivo* from colorectal tumour tissue can enhance an anti-tumour immune response, in fact, only the opposite has been found so far (Kryczek, Liu et al. 2009). However, this studied relied on CD25 as a marker for $Foxp3^+$ T cells, which can also denote a population of highly suppressive $Foxp3^-$ T cells, as demonstrated in Chapter 4.

It seems certain tumour microenvironments, including CRC, facilitate the development and expansion of highly suppressive populations of T cells (Chaput, Louafi et al. 2009; Kryczek, Liu et al. 2009; Camisaschi, Casati et al. 2010) the complexity of which we are only just beginning to appreciate (outlined in Figure 5.2). It is probable that the mucosal environment of the intestine plays a role in determining the constituents of intratumoural T cells. Tregs have a role in ameliorating gut inflammation and therefore could help prevent carcinogenesis by controlling tumour-promoting inflammation (Maggio-Price, Treuting et al. 2006; Mantovani, Allavena et al. 2008). As discussed in the Introduction, mucosal integrity, microbiota, and inflammation all play a role in the pathogenesis of CRC, and Tregs actively impinge on Th17 cells, thus limiting the survival and invasion of aberrant epithelial cells (Grivennikov, Wang et al. 2012; Gallimore and Godkin 2013). However, if a tumour develops despite the control of inflammation, $Foxp3^+$ T cells may now contribute to tumour progression by actively impinging on anti-tumour responses, demonstrating an apparent shift from host protection to tumour protection (Whiteside 2012). It is also likely that regulatory $CD4^+$ T cells other than naturally occurring $CD4^+CD25^{hi}Foxp3^+$ Tregs are contributing to immune suppression; these include inducible Tr1 (IL-10 producing) and Th3 (TGF-β-producing) subsets, which display variable expression profiles of CD25 and Foxp3 and are important mediators of intestinal homeostasis (Maynard, Harrington et al. 2007; Josefowicz, Niec et al. 2012). The work in this thesis identified a highly suppressive population of $Foxp3^-CD4^+$ colorectal TILs; these cells co-express LAP, LAG-3 and CD25, produce the immunosuppressive cytokines IL-10 and TGF-β, and demonstrate marked suppressive properties *in vitro*. TGF-β-producing $CD4^+Foxp3^-$ T cells have recently been shown to impede immunosurveillance in models of prostate and breast cancer

