An Investigation of the Influence of $CD4^+CD25^+$ Regulatory Cells on the Generation of Tumour Immunity in Mice and Human Patients

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Summary

A population of T cells identified as $CD4^+CD25^+FOXP3^+$ (Tregs) are present in rodents and humans. These cells are vital for suppressing the activity of conventional self-reactive T cells. Previous work conducted using tumour cell lines has indicated that Tregs also suppress effective anti-tumour immunity in mice. Existing data derived using immunocompromised strains of mice has strongly suggested that the immune system is capable of recognising malignant cells, a process termed immunosurveillance. It was reasonable to hypothesise therefore that Tregs impinge on this process, effectively hampering elimination of tumour cells by the immune system. This hypothesis was tested in this thesis by analysing the impact of Tregs on tumours induced *in vivo* using the chemical carcinogen, methylcholanthrene. Results of these studies indicated that chemically induced tumours are significantly infiltrated with Tregs and that partial depletion of theses cells prior to exposure to the carcinogen results in a marked reduction in tumour incidence. These findings support a role for Tregs in immunosurveillance.

The role of Tregs during human malignancy was also addressed in this thesis using blood samples obtained from a cohort of patients with colorectal cancer. Specifically, studies were carried out to determine whether Treg frequencies were elevated in this group compared to healthy controls and to address whether these cells suppressed tumour antigen specific T cell responses. Results of these studies indicated that that colorectal carcinoma patients have an elevated frequency of Tregs in peripheral blood compared to age matched controls and that these Tregs suppress $CD4^+$ T cell responses to tumour antigens.

Collectively these data indicate that Tregs downmodulate immune responses to tumours and support strategies aimed at depleting or inactivating the cells for therapeutic purposes.

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Abreviations used

α-gal: α-galactosylceramide
3dTx: Thymectomy three days after birth
APC: Allophycocyanin
BrdU: Bromodeoxyurinde
cpm: counts per minute
CRC: Colorectal Carcinoma
dsDNA: double stranded DNA
HA: Haemaglutinin
ELISpot: Enzyme-Linked Immuno spot assay
EAE: Experimental autoimmune encephalitis
FITC: Fluorescin Isothiocyanate
IBD: Inflammatory bowel disease
i.p.: Intra-peritoneal injection
IFN-γ: Interferon-γ
IL: Interleukin
INTDLN: Inguinal non-tumour draining lymph node
ITDLN: Inguinal tumour draining lymph node
LN : Lymph node
LNIL: Lymph node infiltrating lymphocyte
LNTDLN: Lumber non-tumour draining lymph node
LTDLN: Lumber tumour draining lymph node
mAb: monoclonal antibody
nu/nu: homozygous NUDE immunodeficient mouse strain
MC: Methylcholanthrene
MVA: Modified Vaccinia Ankara
NTDLN: non-tumour draining lymph node
PE: R-Phycoerythrin
PNTDLN: Popliteal non-tumour draining lymph node
PTDLN: Politeal tumour draining lymph node
PPD: Purified Protein Derivative
NRS: Normal rabbit serum
NTDLN: non-tumour draining lymph node
SA: Steptavidin
s.c.: Subcutaneous injection
Sfc: IFN-γ secreting, spot forming cell
TIL: Tumour infiltrating lymphocyte
TDLN: Tumour draining lymph node
T_{EM}: Effector memory T cell
T_{CM}: Central memory T cell
TGF-β: Transforming growth factor-β
Treg: $CD4^+FOXP3^+CD25^+$ Regulatory T cell
UC: Ulcerative Colitis

1.0 Introduction

1.1 Immunosurveillance of tumours

Tumours arise from the outgrowth of cells that have accumulated multiple mutational and epigenetic changes (Hanahan, *et al.*2000). A mutational change referrers to a deletion or modification of the base pair sequence of DNA; an epigenetic change refers to a modification of the chemical structure of a gene, including aberration of the methylation state of a DNA sequence for example. In order to prevent the growth of transformed cells, a number of pathways, collectively referred to as intrinsic cellular control pathways (Smyth, *et al.*2006a), induce cell repair, senescence or apoptosis following cellular injury. A debate has been ongoing over the last several decades as to whether malignant cells may also be eliminated by an extrinsic control mechanism, whereby the unmanipulated immune system can survey and eliminate transformed cells by the recognition of tumour antigens (Smyth, *et al.*2006a).

1.2. Evidence for immunosurveillance in mice

A longstanding observation has been that tumours from a diverse array of histological origins are often infiltrated by immune cells and raises the question of whether these cells are simply bystanders to tumour development or actually recognise and attack the tumour; originally observed by Paul Ehrlich in 1909 (German text) and cited by researchers of the field of tumour immunology a century later (Smyth, *et al.*2006a). Observations using tumour transplantation models have provided strong evidence to support immune recognition of tumours and have also established that tumours may escape immune recognition though a loss of immunogenic antigens, termed immunoediting/immunoselection (reviewed in (Dunn, *et al.*2002, Smyth, *et al.*2006a)). These studies concluded that tumours which developed in immunodeficient mice were exposed to a less vigorous anti-tumour immune response and retained the expression of immunogenic tumour antigens which are lost in tumours that developed in wild type mice. Evidence to support immunoselection was collected in several studies. An initial

insight into immunoselection was observed by comparing growth kinetics of tumours passaged in either WT mice, partly immunosuppressed by UV radiation, or highly immunodeficient nude mice. Tumours were excised from donor mice following one month of passage and transferred to WT mice. Tumours that were passaged in WT mice proceeded to grow faster and demonstrated a higher tumouriogenic potential than those passaged in nude mice (Urban, *et al.*1982). These observations have been complemented with a series of experiments which compared the growth of tumours generated in various immunodeficient strains of mice or WT immunocompetent mice and transplanted into naïve immunodeficient or WT mice. An informative study was performed using multiple carcinogen induced cell lines generated in $Rag^{-/-}$ or WT mice (Shankaran, *et al.*2001). All tumours generated in WT mice grew when transplanted into $Rag^{-/-}$ and WT mice, however, less than 50% of tumours that were generated in $Rag^{-/-}$ mice grew in WT mice. All $Rag^{-/-}$ tumours grew when transplanted into naïve $Rag^{-/-}$ mice, which demonstrated the absence of inherent growth differences between tumours generated in $Rag^{-/-}$ and WT mice. Therefore tumours that developed in immunodeficient mice were more immunogenic than those that developed in immunocompetent mice (Shankaran, *et al.*2001). Similar studies have also found that tumours developing in nude (Svane, *et al.*1996), $perforin^{-/-}$ (Street, *et al.*2002), $J\alpha281^{-/-}$ (NKT cell) (Smyth, *et al.*2000a) immunodeficient mice demonstrate higher immunogenicity than those in immunocompetent mice.

Through the study of carcinogen-induced tumours in mice, the interaction between the immune system and an emerging tumour has been described as comprising three stages, collectively termed immunoselection (Smyth, *et al.*2006a). In the first phase, termed elimination, tumour antigens expressed by newly formed tumour cells are recognised and the cells are rejected. A number of tumour cells may lose expression of the relevant antigen(s) to escape rejection and enter the second phase, described as the equilibrium phase. Tumours may continue to upregulate a number of antigens that are continuously eliminated by anti-tumour responses and malignant cells become increasing genetically unstable. Eventually anti-tumour immune responses sculpture tumour antigen expression until the tumour is refractory to immune attack and enters the final escape

phase of growth. In some circumstances anti-tumour immune responses might be harmful and transform a benign tumour mass into an aggressive tumour (Smyth, *et al.*2006a).

The contribution of different components of the immune system to anti-tumour responses has been partly delineated by sequentially comparing tumour induction in mice deficient of particular cell types or signalling molecules to their wild type counterparts. A series of different models of tumour induction have been employed to observe anti-tumour immune responses and various arms of the immune system appear to be important in controlling tumour development in different models. Some tumour models are considered to be more representative of how the immune system coordinates anti-tumour immunity *in vivo* than others. Many studies inject immortalised cells pre-endowed with the necessary mutational and epigenetic changes required to rapidly produce palpable tumours and largely omit significant interactions between the immune system and cells in the process of accumulating mutations necessary to escape homeostatic control of cellular expansion. Models that either use carcinogens to elicit tumour development or study spontaneous tumour induction do include these early interactions and may be used to deconstruct how the immune system recognises and prevents tumour development. Injection of the carcinogen methylcholanthrene (MC) is one of the most established tumour induction models and has been used to establish the role of a series of cell types and signalling molecules that suppress tumour development. Other studies have compared spontaneous tumour development in immunodeficient mice to their wild type counterparts. Use of the different models of tumour development and important observations made within these studies is summarised below.

1.2.1. The study of immunosurveillance using methylcholanthrene induced tumours

Tumours develop approximately 100 days after the injection of MC and are believed to proceed though the three stages of tumour development, termed immunoselection, as described above (Smyth, *et al.*2006a). Injected MC causes a massive amount of DNA damage which induces significant cell death and inflammation. Fibroblasts are attracted by the inflammation and surround MC crystals that reside at the injection site (Qin, *et*

*al.*2002). Fibroblasts are subjected to the majority of MC crystal mutagenic activity which explains why MC injection induces fibrosarcoma development. Fibroblasts proceed to secrete extracellular matrix to form a barrier that shields MC mutagenic activity from surrounding tissue and preventing further mutagenesis. Mice that do not accumulate a sufficient number of mutations before extracellular matrix has encapsulated MC fibres remain tumour free (reviewed in (Blankenstein *et al.* 2003)). The immune system is believed to contribute to preventing tumour induction by recognising antigens arising as a result of MC induced mutations. Elimination of cells mutated by MC is thought to be hindered in immunodeficient mice thereby the incidence of MC induced tumour development is increased in these mice. The type of tumour antigen recognised by the immune system within MC induced tumours is unclear; however, the random mutagenic activity of MC undoubtedly creates a plethora of tumour specific antigens. Previous research has strongly indicated that tumours that are generated by MC carcinogenesis do not share tumour antigens that are sufficiently immunogenic to generate protective cross-protection (Prehn *et al.* 1957). Cells that have begun to escape control of the cell cycle after MC induced mutation may also upregulate a number of differentiation and shared tumour antigens that the immune system is capable of recognising.*

A variety of cells and signalling molecules have been identified that contribute to the control of MC tumour induction (Table 1.1). IFN-γ signalling, NKT and NK cells appear to complement each other and play a central role in control of MC-induced tumours. IFN-γ is a pluripotent cytokine and the IFN-γ receptor is expressed on nearly all cell types. Signalling through the IFN-γ receptor coordinates both the innate and adaptive immune response (Boehm, *et al.*1997) and acts to directly kill tumour cells and enhance recognition of tumour cells by the immune system (Kaplan, *et al.*1998). IFN-γ may also antagonise MC induced tumouriogenesis by accelerating the encapsulation of MC crystals with a collagen matrix and antagonise angiogenesis (Qin, *et al.*2002). Jα281$^{-/-}$ mice are deficient of NKT cells and have an enhanced rate of MC mediated tumour induction (Smyth, *et al.*2000a, Street, *et al.*2001, Smyth, *et al.*2001) whilst

* Tumour antigens are categorised into groups. The definition of these categories is described in section 1.4

Table 1.1. Susceptibility to MC induced tumourigenesis by immunodeficiency

Treatment/Strain	Deficiency	Susceptibility to MC Induced Tumours
IFN-$\gamma^{-/-}$	IFN-γ cytokine	Enhanced (Takeda, *et al.*2002, Street, *et al.*2001)
IFN-$\gamma R^{-/-}$	IFN-γ signaling	Enhanced (Qin, *et al.*2002, Shankaran, *et al.*2001, Kaplan, *et al.*1998, Dunn, *et al.*2005a)
$STAT1^{-/-}$	IFN-γ, α, β signaling	Enhanced (Shankaran, *et al.*2001, Kaplan, *et al.*1998)
$Rag^{-/-}$ x $STAT1^{-/-}$	T, B, NKT cells, IFN-γ, α, β signaling	Enhanced (Shankaran, *et al.*2001)
IFNAR1 subunit	IFNα, IFNβ signaling	Enhanced (Dunn, *et al.*2005a)
$Perforin^{-/-}$ x IFN-$\gamma^{-/-}$	Perforin cytotoxicity and IFN-γ cytokine	Enhanced (Street, *et al.*2001)
$Perforin^{-/-}$	Perforin cytotoxicity	Enhanced (Smyth, *et al.*2000a, Street, *et al.*2001, van den Broek, *et al.*1996)
IL-12p40$^{-/-}$	IL-12, IL-23	Enhanced (Smyth, *et al.*2000a)
Anti-asialo-GM1antibody	NK cells	Enhanced (Smyth, *et al.*2001)
$J\alpha281^{-/-}$	NKT cells	Enhanced (Smyth, *et al.*2000a, Street, *et al.*2001, Smyth, *et al.*2001)
$TRAIL^{-/-}$	TRAIL	Enhanced (Cretney, *et al.*2002)
Injection of anti-TRAIL antibody	TRAIL	Enhanced (Takeda, *et al.*2002)
$Rag^{-/-}$	T, B, NKT cells	Enhanced (Shankaran, *et al.*2001)
$TCR v\beta^{-/-}$	$\alpha\beta TCR^+$ T cells and NKT cells	Enhanced (Girardi, *et al.*2001)
$TCR v\delta^{-/-}$	$\gamma\delta TCR^+$ T cells	Enhanced (Girardi, *et al.*2001)
SCID	T, B and NKT cells	Enhanced (Smyth, *et al.*2001)
$CD8^{-/-}$	$CD8^+$ T cells, $CD8^+$ DC	No effect (Smyth, *et al.*2001, van den Broek, *et al.*1996)
IL-12 cytokine injection	Th1 to Th2 shift by CD4 and CD8 T cells	Reduced (Smyth, et al.2001, Noguchi, et al.1996)
injection of α-galactosylceramide	NKT cell activation	Reduced (Hayakawa, et al.2003)

activation of NKT cells by injecting α-galactoslyceramide (α-gal) enhances NKT cell mediated tumour rejection through IFN-γ release and TRAIL (Hayakawa, *et al.*2003). TRAIL also contributes to NK mediated tumour rejection (Takeda, *et al.*2002). α-gal enhances NKT cell IL-12 and IFN-γ production which in turn triggers NK cell cytotoxicity and IFN-γ release (Eberl, *et al.*2000, Carnaud, *et al.*1999). IL-12 deficient mice had an elevated incidence of MC mediated tumour induction (Smyth, *et al.*2000a), whilst injection of WT mice with IL-12 reduced MC tumour induction (Smyth, *et al.*2001, Noguchi, *et al.*1996). Tumours that appeared following IL-12 therapy appeared softer and irregular compared to those which formed in control mice and indicated that tumours that developed in IL-12 treated mice were exposed to a more vigorous immune attack (Noguchi, *et al.*1996). Mice that lack perforin (Smyth, *et al.*2000a, Street, *et al.*2001, van den Broek, *et al.*1996) and IFN-γ mediated signalling (Shankaran, *et al.*2001, Qin, *et al.*2002, Kaplan, *et al.*1998, Dunn, *et al.*2005a, Street, *et al.*2001, Takeda, *et al.*2002) have an enhanced susceptibility to tumour induction and mice deficient of both have an equivalent level of tumour induction to that observed in NKT cell deficient Jα281$^{-/-}$ mice (Street, *et al.*2001). The role of T cells in controlling MC mediated tumour induction is uncertain. Tumour induction is elevated in Rag$^{-/-}$ mice which lack T, B and NKT cells (Shankaran, *et al.*2001). The absence of NKT cells might be largely responsible for the enhanced tumour induction in Rag$^{-/-}$ mice and other studies suggest that CD8^{+} T cells do not contribute to anti-tumour immunity of MC induced tumours (van den Broek, *et al.*1996, Smyth, *et al.*2000a). A role for γδTCR^{+} T cells (Girardi, *et al.*2001) and type 1 interferons (Dunn, *et al.*2005a) in controlling MC tumour induction has been implicated. The effect of different immune cells/cytokines is summarised in Table 1.1.

1.2.2. Spontaneous tumour development

Spontaneous tumour development in laboratory animals, without application of carcinogen or transplantation of immortalised cell lines, may provide a closer experimental representation of normal tumour development *in vivo*.

Mice on a p53 deficient background are more cancer prone and have been crossed with immunodeficient mice or received immunodepleting treatment to compare tumour induction with immunocompetent p53 deficient litter mates. Further observations have also established that ageing immunodeficient mice with a full complement of tumour suppressor genes have an accelerated rate of tumour induction compared to WT litter mates. As shown in Table 1.2, several immune functions important for controlling MC-induced tumours also influence development of spontaneous tumours in p53 deficient and ageing animals.

Important roles of NKT cell activation (Hayakawa, *et al.*2003), TRAIL (Takeda, *et al.*2002) and IFN-γ (Kaplan, *et al.*1998, Qin, *et al.*2002) have been identified in inhibiting spontaneous tumour development in p53 deficient mice.An enhanced rate of tumour induction has been observed in ageing mice with IFN-γ (Shankaran, *et al.*2001, Street, *et al.*2002) and/or perforin (Smyth, *et al.*2000b, Street, *et al.*2002, van den Broek, *et al.*1996)deficiency; whilst tumour induction in IL-$18^{-/-}$, IL-$12^{-/-}$, TNF-$\alpha^{-/-}$ (Street, *et al.*2002) and GM-$CSF^{-/-}$ (Enzler, *et al.*2003) mice was comparable to that in ageing WT litter mates.

These studies have also indicated that a particular immunodeficiency predisposes tumour development at distinct anatomical locations. IFN-γ and perforin deficient mice are predisposed to develop lymphomas (Street, *et al.*2002, Smyth, *et al.*2000b) and mice deficient of LMP2 subunit, important in MHC I presentation, are more likely to develop uterine leiomyosarcomas (Hayashi, *et al.*2002).

1.3. Evidence to support immunosurveillance of tumours in humans

The principle of tumour immunosurveillance should be transferable to human tumour development in theory. Evidence available to prove if this is the case appears to be conflicting at times, however, an overall picture has developed to suggest that tumour

Table 1.2. Susceptibility to spontaneous tumourigenesis in either genetically modified or ageing mice by immunodeficiency

Treatment/Strain	Deficiency/effect	Susceptibility to tumour development
IFN-$\gamma^{-/-}$	IFN-γ cytokine	Enhanced (Street, *et al.*2002)
IFN-$\gamma R^{-/-}$	IFN-γ signaling	Enhanced in $p53^{-/-}$ (Kaplan, *et al.*1998) / no difference (Qin, *et al.*2002)
$Rag^{-/-}$	T, B, NKT cells	Enhanced (Shankaran, *et al.*2001)
$Rag^{-/-}$ x $STAT1^{-/-}$	T, B, NKT cells, IFN-γ, α, β signaling	Enhanced (Shankaran, *et al.*2001)
GM-$CSF^{-/-}$ x IFN-$\gamma^{-/-}$	GM-CSF and IFN-γ cytokines	Enhanced (Enzler, *et al.*2003)
GM-$CSF^{-/-}$	GM-CSF cytokine	No difference (Enzler, *et al.*2003)
Injection of α-galactosylceramide	NKT cell activation	Reduced tumour formation in Her-2/neu oncogene transgenic mammary carcinoma and $p53^{-/-}$ (Hayakawa, *et al.*2003)
IL-12 cytokine injection	Enhanced IL-12 activity	Reduced in Her-2/neu oncogene transgenic mammary carcinoma model (Hayakawa, *et al.*2003)
IL-12p40$^{-/-}$	IL-12 + IL-23	No difference (Street, *et al.*2002)
Perforin$^{-/-}$	Perforin mediated cytotoxicity	Enhanced (Smyth, *et al.*2000a, van den Broek, *et al.*1996, Street, *et al.*2002)
Injection of anti-TRAIL antibody	TRAIL	Enhanced development in $p53^{+/-}$ mice (Takeda, *et al.*2002)
IL-$18^{-/-}$	IL-18	No difference (Street, *et al.*2002)
TNF-$\alpha^{-/-}$	TNF-α	No difference (Street, *et al.*2002)
LMP-$2^{-/-}$	Protesome protein degradation	Enhanced (Hayashi, *et al.*2002)
Transgenic expression of CSF-1	Enhanced macrophage recruitment into tumour	Enhanced progression to malignancy in spontaneous breast cancer model (Lin, *et al.*2001)

immunosurveillance does indeed occur in human malignancy. This data has been collected from various fields of research.

Epidemiological studies of tumour incidence during chronic immunosuppression

A substantial part of the evidence to support immunosurveillance in humans is derived from epidemiological studies of tumour incidence in patients on organ transplant registers that receive lifelong immunosuppressive therapy (reviewed in (Penn.1999, Dunn, *et al.*2002)). Patients have higher incidence of several malignancies, including Kaposi's sarcoma, non-Hodgkin's lymphoma and carcinomas of the genitourinary and anogenital regions. However, these particular malignancies have been associated with viral transformation in the presence of chronic immunosuppression and may not be attributed to compromised tumour immunosurveillance (reviewed in (Dunn *et al.* 2002). The incidence of tumours with non-viral origin is contested. A higher incidence of tumours with a non-viral origin has been reported on the Nordic and Australian/New Zealand transplant registers whilst no change from a control population has been reported by the Cincinnati register (reviewed in (Dunn *et al.* 2002). Differences between the populations on each register have been suggested to explain this disparity. The Australian/New Zealand register has a massively elevated incidence of skin cancer; the average age of transplant patients in the Cincinnati register is significantly less than those of other registers and below the typical age of tumour development in the normal population; and lastly genetic difference between populations also account for altered rates of tumour incidence. An overall examination of epidemiological data does suggest an increase in those tumours not thought to be associated with viral infections in subjects receiving immunosuppression, however, this data is difficult to interpret with certainty because commonly used immunosuppressive drugs may themselves be carcinogenic.

Allogenic transplantation of quiescent malignancy by organ transplantation

A large collection of anecdotal cases of transplanted tumour occurrence in organ transplant patients has also contributed to the argument for tumour immunosurveillance in humans.

In a number of cases, patients received organ transplants and proceeded to develop allogeneic melanoma between six months and two years after transplantation (Morris-Stiff, *et al.*2004, Elder, *et al.*1997, MacKie, *et al.*2003, Penn.1996). Organ recipients were treated with standard immunosuppressive therapy to prevent organ rejection and halting immunosuppression induced successful elimination of melanoma in a number cases (Morris-Stiff, *et al.*2004, Elder, *et al.*1997, Penn.1996, Suranyi, *et al.*1998). In one report, melanoma was not eliminated by removal of immunosuppressive treatment alone and required IFN-α immunotherapy with adoptive transfer of activated pooled lymphocytes, PPD adjuvant and irradiated melanoma cells. Pooled lymphocytes were obtained from HLA-mismatched individuals and stimulated with PHA in order generate helper T cell assistance to stimulate effective allograft rejection of melanoma cells (Suranyi, *et al.*1998).

Anti-tumour immunotherapy trials

Further evidence to support tumour immunosurveillance in humans is derived from trials to manipulate and improve the autologous recognition of tumour cells in melanoma patients (Dudley, *et al.*2005, Dudley, *et al.*2002). Dudley *et al* isolated and expanded TILs derived from melanoma patients *in vitro* and transferred TILs back to patients that previously received lymphocyte depleting chemotherapy to enable homeostatic expansion of transferred T cells. TILs survived, expanded *in vivo* and infiltrated melanoma lesions. Transfer of autologous TIL initiated disease regression and vitiligo, suggesting transferred cells also recognised self-antigen that was co-expressed by normal melanocytes and melanoma cells (Dudley, *et al.*2005, Dudley, *et al.*2002).

Correlation of lymphocyte infiltration of tumour with prognosis

A series of studies have established that a strong lymphocyte infiltration of tumour is positively correlated with an improved overall life expectancy and a reduced rate of disease recurrence following surgery. This observation has been made with a diverse array of different types of tumour, including renal, colorectal, breast, bladder, prostate, and ovarian cancer (reviewed in (Dunn, *et al.*2002)). A number of informative studies have dissected the importance of TIL in the prognosis of colorectal cancer. An improved prognosis associated with an increase immune cell infiltration of the tumour in colorectal was first described in the 1930's . In a later study, around 50% of patients with a moderate to high infiltration of tumour stroma survived beyond five years, whilst around 30% of patients with an absence of tumour infiltration survived beyond five years (Nacopoulou, *et al.*1981). The extent of lymphocyte infiltration appeared to be independent to the stage of the disease. A significant lymphocyte infiltration of tumour proximal LNs was also strongly correlated with prognosis. 10% of patients with a paucity of lymphocytes in proximal LN survived beyond 5 years which was elevated to 70% of patients with a preponderance of lymphocytes in the cortical and medullary regions of the LN. These observations have been extended to reveal the importance of a Th1 lymphocyte response and the location of TIL. One study classified the level and location of TIL in colorectal cancer and established that a high infiltration of cancer cell nests by activated $CD8^+$ T cells was associated with an improved prognosis whilst an infiltration of the invasive margin was not (Naito, *et al.*1998). A recent study sampling the centre and invasive margins of tumours resected from a large cohort of patients (Galon, *et al.*2006)revealed that expression of a cluster of T cell associated genes were associated with an improved prognosis. These genes included CD3, CD8, CD45RO and granzyme B. Conversely, a low expression of these genes in both tumour samples was correlated with a poor prognosis, which even extended to patients with minimal tumour invasion. Genomic analysis was confirmed by *in situ* immunohistochemical staining. Thus, the number of TIL appeared to serve as an independent prognostic indicator regardless of the patho-histological staging of disease.

The correlation of lymphocyte infiltration with prognosis is an argument for the role of immunosurveillance in tumours (reviewed in (Dunn, *et al.*2002)). The collective

data provided by epidemiology, allogenic tumour transplantation, immunotherapy trials and improved prognosis with tumour lymphocytes infiltration strongly supports the existence of tumour immunosurveillance in humans.

1.4. Tumour antigens recognised by the immune system

Several distinct families of tumour antigens are believed to be targeted by T cells (reviewed in (Boon, *et al.*1997)). These include: 1) viral antigens expressed following the viral transformation of cells; 2) differentiation antigens which comprise non-mutated antigens that are upregulated in transformed cells; 3) shared tumour antigens that are normally only expressed in the testis, but become expressed in a several different tumours; 4) tumour specific antigens that arise following a mutation which is restricted to a specific malignant cell. Virus- and tumour-specific antigens may be recognised as foreign by the immune system and trigger robust anti-tumour immune responses. Shared and differentiation tumour antigens are self-antigen which are co-expressed by self tissue. Immune responses against self-antigens are limited to avoid autoimmunity and many potential high affinity T cells that recognise these antigens are eliminated or convert to a regulatory T cell phenotype in the thymus to leave a pool of conventional T cells with a lower affinity to self antigen (Hsieh, *et al.*2004, Hsieh, *et al.*2006) [†]. High affinity self reactive T cells that escape deletion in the thymus may ultimately become tolerised after recognition of self-antigen in the absence of co-stimulation or induced to apoptose by activation induced cell death (AICD).

A relatively recent addition to the knowledge of how self-reactive immune responses may be prevented came from the discovery of a population of naturally occurring regulatory T cells (Treg). These cells have proven vital to prevent self-reactive immune responses that drive autoimmune disease (reviewed in (Sakaguchi.2004)). It is therefore logical that Treg are also able to suppress anti-tumour immune responses that recognise self-antigen expressed by tumour cells. In fact, a substantial amount of

[†] T cells with high affinity TCR recognition of self antigen have been observed to enter the regulatory T cell phenotype, described section 1.5.9.

evidence has been assembled to describe a pivotal role for Treg in suppressing anti-tumour immune responses that are otherwise capable of rejecting developing tumours. Various studies have observed that Treg heavily infiltrate tumours and may suppress anti-tumour immunity (reviewed in (Gallimore, *et al.*2002)). These have included tumours from a wide range of histological origins. The mechanism of how Treg are originally recruited to the tumour environment is of central importance. The fact that the tumour environment is considered to be highly tolerising (reviewed in (Finn.2003, Blattman, *et al.*2004)) and studies described in later (section 1.5.5.) suggest that T cells residing in tumour tissue may undergo *de novo* conversion to the Treg phenotype and contribute to inhibit tumour immunosurveillance. The following section of this report describes Treg and discusses a potential role for the cells in tumour development.

1.5. Characterising $CD4^+$ $FOXP3^+$ Treg

Extensive experimental data has established that there is a sub-population of naturally occurring CD4+ T lymphocytes that can be defined by their ability to regulate the activity of other immune cells. These cells, termed Treg can be identified by the constitutive expression of a transcription factor called FOXP3 (discussed in detail later). Treg have been demonstrated to regulate the activation of both the adaptive (Sakaguchi, *et al.*1995) and innate immune system (Maloy, *et al.*2005, Maloy, *et al.*2003). The involvement of Treg to control immune responses to self antigen (Sakaguchi.2004) and foreign antigen (Peters, *et al.*2006, Suffia, *et al.*2006) has been established with important implications for autoimmune disease (Sakaguchi.2004), tolerance of allogenic transplants (Hoffmann, *et al.*2006), pathogens (Peters, *et al.*2006, Suffia, *et al.*2006), allergy (Sakaguchi.2005) and anti-tumour responses (Yamaguchi, *et al.*2006, Betts, *et al.*2006). The phenotypic and functional characteristics of Treg are described in detail below with particular reference to their involvement in controlling anti-tumour immune responses.

1.5.1. $CD4^+$ Treg

Controversy followed the proposed existence of a population of suppressive T cells, whose primary purpose was to regulate pathogenic immune responses. The lack of a cellular marker to identify these "suppressor" cells and the failure to identify the soluble suppressive factor (that was thought to be encoded within a theoretical IJ locus of the MHC region) led to a discrediting of the field of suppressor T cells under the scrutiny of modern molecular biology techniques.

Despite early setbacks, a number of important observations maintained some confidence in the existence of Treg. Experimental evidence in mice established the existence of a separate lineage of T cells with regulatory properties whose emergence from the thymus was delayed approximately three days from the initial output of T cells. Mice that were thymectomised three days after birth (3dTx) appeared to lack a putative population of Treg and developed autoimmune disease (Kojima, *et al.*1976). In a separate study administration of cyclosporine induced autoimmune disease in normal mice (Sakaguchi, *et al.*1989), cyclosporine appeared to remove the Treg population and trigger the activation of self-reactive T cells and autoimmune disease. Autoimmune disease was prevented by the transfer of splenocytes purified from a normal syngenic mouse that did not receive cyclosporine. Several studies began to search for a cell marker that could identify the cells that controlled self-reactive T cells. Early studies demonstrated tolerance mediated by $CD4^+CD5^+$ (Sakaguchi, *et al.*1985, Sakaguchi, *et al.*1982) and $CD4^+CD45RB^{lo}$ cells (Powrie, *et al.*1990), however, the heterogeneity of these populations prevented a definitive identification of Treg.

1.5.2. Refining the Treg phenotype as $CD4^+CD25^+$

Subsequent studies proceeded to show the cells that emerged from the thymus three days after birth and controlled autoimmunity could be identified by expression of the CD25 antigen (Asano, *et al.*1996). Mice that received 3dTx had a delayed emergence of $CD4^+CD25^+$ T cells in the periphery and adult mice that received 3dTx had a log fold reduction of $CD4^+CD25^+$ T cells compared to mice with a functional thymus (Asano, *et al.*1996). Splenocytes purified from naïve euthymic mice younger than three days old

transferred autoimmunity to nude mice and the transfer of $CD4^+CD25^+$ cells from euthymic mice prevented autoimmunity, whilst $CD4^+CD25^-$ T cells did not (Asano, *et al.*1996). In separate experiments, $CD4^+CD25^+$ cells are also able to transfer tolerance to lymphopenic mice as well to as thymectomised mice (Sakaguchi, *et al.*1995). $CD4^+$ lymphocytes were isolated from normal mice and depleted of $CD25^+$ cells. When transferred to nude lymphopenic mice, T cells were hyperactive to stimulation with self and non-self antigen and generated serological and histological autoimmunity and in some cases a graft-vs-host like wasting disease (Sakaguchi, *et al.*1995). The early replacement of $CD4^+CD25^+$ cells prevented the induction of autoimmunity, however, no protection was afforded by the transfer of a comparable quantity of $CD4^+CD25^-$ or $CD8^+$ T cells (Sakaguchi, *et al.*1995). Self-reactive T cells elicited a broad range of organ specific pathologies, including gastritis, oophoritis, thyroiditis, sialoadenitis, adrenalitis, insulitis, glomerulonephritis and arthritis; however, various mouse strains appeared to have an altered frequency and hierarchy of disease onset (Sakaguchi *et a*l 1996). BALB/c mice are described to have an elevated frequency of gastritis, A/J mice have an elevated level of oophoritis, whilst C57/BL6 mice have very little autoimmune onset. Distinctions between the type of MHC molecules expressed in these stains of mice generate different repertoires of self-antigen that is presented to self-reactive T cells and is believed to account for the differences of autoimmune diseases that occur in different strains of mice (Sakaguchi *et al.* 1996). This explanation indicated that Treg suppress in an antigen specific manner and further data generated using Treg obtained from transgenic mice proved this point (discussed in further detail in section 1.5.8).

The identification of $CD4^+CD25^+$ Treg converged with the identification of a population of $CD4^+$ regulatory T cells identified as $CD45RB^{lo}$ that could, upon transfer, inhibit colitis. Subsequent studies proved that the majority of the regulatory activity within the $CD4^+CD45RB^{lo}$ population was restricted to the $CD25^+$ subpopulation of $CD4^+CD45RB^{lo}$ cells (Read, *et al.*2000).

The suppressive capacity of $CD4^+CD25^+$ T cells has also been demonstrated *in vitro*. $CD4^+$ splenocytes isolated from normal mice were separated according to their

expression of the CD25 antigen. $CD25^+$ and $CD25^-$ T cells received polyclonal TCR stimulation and contact with antigen presenting cells to provide co-stimulation. $CD25^-$ T cells underwent robust proliferation in isolation but $CD25^+$ cells remained anergic. When these populations were mixed, $CD4^+CD25^+$ cells demonstrated their regulatory phenotype and suppressed the proliferation of $CD25^-$ T cells (Thornton, *et al.*1998). These experiments therefore established that $CD4^+CD25^+$ T cells could act as regulatory cells.

1.5.3. Identification of $CD4^+CD25^+$ using CTLA-4 and GITR

The identification of Treg by constitutive expression of CD25 was a major advancement in the study of these cells; however, activated T cells also transiently express CD25. A closer examination of other surface markers was therefore conducted to improve the identification of Treg. These studies identified that Treg also constitutively express GITR (Shimizu, *et al.*2002) and CTLA-4 (Takahashi, *et al.*2000). Identification of Treg through a combination of these markers enhanced the identification of Treg but making a definitive distinction between Treg and activated T cells remained difficult. Surface expression of CTLA-4 (described in further detail in section 1.5.8.) is enhanced during T cell activation and its ligation by B7 molecules delivers an inhibitory signal to T cells that is important to arrest aberrant T cell responses (reviewed in Teft *et al* 2006). GITR is expressed predominantly by Treg in naïve mice but expression is rapidly up-regulated on conventional T cells after activation (Shimizu 2002). The normal physiological function of GITR has not been fully elucidated (reviewed in Shevach and Stephens 2006) but ligation of GITR has been shown to activate conventional T cells and break self-tolerance.

1.5.4. Identification of $CD4^+CD25^+$ using FOXP3

The fact that CD25 antigen is upregulated by activated T cells impairs the characterisation of Treg during normal stimulation of the immune system by self and

non-self antigen. A major advance in the field of Treg immunology came with the identification of the transcription factor FOXP3 that was unique to the Treg lineage and could be used to identify and track Treg during immune activation and quiescence. FOXP3 was identified from observations of the lethal X-linked immunodeficiency syndrome IPEX (Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome) in humans, associated with autoimmune disease of multiple endocrine organs, originally described in 1982 (Powell, *et al.*1982); and the scurfy mouse strain that displays an identical pathology, originally described in 1959 (Russell, *et al.*1959). The cause of IPEX human syndrome (Brunkow, *et al.*2001) and scurfy mouse phenotype (Bennett, *et al.*2001) was isolated to mutations within the transcription factor FOXP3 in close succession. The mutations of FOXP3 abrogated the development of the Treg lineage and ultimately triggered a lethal autoimmune pathology. The intracellular location of FOXP3 necessitates the intracellular staining of Tregs to observe FOXP3 expression and prevents the identification of FOXP3 in live cells, which presents a major drawback to the study Tregs. However, Fontenot *et al* have recently developed a mouse strain expressing GFP under the control of the FOXP3 promoter which negated the need to permeabilise Treg and enabled single cell analysis of FOXP3 expressing cells (Fontenot, *et al.*2005c). Using this mouse, the investigators observed that $FOXP3^+$ cells were restricted to the $CD4^+$ T cell compartment. Interestingly there is a broad variation of CD25 expression linked to the anatomical locations of Treg (Fontenot, *et al.*2005c). Both $CD4^+CD25^{hi}FOPX3^+$ cells and $CD4^+FOXP3^+CD25^{lo/int}$ cells acted as suppressors, showing the FOXP3 molecule to be a more accurate marker of regulatory T cells. However, due to the intracellular location of FOXP3, the CD25 antigen remains the most well used Treg marker to perform functional analyses of Treg activity.

1.5.5. Thymic generation of Treg

As described above, previous studies have shown that mice thymectomised three days after birth suffer from autoimmune disease (Kojima, *et al.*1976) which suggested that self-reactive T cells emerged from the thymus before day three and that the production of Treg was delayed until after day three. Single cell analyses of FOXP3-GFP expressing

mice demonstrated a large disparity in the percentage of $CD4^+$ cells that expressed FOXP3 at 12hr after birth compared to four day old neonates (Fontenot, *et al.*2005a). The percentage of $CD8^-CD4^+$ thymocytes that expressed FOXP3 rose incrementally from <0.1% 12 hours after birth to 4% at 21 days after birth. The single largest increase in the percentage of $CD4^+$ SP thymocytes that expressed FOXP3 occurred between day 3 and 4 (0.7% to 1.9%) and correlated with induction of autoimmunity, observed in earlier studies, following 3dTx (Asano, *et al.*1996, Kojima, *et al.*1976).

Table 1.3. *In vitro* culture requirement to induce *de novo* FOXP3 expression

Isolation of $CD4^+FOXP3^-$ Teff	Primary mediator of FOXP3 conversion	Other requirement for FOXP3 conversion	Reference
$CD4^+CD25^-$ purification	TGF-β	Anti-CD3 stimulation + APC ‡Absence of IL-2	(Chen, *et al.*2003)
$CD4^+CD25^-$ purification	TGF-β	‡IL-2 and anti-CD3 stimulation Absence of strong anti-CD28 stimulation	(Fu, *et al.*2004)
FOXP3-Fluorescent reporter	TGF-β	Anti-CD28 stimulation	(Wan, *et al.*2005)
FOXP3-Fluorescent reporter	TCR activation / APC stimulation	TGF-β enhances conversion	(Kim, *et al.*2006a)

‡ Discrepancies between culture conditions used to propagate *de novo* FOXP3 conversion exist. Further experimentation appears necessary to establish robust guidelines to reproducibly derive $FOXP3^+$ cells from conventional $CD4^+FOXP3^-$ T cells

1.5.6. Extrathymic generation of Treg

The thymic commitment of $CD4^+FOXP3^+$ Treg is independent of environmental stimulation and has led to the description of Treg as "naturally occurring". This feature also distinguishes $CD4^+FOXP3^+$ Treg from the Tr1 and Th3 lineages of $CD4^+$ regulatory T cells that differentiate from T cells in the periphery. FOXP3 expression has not been observed in peripheral $CD4^+$ T cells following stimulation with viral or bacterial antigens and standard mitogens (Khattri, *et al.*2003, Fontenot, *et al.*2003, Hori, *et al.*2003). However, peripheral, *de novo* FOXP3 expression has recently been shown to occur under certain conditions, whereby tolerised T cells have been shown to express FOXP3. Extra-thymic generation of Treg within tumours may by an important mechanism to attenuate anti-tumour immune responses (see later).

In vitro

Various studies purified and incubated T cells in culture with various concentrations of TGF-β and reported *de novo* expression of FOXP3 in up to 30% of cells (Wan, *et al.*2005, Kim, *et al.*2006a, Fu, *et al.*2004, Chen, *et al.*2003). .
The precise experimental conditions under which TGF-β mediates *de novo* FOXP3 up-regulation *in vitro* has not been fully defined and a number of culture conditions have been found to trigger FOXP3 expression (Table 1.3.).

In vivo

Exposure of naïve $CD4^+$ T cells to their cognate antigen within a tolerising environment can drive the conversion of T cells to $FOXP3^+$ Treg *in vivo* (Apostolou, *et al.*2004, Kretschmer, *et al.*2005). *De novo* expression of FOXP3 was examined in $Rag^{-/-}$ TCR-HA transgenic mice. Thymic production of Treg has previously been shown to require high affinity recognition of cognate antigen in the thymus (Kawahata, *et al.*2002, Hsieh, *et al.*2004); and thus, in the absence of HA antigen and functional Rag genes, TCR-HA transgenic mice fail to produce either HA or self-antigen specific $FOXP3^+$ Treg. Osmotic

mini-pumps were installed in mice to deliver a low dose of HA antigen under the skin. T cells proceeded to recognise HA antigen in the absence of activation signals and converted to the $FOXP3^+$ Treg phenotype. Treg persisted for several months after antigen delivery was halted (Apostolou, *et al.*2004).

De novo expression of FOXP3 was also demonstrated in $Rag^{-/-}$ TCR-HA mice by delivering HA antigen to DC using a HA-anti-DEC205 antibody fusion protein (Kretschmer, *et al.*2005). DEC205 is an endocytosis receptor that is abundantly expressed on DC within the T cell areas of lymphoid organs. Targeting antigen to DC using this fusion protein initiated DC to present cognate antigen to T cells within an anergic environment devoid of co-stimulatory signals. In separate experiments, T cells were isolated and exposed to TGF-β in the absence of IL-2 *in vitro* before being transferred back into mice (Kretschmer, *et al.*2005). Prior exposure to TGF-β enhanced FOXP3 conversion beyond that observed in mice that received DC targeted antigen alone. In a complementary experiment, $Rag^{-/-}$TCR-HA mice were bred onto a truncated TGF-β receptor background (Kretschmer, *et al.*2005). T cells were then purified and injected into WT mice. T cells refractory to TGF-β signalling underwent a markedly reduced rate of conversion to the $FOXP3^+$ phenotype compared to HA specific cells isolated from mice that expressed a functional TGF-β receptor. These studies showed that T cells that receiving TCR stimulation in a tolerising environment can permanently convert to the Treg phenotype. These observations are of great importance in the context of tumour development (discussed in detail later).

1.5.7. Peripheral survival of Treg

A number of signals have been found to be important for maintaining Treg numbers and/or function in the periphery following migration of the cells from the thymus. These signals have largely been identified following observations of impaired Treg number and/or function in mice deficient of B7, CD28, IL-2, TGF-β or IFN-γ.

IL-2 and CD28

Unlike conventional T cells, Treg are strictly dependent on IL-2 for survival in the periphery and IL-$2^{-/-}$ mice are deficient of Treg (Fontenot, *et al.*2005b, D'Cruz, *et al.*2005, Sadlack, *et al.*1993). The survival of T cells in the absence of Treg within IL-$2^{-/-}$ mice drives lethal colitis early on in life. The pathology appears to be driven more by commensal gut bacteria and treatment of mice with antibiotics eliminates major pathology to leave residual disease driven by autoreactivity (Sadlack, *et al.*1993).

$B7^{-/-}$ and $CD28^{-/-}$ mice are deficient of Treg due to a global reduction of IL-2 production (Tang, *et al.*2003, Salomon, *et al.*2000). Quiesent T cells express CD28 which binds B7 molecules to stimulate homeostatic IL-2 production. In order to demonstrate the importance of CD28/B7 interaction for Treg survival, normal mice were injected with a CTLA-4-Ig fusion protein that blocked CD28 binding B7 molecules (Tang, *et al.*2003, Salomon, *et al.*2000). Injection of this fusion protein triggered Treg death in the periphery of mice. Consistent with these results, Treg isolated from $CD28^{+/+}$ mice perished when injected into $CD28^{-/-}$ mice, an effect that was reversible by administration of IL-2. (Tang, *et al.*2003). CD28 signalling is itself important for Treg survival, independent of endogenous IL-2, since blocking CD28 on Treg from interacting with B7 diminishes the survival of Tregs even in the presence of IL-2 (Tang, *et al.*2003). In addition, no Treg can be generated from $CD28^{-/-}$ bone marrow even in the presence of IL-2 (Tai, *et al.*2005).

TGF-β

TGF-$\beta^{-/-}$ mice are deficient of Treg and succumb to lethal lymphoproliferation and autoimmune disease at 4-5 weeks of age (Kulkarni, *et al.*1993, Shull, *et al.*1992). The aetiology of Treg deficiency has been dissected using mice 8-12 days old before the initiation of systemic non-specific activation and expansion of $CD4^+$ T cells (Marie, *et al.*2005). Normal thymic production of $CD4^+FOXP3^+$ Treg was observed in TGF- $\beta^{-/-}$ mice, however, in the absence of TGF- β, Tregs down-regulated FOXP3 expression and failed to survive in the periphery after migration from the thymus (Marie, *et al.*2005). To

determine whether Tregs survive using an exogenous source of TGF-β, Treg that recently emigrated from the thymus were isolated from TGF-$\beta^{-/-}$ mice and injected into TGF-$\beta^{+/+}$ mice. TGF-$\beta^{-/-}$ Treg survived and re-expressed FOXP3 and reciprocally, Treg from a TGF- $\beta^{+/+}$ donor down-regulated FOPX3 and died in TGF- $\beta^{-/-}$ recipients.

IFN-γ

Treg function is partially impaired in IFN-$\gamma^{-/-}$ mice (Kelchtermans, *et al.*2005, Wang, *et al.*2006). Data collected using IFN-$\gamma^{-/-}$ mice failed to observe an obvious impairment of Treg suppressive function in naïve animals but a defective capability to suppress was clearly demonstrated following experimental induction of autoimmune disease *in vivo* (Kelchtermans, *et al.*2005, Wang, *et al.*2006). IFN-$\gamma^{-/-}$ mice developed a more severe pathology following induction of rheumatoid arthritis (Kelchtermans, *et al.*2005) and experimental autoimmune encephalitis (Wang, *et al.*2006) compared to WT littermates. IFN-$\gamma^{-/-}$ Treg did not demonstrate impaired TGF-β production and the impairment of Treg function appeared to be directly due to a lack of IFN-γ signalling (Kelchtermans, *et al.*2005). *In vitro* incubation of IFN-$\gamma^{-/-}$ Treg with IFN-γ re-established full suppressive function and Treg proceeded to control rheumatoid pathology when injected into mice (Kelchtermans, *et al.*2005).

1.5.8. The mechanism of Treg mediated suppression

The exact method of how Treg exert their suppressive effects is currently unknown. Studies have been performed both *in vivo* and *in vitro* and results imply that more than one mechanism, including cell-cell contact and production of immunosuppressive cytokines, may account for the activity of Treg cells.

Cell-cell contact and IL-2 starvation

An early study of how Tregs suppress was performed by purifying conventional T cells and Tregs from transgenic mice that recognized distinct OVA peptides (Takahashi, *et al.*1998). CD25 expression was used to distinguish Treg from other T cells. Cells were incubated together with titrated concentrations of each population's cognate peptide and cell proliferation of conventional T cells was measured. Two important observations were made. Firstly, Treg suppression of T cell proliferation only occurred once Treg recognised their cognate antigen. When only the antigen recognised by the conventional T cells was present, Treg did not mediate suppression. Secondly, the concentration of antigen required to activate Treg was a log-fold less that that required to activate other T cells indicating that, as a population, Treg are endowed with higher affinity TCR than conventional T cells (Takahashi, *et al.*1998).