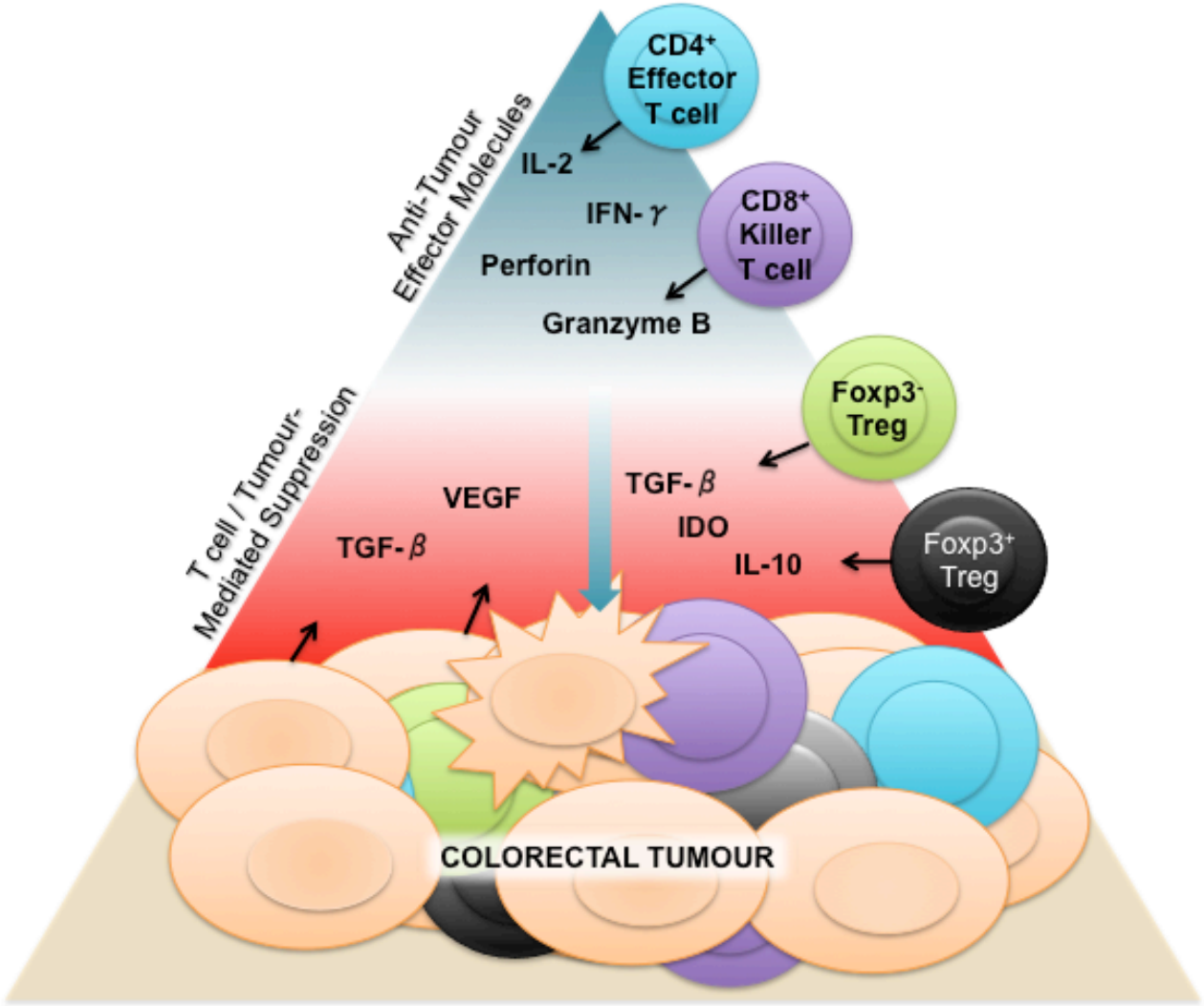

Figure 5.2 Colorectal tumour-infiltrating T cells exert opposing effects, reflecting their heterogeneous nature. $CD4^+$ and $CD8^+$ T cells produce effector molecules capable of anti-tumour effects. However, the tumour microenvironment consists of many cells capable of potent suppression, including both $Foxp3^+$ and $Foxp3^-$ regulatory T cells. Hence, tumours are able to grow even in the presence of large numbers of T cells.

(Donkor, Sarkar et al. 2011; Sarkar, Donkor et al. 2011), by directly inhibiting cytotoxic T cell responses to tumours. The removal of the TGF-β-producing $CD4^+$ T cells resulted in markedly enhanced tumour control and almost completely diminished the metastatic spread of the tumour. This is in accordance with studies demonstrating the importance of TGF-β in inducing epithelial-to-mesenchymal transition, an integral process in the invasion and metastatic potential of all carcinomas (Xu, Lamouille et al. 2009). The TGF-β-producing $CD4^+Foxp3^-$ cells discovered infiltrating oncogene-induced prostate cancer most likely represent a very similar population of cells identified here in human colorectal TILs and almost certainly contribute towards tumour progression. This work, alongside the recent dispute over the prognostic effect of $Foxp3^+$ Tregs, means that we may be placing too much emphasis on this transcription factor to denote the regulatory T cell populations in tumours, since clearly other regulatory populations not expressing Foxp3 exist.

5.3 Novel Immunotherapeutic Strategies for CRC

Successful immunotherapy for CRC will most likely require a multi-faceted approach to boost tumour-specific effectors with concurrent depletion or blockade of tumour-specific regulators. For tumour cell eradication to occur, sufficient numbers of effector T cells with the ability to recognize tumour antigens with high avidity must be generated. These cells must then be able to infiltrate the tumour to enact effector mechanisms and not be adversely influenced by regulatory mechanisms that T cells can be induced to express within the tumour microenvironment (Figure 5.3). Whilst adoptive therapy of tumour-specific T cells holds significant promise (Parkhurst, Yang et al. 2011), an ideal scenario would be to administer an anti-cancer vaccine to boost the patients' own anti-tumour T cell responses. However, vaccinating patients