Other studies discovered that Treg mediated suppression of T cells *in vitro* was conditional on cell-cell contact between the cells. Treg and conventional $CD4^+$ T cells were purified from WT mice according to CD25 expression and cultured *in vitro* with anti-CD3 antibodies and APC. When cultured in isolation, $CD4^+CD25^-$ T cells underwent robust proliferation and $CD4^+CD25^+$ Treg remained anergic. Treg could inhibit T cell proliferation unless the cells were separated by a permeable membrane thus demonstrating the necessity of cell-cell contact (Thornton, *et al.*1998, Shevach.2002). T cells were then activated with concanavalin A to induce CD25 expression and incubated with freshly isolated T cells. Activated $CD4^+CD25^+$ T cells did not suppress $CD4^+CD25^-$ proliferation and demonstrated that CD25 expression alone did not confer the suppressive Treg phenotype (Thornton, *et al.*1998). An analysis of IL-2 transcripts in T cells previously incubated with Treg implied that IL-2 expression was suppressed in these cells (Shevach.2002). However, this method of suppression does not appear to correlate with how Treg suppress *in vivo*, whereby conventional T cells have been demonstrated to be fully capable of being activated and mediating lethal wasting disease in the absence of IL-2 or IL-2Rβ. These studies suggest that Treg do not suppress T cells solely through inhibiting IL-2 expression.

Production of Immunosuppressive Cytokines

IL-10

A mouse model of colitis has been established whereby adoptive transfer of T cells without Treg into immunodeficient mice induces colitis. To determine whether IL-10 is involved in Treg mediated suppression, Treg derived from IL-$10^{-/-}$ mice were tested for their ability to inhibit colitis. Treg derived from IL-$10^{-/-}$ mice were unable to transfer suppression of colitis and suppression mediated by WT Treg was abolished by the injection of anti-IL-10 receptor binding antibody (Asseman, *et al.*1999).
The requirement of IL-10 appeared to be dictated by the microenvironment of disease, whereby IL-10 was necessary to prevent colitis yet was dispensable for Treg suppression of gastritis (Suri-Payer, *et al.*2001). The difference between these pathologies was deemed to result from a stronger activation of $CD4^{+}CD25RB^{hi}$ cells in the gut, driven by commensal bacteria, whereby germ free mice treated in the same manner developed significantly milder colitis(Sadlack, *et al.*1993). Following the removal of commensal bacteria, residual mild colitis was hypothesised to be mediated by self-antigen. It therefore appeared that certain mediators of Treg suppression were dispensable according to the potency of suppression required to tolerise T cells exposed to varying strength of activation signals.

TGF-β

Multiple investigations have identified a role for TGF-β in mediating Treg suppression. One study purified Treg using expression of CD4 and CD25 from TGF-$\beta^{-/-}$ mice and placed cells in culture with APC and T cells derived from WT mice and polyclonal anti-CD3 antibodies (Marie, *et al.*2005). TGF-$\beta^{-/-}$ Treg were able to suppress T cell proliferation when APC were derived from TGF-$\beta^{+/+}$ mice, however suppression was attenuated if Treg and APC were both isolated from TGF-$\beta^{-/-}$ mice. This demonstrated that TGF-β must be present for Treg to suppress but that Treg mediated TGF-β production is not strictly necessary. Further studies have looked at the role of TGF-β to act upon both Treg and conventional T cells (Fahlen, *et al.*2005). The first possibility was addressed by co-transferring conventional T cells that expressed a truncated TGF-β

receptor and Treg purified from WT mice into immunodeficient mice. TGF-$\beta R^{-/-}$ T cells were refractory to suppression and mice that received these cells developed lethal colitis. Mice receiving T cells isolated from WT mice did not develop colitis; however, if Tregs that expressed a truncated TGF-β receptor were co-injected with WT T cells into immunodeficient mice, a lethal colitis also developed. These experiments demonstrated that TGF-β performs a dual role by both activating Tregs to suppress and by directly tolerising conventional T cells (Fahlen, *et al.*2005).
The central role of TGF-β has been confirmed in similar co-transfer experiments that injected Treg and conventional T cells into immunodeficient mice and observed an abrogation of tolerance when recipient mice where injected with TGF-β blocking antibody (Read, *et al.*2000). The role of TGF-β produced by Treg in the tumour environment has also been examined, whereby TGF-β was seen to specifically inhibit cytotoxicity of anti-tumour CTL, without an inhibition of either IFN-γ production or proliferation (Chen, *et al.*2005a). (The role of Treg in the tumour environment is described in further detail in section 1.6.)

CTLA-4

CTLA-4 may also contribute to Treg mediated suppression. The importance of CTLA-4 in down-regulating aberrant immune responses has been well established. CTLA-4 deficient mice succumb to a lethal lymphoproliferative disease in early life (Tivol, *et al.*1995). Pathology is associated with a multi-organ mononuclear infiltrate and T cells purified from these CTLA-$4^{-/-}$ mice undergo spontaneous proliferation *in vitro*.
CTLA-4 is upregulated during T cell activation and on binding its ligand B7, transmits an inhibitory signal to the cell. A specific role for CTLA-4 in modulating Treg function has been difficult to identify but constitutive CTLA-4 expression suugests a role for the molecule in Treg function (Takahashi, *et al.*2000); In addition, constitutive CTLA-4 expression is induced when $CD4^{+}CD25^{-}$ T cells are transfected with FOXP3 (Hori, *et al.*2003), a transcription factor considered to be the master control gene that confers the Treg phenotype.

Some findings have indicated that CTLA-4 expressed by Tregs may confer a negative signal to conventional T cells that express B7 molecules during activation. If Tregs and conventional T cells are isolated from WT mice and incubated together *in vitro*, on addition of an anti-CTLA-4 blocking antibody abrogates Treg mediated suppression of proliferation (Takahashi, *et al.*2000). Complementary experiments were performed *in vivo* by injecting immunodeficient mice with Treg and T cells isolated from WT mice and co-injecting non-depleting anti-CTLA-4 antibody. Antibody treatment interrupted CTLA-4 interaction with B7 molecules and mice developed colitis, whilst mice treated with an isotype control antibody did not develop pathology (Read, *et al.*2000). Further research has suggested a role of CTLA-4 in the control of gastritis, as WT mice that were injected with a non-depleting CTLA-4 antibody developed gastritis (Takahashi, *et al.*2000). Constitutive CTLA-4 expression by Treg suggests that anti-CTLA-4 antibody treatment may initiate pathology by interfering with Treg mediated suppression. However, the role of CTLA-4 expressed by conventional T cells would also be abrogated by injecting this antibody so the overall contribution of CTLA-4 on Tregs versus conventional T cells is unclear. Observations performed using $B7^{-/-}$ mice overcame this problem and indicated that CTLA-4 expressed by Treg confer a negative signal to T cells that express B7 molecules during activation (Paust, *et al.*2004). These experiments observed that activated $CD4^+$ and $CD8^+$ T cells isolated from WT mice expressed B7, although to a much lower extent than APC. The study progressed to isolate and incubate Treg from WT mice in culture with T cells isolated from either WT or $B7^{-/-}$ mice. $B7^{-/-}$ T cells were refractory to Treg suppression *in vitro* and complementary observations *in vivo* demonstrated that $B7^{-/-}$ T cells elicited lethal wasting disease when co-injected with WT Treg into immunodeficient mice (Paust, *et al.*2004). This finding presented a novel mechanism of how CTLA-4 expressed by Treg operates, since the conventional role of CTLA-4 is to transmit a negative signal to activated T cells that express CTLA-4 when binding B7 molecules on APC.

1.5.9. Identification of Treg target antigens

The fact that Tregs need to be activated via the TCR (e.g. with anti-CD3 antibody) in order to regulate immune responses has raised the question of what antigens Tregs recognize *in vivo*. A preliminary study of Vα and Vβ TCR usage identified that Treg TCRs comprised every TCRα and TCRvβ chain in the genome which suggested the range and quantity of antigen recognised by Treg was at least as broad as for conventional T cells (Takahashi, *et al.* 1998).

Previous attempts to characterise the affinity and identity of antigens recognised by Treg TCRs was hampered by the innate anergic phenotype of Treg. A more recent study employed more sophisticated techniques to compare the TCR usage in regulatory ($CD25^+$) and nonregulatory (CD25-) $CD4^+$ T cells. (Hsieh, *et al.*2004, Hsieh, *et al.*2006). To facilitate such a study the authors exploited a mouse strain expressing a transgenic TCRβ on a $TCR\alpha^{+/-}$ background. Specificity of the TCR of T cells generated in these mice was only attributable to the rearrangement of the functional TCRα chain that associated with the transgenic TCRβ chain. T cells were isolated from the Treg and conventional T cell lineages of these mice and TCRs were cloned. Sequencing the CDR3 region (i.e. the region of the TCR that confers antigen specificity) of the TCRα chain isolated from these T cells revealed that the regulatory and conventional T cell populations were equally diverse but that the actual TCR repertoires between the two populations were overlapping. Cloned TCRs were inserted into conventional T cells that expressed a non-functional TCR. The affinity and antigen recognised by TCR isolated from Treg could therefore be examined without the constraints of Treg anergy conferred by normal thymic Treg production. The authors of this study tested the hypothesis that regulatory T cell TCR recognise self-antigen with higher avidity than those of T cells. The T cells with clones TCRs were adoptively transferred into $Rag^{-/-}$ mice and the extent of homeostatic expansion of transgenic $Rag^{-/-}$ T cells transduced with regulatory cell derived TCR and nonregulatory cell derived TCR was compared. The extent of expansion was believed to correlate with the avidity of the TCR / MHC-self-peptide interaction. Those T cells transduced with regulatory cell TCR underwent much more rapid proliferation compared to T cells transduced with nonregulatory cell TCR and those

transduced with empty vector. The latter population of cells was included to show that the transgenic TCR expressed by the T cells that received cloned TCR (which recognises a peptide derived from human CLIP) did not expand appreciably upon transfer. These data support the premise that a higher frequency of self-reactive TCR is found within the regulatory T cell population. In a second study, a comparison of TCR repertoires of regulatory T cells and potentially autoreactive T cells derived from Foxp3-deficient mice revealed similarities between the two populations indicating that in the absence of Foxp3 expression, T cells expressing these TCRs are pathogenic.

A third finding relating to these studies was that the diversity of the TCR repertoire of peripheral Treg and Treg thymocyte precursors was equivalent yet distinct. There was a greater similarity in TCR usage by Treg thymocyte precursors between separate mice than between the peripheral and thymocyte TCR usage within individual mice. The TCR repertoire of peripheral Treg was believed to diverge from that of Treg thymocyte precursors by an enhanced proliferation of peripheral Treg with higher avidity for self antigen and the loss of clones with relatively low affinity for self-antigen following emigration from thymus to the periphery.

A question remains as to whether antigen recognition by Treg is restricted to self antigen or if the cells can also respond to foreign antigen. To address this issue a well characterised mouse model with *Leishmania major* infection was used (Suffia, *et al.*2006). Mice were infected with and the infiltration of Treg to the infection site was analysed. These local Treg appeared to inhibit the T cell response to *L. major* by increasing the parasite survival and expansion locally. *L. Major* specific Treg were also found in draining lymph nodes but did not disseminate. Investigators continued to purify Treg from the dLN and incubated T cells *in vitro* in the presence of *L.major* infected DC. Treg were activated through the recognition of their cognate *L. major* antigen and suppressed conventional T cell proliferation. *L major* specific Treg lines transferred into mice with quiescent disease accumulated at the dLN and reactivated *L. major* infection. Treg were judged not to be activated through bystander activation during inflammation as Treg failed to accumulate in the dLN of T.gondii infection (Suffia, *et al.*2006). These

experiments demonstrate the Treg do recognise foreign antigen and mediate the control of T cells responding to foreign antigen.[§]

1.6. Evidence that Treg suppress immunosurveillance of tumours in mice

Tumour development may be observed following the injection of immortalised cells that have been maintained *in vitro* into mice. These tumour cell lines are usually derived from tumours that have occurred spontaneously or following the administration of carcinogen to mice and form palpable tumours after injection *in vivo*. To date, the interaction of Treg with anti-tumour responses has largely been studied in most detail using transplantable tumour cell lines. A collection of observations, summarised below, using transplantable cell lines has indicated that Treg may inhibit anti-tumour responses.

1.6.1. Early studies

Developing tumours frequently contain an inflammatory infiltrate and it has long been hypothesised that the immune system is able to control and eliminate tumours (Burnet.1957). Despite these observations, malignant cells derived from a wide range of histological origins in patients and animal models appear to negate the immune system and establish palpable tumours. Robert North and colleagues performed a series of seminal experiments in the 1980's to attempt to identify why effective anti-tumour immunity often failed to reject tumours (Berendt, *et al.*1980). Data collected from these studies demonstrated for the first time transferable T cell mediated tolerance that suppressed anti-tumour immunity.

Initially, anti-tumour effector cells capable of rejecting tumour cells were generated to serve as targets for suppression. Naïve mice injected with the MethA tumour cell line developed tumours but mice co-injected with tumour cells and bacterial endotoxin adjuvant proceeded to reject inoculated tumour cells. Injection of endotoxin adjuvant appeared to either inhibit the expansion of Treg or activated and rendered T cells refractory to suppression, thereby creating a window to enable the rejection of

[§] Various studies have also identified Treg specific for tumour antigens and are discussed in section 1.6.4.

tumour cells. Splenocytes were obtained from mice that rejected tumour and transferred to a second group of naïve mice that were subsequently injected with tumour cells, without co-injection of endotoxin. Tumour primed splenocytes eliminated tumour cells as long as the recipient mice either received prior thymectomy and irradiation (Berendt, *et al.*1980) or cyclophosphamide (North.1982) to destroy the resident T cell population. These experiments demonstrated a population of T cells within naïve mice that were able to suppress the anti-tumour function of otherwise effective anti-tumour splenocytes. Further experimentation supported these findings. Mice were injected with endotoxin and tumour cells to prime tumour specific effector cells as before. A separate group of mice were injected with tumour cells without endotoxin. A third group of mice were challenged with tumour cells after they were injected with endotoxin primed anti-tumour splenocytes along with splenocytes derived from mice injected with tumour cells alone. The latter population inhibited the anti-tumour function of endotoxin plus tumour primed splenocytes but depletion of T cells from this latter population inhibited the transfer of tolerance.

This study therefore pin-pointed a population of cells that normally inhibit anti-tumour immune responses and demonstrated the T cell phenotype of these suppressor cells (Berendt, *et al.*1980).This transferable suppression from tumour bearing mouse T cells has been replicated using other tumour cell lines (Dye, *et al.*1981) and shown to be restricted to $CD4^+$ ($Ly\text{-}1^+$) T cells (North, *et al.*1984).

The identification of suppressive $CD4^+$ T cell involvement in tumour growth presented by these studies fundamentally progressed the understanding of how tumours are able to escape immune-mediated rejection. Later studies built on this foundation to characterise the nature of regulatory T cells identification of Treg and to better understand how these cells suppress anti-tumour immunity.

1.6.2. The role of $CD4^+CD25^+$ Treg in the rejection of transplantable tumour cell lines in mice

Seminal studies performed by Sakaguchi and co-workers established that Treg could be identified using CD25 (described earlier). Important studies performed by the same group

established the role of $CD25^+$ Treg in tumour progression which built upon the studies by North and co-workers described above. Investigators injected mice with $CD25^+$ cell-depleting antibodies or isotype control antibodies prior to inoculation with tumour cells (Shimizu, *et al.*1999, Onizuka, *et al.*1999). Mice depleted of Treg using $CD25^+$ depleting antibody mounted T cell mediated anti-tumour responses capable of rejecting a panel of tumour cells lines, including the MethA tumour cell line used by North and co-workers. A number of other groups continued to show Treg mediated suppression of anti-tumour immune responses and elimination of tumour cells by the depletion of Treg using CD25-specific antibodies (Sutmuller, *et al.*2001, Golgher, *et al.*2002).

Further investigations have proceeded to examine the distribution of Treg in the tumour environment and to characterise anti-tumour responses suppressed by Treg.

Location of Treg mediated suppression

Several studies have explored the premise that Treg must migrate into the tumour environment and closely associate with those cells that mediate anti-tumour function in order to mediate their suppressive function. Investigations, conducted using several tumour cell lines and strains of mice, have analysed Treg infiltration in the tumour, TDLN** and spleen of tumour bearing mice (Needham, *et al.*2006, Ghiringhelli, *et al.*2005a, Ghiringhelli, *et al.*2005b, Ko, *et al.*2005). The extent of Treg infiltration of the tumour environment varies between models and probably reflecting differences in immunogenicity between tumour cell lines and differences amongst the strains of mice used. Investigators injected C57BL/6 mice with a mesothelioma tumour cell line subcutaneously (this tumour cell line was originally derived from the peritoneal cavity of a mouse injected with asbestos fibers) to observe Treg infiltration of the developing tumour environment. Tumours contained a $CD4^+$ T cell infiltrate that comprised of approximately 60% $FOXP3^+$ Treg cells (Needham, *et al.*2006). This study and others (Ghiringhelli, *et al.*2005a, Ghiringhelli, *et al.*2005b, Ko, *et al.*2005) demonstrated that significant numbers of Treg accumulate within tumours. A particularly informative study investigated the location and intensity of Treg proliferation using flow cytometry BrdU

** TDLN: tumour draining lymph node; NTDLN: non-tumour training lymph node

incorporation (Ghiringhelli, *et al.*2005b). Mice were injected s.c. with the B16 melanoma cell line and Treg accumulation within the developing tumour environment was examined by removing TDLN, NTDLN, tumour and spleen samples. TDLN contained an enhanced proportion of Treg within the $CD4^+$ compartment and Treg residing in tumours and TDLN were found to rapidly proliferate compared to those in the spleen and NTDLN (Ghiringhelli, *et al.*2005b). Investigators proceeded to examine what instructed Treg recruitment into the tumour environment and established that immature DC that resided in TDLN were instructed by tumour cells to secrete TGF-β which in turn initiated the expansion of tumour infiltrating Treg (Ghiringhelli, *et al.*2005b).

1.6.3. Targets and mechanism of Treg mediated suppression

Treg express CD4, CD25, CTLA-4, GITR and FOXP3 (described previously). A number of investigations have expanded on those studies that used CD25 -specific antibodies to deplete Treg by using anti-GITR and -CTLA-4 non-depleting antibodies to antagonise Treg mediated suppression of tumour immunity. Experiments performed using these antibodies have identified cell types that coordinate the anti-tumour immune response unleashed by the abrogation of Treg function.

T cells

Investigators immunised mice with activated DC pulsed with B16 melanoma tumour cells that has been stressed by heat shock and gamma irradiation and subsequently inoculated the mice with viable B16 tumour cells (Prasad, *et al.*2005). Immunisation retarded tumour growth and was dependent on lymphocyte mediated anti-tumour function. Tumour cells grew vigorously when mice were depleted of either $CD4^+$ or $CD8^+$ T cells. The efficiency of immunisation was significantly enhanced by depletion of Treg and elicited complete rejection of tumour cells and generated anti-tumour memory capable of rejecting a second inoculation of B16 tumour cells 90 days later (Prasad, *et al.*2005). Additional studies depleted mice of Tregs using PC61 prior to inoculation with

the CT26 tumour cell line originally derived from a colon carcinoma (Golgher, *et al.*2002). In the absence of Tregs, mice rejected CT26 tumour cells and could then reject a second inoculation six months later without first depleting Tregs, suggesting a robust anti-tumour immune response had been generated (Golgher, *et al.*2002).

In further tumour experiments, mice were administered a blocking anti-GITR antibody that is known to inhibit Treg function (Turk, *et al.*2004, Ko, *et al.*2005). Mice were injected with anti-GITR antibody and either melanoma (Turk, *et al.*2004) or fibrosarcoma tumour cells (Ko, *et al.*2005). Mice injected with melanoma cells and anti-GITR antibody developed concomitant immunity[††] to melanocyte antigens, whereby a $CD8^+$ T cell mediated immune response was elicited by an initial inoculation of tumour cells that could reject a second challenge of tumour cells (Turk, *et al.*2004).
These experiments were extended to elicit complete rejection of a primary tumour challenge by manipulating Treg function with non-depleting anti-GITR and –CTLA-4 depleting antibodies. Investigators injected mice with tumour cells and anti-GITR antibody to elicit complete rejection of fibrosarcoma cells (Ko, *et al.*2005). Injection of anti-GITR antibody enhanced IFN-γ dependent infiltration of tumours by $CD4^+$ and $CD8^+$ T cells. Co-injection of anti-CTLA-4 and anti-GITR antibodies further enhanced anti-tumour immunity (Ko, *et al.*2005). It is not clear how the antibody binding of Treg receptors alters their function. One interpretation of these findings is that binding of Treg surface receptors by anti-GITR and –CTLA-4 antibody inhibited Treg function to reveal and potentiate anti-tumour immunity; and in the case of anti-CTLA-4 and –GITR antibodies, this may be an additive effect.
Other studies examined the ability of Treg derived TGF-β to inhibit $CD8^+$ T cell mediated tumour rejection (Chen, *et al.*2005a). Mice were injected with CT26 tumour cells that were modified to express haemaglutinin antigen, an immunogenic protein of the influenza virus. The transfer of HA specific CD8 cells from transgenic mice elicited complete rejection of tumours. Transfer of naïve HA specific Tregs abrogated tumour

[††] Concomitant immunity describes the development of an anti-tumour immune response capable of rejecting a syngenic tumour at a distant anatomical location but is unable to reject the primary tumour. This is usually because the primary tumour has grown to beyond the size that the immune response is capable of destroying during the delay between initial inoculation and the development of an effective anti-tumour immune response.

rejection, however anti-tumour function was not interrupted when HA specific $CD8^+$ cells that lacked a functional TGF-β receptor were transferred. Further investigation demonstrated that Tregs specifically inhibited $CD8^+$ cell cytotoxicity, whilst $CD8^+$ cells continued to infiltrate, proliferate and secrete IFN-γ at the tumour site (Chen, *et al.*2005a). $CD8^+$ T cells were not able to reject tumour in the absence of functional cytoxicity.

The extent to which Treg suppressed $CD4^+$ cell mediated anti-tumour responses capable of rejecting tumour has also been examined (Casares, *et al.*2003). Mice were depleted of Treg using an anti-CD25 specific antibody and co-depleted of $CD8^+$ T cells and subsequently injected with CT26 tumour cells. Tumour specific $CD4^+$ cells conferred tumour protection in the absence of $CD8^+$ T cells and could be transferred to mediate tumour rejection in $Rag^{-/-}$ mice. $CD4^+$ mediated protection was abrogated by the injection of anti-IFN-γ antibody. Mice were then injected with protein matrix loaded with tumour cells to establish how IFN-γ initiated tumour rejection and observed a significant inhibition of tumour stimulated angiogenesis. These experiments therefore established an important role of $CD4^+$ cells to inhibit tumour stimulated angiogenesis which is inhibited by Treg (Casares, *et al.*2003).

NK cells

A number of studies have demonstrated that Treg directly suppress NK cell anti-tumour function, primarily by inhibiting NK cell activation following recognition of NKG2D ligands expressed by tumour cells. Investigations using WT and TGF-$\beta^{-/-}$ mice demonstrated the importance of Treg TGF-β production in inhibiting NK tumour rejection (Ghiringhelli, *et al.*2005a). Nude mice were co-injected with NK cells and the YAC-1 tumour cell line, which presents a robust target of NK mediated tumour cell lysis. Mice were also injected with Treg from either WT or TGF-$\beta^{-/-}$ mice. Treg derived from WT mice proceeded to inhibit NK mediated tumour lysis but NK function was not impaired by TGF-$\beta^{-/-}$ Treg (Ghiringhelli, *et al.*2005a). It is possible Tregs may inhibit NK function by reducing the expression of NKG2D. Mice were injected with the metastatic melanoma cell line transfected with the NKG2D ligand Rae-1. NK cells reduced the

number of metastasis following the injection Rae-1 expressing tumour cells compared to unmodified tumour cells. However, NKG2D mediated reduction of metastasis was inhibited in mice co-injected with WT Treg (Ghiringhelli, *et al.*2005a). This suggests that Tregs impinge on NK cell function rather than altering tumour Rae-1 expression. Observations performed by others have supported these findings (Smyth, *et al.*2006c, Simon, *et al.*2007). Investigators studied NK cell anti-tumour function by comparing the rejection of closely related tumour cell lines distinguished by differential expression of the NKG2D ligand Rae-1. Initial experiments developed a metastatic tumour model and demonstrated a reduction of metastasis following Treg depletion, that was lost if NK cells were co-depleted (Smyth, *et al.*2006c). Further analysis showed an enhanced rejection of melanoma cells following depletion of Treg that was conditional of Rae-1 expression by tumour cells. Similar experiments conducted in $Rag^{-/-}$ mice demonstrated NK activation was independent of T cell activation and co-injection of Treg abrogated NK anti-tumour function. The mechanism of Treg suppression of NK function was deconstructed *in vitro* to show Treg must be activated to suppress NK mediated killing and once activated, Treg suppress through membrane bound TGF-β (Smyth, *et al.*2006c).

1.6.4. Tumour antigen recognised by Treg

Studies performed in mice and with patient T cells suggest that Tregs may recognise tumour antigens and suppress anti-tumour responses. Tumour antigens recognised by these Treg were often self-antigen expressed by tumour and self-tissue. One group (Nishikawa 2001) described the activation of Treg by vaccinating mice with a group of SEREX defined autoantigens. These autoantigens were detected using IgG recognising tumour cDNA expression libraries, obtained from the serum of mice transplanted with cognate tumour cell lines. Antigens that elicited robust and consistent recognition were used within experiments and none were mutated proteins and therefore represented authentic autoantigen. Mice vaccinated with these antigens developed a population of Treg with potent suppressive activity and FOXP3 expression elevated 5-10 fold higher than naïve $CD4^{+}CD25^{+}$ Treg (Nishikawa 2005 JEM). Treg expanded after vaccination with these autoantigens inhibited of anti-tumour immune responses to transplanted

tumour cell lines (Nishikawa 2003) and carcinogen induced tumours (Nishikawa 2005 PNAS). Evidence of Treg specific to tumour antigens has also been described in human samples. TIL have been isolated from human tumours which possess the $GITR^+$, CTLA-4^+, $FOXP3^+$ suppressive Treg phenotype and recognised the shared tumour antigens LAGE (Wang et al 2004) and ARTC-1 (Wang 2005a).

1.6.5. Thymectomy of three day old neonatal mice enhances MCA induced tumour induction

The above studies used transformed tumour cell lines to demonstrate that Tregs may inhibit anti-tumour immune responses. However, earlier studies suggested that Tregs may inhibit immune recognition of tumours elicited *de novo* by injection of carcinogens. An investigation of MC mediated tumour development indicated that ablation of Treg development by three day thymectomy reduced tumourigenesis induced by a high dosage of MC (Prehn, *et al.*1986). These experiments were performed before the advent of cell markers to clearly identify Treg, however, seminal experiments had previously identified that three day thymectomy interrupted normal regulatory T cell development (Kojima, *et al.*1976). In the light of current knowledge, we can hypothesize that the absence of regulatory T cells in mice thymectomised three days after birth enhanced the recognition and elimination of mutated tumour cells.

1.7. Colorectal carcinoma in humans

Extensive studies of malignancy in mice have improved the understanding of how the immune system may recognise and attack malignant disease. This research has indicated the cell types and signalling molecules that mediate anti-tumour immunity and also identified the fundamental role for Treg in inhibiting effective anti-tumour immunity. Studies in mice have laid a framework for identifying the shortcomings of tumour immunosurveillance in patients and how new therapies may target mechanisms that impede effective anti-tumour immunity. The field of tumour immunology is in the process of exploring observations described in murine tumour studies in patients with

malignant disease. An important part of this process involves the analysis of the extent to which Treg inhibit anti-tumour immunity in a commonly diagnosed type of malignant disease in patients.

CRC is the third most common malignancy in the UK and second leading cause of cancer deaths, accounting for around 16 000 deaths a year. One in twenty UK residents is estimated to develop CRC in their lifetime. The incidence of disease is equal in males and females but increases with age, whereby approximately 8/10 people diagnosed with CRC are aged over 60. The disease might be identified by acute abdominal pain, bleeding and abnormal bowel habit. Diagnosis is usually made following a positive faecal occult blood test and subsequent colonoscopy. A sample of abnormal bowel tissue may be collected during colonoscopy for histology to identify malignant cells. Following diagnosis, the standard treatment of CRC involves elective surgery (to remove malignant segments of the bowel and surrounding lymph nodes) and adjuvant chemotherapy. Histological analysis of resected bowel is used to stage the extent of disease using the Duke's classification (A-D) and provides an accurate assessment of prognosis. Duke's stage A classification corresponds to the earliest stage of disease, with the best prognosis and comprises disease that involves the inner lumen wall of the bowel without penetration to the outside wall. Disease that has penetrated to the outside wall of the bowel but does not involve regional lymph nodes is referred to as Duke's stage B; Dukes stage C refers to disease that has also spread to lymph nodes; and Duke's stage D describes disease that has metastasised to distant organs.

Half of patients that undergo potentially curative treatment either relapse or develop metastatic disease within five years. A potential way to improve treatment of CRC might be to enhance immune recognition and clearance of malignant cells after debulking of tumour tissue by surgery. There is evidence to suggest the immune system does normally recognise colorectal tumours and several studies have indicated that colorectal tumours are infiltrated with T cells and that the extent of T cell infiltration correlates with survival (Dunn, *et al.*2002, Nacopoulou, *et al.*1981, Naito, *et al.*1998, Galon, *et al.*2006). Despite this, CRC is deemed to be poorly immunogenic compared to other malignancies such as melanoma (reviewed in (Dalerba, *et al.*2003)) and CRC patients have been reported to

exhibit a degree of immunosuppression (Ordemann *et al* 2006). These finding provide a rational to study immune recognition of CRC and investigate why normal immune recognition of tumour tissue is not sufficient to clear malignant cells remaining after surgery. One possibility is that potentially curative immune recognition of malignant cells is suppressed by mechanisms in place to suppress auto-reactive immune responses. Tregs have been shown to suppress self-reactive responses and studies previously described in mice using transplanted tumour cell lines have shown Tregs suppress anti-tumour immune responses.

1.8. Summary

The aforementioned studies have presented significant evidence to help substantiate the existence of immunosurveillance of tumours that develop in mice and humans. In fact, in the presence of a functional immune system, many tumours may be eliminated in the early stages of development. However, tumours that do progress to generate lethal pathology appear to do so, in part, by evading elimination by the immune system. The ability of tumour cells to lose the expression of immunogenic antigens may be important to escape rejection, however, Treg may also be make an important contribution to inhibit the function of anti-tumour immune responses and therefore, to tumour progression. Collectively, the studies performed in rodent models using transplanted tumour cell lines support a role for Treg in inhibiting immune responses to tumours. However, it is important to establish the extent that Tregs inhibit anti-tumour immune responses that develop over an extended period of time during *de novo* tumour induction, that more closely reflects the long time scale that tumours are believed to develop in patients. Although less research has been performed on the role of Tregs during *de novo* tumour development, existing studies in rodent models does provide a sound rationale for investigating a role for these cells in human malignancy. Many recent studies (described in detail later in this thesis) have, as such, addressed the hypothesis that Treg suppress anti-tumour immune responses and therefore contribute to the progression of cancer in humans. In this thesis, a study of the impact of Treg on immune responses in patients

with colorectal cancer is described and discussed in the context of other similar studies investigating the effects of Treg on a variety of human tumours.
The experiments described in this thesis were performed with the following hypotheses:

1) Tregs enhance *de novo* tumour formation in mice

2) Patients with CRC have an elevated frequency of Tregs in PBMC

3) Patients with CRC exhibit a degree of immunosuppression

4) Tregs inhibit tumour antigen specific immune responses

1.9. Objectives

Experiments described in this thesis were performed to fulfil the following objectives:

1) Induce *de novo* tumour development in mice and examine the extent that Tregs infiltrate these tumours and whether Tregs enhance the frequency of *de novo* tumour development.

2) Establish methods to measure the frequency of Tregs in PBMC and examine if patients with CRC have an enhanced frequency of Tregs compared to healthy controls.

3) Establish functional T cell assays to measure antigen specific immune responses and firstly examine if CRC patients exhibit signs of immunosuppression; and secondly examine if Tregs suppress tumour antigen specific immune responses.

2.0. Materials and methods

2.1. Patient recruitment

Patients that presented with the first case of an adenocarcinoma and had no distant metastases on cross sectional imaging (TNM stage I-III; Duke's stage A – C) were identified from multi-disciplinary team meetings.

Ex-vivo IFN-γ release analysis was performed on colorectal cancer patients (n=39, age range 45-95, mean age 68). Flow cytometry was performed on a number of these patients (n=12, age range 58-80, mean age 71) whilst six-day proliferation assays were performed on a separate subset of these patients (n=13, age range 55-88, mean age 74). A cohort of age matched controls was also recruited for analysis of PBMC by flow cytometry (n=17, age range 48-93, mean age 64). *Ex-vivo* IFN-γ release analysis was performed on a proportion of controls (n=11, age range 48-63, mean age 57) and six-day proliferation assays were performed with these controls too (n=9, age range 48-64, mean age 56) Local ethical committee approval was granted for the study by the Bro Taf Local Research Ethics Committee.

2.2. Cell preparation

2.2.1 Purification of human lymphocytes

50ml of blood was collected from patients with colorectal cancer one day before surgery and heparinsed (10U/ml). Blood was layered onto lymphoprep (Axis-Shield, Oslo Norway) and centrifuged at 2000rpm for 20 minutes with brake off. PBMC were then washed in RPMI by centrifugation at 2000rpm for 10 minutes, 1600rpm for 10 minutes and 1400rpm for 5 minutes with the brake on.

2.2.2 Preparation of murine tissue samples

Single cell suspensions of spleens, LNs and methylcholanthrene induced tumours were prepared by filtering mashed tissues through 70μm nylon strainers (BD Biosciences, Franklin Lakes, NJ, USA). Protein aggregates formed within freshly prepared TIL after centrifugation and were therefore re-filtered through a second mesh. Collagenase

treatment was not used to retrieve TIL. Blood was collected in heparinised tubes. Blood and spleen cells were incubated with RBC lysis solution (BD PharMingen, San Diego, CA, USA) for five 2 minutes on ice and washed in FACS buffer (PBS + 2% FCS + 2mM EDTA).

2.3. Flow Cytometry

2.3.1. Antibodies

Anti-human-CD4 FITC, -CD25 APC, -CD8 PE, -CD19 FITC, -CD45RO PE, -CD45RA PE and -CTLA-4 specific antibodies were purchased from BD PharMingen (San Diego, CA, USA). Anti-human-CD25 PE was purchased from Miltenyi Biotech (Auburn, CA, USA). Anti-human-CD3 APC was purchased from Serotech (Raleigh, NC, USA). Mouse IgG1 FITC, IgG1 PE, IgG1 APC, IgG2aκPE, IgG2bκ PE and rat IgG2a PE isotype control antibodies were purchased from BD PharMingen (San Diego, CA, USA). Anti-mouse-CD4 Cy5, -CD4 FITC, -CD11b PE, –NK1.1 FITC was purchased from Caltag Laboratories (Burlingame, CA, USA); anti-mouse-CD25 biotin (74D), -CD16/CD32 (FcγIII/II Receptor), -CD4 purified, -CD8 purified, -CD45R/B220 purified, SA-PerCP-Cy5.5, -CD4 Alexa 410, -CD45R/B220 PerCP-Cy5.5, -TCRαβ APC, -CD8 PerCP-Cy5.5, -NK1.1 PE, -CD107a FITC, -CD69 FITC, -Gr1 PerCP-Cy5.5, -CD8 PE Alexa 647 and –Goat-anti-rat FITC was purchased from BD PharMingen (San Diego, CA, USA); anti-mouse TCRvβ 4, 8.1/2, 8.3, 11 and 12- FITC specific antibodies were donated by Dr Alison Banham (Imperial College, London, UK); normal rat serum, anti-mouse FOXP3 PE antibody and staining kit and anti-human FOXP3 FITC and staining kit was purchased from Ebioscience (San Diego, CA, USA); rabbit anti-mouse FOXP3 polyclonal Ab was donated by Dr Janine Coombs (Oxford University, Oxford, UK); swine anti-rabbit biotin was purchased from Dakocytomation (Glostrup, Denmark); Alexa 594 conjugated streptavidin and Alexa488 conjugated goat anti-rat IgG where purchased from Invitrogen (Calsbad, CA, USA).

2.3.2. Cell Surface staining

Cells were resuspended in FACS buffer (PBS + 2% FCS + 2mM EDTA) and incubated with 0.5μg/ml antibodies for 30 minutes at 4°C and then washed twice in FACS buffer. Stains requiring secondary staining with SA-PerCp-Cy5.5 were incubated for 15 minutes at 4°C and washed twice in FACS buffer.

2.3.3. Intracellular staining

Anti-human-CTLA-4 staining was performed using a kit purchased from BD PharMingen (San Diego, CA, USA) in accordance with the manufacturer's instructions. Anti-mouse-FOXP3 and anti-human FOXP3 staining was performed using separate kits purchased from ebioscience (San Diego, CA, USA) according to the manufacturer's instructions). In both cases, cells were stained with 0.5μg/ml antibodies for 30 minutes at 4°C. Cells were then resuspended in FACS fix (PBS + 2% FCS + 2mM EDTA + 2% formalin).

2.3.4. Flow cytometric analysis

Samples were analysed by flow cytometry (FACS-CALIBER ®; Beckton Dickinson, CA, USA). Human data was analysed using the Summit v3.1 analysis software (build 839 Dakocytomation, Fort Collins, CO, USA) and murine data was analyzed using the Winmdi software package.

2.4. Murine $CD4^+CD25^+$ suppression assay

$CD4^+CD25^+$ cells were derived from single cell suspensions of splenocytes using the murine regulatory T cell purification kit (Miltenyi Ltd, Auburn, CA, USA). $CD4^+$ cells were purified by negative selection to derive a $CD4^-$ population that was typically 98% pure. $CD25^+$ cells were positively selected from $CD4^+$ cells to derive $CD4^+CD25^-$ and $CD4^+CD25^+$ populations that were typically 80% and 90% pure respectively. $2x10^4$ $CD4^+CD25^-$ cells were stimulated with 1μm anti-CD3 antibody (Leinco, St Louis, Missouri, USA) and $1x10^5$ mitomycin (Sigma-Aldrich, Gillingham, Dorset, UK) treated $CD4^-$ splenocytes and incubated with titrated numbers of $CD4^+CD25^+$ T cells. Cells were incubated in RPMI 1640 supplemented with 100 U/ml penicillin, 10% heat-treated FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (Invitrogen,

Carlsbad, CA, USA). Cells were incubated for 3 days and pulsed with [methyl-3H]-thymidine (GE Healthcare, Buckinghamshire, UK) for a further 18hrs.

2.5. Depletion of human $CD25^+$ cells

Two methods of depleting $CD4^+CD25^+$ Regulatory T cells from PBMC using anti-human CD25 microbeads (Miltenyi Ldt, Auburn, CA, USA) were performed in parallel. 10^7 PBMC were incubated with 10µl anti-human-CD25 microbeads (Miltenyi, Ldt, Auburn, CA, USA) and 90ul MACS buffer and incubated for 15 min at 4^0C. Cells were then washed once in MACS buffer and centrifuged at 1400rpm before being resuspended in 500µl MACS buffer and passed down a positive selection column (MS column Miltenyi Ltd, Auburn, CA, USA). Alternatively, 10^7 PBMC were incubated with 20µl anti-CD25 microbeads in 80µl buffer, washed and passed down a depleting column (LD column, Miltenyi Ltd, Auburn, CA, USA). For all patient samples, $CD4^+CD25^+$ regulatory T cells were depleted from PBMC using 10µl anti-CD25 microbeads and passing cells down a MS column.

2.6. Human T cell assays

2.6.1. Antigens

Haemaglutinin protein (HA) was obtained from Dr John Skehel (NIMR, Mill Hill, UK), purified protein derivative (PPD) (Statens Serum Institute, Denmark), 5T4 (Oxford Biomedica, Oxford, UK), CEA (Calbiochem, Darmstadt, Germany), Phytohaemagglutinin (PHA) (Sigma-Aldrich, Gillingham, Dorset, UK).

2.6.2. IFN-γ ELISpot assays

50 µl of anti-IFN-γ capture antibody (1-D1K, Mabtech, Sweden) (1:1000 dilution) was coated onto polymer-backed 96-well filtration ELISpot plate (MAIP-S-4510) (Millipore, Moslheim, France) and incubated at 37^0C for 2hrs. Plates were washed with 6x PBS to remove excess coating antibody and blocked by coating wells with 10% FCS RPMI for 1hr at 37^0C. The block was discarded and 3.5 $x10^5$ PBMC resuspended in 100µl of 5% FCS RPMI were added to each well. Cells were stimulated with 1µg/ml PPD, HA, CEA

and 5T4 and 5μg/ml PHA for 18hrs at 37^0C. A positive PHA response was necessary to ensured that PBMC were viable and PBMC that did not respond to were excluded. Undepleted and $CD25^+$ cell depleted PBMC were assayed on the same plate and stimulated with antigen in duplicate wells. Wells containing cells incubated with media alone were included to establish background IFN-γ release. After 18hr incubation with antigen, cells were discarded and plates were washed with 4x PBS. Plates were then incubated with dH_2O for 10min to lyse any remaining cells and then washed with a further 4x PBS.

Wells were then incubated with 50μl of 1μg/ml secondary biotinylated anti-IFN-γ antibody (7-B6-1, Mabtech, Nacka Strand, Sweden), which recognizes a separate epitope to the 1-D1K coating antibody and incubated for 2hrs at RT. Excess secondary antibody was discarded and wells were washed with 6x PBS. Wells were then incubated with 50μl of streptavidin-alkaline phosphatase (Mabtech, Nacka Strand, Sweden) conjugate at 1:1000 dilution, as recommended by the manufacture, for 2hs at RT. Wells were then washed with 6x PBS and incubated with 100μl colour development solution for at least 30 min at RT in the dark and until spots were visible. Colour development solution comprised of 4% AP colour development buffer (BIO-RAD, x25 stock) (Bio-Rad, Hercules, CA, USA, 1% substrate A and 1% substrate B (AP conjugate substrate kit, BIO-RAD) in dH2O. The colour development was stopped by washing the plate in tap water. Plates were dried and spots were counted using an ELISpot counter (Autoimmun Diagnostika GMBH (AID), Strasberg, Germany) and analysed with the ELISpot 3.4 software package to ensure the consistent analysis of spots between wells (AID). Examples of images of *ex-vivo* IFN-γ release ELISpot wells of purified PBMC to various antigens are displayed in Figure 2.1.

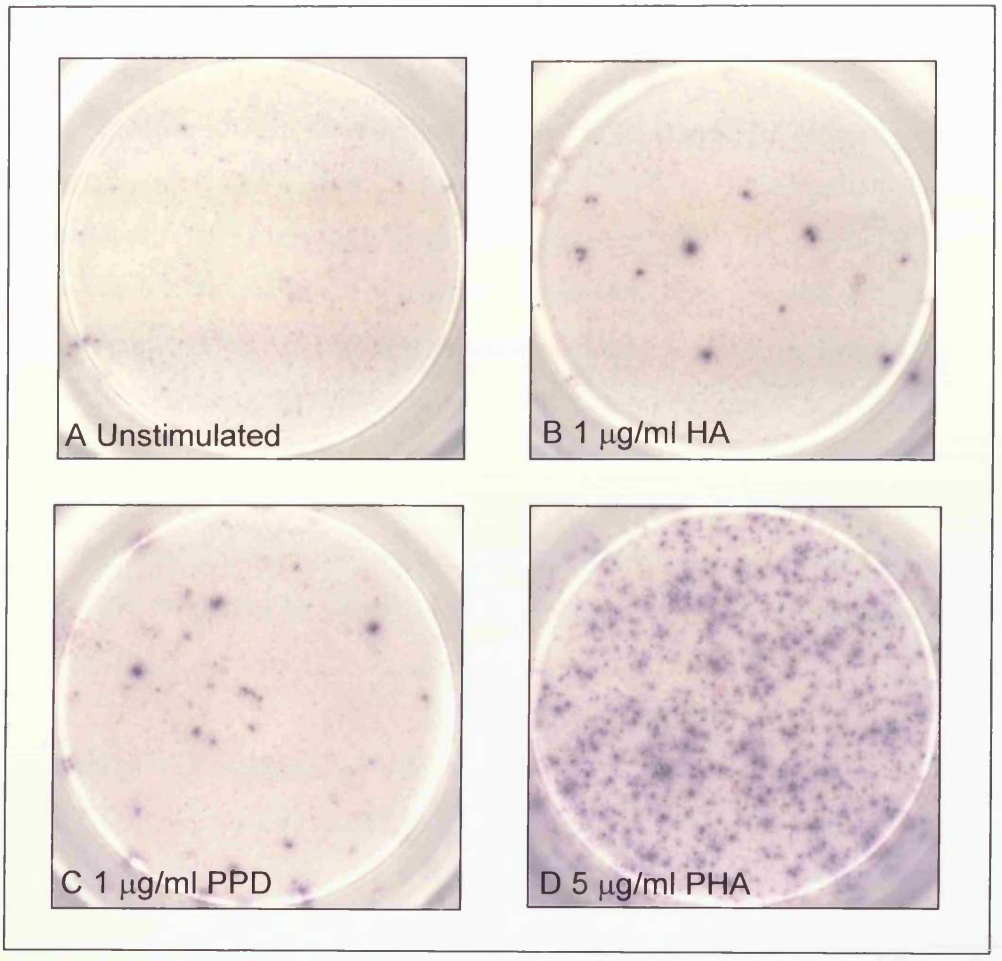


Figure 2.1. *Ex-vivo* IFN-γ release of purified PBMC to antigenic stimulation

$3.5x10^5$ purified PBMC were incubated in 5% FCS RPMI and either unstimulated (A) or stimulated with 1μg/ml HA (B), PPD (C) or 5 μg/ml PHA (D) protein. IFN-γ release was measured 18hr after antigen stimulation. Each antigen was measured in duplicate wells.

2.6.3. Six day thymidine incorporation proliferation assays

$2x10^5$ PBMC were stimulated with 1μg/ml PPD, HA, CEA and 5T4 antigens or with media alone in triplicate wells for six days. Pooled AB serum (Welsh blood service, Llantrisant) was heat inactivated by incubation at 56°C for 30 min and centrifuged at 2000rpm for 10 min. The surface lipid layer was removed. Serum was then filtered using a 0.2μm high-flow filter (Millpore, Moslheim, France) and diluted to 5% or 10% in RPMI 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA). Autologous serum was collected by incubating 10ml blood for 30min at 37°C in non-heparinised tubes. Serum was isolated from the clotted blood fraction by centrifugation at 2000rpm for 10min. Serum was heat inactivated, filtered and made up to 5% and 10% RPMI the same as for pooled AB serum. Cells were incubated for 6 days and pulsed with [methyl-3H]-thymidine (GE Healthcare, Buckinghamshire, UK) for a further 6 or 18hrs.

2.6.4. $CD4^+CD25^+$ cell suppression assay

$CD4^+CD25^+$ cells were derived from PBMC using the human regulatory T cell purification kit (Miltenyi Ltd, Auburn, CA, USA). $CD4^+$ cells were purified from PBMC by negative selection to derive a $CD4^-$ population that was typically 90% pure. $CD25^+$ cells were positively selected from $CD4^+$ cells to derive $CD4^+CD25^-$ and $CD4^+CD25^+$ populations that were typically 98% and 95% pure respectively.