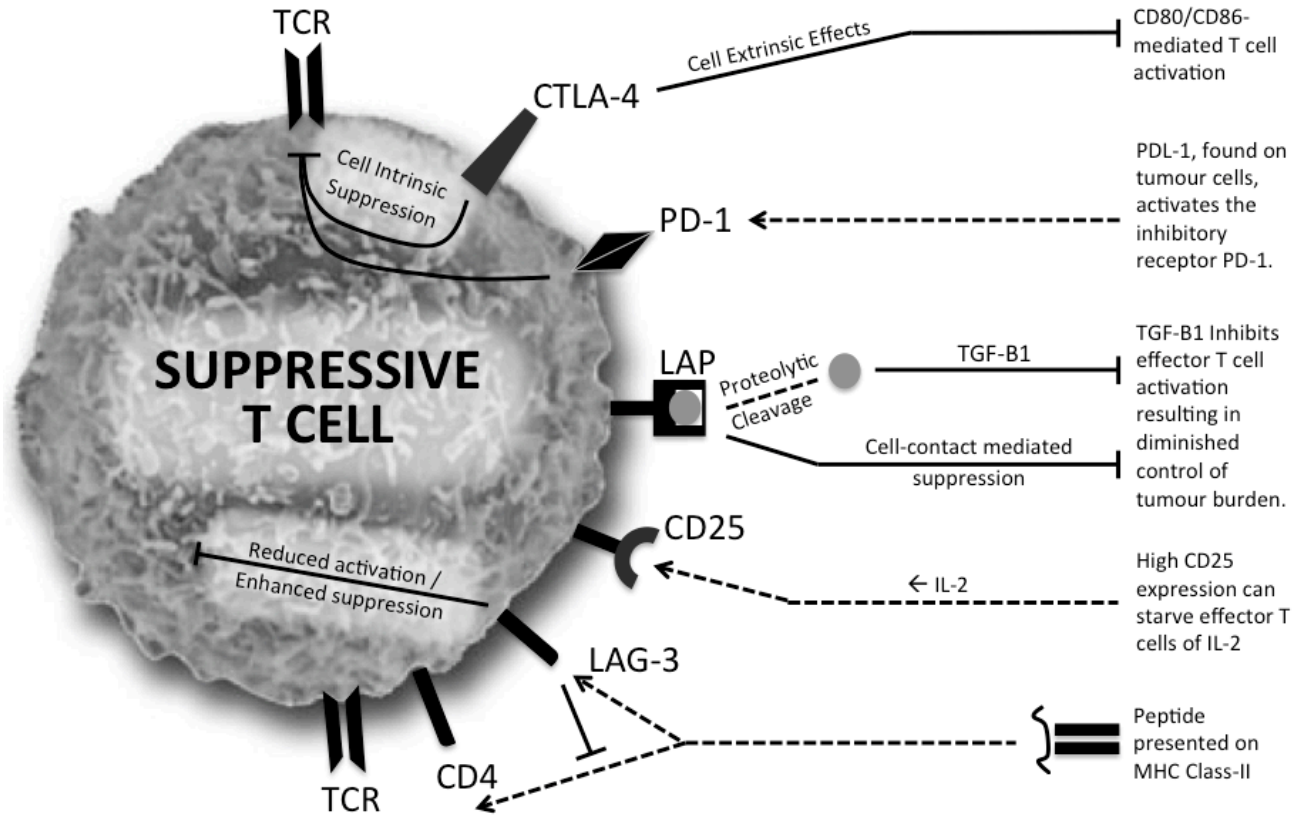

Figure 5.3 Regulatory Functions Employed by Tumour-Infiltrating CD4+ T cells. Many antigens with immunosuppressive functions exist in humans to regulate immune responses, and many are expressed in abundance upon intratumoural T cells, which can act to intrinsically suppress cellular activation, or extrinsically regulate other cells, as indicated. These include (but is not limited to): Cytotoxic T lymphocyte antigen-4 (CTLA-4 / CD152); the negative immunomodulator PD-1 (CD279); the membrane bound latent form of TGF-β (LAP); the IL-2 receptor (CD25); and LAG-3 (CD223) which inhibits TCR-mediated signaling and regulates homeostatic T cell expansion.

with whole tumour antigens has yielded disappointing data from phase II/III trials for a variety of reasons (Amato, Hawkins et al. 2010). One issue that arises from targeting T cells in this manner is the activation of TAA-specific Tregs (Vence, Palucka et al. 2007; Bonertz, Weitz et al. 2009; Ebert, Macraild et al. 2012), which will in turn suppress any beneficial response. Preliminary evidence for this occurring in metastatic CRC patients following TroVax® vaccination is also demonstrated here (Chapter 3). Depleting Tregs whilst stimulating the anti-tumour T cells has already been shown to induce potent anti-tumour responses (Dannull, Su et al. 2005; Walter, Weinschenk et al. 2012). Indeed, depleting Tregs alone using low-dose cyclophosphamide unmasks significant anti-tumour responses in metastatic cancer patients (Chapter 3 and ((Ghiringhelli, Menard et al. 2007; Ge, Domschke et al. 2011)). The activation of tumour-specific Tregs could also be avoided with knowledge of tumour-specific epitopes recognised by both effector and regulatory T cell subsets. In Chapter 3, depletion of $CD25^{hi}$ Tregs prior to culture with 5T4 peptide pools revealed that in the donors tested, some effector T cell responses were augmented, whilst others remained unaffected. This indicates that some Tregs recognize the same 5T4 epitopes as effector T cells, but crucially, some effector T cell epitopes are not recognised. These specific epitopes could be exploited to stimulate an anti-tumour response, whilst minimizing the activation of Tregs and subsequent suppression of responses. Work is now ingoing to study a much larger group of individuals to pinpoint more precise HLA-restricted 5T4 epitopes recognised by regulatory and effector T cells.

Even if we can negate the function of Tregs by depletion or by specifically activating effector T cell subsets, tumour-specific T cells are further constrained by a number of highly immunosuppressive factors within the tumour microenvironment.

Recently it has been shown that inhibition of dendritic cell (DC) maturation via high levels of intratumoural CCL2, CXCL1, CXCL5 and VEGF can lead to a reduction in antigen presentation to T cells in CRC (Michielsen, Hogan et al. 2011) and crucially can skew an effective Th1 response (Minkis, Kavanagh et al. 2008). Furthermore, this tumour-induced immunosuppression is heightened at later stages of tumour development, further compounding the use of beneficial immunotherapeutic agents in patients with end-stage CRC. It is probable that agents to reduce these other mechanisms of tumour-induced immunosuppression, such as targeted blocking of suppressive antigens, cytokines and regulatory chemokine molecules, may be necessary for immunotherapeutic success.

The latency-associated peptide (LAP) displayed significant expression amongst colorectal $CD4^{+}Foxp3^{-}$ TILs and produced potent immunosuppressive effects. This homodimeric molecule non-covalently encases TGF-β to form a latent, inactive TGF-β complex on the cell surface. This latent form is unable to bind to its receptor until proteolytic cleavage (or structural modification) causes a conformational change to LAP that exposes active TGF-β (Tran 2012). A number of studies, including the work in this thesis, show that within the tumour microenvironment, TGF-β is having an adverse effect on anti-tumour T cell responses; so finding ways of inhibiting the release of active TGF-β could enhance effector T cell responses. However, potential targeting of TGF-β signalling in humans to treat cancer is fraught with danger given its pleiotropic properties; whilst TGF-β can promote cancer progression by inhibiting beneficial immune responses, it also serves to suppress tumour cell growth (Pu, Collazo et al. 2009) and maintain homeostasis in a whole host of systems throughout the body (Taylor 2009).

Nevertheless, a number of therapies are currently being developed in an effort to target this pathway in cancer. Some of the most promising include a TGF-β1 neutralizing antibody (Genzyme, GC-1008; (J. C. Morris 2008)), the silencing oligonucleotide, Trabedersen (Antisense Pharma, AP12009) and a TGF-β1 receptor kinase inhibitor (Lilly Research Laboratories, LY364947). (Other novel therapies targeting TGF-β in cancer are reviewed in (Flavell, Sanjabi et al. 2010) and (Akhurst and Hata 2012)).

More targeted therapies could arguably arise from the modulation of LAP by disabling processes allowing for the activation of TGF-β by cells that produce it, such as the $CD4^+LAP^+$ TILs identified here. Inhibition of LAP cleavage by certain integrins, e.g. αvβ6, has garnered recent interest, since this integrin is essential for the release and activation of TGF-β in human tumours, and greater expression of this integrin in colorectal, lung, breast and skin carcinomas is associated with metastatic spread (Bandyopadhyay and Raghavan 2009). Indeed, antibody blockade of αvβ6 has been shown to inhibit tumour progression *in vivo* (Van Aarsen, Leone et al. 2008). Given that αvβ6 is not expressed on normal epithelial cells, this may be one way to selectively target T cell-mediated suppression by TGF-β production within tumours.

Phenotypic analysis of the $CD4^+LAP^+$ suppressive TILs found that these cells co-express the MHC class-II binding CD4 homologue, LAG-3. This marker has previously been shown to confer suppressive activity by T cells (Huang, Workman et al. 2004) and by $CD4^+Foxp3^-$ T cells infiltrating Hodgkin's lymphomas (Gandhi, Lambley et al. 2006). Furthermore, dual expression with PD-1 negatively regulated NY-ESO-1-specific $CD8^+$ T cell responses in ovarian cancer (Matsuzaki, Gnjatic et al. 2010) and dual anti-LAG-3/anti-PD-1 antibody treatment cured most mice of

established tumours that were largely resistant to single antibody treatment (Woo, Turnis et al. 2012). Here, over 90% of colorectal $CD4^{+}LAG\text{-}3^{+}$ TILs expressed PD-1, and these cells were shown to induce significant inhibitory effects upon effector T cells. Other regulatory markers such as CTLA-4 are also found on these cells; however, CTLA-4 serves as a dominant off-switch by blocking CD28 signalling and consequent T cell activation (Walker and Sansom 2011). Therefore it comes as no surprise that the anti-CTLA-4 antibody Ipilimumab (recently approved for the treatment of malignant melanoma) can cause severe autoimmunity since it also inhibits homeostatic functions of regulatory T cells throughout the body, especially in the skin and gut (Weber 2009). Hence, a key challenge for future immunotherapy is to achieve increased efficacy without enhanced toxicity. PD-1 and LAG-3 appear to serve a more subtle and precise down-regulation of immune functions and their expression is more restricted to TILs (Chapter 4 and (Turnis M.E. 2012), thus antibodies to PD-1 and LAG-3 may promote potent anti-tumour immune response but exhibit less systemic toxicity.

Emerging evidence from multiple clinical trials blocking certain immunosuppressive checkpoints, such as CTLA-4 (Ipilimumab) or PD-1 (BMS-936558) indicates that preventing tumour-induced T cell suppression or exhaustion restores protective immunity against established cancers, hence advanced tumours remain somewhat immunogenic. This is counter-intuitive to the immunoediting hypothesis described earlier, since tumours were thought to escape immune recognition by becoming less immunogenic. Instead, it seems that the tumours can exhaust effector T cells, but the potential for reigniting an immunological anti-tumour response still exists. The PD-1 antibody currently being tested in clinical trials was found to produce objective responses in ~28% of patients with renal cell carcinoma or

melanoma (Topalian, Hodi et al. 2012). An antagonist LAG-3 antibody has also been developed (ImmuTune IMP701, Immutep), specifically to block LAG-3-mediated immune down-regulation in the treatment of cancer and chronic infectious diseases. This therapy is currently in the preclinical phase of development but the first in-human trials are expected to start in the coming years.

5.4 Concluding Remarks

Research in the field of cancer immunology has intensified in recent years with the realization that the immune system offers a viable approach to eradicating tumour cells, which could one day lead us to a cure for cancer. In 2011, the anti-CTLA-4 antibody, Ipilimumab, became the first immune therapy approved by the FDA for use in advanced melanoma, being the first drug ever proven to prolong survival in advanced melanoma. This is only the first example in a growing list of promising immune therapies that aim to break tumour tolerance and enhance anti-tumour immunity; a number of other prospective treatments in the pipeline are discussed here, with experimental evidence to reinforce their usage presented in this thesis. A key focus for this work was to definitively identify some of the regulatory processes that hinder anti-tumour specific T cell responses, by analysing the phenotype and function of T cells found in the tumour, colon and peripheral blood in patients with cancer. It is hoped that such knowledge will aid strategies to enhance novel immunotherapies by maximizing beneficial anti-tumour specific effector T cell responses and minimise the effects of suppressive T cells. Identifying these regulatory populations and pathways is the beginning of the battle: working out how to control or remove these cells and whether this benefits patient management are the future challenges.