$2x10^4$ $CD4^+CD25^-$ cells were stimulated with 1μm anti-CD3 antibody (OKT3, American Type culture collection (ATCC) Middlesex, UK) and 1 μg/ml soluble anti-CD28 (clone 28.2) (BD Pharmingen, San Diego, CA, USA) antibodies in RPMI 1640 supplemented with 100 U/ml penicillin, 10% heat-treated FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 1 mM sodium pyruvate in the presence of titrated numbers of $CD4^+CD25^+$ cells. Cells were incubated for 3 days and pulsed with [methyl-3H]-thymidine (GE Healthcare, Buckinghamshire, UK) for a further 18hrs.

2.7. Thymide incorporation assays: plate pulsing and harvesting

Proliferation was measured on by adding 0.037 MBq/well (1 μCI) of [methyl-3H]-thymidine ((GE Healthcare, Buckinghamshire, UK)) to each well and incubating the

plates for 6 or 18hrs.Thymidine incorporation was assessed after harvesting cells onto printed A filtermats (Perkin Elmer Wallac, Turku, Finland) using a TomTek harvester (Hamden, CT, USA) and coating dried filtermats with meltilex A melt-on scintillator sheets (Perkin Elmer Wallac, Turku, Finland). Filtermats were read on a Trilux 1450 microbeta liquid scintillation and luminescence counter (Perkin Elmer Wallac, Turku, Finland).

2.8. Immunofluorescent staining of FOXP3 in human $CD25^+$ PBMC

$CD25^+$ cells were removed from purified PBMC as described above. Undepleted, $CD25^+$ depleted and $CD25^+$ selected PBMC were resuspended at $2x10^5$ cells/ml in 10% FCS RPMI and incubated at 37°C on poly-L-lysine coated glass slides (Sigma-Aldrich, Gillingham, Dorset, UK) for 30 minutes. Slides were observed under the microscope to ensure cells had adhered and washed once in 1x PBS. Cells were then permeablised in ice cold methanol for 5 minutes and then washed with 1xPBS. Slides were subsequently blocked with 1% BSA (Sigma-Aldrich, Gillingham, Dorset, UK) PBS for 30 minutes and then incubated with mouse IgG1 anti-human FOXP3 hybridoma supernatant for 1hr at RT (Clone 236/AE7, Oxford University, Oxford, UK)) or mouse IgG1 isotype control. Slides were then washed 3 times with 1% BSA PBS and incubated with donkey-anti-mouse Alexa 594 at and DAPI anti-nuclear stain at for 30 minutes at RT. Slides were then washed 3 times with PBS and mounted using Vector shield fluorescent mounting solution (Vector Labs, Burlingame, CA, USA). FOXP3 staining was examined using a DM LB2 microscope (Leica Microsystems, Ernst-Leitz-Strasse, Germany). Five high power images were taken of undepleted, $CD25^+$ cell depleted and $CD25^+$ cell selected PBMC. Using the openlab software package (Improvision Ltd, Coventry, UK), DAPI stained cells were counted to provide a total cell count in each high power field. The fluorescent intensity of $FOXP3^+$ cells was established and the density slicing program of the open lab software package was used to count all $FOXP3^+$ cells within high powered images of each PBMC fraction.

2.9. Mice

WT C57BL/6 (H-2^b) 6 -12 wk old and 25 wk old female mice were sourced on site for most experiments. IFN-γ KO mice on a C57BL/6 (H-2^b) background were received from Professor N. Topley (Charles River); and when performing experiments with these mice, WT C57BL/6 (H-2^b) were also sourced from Professor N. Topley (Charles River) to eliminate environmental differences between mice strains. Mice were housed in filter top cages and supplied with sterile food and bedding. Experiments were performed in compliance with Home Office regulations.

2.10. Methylcholanthrene injections

400μg methylcholanthrene (Sigma-Aldrich, Gillingham, Dorset, UK) suspended in 100μl olive oil was injected s.c. into the hind leg of mice. Mice were monitored weekly for tumour development.

2.11. Murine Immunohistochemistry

Pieces of normal muscle, spleen and tumour were mounted in OCT (RA Lamb Ltd, Sussex, UK) and frozen in liquid nitrogen. OCT blocks were stored at -80°C. 10μm cryostat-frozen sections were cut from OCT blocks, mounted on positively coated slides (RA Lamb Ltd, Sussex, UK) and dried overnight at room temperature. Slides fixed with cold acetone (Fisher Scientific Ltd, Leicestershire, UK) for 15minutes on ice and allowed to dry. Sections were permeabilized with 0.2% Tween 20(Fisher Scientific Ltd, Leicestershire, UK), 1% BSA (Sigma-Aldrich, Gillingham, Dorset, UK) for 30 minutes prior to a 1 hour incubation with primary antibodies. Sections were subsequently incubated with biotinylated swine anti-rabbit polyclonal Ab followed by Alexa 594 conjugated streptavidin and Alexa488 conjugated goat anti-rat IgG. Sections were mounted using Vector shield fluorescent mounting solution (Vector Labs, Burlingame, CA, USA). Slides were then examined with using a DM LB2 microscope (Leica Microsystems, Ernst-Leitz-Strasse, Germany) and analyzed using the Openlab software package (Improvision Ltd, Coventry, UK).

2.12. $CD25^+$ cell depletion

Mice were injected i.p. with 500μg doses of CD25-depleting PC61 antibody (Lowenthal, *et al.*1985) or isotype control GL113 (Golgher, *et al.*2002) (produced on site) on d-3 and d-1 in 100ul PBS prior to methylcholanthrene administration.

2.13. Auto-antibody detection

Serum was collected from mice and analyzed for circulating anti-dsDNA IgM auto-antibody using the an anti-dsDNA IgM-specific ELISA Kit (Cat. No. 5130) (Alpha Diagnostic San Antonio, TX, USA. Serum from a naïve animal was diluted at 1:25, 1:50, 1:100, 1:250 using the supplied diluent to determine the optimal serum dilution to perform the ELISA. Serum was ultimately diluted 1:100 and assayed according to the manufactures instructions. The plate was read on a DYNEX MRX analyzer (GMI Inc, Ramsey, MN, USA.

3. An Investigation of the Impact of Regulatory T Cells on Tumour Rejection

3.1. Introduction

As discussed in detail in Chapter 1, there is a substantial body of evidence supporting a role for the immune system in recognition and targeting of tumours. Administration of the carcinogen MC induces *de novo* tumour development in mice and this model has provided a means with which the interaction between growing tumours and the immune system can be studied. Observations using this model have highlighted important roles for a number of immune cell types and inflammatory molecules in tumour rejection. Various studies have used transplantable cell lines to suggest that Tregs are capable of suppressing the anti-tumour immune response; however, the role of Tregs during *de novo* tumour development has not been clarified.

The aim of the work described in this chapter was to analyse the impact of Tregs on tumours developing *in vivo*. For this purpose, mice were injected with MC to induce *de novo* tumour development. Subsequently, tumours were excised and analysed for the presence of Tregs both within the tumours and the tumour draining lymph nodes. In addition, the impact of Tregs on tumour development was assessed by comparing tumour incidence in mice transiently depleted of Tregs and control mice.

3.2. Results

3.2.1. Treg infiltration of MC induced tumours

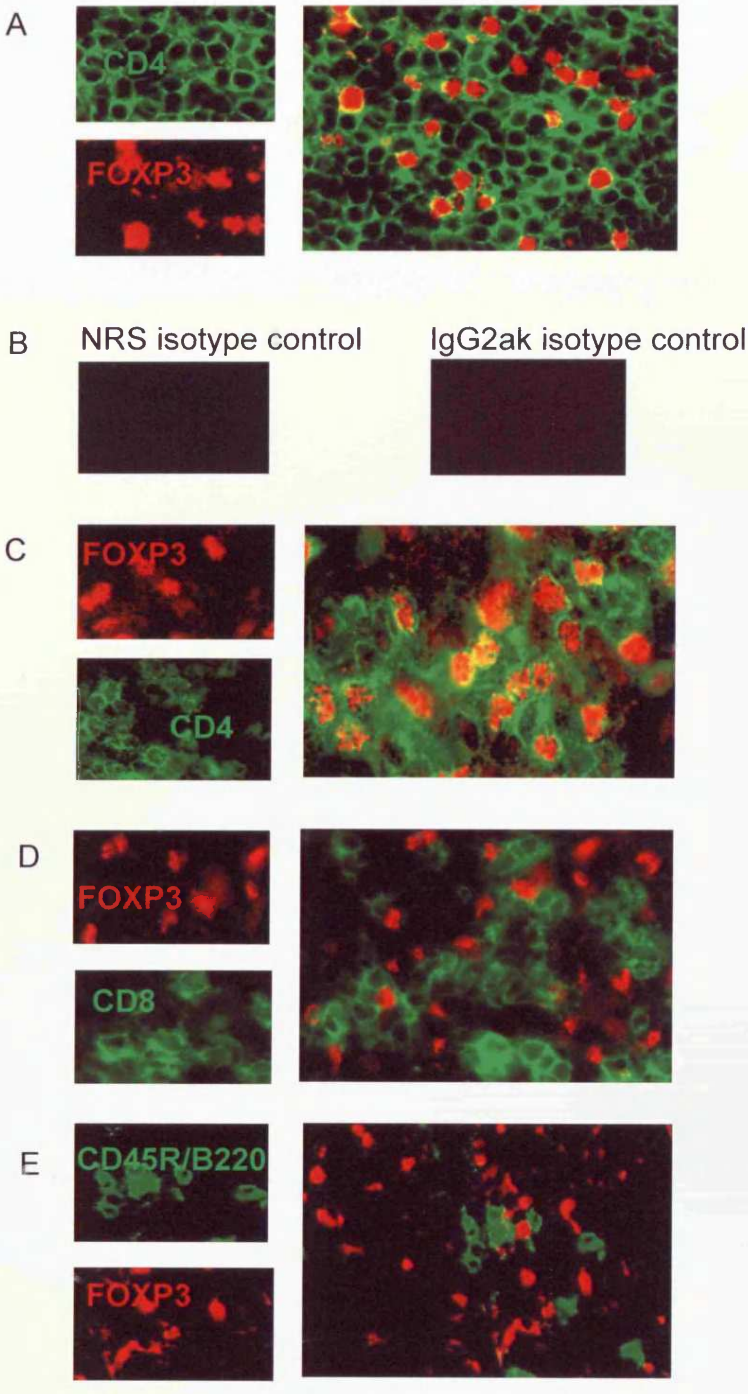
Assessment of Treg infiltration by immunohistochemistry

The first aim of this study was to determine, by immunohistochemistry, whether Tregs were present in MC-induced tumours. Mice were injected with MC subcutaneously and sacrificed when palpable tumours emerged, approximately 100 days after injecton. Immunofluorescent staining of Tregs was initially established on sections of mouse spleen. Spleen tissue was stained with anti-CD4 Alexa 488 and FOXP3 Alexa 594

specific antibodies and intracellular FOXP3 staining was observed in $CD4^+$ splenocytes in the $CD4^+$ T cell follicles (Figure 3.1 A). Non-specific staining of spleen tissue with isotype control antibodies was absent (Figure 3.1. B). A spleen section was included in subsequent FOXP3 staining experiments to serve as a positive control.
MC induced tumours were excised from the hind legs of mice, sectioned and stained with anti-CD4, -CD8, -CD45R/B220 (B cell marker) and –FOXP3 specific antibodies. Tregs infiltrated tumours, making cell to cell contact with conventional $CD4^+FOXP3^-$ effector cells (Figure 3.1 C) and were in close proximity to $CD8^+$ (Figure 3.1 D) and $CD45R/B220^+$ cells (Figure 3.1 E). Non-specific staining of antibodies was excluded using isotype controls (Figure 3.1 F). The preponderance of the inflammatory and regulatory cell infiltrate was present at the interface of the tumour with normal striated muscle tissue. A section of tumour at the interface with striated muscle tissue is displayed (Figure 3.1 G). The matrix of tumours was densely packed with cells, which could be identified with the DAPI nuclear stain. Collectively these data indicate that Tregs do infiltrate MC induced tumours and appear to interact with other immune cells infiltrating the tumour.

Assessment of Treg infiltration by Flow cytometry

In order to gauge the extent of Treg infiltration of MC induced tumours, the proportion of Tregs to $CD4^+FOXP3^-$ conventional T cells was analysed by flow cytometry and compared to Treg numbers in the TDLNs and spleen. The popliteal, lumbar and inguinal lymph nodes all drained the tumour site, collectively termed TDLNs. Lymph nodes collected from the healthy flank, not injected with MC, are referred to as the NTDLNs. Single cell suspensions of tumours were prepared and stained with anti-CD4, -CD25 and FOXP3 specific antibodies. The proportion of Tregs within TILs was compared to that in blood, spleen, TDLNs and NTDLNs. 80-90% of $CD4^+FOXP3^+$ cells isolated from tumours and lymph nodes expressed CD25 (Figure 3.2 A), whilst approximately 60-70% of $CD4^+FOXP3^+$ splenocytes expressed CD25 (data not shown).
On average, 50% of all $CD4^+$ tumour infiltrating cells were $FOXP3^+$ (Figure 3.2 B), significantly more than in blood (5.5%), spleen (19.0%), NTDLN (14.1%) and TDLNs


A
CD4
FOXP3
B
NRS isotype control
IgG2ak isotype control
C
FOXP3
CD4
D
FOXP3
CD8
E
CD45R/B220
FOXP3

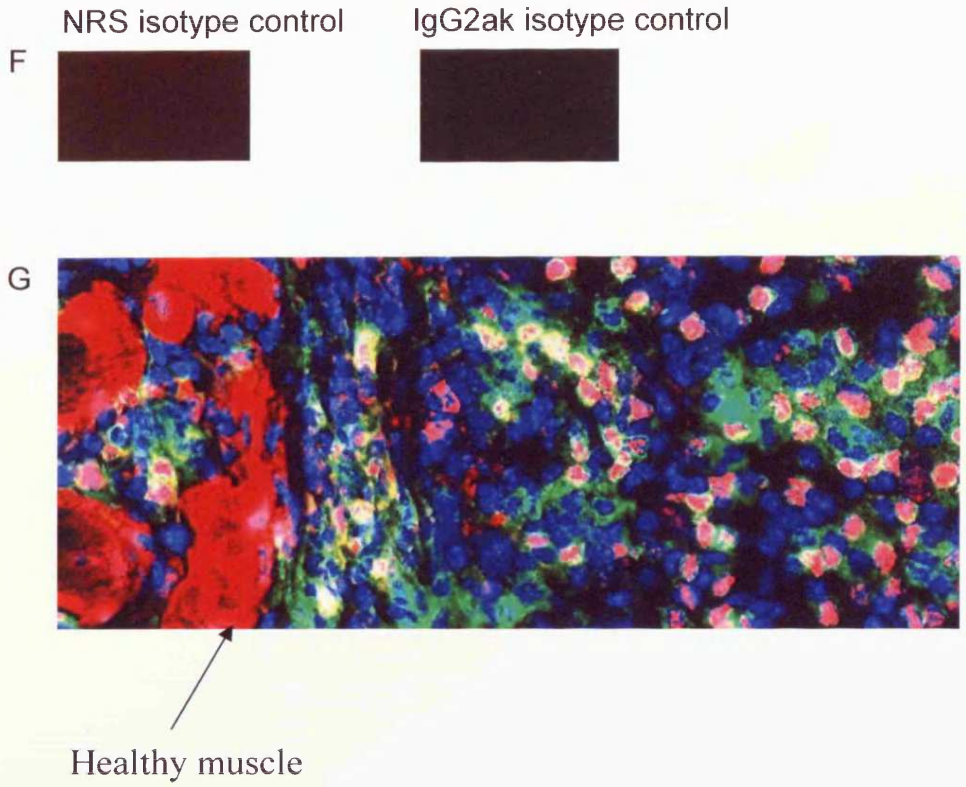
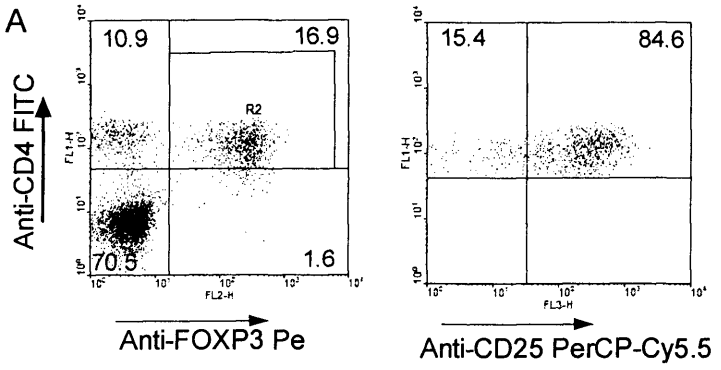
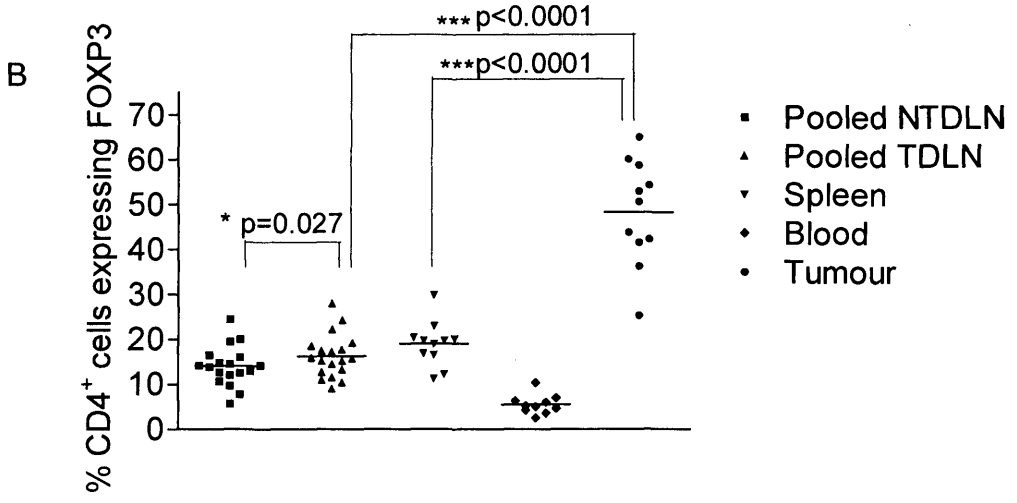
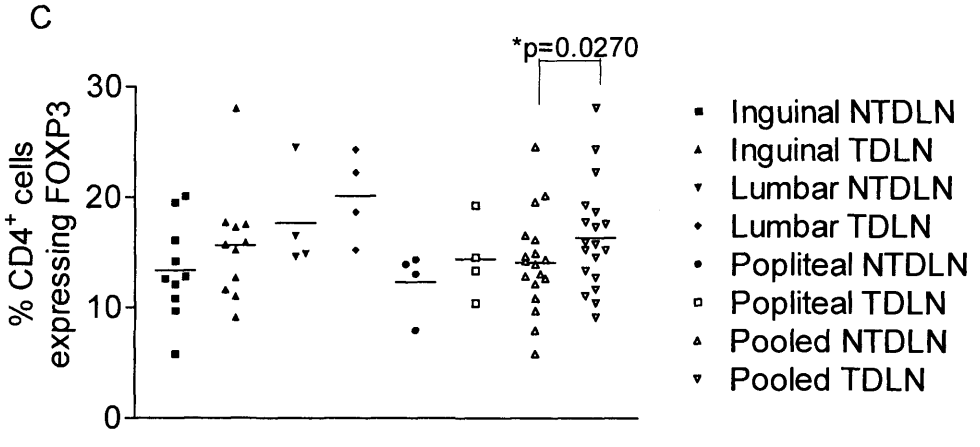


Figure 3.1. Immunofluorescent visualisation of the cellular infiltrate of MC induced tumours.
Treg infiltration of MC induced tumours was assessed by staining with rat anti-CD4 Alexa 488 specific antibody and rabbit anti-FOXP3 Alexa 564 polyclonal antibody. Staining was optimised on sections of spleen (A). Spleen tissue was used from then on as positive control to observe Treg infiltration of tumours. Isotype control antibodies did not bind non-specifically to sections of spleen (B). Tumour sections were stained with anti-FOXP3 polyclonal antibody and either anti-CD4 Alexa 488 (C); anti-CD8 488 (D), anti-B220/CD45R 488 (E). Isotype control antibodies did not bind to sections of tumour (F). Tumour sections were stained with anti-CD4 (green) and -FOXP3 (red) specific antibodies and the distribution of the inflammatory infiltrate in tumours was observed, whereby all nucleated cells were observed with the DAPI nuclear marker (blue) and the tumour interface with healthy tissue was highlighted by the autofluorescent normal muscle (G).


A
10.9
16.9
R2
70.5
1.6
Anti-CD4 FITC
Anti-FOXP3 Pe
15.4
84.6
Anti-CD25 PerCP-Cy5.5


B
*** p<0.0001
***p<0.0001
* p=0.027
% CD4+ cells expressing FOXP3
70
60
50
40
30
20
10
0
Pooled NTDLN
Pooled TDLN
Spleen
Blood
Tumour


C
*p=0.0270
% CD4+ cells expressing FOXP3
30
20
10
0
Inguinal NTDLN
Inguinal TDLN
Lumbar NTDLN
Lumbar TDLN
Popliteal NTDLN
Popliteal TDLN
Pooled NTDLN
Pooled TDLN

D

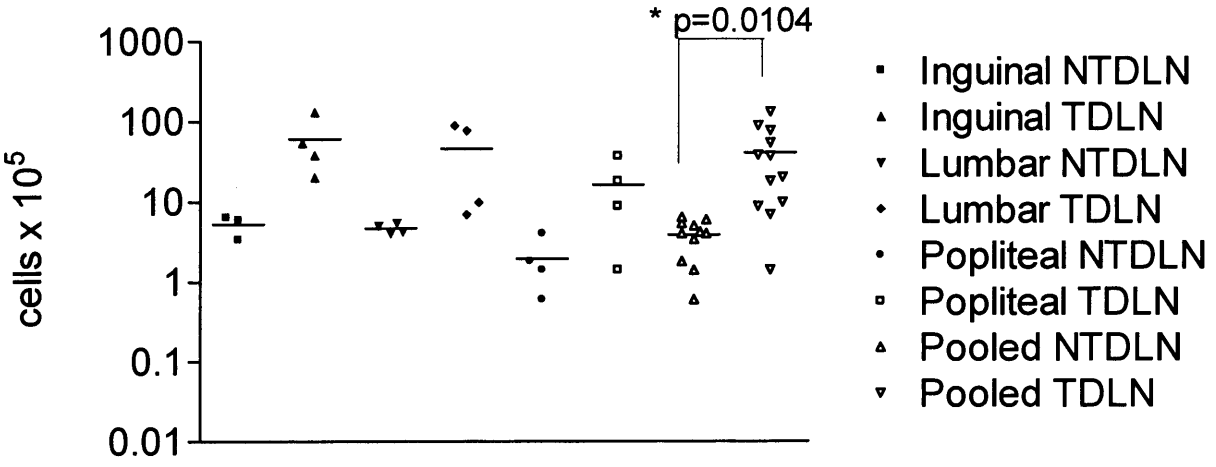


Figure 3.2. Flow cytometric analysis of the cellular infiltrate of MC induced tumours.

Single cell suspensions were stained with rat anti-FOXP3 Pe, -CD4 FITC and CD25 biotin / SA-PeCP-Cy5.5 specific antibodies.

A) Representative FACS plot of tumour tissue $CD4^+FOXP3^+$ Treg infiltration and the CD25 expression on Tregs is displayed.