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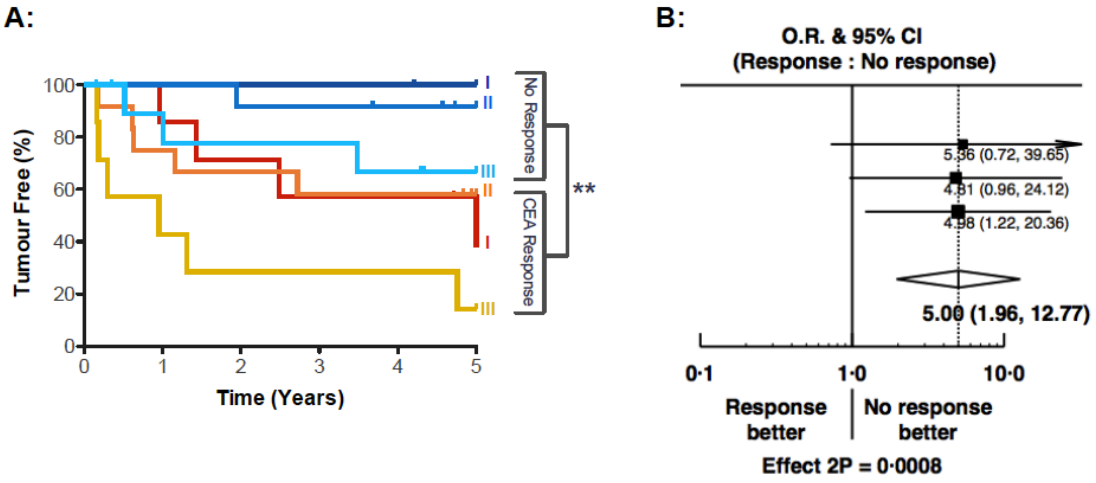
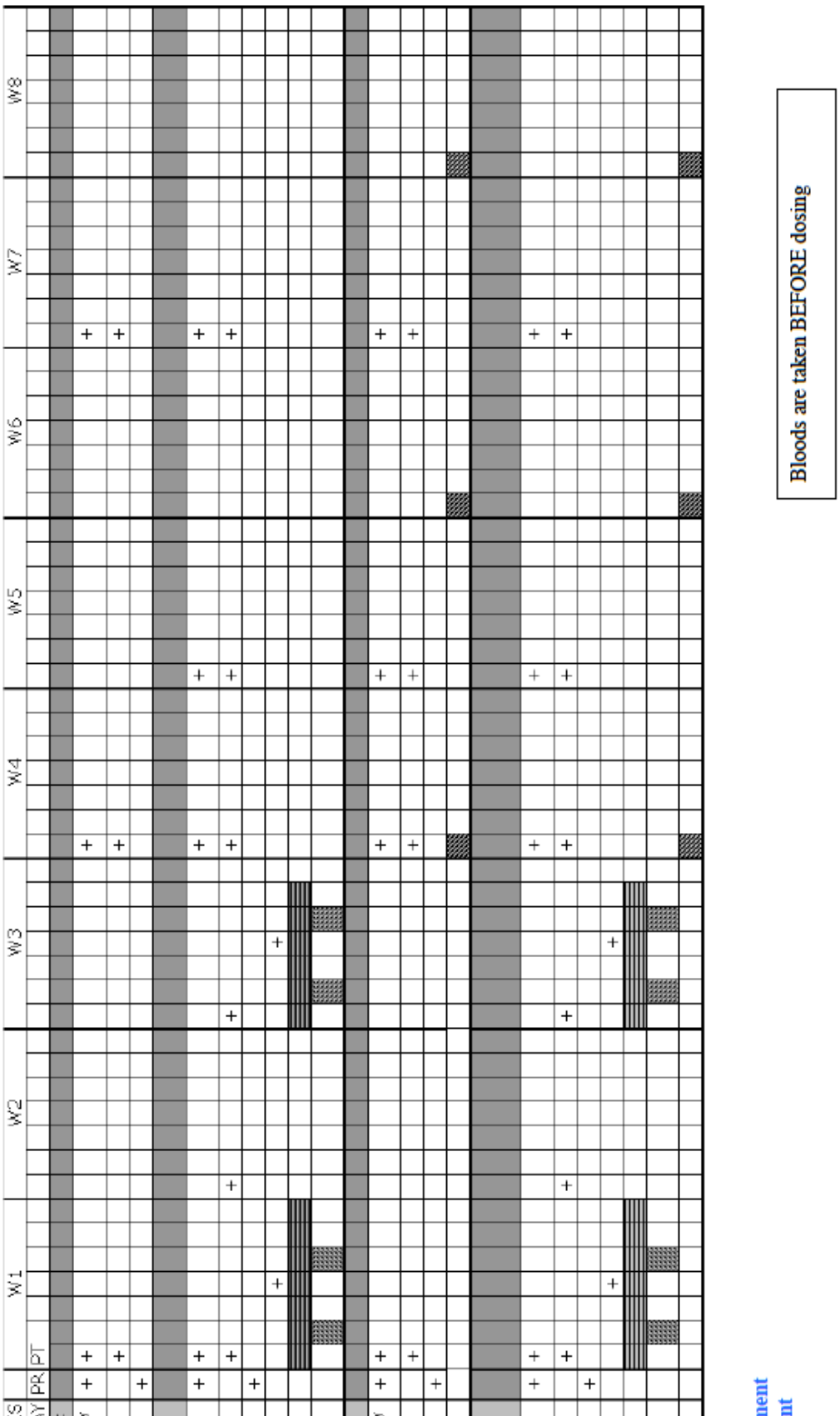
Appendix



CEA-specific T cell responses and 5-year tumour recurrence. Irrespective of the tumour stage, patients with a pre-operative anti-CEA T cell response were more likely to suffer a tumour recurrence (A and B). Significant difference is indicated (** $P < 0.01$).