B) The percentage of $CD4^+$ cells that are $FOXP3^+$ in pooled TDLNs and NTDLNs, spleen, blood, and MC induced tumours. Data was analysed using a paired *t-test.*

C) A breakdown of the percentage of $CD4^+$ cells that are $FOXP3^+$ within individual inguinal, lumbar and popliteal TDLNs obtained from various locations that drained MC induced tumours in the hind leg of mice and NTDLN obtain from the opposite flank of mice. Data was analysed using a paired *t-test.*

D) The number of cells that infiltrate the inguinal, lumbar and popliteal TDLN and NTDLN is displayed. Data was analysed using a paired *t-test.*

(16.3%). The proportion of $CD4^+$ cells that expressed $FOXP3^+$ was marginally elevated in TDLNs compared to NTDLNs (Figure 3.2 B, C), however, the elevation of Tregs within the $CD4^+$ T cell population, was not as pronounced as in tumours. Recruitment of cells to TDLNs was observed, whereby TDLNs contained approximately a log fold increase in total cell infiltrate compared to NTDLNs (Figure 3.2 D).

In MC induced tumour bearing mice, approximately 20% of the $CD4^+$ splenocyte pool were $FOXP3^+$ (Figure 3.2 B), a percentage higher than that previously reported using CD25 expression to identify Treg (Sakaguchi, *et al.* 1995). Palpable tumours appeared in mice over a period of 90 and 240 days after the injection of MC, thus Treg frequencies were being measured in mice over 20 weeks old; and in this time mice became relatively old. To distinguish whether the older age of tumour bearing mice or the presence of the tumour was accountable for the elevation in the proportion of Tregs in the $CD4^+$ splenocyte pool, the proportion of Tregs in tumour bearing mice was compared to those in age-matched tumour free mice. There was no significant difference in the total cell count of spleens derived from each group of mice (Figure 3.3. A); however, a significantly lower percentage of $CD4^+$ splenocytes expressed FOXP3 in young mice compared to tumour bearing and age matched mice (Figure 3.3. B). Tumour bearing and age matched mice did not have elevated total numbers of Tregs compared to young mice (Figure 3.3. C); in fact, tumour bearing and age matched mice had a reduced $CD4^+FOXP3^-$ total cell count, which accounted for the elevation in the proportion of Tregs cells of total $CD4^+$ cells compared to young mice (Figure 3.3. D). Tumour bearing mice had a subtle but insignificant decrease in the total number of $CD4^+FOXP3^-$ cells compared to age matched mice (Figure 3.3. D), possibly due to the migration of $CD4^+FOXP3^-$ cells out of the spleen into emerging tumours.

Overall these data indicate that tumours developing *de novo* have a considerable enrichment of Tregs, forming a large proportion of the total $CD4^+$ lymphocyte tumour infiltrate. The enrichment of Tregs is most apparent within the immediate tumour environment but higher numbers are also observed in the TDLN compared to NTDLNs. These data imply that Tregs may influence immune responses to the tumour and therefore tumour development.

A
Total splenocytes x 10^7
14
12
10
8
6
4
2
0
B
% CD4+ cells expressing FOXP3
30
20
10
0
**p=0.0039
p=0.1115ns
C
CD4+FOXP3+ splenocytes x 10^5
75
50
25
0
D
CD4+FOXP3- cells x10^6
30
20
10
0
Young naive
Old naive
Old tumour bearing

Figure 3.3. Flow cytometric analysis to compare the number of $CD4^+$ cells that are $FOXP3^+$ in old versus young mice.
Single cell suspensions were prepared from spleens of young and old mice and total cell counts were performed (A). Splenocytes were stained with anti-CD4 FITC and anti-FOXP3 antibodies. The percentage of $CD4^+$ splenocytes that express FOXP3 is shown (B), alongside the total number of $CD4^+FOXP3^+$ (C) and $CD4^+FOXP3^-$ splenocytes (D). Data was analysed using an *unpaired t-test.*

3.2.2. Anti-CD25 specific antibody PC61 depletes Treg

The overall aim of the following experiments was to determine whether Tregs influence the development of MC-induced tumours. To address this, MC mediated tumour induction was compared between mice depleted of Tregs and mice with a normal Treg repertoire. Tregs were depleted *in vivo* by injecting the CD25 specific antibody, PC61. The efficiency of Treg depletion by the CD25-specific antibody, PC61 was assessed before tumour induction experiments were performed. The investigation was divided into several objectives.

i. To analyse how effectively PC61 depleted $CD4^+CD25^+$ cells.
ii. Analyse how efficiently PC61 depleted $FOXP3^+$ T cells, as not all $FOXP3^+$ T cells are $CD25^+$ (approximately 30% , Figure 3.4)
iii. Analyse the kinetics of Treg return following the injection of PC61
iv. Analyse whether Tregs that either remained or returned following PC61 injection were functionally suppressive.

Objective 1: Analysis of the ability of PC61 to deplete $CD4^+CD25^+$ cells.

Mice were injected with two 500μg doses, administered one day apart, of either PC61 or the isotype control antibody, GL113. Three days after the final injection, mice were sacrificed and splenocytes stained with anti-CD4, -CD25 and –FOXP3 antibodies. As shown in a representative FACS plot, PC61 effectively depleted a substantial proportion of $CD4^+CD25^+$ cells (Figure 3.5 A). PC61 depleted over half of the total number of $CD4^+CD25^+$ splenocytes (Figure 3.5 B) and $CD4^+$ cells that express CD25 (Figure 3.5. C). As expected numbers of $CD4^+CD25^-$ cells remained similar in both groups of mice (Figure 3.5. D).

Objective 2: Investigating the ability of PC61 to deplete $CD4^+FOXP3^+$ cells.

As described in Objective 1 above, splenocytes from mice injected with either PC61 or the control antibody, GL113 were stained with CD4-, CD25- and FOXP3-specific

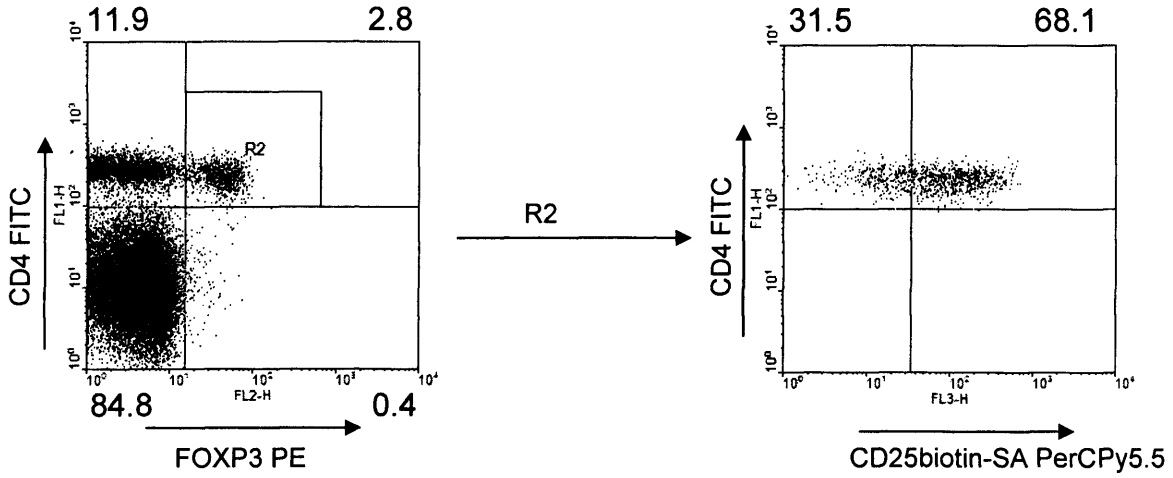
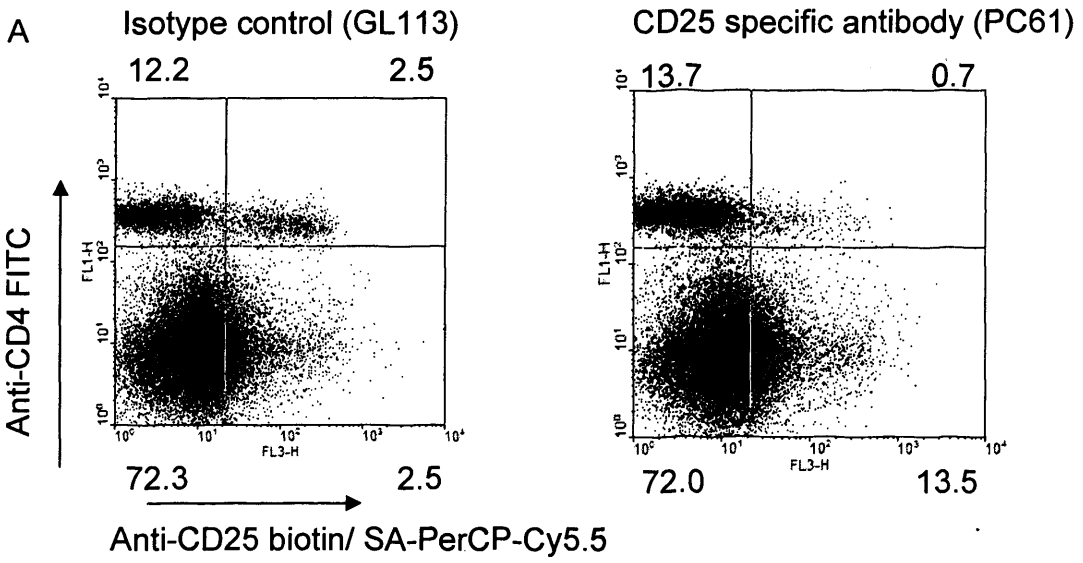


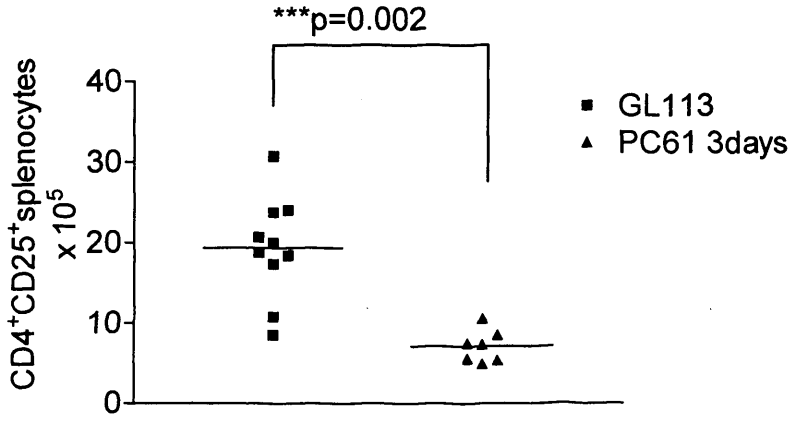
Figure 3.4. CD4$^+$FOXP3$^+$ splenocytes and their expression of CD25 in a naïve mouse.
Splenocytes were obtained from a naïve mouse and stained with anti-CD4 FITC, -FOXP3 PE and -CD25biotin-SA PerCP-Cy5.5 specific antibodies and analysed by flow cytometry. CD4$^+$FOXP3$^+$ splenocytes were gated and the percentage of cells that expressed CD25 was evaluated. Data is representative of multiple experiments.

Three days antibody injection

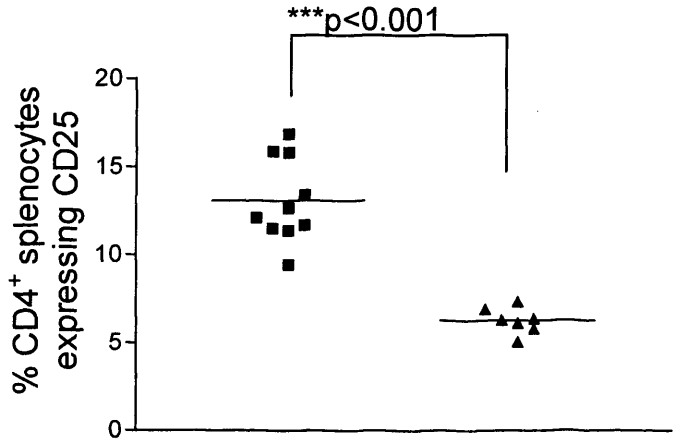
A



B



C



D

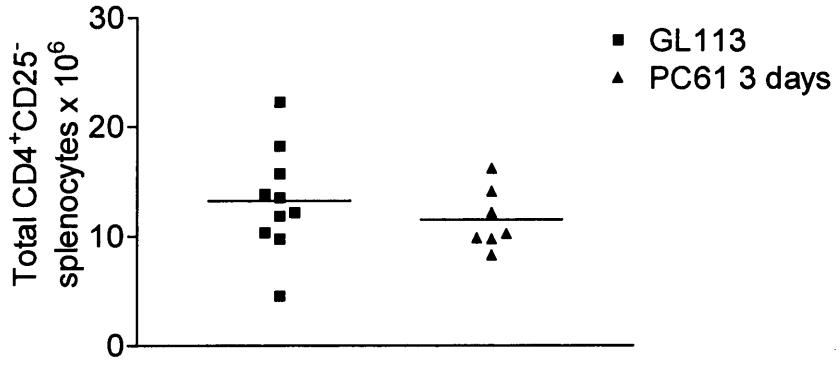


Figure 3.5. Analysis of the capability of antibody PC61 to deplete $CD4^+CD25^+$ splenocytes.

Spleens were harvested from mice that received two 500μg doses of either αCD25 specific depleting PC61 antibody or isotype control GL113 antibody three days earlier. Cells were stained with anti-CD4 FITC and -CD25biotin-SA PerCP-Cy5.5 antibodies. A representative FACS plot of splenocyte staining with anti-CD4 and –CD25 antibodies following the injection of either PC61 or GL113 is shown (A). The total number of $CD4^+CD25^+$ (B), the percentage of $CD4^+$ cells that express CD25 and (C) and the total number of $CD4^+CD25^-$ splenocytes (D) at the indicated time point is shown. Data was analysed using an *unpaired t-test.*

antibodies (representative FACS plots Figure 3.6 A). Overall, PC61-treated mice had approximately half the number of $CD4^+FOXP3^+$ cells observed in the control mice (Figure 3.6 B) and a substantial reduction in the percentage of $CD4^+$ cells that expressed FOXP3 (Figure 3.6 C). A small reduction in the number of $CD4^+FOXP3^-$ cells was observed (Figure 3.6 D), which probably reflects the fact that not all cells expressing CD25 also express FOXP3 (Figure 3.6 E). In addition, it is possible that upon injection of PC61 there is some up-regulation of CD25 on T cells therefore rendering these cells susceptible to the depleting effects of the antibody.

Objective 3: Analysis of Treg return after PC61 injection.

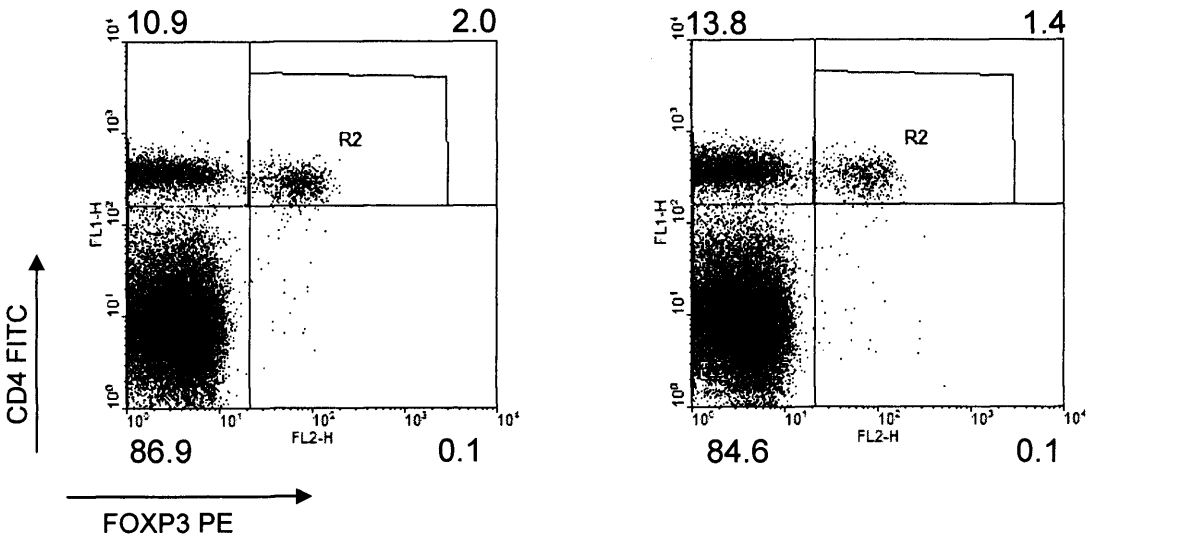
Mice were injected with PC61 as described above and sacrificed at various time points after injection. Splenocytes were stained with anti-CD4, -CD25 and -FOXP3 specific antibodies and analysed by flow cytometry. At one week post-injection the levels of Treg, as measured by FOXP3 expression, were still substantially reduced (Figure 3.7 A) whereas at three weeks Treg began to return, which coincides with when PC61 becomes undetectable in the serum (Jones, *et al.*2002). The expression of CD25 by Treg also begins to return at three weeks after PC61 injection and is completely returned by six weeks after injection (Figure 3.7 B).

Objective 4: Analysis of whether Tregs present after PC61 injection are suppressive.

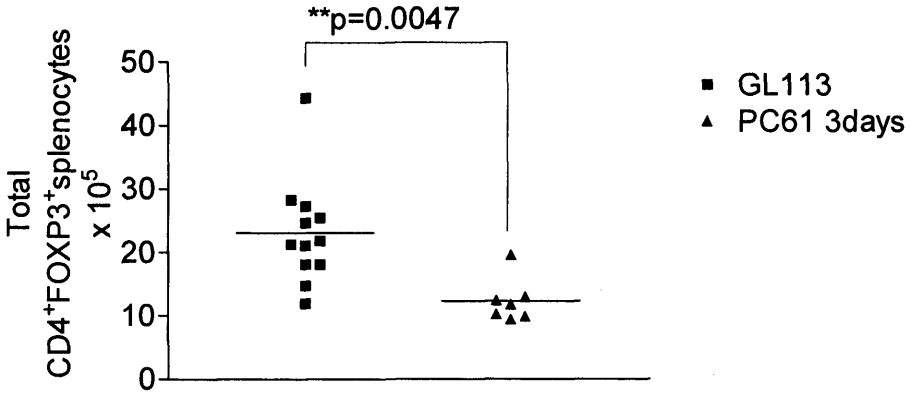
To confirm that the $CD4^+FOXP3^+CD25^+$ cells that were present after PC61 injection were functionally suppressive, $CD4^+CD25^+$ cells were isolated and tested for their ability to suppress $CD4^+CD25^-$ cell proliferation following polyclonal stimulation. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were isolated using magnetic microbeads (Figure 3.8. A-C). Various quantities of $CD4^+CD25^+$ cells were added to $CD4^+CD25^-$ cells and stimulated with anti-CD3 antibodies and irradiated APCs. Cells were isolated from mice 3wks and 6wks after PC61 injection. Cells isolated from mice that were injected with isotype control served as controls; where polyclonal stimulation of $CD4^+CD25^-$ cells induced robust proliferation, $CD4^+CD25^+$ cells were anergic and suppressed $CD4^+CD25^-$

Three days post antibody injection

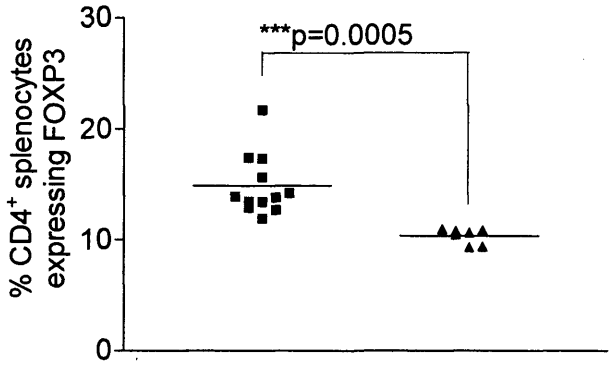
A



B



C



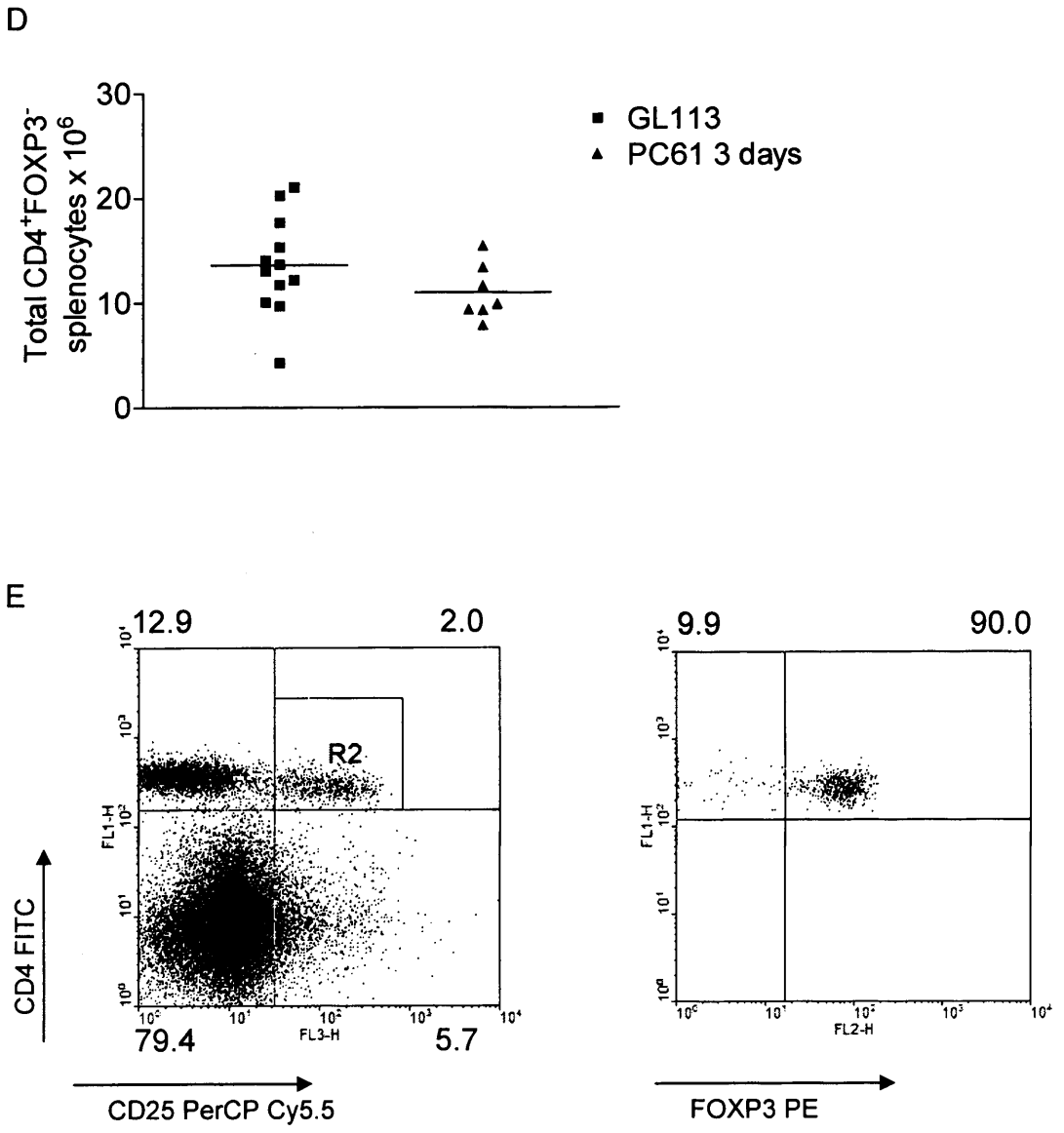


Figure 3.6. Analysis of the capability of the antibody PC61 to deplete $CD4^+FOXP3^+$ splenocytes.