Trial Flowchart: Weeks 1-8



Patient Information Sheet:

An Investigation into the role of lymphocytes on the generation of tumour immunity in humans.

You are being invited to take part in a research study. Please ask us if anything is unclear or if you need more information. Take time to decide whether or not you wish to take part.

Purpose of the Study:

Your immune system is involved in fighting infections such as bacteria and viruses using white blood cells. However, there is strong evidence that the immune system is important in causing inflammatory conditions such as Crohn's disease or ulcerative colitis, and also in fighting abnormal cancer cells, which might arise in normal or inflamed tissue. The purpose of this study is to examine the way your white blood cells recognise and attack cells of the body, including malignant tumour cells.

The Volunteers

We are interested in patients who are about to undergo a resection of their colon for malignancy or a complication of inflammatory bowel disease.

Do I have to take part?

It is up to you whether or not to take part. If you do decide to take part, you will keep this information sheet and be asked to sign a consent form. If you decide not to take part, it will **not** affect the standard of your care in any way.

What does it involve?

To carry out this research, we would require a small sample of blood (30-40 mls, i.e. one syringe full) before your operation. After your operation, it is usual for the resected bowel to be sent down to a pathologist for examination. In the course of this standard examination, the pathologist will remove a small sample of the abnormal bowel and adjacent lymph nodes, required for use in this study. The white cells in these samples will also be analysed. After you have made a full recovery from your operation, 2-3 further blood samples would be analysed over a 12 month period. These samples will usually be obtained by us when you are attending outpatients.

Are there any risks?

There are no extra risks in taking part. **This study in no way impinges or alters your treatment**. There is only the inconvenience of an extra blood sample.

What are the benefits of taking part?

This is a valuable study in allowing us to understand how and why our white blood cells attack our own tissues. This knowledge would potentially allow new therapies to be designed in the future that manipulate this response to treat certain cancers or inflammatory conditions.

What happens when the research is over?

When we have all the results, we will aim to present the data at local, national and international meetings. At these meetings, experts in the field will be able to review and discuss the new information. The work will also be published in journals so that the information can reach a wide audience.

Patient Confidentiality

All information collected during the study will be kept confidential. Any information about you that leaves the hospital will have your name and address removed so that it cannot be recognised. All results obtained from blood or tissue samples that you provide will be completely anonymous.

Who is organising the research?

This study is being supervised by Dr Andrew Godkin (Consultant Gastroenterologist) in close liason with Mr Mike Davies or Miss Rachel Hargest (Consultant Surgeons) who is performing your operation and looking after you whilst you are in hospital. The research is carried out in the Henry Wellcome Building (Cardiff University) at the Heath Hospital site.

Who has reviewed the study?

This study has been reviewed by the Cardiff and Vale NHS Trust Research and Development Committee and the Local Research Ethics Committee. The study is being funded by a grant from Cancer Research Wales and was initially sent out for external review.

Who to contact

If you wish to discuss any issues, please ask the houseman looking after you on the ward. If issues arise that he / she cannot deal with, Mr Davies or Dr Godkin will be able to discuss these further.

Centre Number:

Study Number: ***SPON 900-10***

Patient ID Number: ……

CONSENT FORM

An Investigation into the role of lymphocytes on the generation of tumour immunity in humans.

Name of Researcher: Martin Scurr (PhD Student)

Research Supervisor: Dr Andrew Godkin (Consultant Gastroenterologist)

	Please tick box
1. I confirm that I have read and understand the information sheet dated 3/2/2011 for the above study and have had the opportunity to ask questions.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by responsible individuals from Cardiff University or the Cardiff and Vale NHS Trust. I give permission to these individuals to have access to my records. All information will remain confidential.	☐
4. I consent for my anonymized results of this study to be published in scientific / medical journals.	☐
5. I consent for obtained samples to be stored for future research.	☐
6. I agree to take part in the above study.	☐

Name of Patient	Signature	Date

Name of Person taking consent	Signature	Date

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes / histopathology.

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Review

T cell subsets and colorectal cancer: Discerning the good from the bad

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ABSTRACT

Tumor-specific T cells must overcome a multitude of suppressive mechanisms to destroy cancerous cells effectively. Furthermore, it appears that the tumor microenvironment facilitates the development of highly immunosuppressive T cells, which may also allow subsequent tumor progression. In colorectal cancer, the relationship between regulatory T cells (e.g. FoxP3$^+$ Tregs) and tumor prognosis and progression is less clear, despite their well-documented ability to impinge on anti-tumor immune responses. Here we explore our current knowledge of colorectal TIL heterogeneity, deciphering subsets which may be of benefit or detriment.