Spleens were harvested from mice that received two 500μg doses of either αCD25 specific depleting PC61 antibody or isotype control GL113 antibody three days earlier. Cells were stained with anti-CD4 FITC and –FOXP3 PE antibodies. A representative FACS plot of splenocyte staining with anti-CD4 and –FOXP3 antibodies following the injection of either PC61 or GL113 is shown (A). The total number of $CD4^+FOXP3^+$ (B), the percentage of $CD4^+$ cells that express FOXP3 (C) and the total number of $CD4^+FOXP3^-$ splenocytes (D) at the indicated time point is shown. Data was analysed using an *unpaired t-test.*

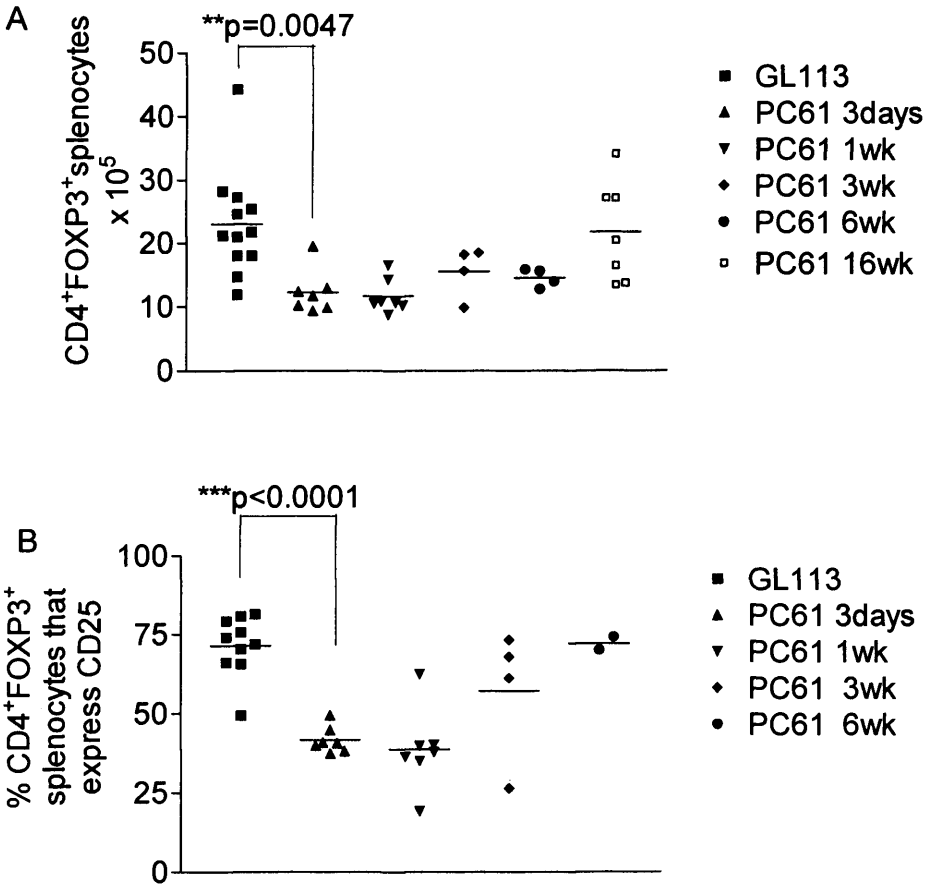
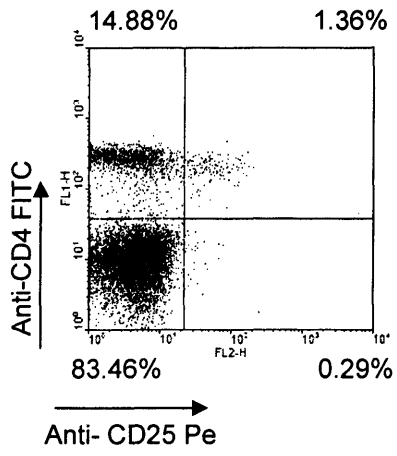
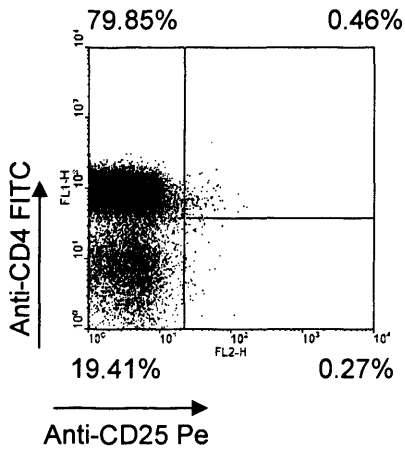


Figure 3.7. Analysis of the return of CD4⁺FOXP3⁺CD25⁺ splenocytes following the injection of the CD25 specific depleting antibody PC61. Spleens were harvested from mice at various time points following the injection of two 500μg doses of either αCD25 specific depleting PC61 antibody or isotype control GL113 antibody. Cells were stained with anti-CD4 FITC, -CD25 biotin-SA PerCP-Cy5.5 and –FOXP3 PE antibody. The total number of CD4⁺FOXP3⁺ cells (A) and the percentage of CD4⁺FOXP3⁺ cells that express CD25⁺ (B) is shown. Data was analysed using an *unpaired t-test.*

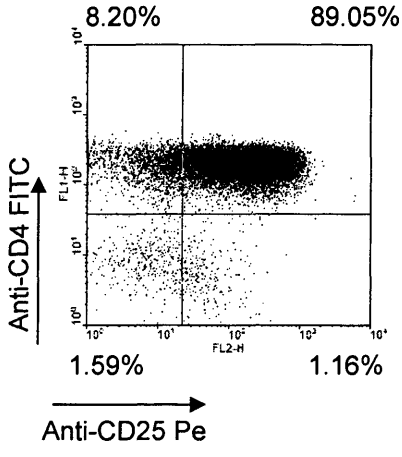
A Undepleted splenocytes



B CD4+CD25- splenocytes



C CD4+CD25+ splenocytes



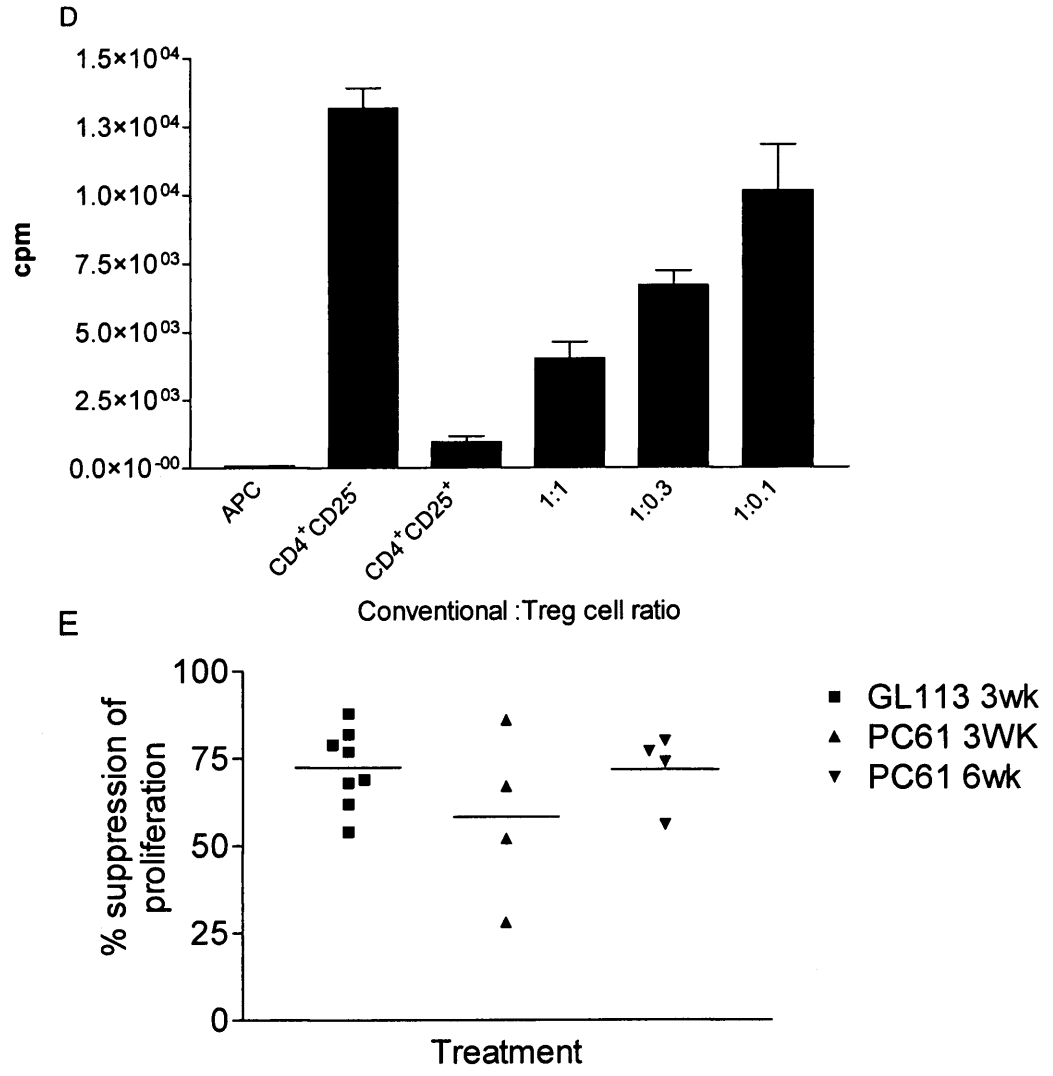


Figure 3.8. Investigation of the suppressive capacity of $CD4^+CD25^+$ splenocytes that return following injection with PC61 antibody.
Single cell suspensions of splenocytes were prepared and incubated with $CD4^+$ enriching beads (to deplete non-$CD4^+$ cells). $CD4^+$ cells were separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations. Undepleted (A), $CD4^+CD25^-$ (B) and $CD4^+CD25^+$ splenocytes (C) were stained with anti-CD4 FITC and CD25 PE specific antibodies to check the purity of cell populations. $CD4^+CD25^-$ (B) and $CD4^+CD25^+$ (C) populations that were typically 80% and 90% pure respectively. To investigate the functional capacity of the $CD4^+CD25^+$ splenocytes that return following administration of PC61, $2x10^4$ $CD4^+CD25^-$ splenocytes were stimulated with $1x10^5$ mitomycin treated $CD4^-$ splenocytes and 1μg/ml αCD3 for 3 days and mixed at various ratios with $CD4^+CD25^+$ splenocytes (D). The percentage of $CD4^+CD25^-$ cell proliferation that was suppressed by $CD4^+CD25^+$ cells at a 1:1 ration was determined for individual mice at each time point after injection of PC61 (E). Data was analysed using *unpaired t-test.*

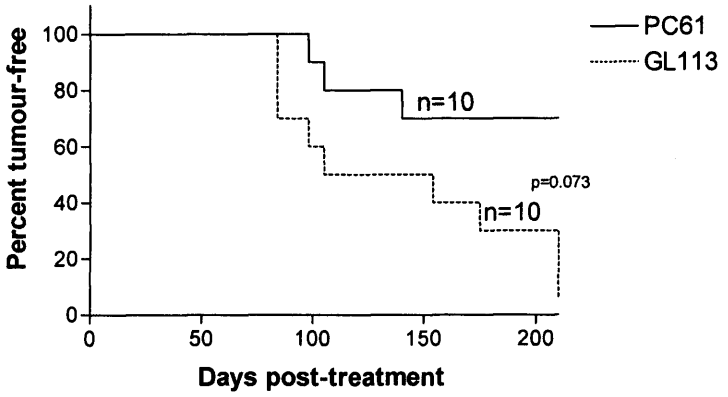
proliferation (Figure 3.8. D). $CD4^+CD25^+$ that returned three weeks and six weeks after PC61 injection had suppressive capacity equivalent to those isolated from mice injected with isotype control antibody (Figure 3.8. E). $CD4^+CD25^+$ isolated from mice three weeks after PC61 injection has slightly reduced suppressive capacity (Figure 3.8.E), although, this group of mice did demonstrate small trend of a reduced suppressive capacity, which correlated with the delay in the full return of CD25 expression on $CD4^+FOXP3^+$ until 6wk after PC61 injection (Figure 3.7. B).
In conclusion PC61 depletes a significant percentage of Tregs, but these cells do gradually return in number over six weeks.

3.2.3. Depletion of Tregs reduces the incidence of MC induced tumours

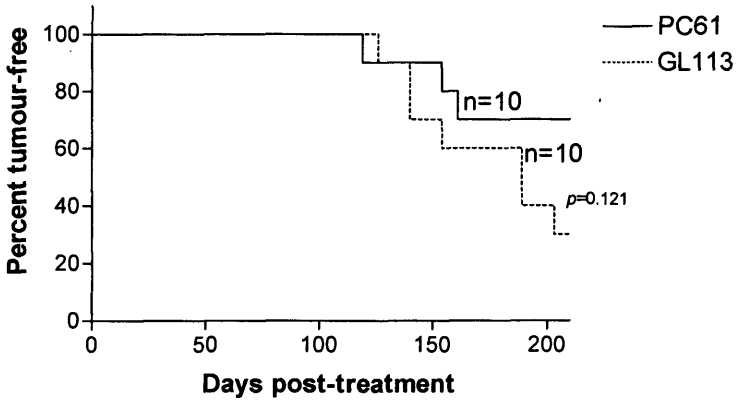
Following on from the observations above that describe depletion of a proportion of Tregs using PC61, experiments were performed to determine whether partial depletion of Tregs affects the development of MC-induced tumours in mice.

Mice were injected with 1mg of either isotype control or PC61 antibody before the injection of 400μg of MC into the hind leg. Tumour development occurred between 80 and 220 days after MC injection.
In two separate experiments performed in the Cardiff animal housing facility, the total number of tumours induced after MC administration was reduced in mice injected with PC61 compared to those treated with isotype control antibody (Figure 3.9. A, B). The tumour induction data of these two experiments was combined (Figure 3.9. C). Log-rank statistical analysis was used to identify a combined reduction of final tumour development and a delay in tumour occurrence in mice treated with PC61 compared to mice treated with isotype control
An identical experiment was performed within a separate animal housing facility in Switzerland (Figure 3.9. D), where similar results were obtained to those in Cardiff. The combined Cardiff and Switzerland data showed a highly significant reduction in total tumour development (Figure 3.9. E)

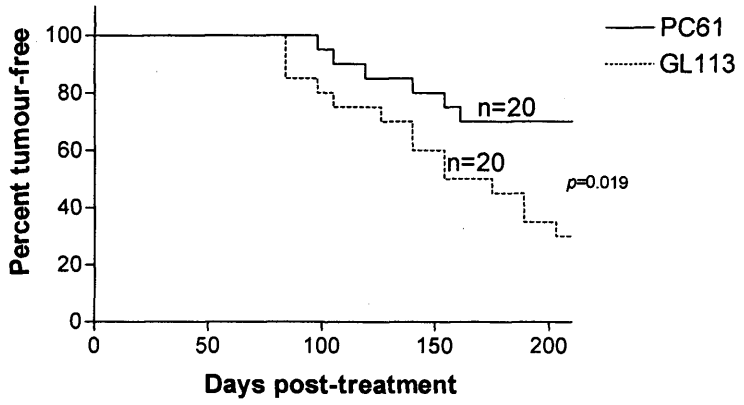
A


PC61
GL113
Percent tumour-free
100
80
60
40
20
0
n=10
p=0.073
n=10
0
50
100
150
200
Days post-treatment

B


PC61
GL113
Percent tumour-free
100
80
60
40
20
0
n=10
n=10
p=0.121
0
50
100
150
200
Days post-treatment

C


PC61
GL113
Percent tumour-free
100
80
60
40
20
0
n=20
n=20
p=0.019
0
50
100
150
200
Days post-treatment

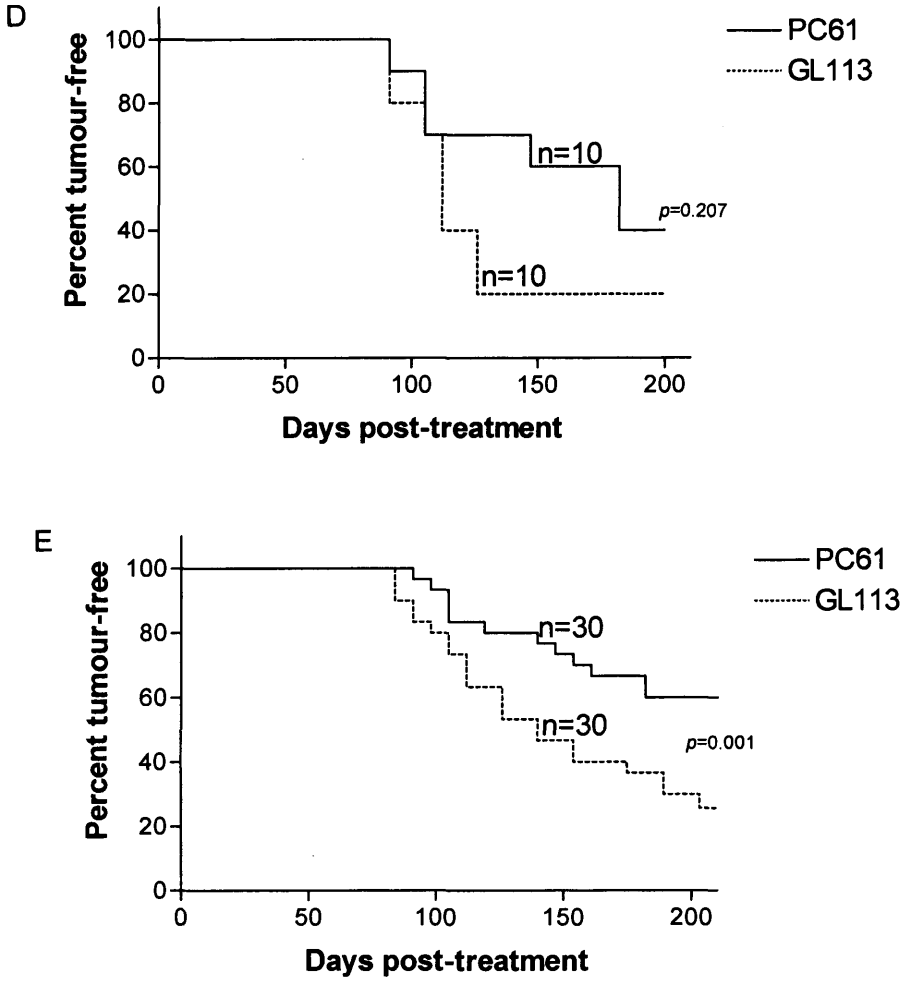


Figure 3.9. MC induced tumour induction following the injection of either PC61 or isotype control GL113 antibody.
In two separate experiments, mice were injected with MC after the injection of two 500μg doses of either PC61 or isotype control GL113 antibody (A, B). The pooled data from these experiments is displayed (C). The same experiment was also performed in mice housed in a separate animal facility (D). The pooled data from the three experiments is displayed (E). The rate of tumour incidence between experimental groups was analysed using *log-rank statistical analysis*.

3.2.4. Mice depleted of Tregs have elevated circulating dsDNA-specific auto-antibodies

The normal peripheral T cell pool contains self reactive cells that escape deletion in the thymus. Several reports indicate that Tregs are essential to suppress the activity of these self-reactive T cells in the periphery.

It was therefore important to determine whether administration of PC61 and MC resulted in autoreactivity in the mice used in the study above.

Sakaguchi *et al* observed a loss of tolerance when $CD4^+CD25^-$ cells were injected into immunodeficient mice (Sakaguchi, *et al.*1995). In the absence of Tregs, conventional $CD4^+CD25^-$ cells induced autoimmunity. Autoimmunity was characterised by the induction of a large panel of auto-antibodies and in some cases a graft-versus-host like disease was initiated. These observations suggested that mice injected with PC61 may also develop autoimmunity due to transient depletion of Tregs.

In order to detect early, developing auto-reactive immune responses, serum was collected from tumour free mice that received PC61 and analysed for anti-ds-DNA IgM auto-antibodies.

Serum collected from naïve and tumour bearing mice that received isotype control antibodies did not have raised levels of circulating anti-ds-DNA specific IgM. The combined data from tumour bearing and tumour free mice that received PC61 showed a significantly elevated level of circulating anti-ds-DNA specific IgM auto-antibody compared to the pooled data derived from mice that did not receive PC61 (Figure 3.10.). Tumour bearing mice that received PC61 had a comparable level of auto-antibody compared to mice treated with PC61 and that remained tumour free (Figure 3.10.). Thus, auto-antibody production is a consequence of treatment with PC61 and is likely to be attributable to the partial ablation of Treg activity. Whether these antibodies have any bearing on tumour growth is unclear.

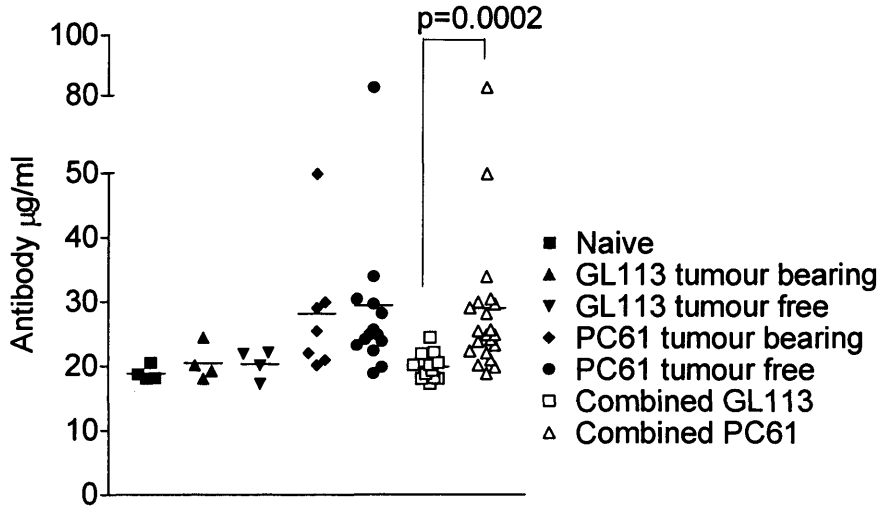


Figure 3.10. Examination of circulating dsDNA-specific IgM in mice following injection of MC

Mice were injected with PC61 or isotype control GL113 mAb before injection of MC. Serum was collected from tumour bearing, naïve and tumour free mice when experiments were terminated and examined for dsDNA-specific IgM antibody. Data was analysed using an *unpaired t-test.*

3.2.5. Treg depletion does not reduce MC induced tumour formation in the absence of IFN-γ

MC induced tumour induction in WT mice was reduced following the depletion of Tregs (Figure 3.9) implying that Treg normally suppress anti-tumour immune responses and that their depletion subsequently uncovers new or enhances existing anti-tumour immune responses. To gain an insight into the immune mechanisms responsible for reduced tumour development in the absence of Tregs, experiments were performed to determine whether the reduced tumour incidence, observed after injection of WT mice with PC61, was lost in mice lacking IFNγ. IFNγ was considered a good candidate for mediating anti-tumour immunity in the absence of Treg since previous studies have shown that MC induced tumour induction is elevated in mice with deficient IFN-γ signalling (Kaplan, *et al.*1998, Shankaran, *et al.*2001) (see section 1.2.).
WT mice (data not shown) and IFN-$\gamma^{-/-}$ mice were injected with either PC61 or the isotype control antibody GL113 prior to the injection of MC as described previously and tumour development was monitored. Overall the number of mice used in this experiment did not enable statistically significant conclusions to be drawn. However, a number of interesting preliminary observations were made.

i) Injecting PC61 delayed and reduced tumour development in WT mice (data not shown) in a similar manner as described previously (Figure 3.9) but this effect was absent in mice lacking IFN-γ (Figure 3.11.). This result implies that IFN-γ dependent anti-tumour effector mechanisms are enhanced by the depletion of Treg in WT mice.

ii) Tumour development may possibly be accelerated in IFN-$\gamma^{-/-}$ mice treated with PC61 compared with mice that received isotype control antibody (Figure 3.11.). This may reflect a loss of Treg function in IFN-$\gamma^{-/-}$ mice (Wang, *et al.*2006, Kelchtermans, *et al.*2005) or that depletion of $CD25^{+}$ effector T cells by PC61 is more important in IFN-$\gamma^{-/-}$ mice. Further experiments are required to see if this effect is significant.