1. Introduction

The role of host immunity in response to human colorectal carcinoma has garnered significant interest since the discovery of a correlation between the degree of infiltrating lymphocytes into tumors and enhanced patient survival [1–3]. Recently, Galon and colleagues made the observation that higher densities of intratumoral CD3$^+$CD45RO$^+$ T cells resulted in a longer median patient survival, *regardless* of pathological tumor stage [4]. This is a striking finding as previously the staging of the tumor had been the gold standard for prognosis. These results suggest some T cells possess the capability of tumor control and can presumably mediate effective deletion of cancerous cells in the colon. However, CD4$^+$ T cell populations vary in function and a number of subsets exist with the capacity to actually suppress antigen-specific T cell responses, including the thymus-derived Foxp3$^+$ regulatory T cells (Tregs) which are particularly abundant in cancer patients and may also be enriched within the tumor microenvironment [5]. Whilst there seems to be a logical rationale to deplete Tregs to enhance anti-tumor immunity [6], the observation that early stage colorectal cancer (CRC) is enriched for Tregs raises a cautionary note [7]. This short review will discuss our understanding of how different CD4$^+$ T cells interact with CRCs, both to our benefit and detriment, and how we might manipulate particular T cell subsets to treat CRC.

2. CRC-specific effector T cell responses

T cells are capable of attacking neoplastic cells through recognition of tumor-associated antigens (TAA) presented to them by antigen-presenting cells (APCs). Numerous TAAs for human carcinomas have been identified and current strategies for prospective immunotherapy include the identification of TAA-derived epitopes presented on MHC class I and class II molecules to CD8$^+$ and CD4$^+$ T cells, respectively, since both subsets of T cell are important for effective anti-tumor immunity [8–10]. The oncofoetal antigens CEA and 5T4 represent excellent targets for immunotherapy as they are abundantly expressed on colorectal cancer cells but less so on healthy tissue [11,12]. T cells specific for such antigens, identified using the highly sensitive IFN-γ ELISpot assay, are found in CRC patients [13–16] and interestingly, in healthy donors, suggesting a role in immunosurveillance. For such cells to exert maximal anti-tumor effects, a Th1-associated helper and cytotoxic response has been shown to be most beneficial, whereby the expression of IFN-γ, CD28, granzyme B and perforin results in prolonged disease-free survival [17]. Indeed for CRC, a skewed Th17 profile appears to be detrimental: patients with high levels of IL-17 in tumors, produced by Th17 T cells and macrophages, have a significantly worse disease-free survival than patients with low IL-17 levels [17]. This may be a result of IL-17 encouraging local production of VEGF, facilitating angiogenesis and tumor growth [18].

3. Regulatory T cells and CRC

Since many TAAs are also self-antigens, mechanisms of immunological tolerance can restrict the effectiveness of tumor-specific effector T cells. In order to maintain tolerance, Tregs are capable of suppressing such responses [19] and it is apparent that tumors

Abbreviations: APC, antigen presenting cell; CRC, colorectal cancer; ELISpot, enzyme-linked immunospot; Foxp3, forkhead box P3; IFN-γ, interferon-γ; IL-10, interleukin-10; IL-17, interleukin-17; TAA, tumor-associated antigen; TGF-β, transforming growth factor-β; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell.

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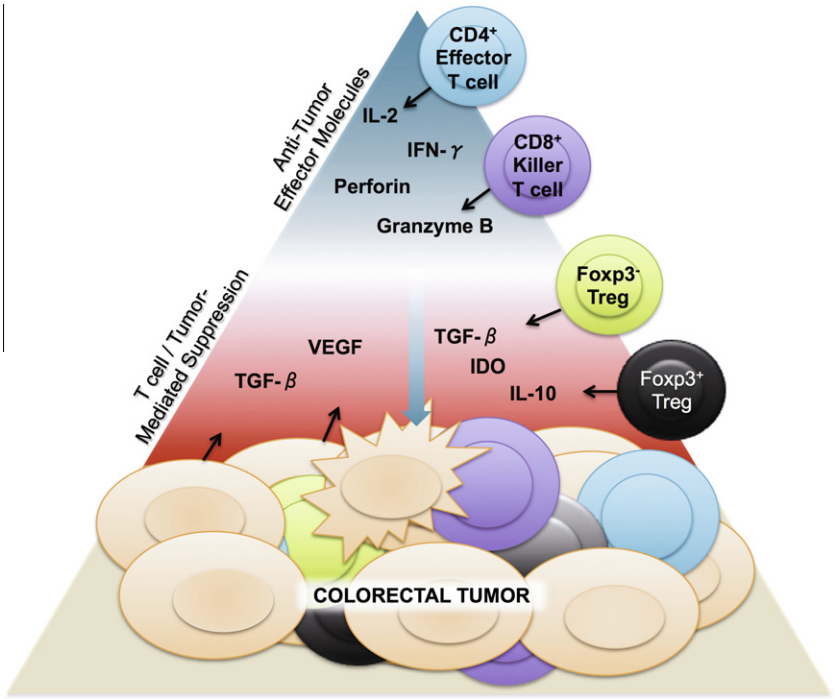

Fig. 1. Colorectal tumor-infiltrating T cells exert opposing effects, reflecting their heterogeneous nature. $CD4^+$ and $CD8^+$ T cells produce effector molecules capable of anti-tumor effects. However, the tumor microenvironment consists of many cells capable of potent suppression, including both $Foxp3^+$ and $Foxp3^-$ regulatory T cells. Thus, tumors are able to grow even in the presence of large numbers of T cells.

employ Tregs as a defense mechanism against host immune surveillance [20]. Intratumoral Tregs appear to play a role in cancer progression, since their selective depletion results in tumor rejection in many models of cancer [21]. In CRC, patients have increased proportions of Tregs in peripheral blood and amongst tumor-infiltrating lymphocytes (TILs) [5], and mounting evidence suggests that these cells impinge upon the beneficial TAA-specific responses [22,23]. We recently identified that surgical removal of the primary tumor is effective in normalizing Treg levels in CRC patients and in unmasking previously suppressed anti-tumor T cell responses. Furthermore, Treg-mediated suppression of tumor-specific $CD4^+$ T cells prior to surgery was associated with tumor recurrence at 12 months [16]. This finding implies depletion of Tregs in CRC patients post-surgery might enhance patient survival.

Yet the role of Tregs that infiltrate colorectal tumors is under debate [24], as some reports demonstrate that a favorable prognosis i.e. early tumor stage is associated with a higher density of tumor-infiltrating $Foxp3^+$ cells [7,25], in contrast to other groups that did not find this [26]. This paradox could be explained by aberrant expression of Foxp3 that may or may not be denoting a truly homogenous population of suppressive T cells. Indeed, Foxp3 can become upregulated on activated effector T cells with apparently no regulatory function [27,28]. Other theories have been proffered for this counter-intuitive observation. Matera et al. speculated that $Foxp3^+$ Tregs do not possess immunosuppressive function in the context of CRC and instead their presence might just be a homeostatic mechanism to control a robust immune response [29]. Others have hypothesized that Tregs can attenuate a Th17-mediated pro-inflammatory and tumor-enhancing response [24]. Whilst this may be plausible, there is currently no evidence to suggest that Tregs isolated *ex vivo* from colorectal tumor tissue can enhance an anti-tumor immune response, in fact, only the opposite has been found so far [30].

It is probable that the mucosal environment of the intestine plays a role in determining the constituents of intratumoral T cells. Tregs have a role in ameliorating gut inflammation and therefore could help prevent carcinogenesis by controlling tumor-promoting inflammation [31,32]. However, if a tumor develops despite the control of inflammation, $Foxp3^+$ T cells may now contribute to tumor progression by actively impinging on anti-tumor responses, demonstrating an apparent shift from host protection to tumor protection [33]. It is also likely that regulatory $CD4^+$ T cells other than naturally occurring $CD4^+CD25^{hi}Foxp3^+$ Tregs are contributing to immune suppression; these include inducible Tr1 (IL-10 producing) and Th3 (TGF-β-producing) subsets, which display variable expression profiles of CD25 and Foxp3 and are important mediators of intestinal homeostasis [34,35]. We have recently identified a highly suppressive population of $Foxp3^-CD4^+$ TILs in CRC, which demonstrate marked suppressive properties *in* vitro (unpublished data). TGF-β-producing $CD4^+Foxp3^-$ T cells have been shown to impede immunosurveillance in a model of prostate cancer [36] and spontaneous mammary cancer [37]. It seems certain tumor microenvironments, including CRC, facilitate the development and expansion of highly suppressive populations of T cells [30,38,39] the complexity of which we are only just beginning to appreciate (outlined in Fig. 1).

4. T cell-mediated immunotherapeutic strategies for treating CRC

Successful immunotherapy for CRC will most likely require a multi-faceted approach to boost tumor-specific effectors with concurrent depletion of tumor-specific regulators. Whilst adoptive T cell therapy holds significant promise [40], an ideal scenario would be to administer an anti-cancer vaccine to boost the patients' own anti-tumor T cell responses. However, vaccinating patients with whole tumor antigens has yielded disappointing data from phase II/III trials for a variety of reasons [41]. One issue that arises from targeting T cells in this manner is the activation of TAA-specific

Tregs [22,23], which will in turn suppress any beneficial response. Depleting Tregs whilst stimulating the anti-tumor T cells has already been shown to induce potent anti-tumor responses [42]. Indeed, depleting Tregs alone using low dose cyclophosphamide unmasks significant anti-tumor responses in cancer patients [43,44]; thus we have started a clinical trial using a vaccine and low dose cyclophosphamide in CRC patients in anticipation of similar beneficial findings [45].

Even if successful depletion of Tregs from colorectal tumors occurs, effective tumor-specific T cell responses are further constrained by a number of highly immunosuppressive factors within the tumor microenvironment. Recently it has been shown that inhibition of dendritic cell (DC) maturation via high levels of intratumoral CCL2, CXCL1, CXCL5 and VEGF can lead to a reduction in antigen presentation to T cells in CRC [46] and crucially can skew an effective Th1 response [47]. Furthermore, this tumor-induced immunosuppression is heightened at later stages of tumor development, further compounding the use of possible beneficial immunotherapeutic agents in patients with end-stage CRC. It is possible that agents to reduce these other mechanisms of tumor-induced immunosuppression, such as targeted blocking of suppressive cytokines and regulatory chemokine molecules, may be necessary for immunotherapeutic success.

5. Conclusion

Deciphering the phenotype and function of T cells infiltrating CRC is a priority for understanding the role of both Tregs and other regulatory $CD4^+$ T cell populations. The recent question over the prognostic effect of $Foxp3^+$ Tregs means that we may be placing too much emphasis on this transcription factor to denote the regulatory T cell populations in tumors, since clearly other regulatory populations not expressing Foxp3 exist. Identifying these regulatory populations is the beginning of the battle: working out how to control or remove these cells and whether this benefits patient management are the future challenges.

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