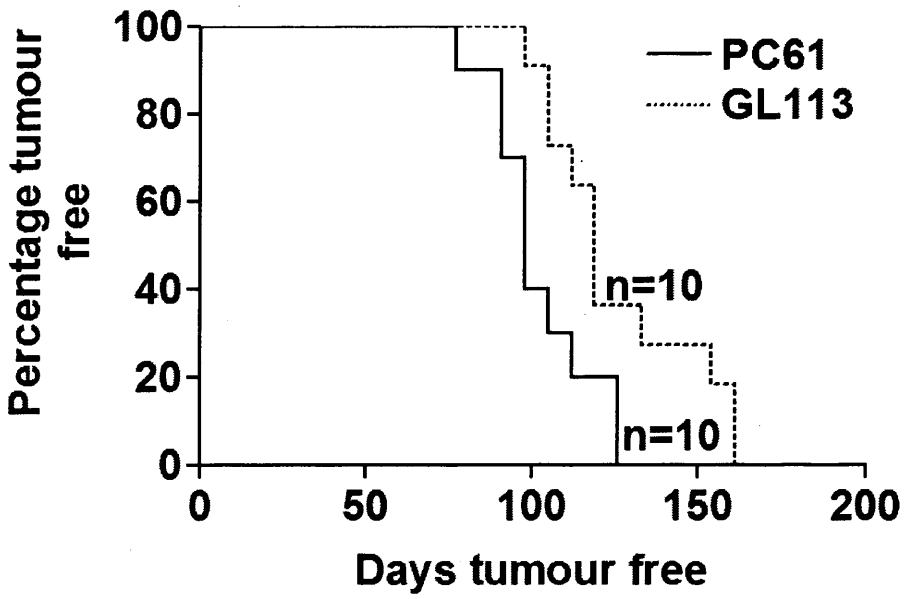


Figure 3.11. MC induced tumour induction following the injection of either PC61 or isotype control GL113 antibody to IFN-$\gamma^{-/-}$ mice
Mice were injected with MC after the injection of two 500μg doses of either PC61 or isotype control antibody GL113. The rate of tumour incidence between experimental groups was analysed using *log-rank statistical analysis*.

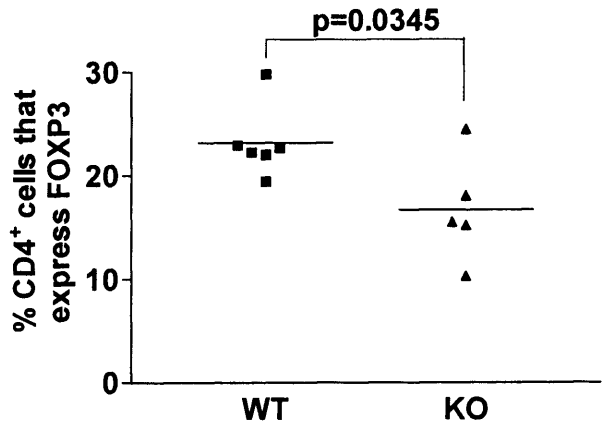
3.2.6. Tregs infiltrate MC induced tumours within IFN-$\gamma^{-/-}$ mice

Experiments discussed in this study have indicated that Treg normally suppress anti-tumour immunity as their depletion reduced tumour development in WT mice. Preliminary experiments suggest that IFN-γ dependent mechanisms contribute to the reduction of tumour development following Treg depletion in WT mice but that Treg function might be reduced in IFN-$\gamma^{-/-}$ mice, to reduce the benefit of Treg depletion. Further experiments were subsequently performed to characterise Treg infiltration of tumours that developed in IFN-$\gamma^{-/-}$ mice.

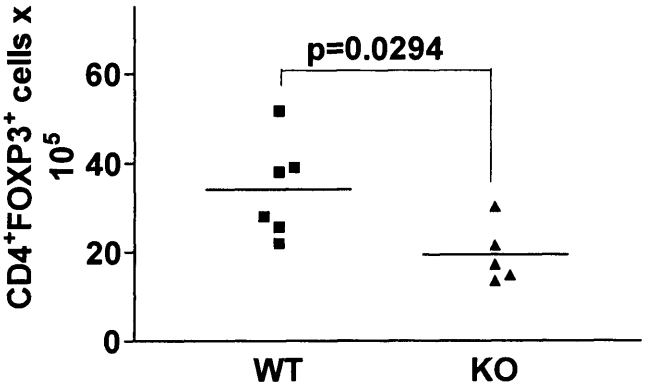
An initial investigation was performed to compare Treg numbers in age-matched naive and tumour-bearing WT and IFN-$\gamma^{-/-}$ mice. Single cell suspensions of splenocytes were prepared from IFN-$\gamma^{-/-}$ and WT mice and stained with anti-CD4, -CD25 and –FOXP3 specific antibodies. Different patterns were observed in naïve compared to tumour-bearing mice. In naïve animals, a lower proportion of $CD4^+$ cells isolated from IFN-$\gamma^{-/-}$ mice expressed FOXP3 compared to WT mice (Figure 3.12 A) and spleens of the IFN-$\gamma^{-/-}$ mice contained fewer Treg (Figure 3.12. B) although a similar number of conventional T cells (Figure 3.12 C). Either Treg production or survival therefore appears impaired in IFN-$\gamma^{-/-}$ mice.
In tumour-bearing mice, the number of Treg in spleen of both groups of mice was similar (Figure 3.12 D) and a similar percentage of $CD4^+$ splenocytes expressed FOXP3 (Figure 3.12. E). These data indicate that the presence of the tumour increases the number of Treg in the spleens of IFN-$\gamma^{-/-}$ mice to a level equivalent to tumour bearing WT mice.

The next objective was to assess whether Tregs were enriched in TIL and TDLN of IFN-$\gamma^{-/-}$ mice, in a similar fashion to the enrichment seen in WT mice (Figure 3.2. B). Although a trend of more Treg infiltrtation within TDLN was observed, the difference in the percentage of Treg infiltrating the TDLN compared to the NTDLN of IFN-$\gamma^{-/-}$ mice was not significant (3.13), compared to the modest increase of Treg infiltration observed

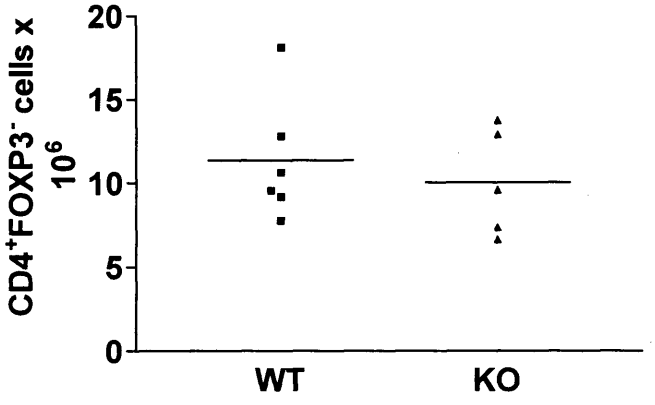
A


p=0.0345
% CD4+ cells that express FOXP3
30
20
10
0
WT
KO

B


p=0.0294
CD4+FOXP3+ cells x 10^5
60
40
20
0
WT
KO

C


CD4+FOXP3- cells x 10^6
20
15
10
5
0
WT
KO

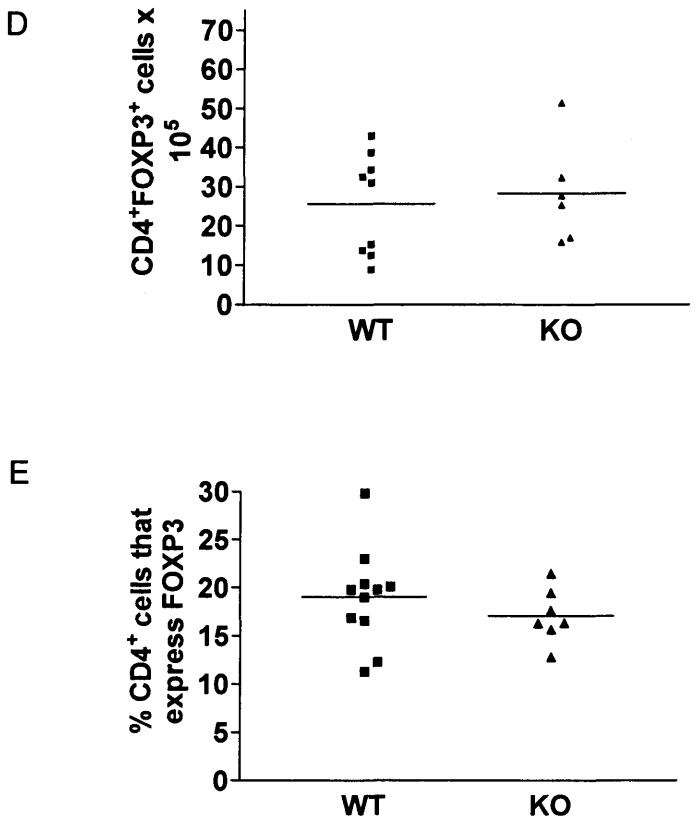
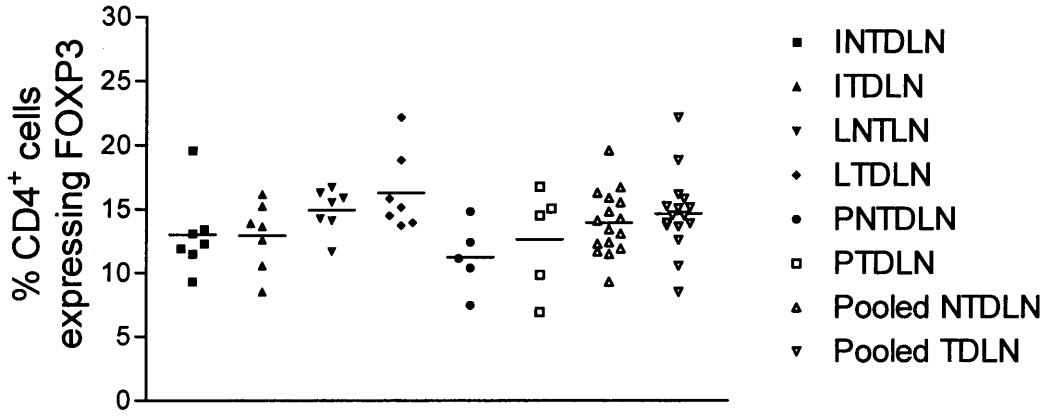


Figure 3.12. Treg in IFN-$\gamma^{-/-}$ and wt spleens obtained from naïve and tumour bearing mice.

Single cell suspensions of splenocytes were stained with anti-CD4 FITC, CD25 biotin-SA PerCP-Cy5.5 and FOXP3 PE specific mAb. Splenocytes were derived from naïve IFN-$\gamma^{-/-}$ and WT mice aged matched to tumour bearing mice (A-C) and the percentage of $CD4^+$ cells that expressed FOXP3 (A), the total number of $CD4^+FOXP3^+$ cells (B) and $CD4^+FOXP3^-$ cells (C) was calculated.

Splenocytes were also derived from tumour bearing IFN-$\gamma^{-/-}$ and WT mice (D-E) and the number of $CD4^+FOXP3^+$ cells and percentage of $CD4^+$ cells that expressed FOXP3 was calculated.



3.13. Flow cytometric analysis of $CD4^+FOXP3^+$ infiltration of MC induced tumours in IFN-$\gamma^{-/-}$ mice.

Single cell suspensions were prepared from inguinal (ITDLN), lumber (LTDN), poplateal (PTDLN) and pooled TDLNs and NTDLNs and stained with anti-CD4 FITC and -FOXP3 PE specific antibodies. The percentage of $CD4^+$ cells that express FOXP3 obtained is shown. Data was analysed using *paired t-test.*

in TDLN from WT mice (Figure 3.2. B). To determine whether Treg infiltration of developing tumours occurred to the same extent as in WT mice, the percentage of $CD4^+$ TIL that expressed FOXP3 in tumour was also compared. An equal percentage of circulating $CD4^+$ cells expressed FOXP3 in IFN-$\gamma^{-/-}$ mice compared to in WT mice in blood, spleen and TDLNs (Figure 3.14 A-C); however, a significantly smaller percentage of $CD4^+$ TIL expressed FOXP3 in IFN-$\gamma^{-/-}$ compared to that observed in WT mice (Figure 3.14. D), which was derived through a reduction of the percentage of Treg of total TIL and not via an elevation of conventional $CD4^+FOXP3^-$ cells (data not shown). It is clear that Treg accumulation at the tumour site is impaired in the absence of IFN-γ and it is possible that Treg require IFN-γ to coordinate Treg trafficking to or proliferate within the tumour site.

In light of a reduced proportion of Treg amongst TIL in IFN-$\gamma^{-/-}$ tumours and previous reports of defective Treg function in the absence of IFN-γ (Kelchtermans, *et al.*2005, Wang, *et al.*2006), it is possible that the Treg TIL population infiltrating in IFN-$\gamma^{-/-}$ tumours exerted less suppression than in tumours that developed in WT mice. PC61 may also deplete activated $CD25^+$ T cells and anti-tumour function of IFN-$\gamma^{-/-}$ mice is less able to recover compared to that of WT mice, even though these IFN-g-/- mice appear to have less Tregs amongst TIL.

3.3. Discussion

Burnet and co-workers originally proposed that the immune system recognises and targets tumours (Burnet.1957). Research conducted more recently has contributed a substantial quantity of data to support this premise, leading to the term "immunosurveillance" (discussed in detail in Chapter 1).

This study began to investigate the impact of Tregs on immunosurveillance using the MC tumour induction model. This model presented the opportunity to study *de novo* tumour development that included the preliminary cell transformation events and the progression of malignancy to the development of palpable tumours. The infiltration of Treg into these tumours was studied and the effect of depleting Treg prior to MC injection enabled the

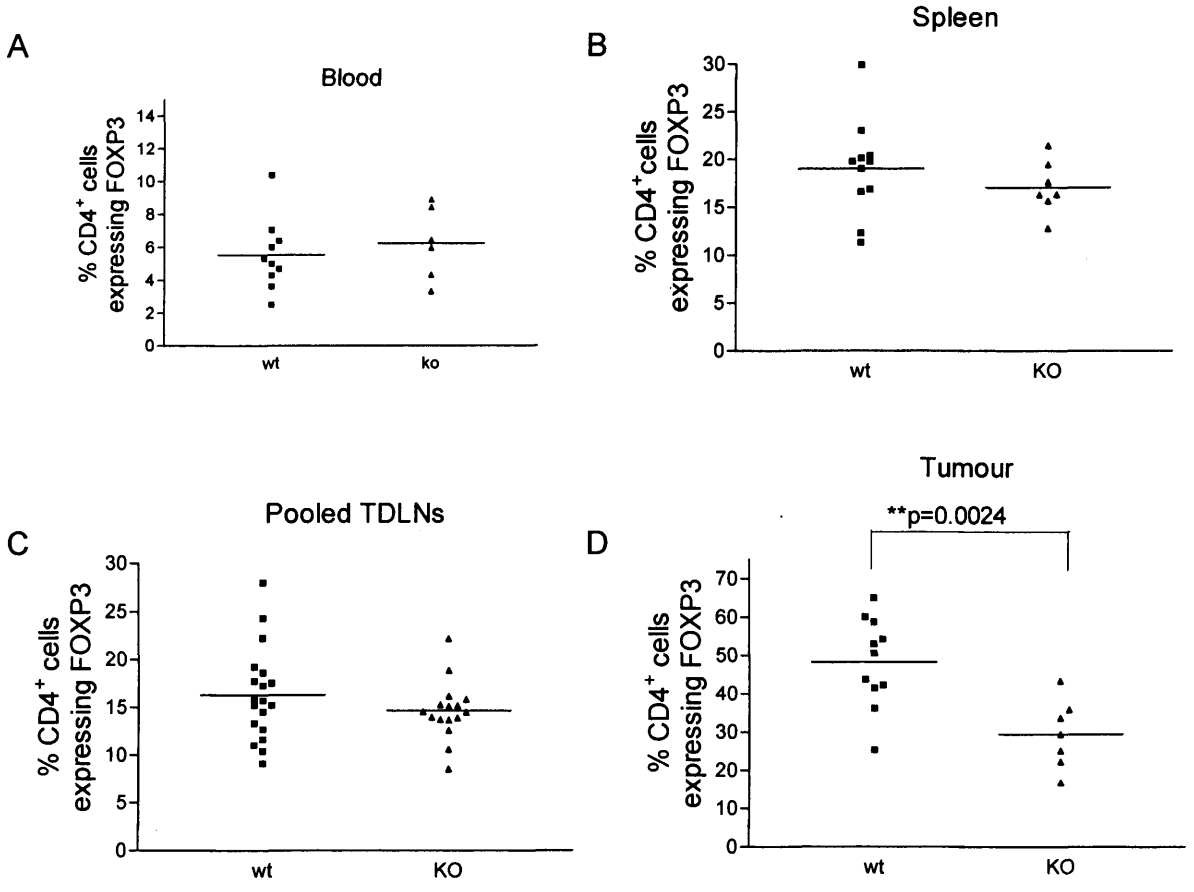


Figure 3.14. Comparison of Treg infiltration in WT and IFN-$\gamma^{-/-}$ mice.
Single cell suspensions were prepared from blood, spleen, TDLNs, and tumour of tumour bearing WT and IFN-$\gamma^{-/-}$ mice. Cells were stained with anti-CD4, -FOXP3 and CD25 specific antibody.
The percentage of $CD4^+$ that express FOXP3 in blood (A), spleen (B) TDLN (C) and tumour (D) of tumour bearing mice is compared between WT and IFN-$\gamma^{-/-}$ mice. Data was analysed using an *unpaired t-test.*

extent to which Tregs inhibited immunosurveillance of developing tumours to be analysed.

Treg are enriched within MC induced tumours

Several studies have observed Treg infiltration of tumours derived from injected tumour cell lines. An important part of the research in this report was to confirm that Treg also infiltrate MC induced tumours and to establish the extent of Treg infiltration. MC induced tumours were sectioned and analysed by histology for staining of CD4 and the Treg specific marker FOXP3. Treg were observed to infiltrate tumours and reside in close proximity to $CD8^+$ cells and formed cell-cell contact with $CD4^+FOXP3^-$ T cells. An analysis of tumours using flow cytometry was found to be the most informative method of assessing Treg infiltration. Flow cytometry enabled the proportion of $CD4^+$ cells that expressed FOXP3 to be established and permitted a comparison of the proportion of $CD4^+$ TIL that expressed FOXP3 to those within TDLN and spleen. Flow cytometry revealed that 50% of $CD4^+$ TIL expressed FOXP3, which represented a significant enrichment of Treg compared to within the TDLN and spleen. A similar comparison between the TDLN and NTDLN revealed a smaller yet significant increase of Treg infiltration within the TDLN.

Observations of Treg in patient tumours and in mice with transplanted cell lines have detailed a number of mechanisms that may operate to cause the accumulation of Treg in tumours. Investigators injected mice with tumour cell lines and established that processing of tumour antigens by myeloid derived DC in TDLN released TGF-β which promoted robust proliferation of Treg in TDLN and tumour (Ghiringhelli, *et al.*2005b). A similar process may occur within MC induced tumours. In studies of ovarian (Curiel, *et al.*2004) and non-Hodgkin's (Yang, *et al.*2006) lymphoma, tumours resected from patients demonstrated the importance of CCL22 to attract $CCR4^+$ Treg into tumours. In the case of ovarian carcinoma, tumour cells and tumour infiltrating macrophages secreted CCL22 but PBMC and normal ovarian cells did not. Secretion of CCL22 by tumour cells

may be part of a range of mechanisms that normally operate to control ongoing inflammation and prevent tissue damage.

Chronic exposure to antigen during viral infection has been described to exhaust conventional T cells and ultimately trigger their apoptosis and is thought to occur to reduce damage to self tissue following the failure of pathogen clearance during acute infection (Wherry, *et al.*2003). Although significant differences exist between persistent anti-tumour and anti-viral immune responses, a similar mechanism of T cell exhaustion may occur in tumours, whereby chronic exposure to tumour antigens may trigger apoptosis of conventional T cells thereby indirectly enriching the Treg population. Presentation of tumour antigens to T cells under highly tolerogenic conditions may cause conventional T cells to convert to Treg. Studies in mice have observed conversion to the Treg phenotype by delivering cognate antigen in the absence of activation signals or targeting antigen to immature DC (Chapter 1). Previous research has also demonstrated the conversion of conventional T cells to Treg during T cell activation and the importance of IFN-γ in this process (Wang, *et al.*2006) (described earlier); whilst IFN-$\gamma^{-/-}$ mice display more severe pathology during chronic inflammation of experimental autoimmune encephalitis and rheumatoid arthritis pathology that is correlated to impaired Treg function (Wang, *et al.*2006, Kelchtermans, *et al.*2005).

It was found using Flow cytometry that tumour infiltrating Treg and conventional T cell TCRvβ usage was the same (data not shown), suggesting that conventional T cells that migrate into the TDLN and tumour tissue may convert into Treg when exposed to tolerising environmental stimulus.

Further analysis of tumours was performed to understand what components of the immune system could potentially recognise and attack developing tumours and to identify candidate cell types that might be exposed to Treg mediated suppression. Evidence has been collected to show that Treg may suppress both the adaptive (Sakaguchi, *et al.*1995) and innate arms of the immune system (Maloy, *et al.*2003). Analysis of MC induced tumours identified infiltration of $TCR\alpha\beta^{-}CD45R/B220^{+}$ B cells, $TCR\alpha\beta^{+}CD8^{+}$ T cells, $TCR\alpha\beta^{+}NK1.1^{+}$ NKT cells, $TCR\alpha\beta^{-}NK1.1^{+}$ NK cells; $TCR\gamma\delta^{+}TCR\alpha\beta^{-}$ T cells and $CD11b^{+}Gr1^{hi}$ neutrophils (data not shown).

The frequency of MC-induced tumours is lower in mice depleted of Tregs

As described above, Tregs were enriched within the TILs of MC-induced tumours, which may be controlled, to the detriment of the host. This hypothesis was tested by depleting Treg prior to injection of the mice with MC.

CD25 is the most specific cell surface marker available to distinguish Treg and therefore injection of mice with anti-CD25 depleting antibody PC61 was chosen as the most suitable method to deplete Treg. Despite the failure of PC61 to deplete the entire Treg population, injection with the antibody did result in a significant reduction in tumour incidence compared to mice receiving the control antibody. Depletion of Treg significantly reduced MC mediated tumour induction and depletion of Treg prior to injection of MC presented as the best available strategy to reduce tumour induction. Further doses of antibody failed to efficiently remove Treg that returned after the first depletion or inhibit tumour induction in mice beyond those injected with one dose of antibody and consequently failed to further reduce MC induced tumour formation (Appendix Table 1 and Table 2). Experiments established that additional doses of PC61 were not removed by mouse anti-rat antibodies and depletion of $CD25^+$ cells occurred normally; however, further doses of PC61 failed to deplete $FOXP3^+$ cells that returned after the first dose of PC61 treatment (data not shown). Alternative methods to either inactive or deplete Treg were trialled in this study. Ligation of GITR by injection of non-depleting DTA-1 antibody has been reported to enhance tumour rejection by deactivating the suppressive function of Treg (Ko, *et al.*2005). In this study, mice were injected with PC61 prior to MC administration and later injected with DTA-1 antibody. DTA-1 failed to further reduce tumour development beyond that of mice that received one dose of PC61 (data not shown). Preliminary experiments were also performed using denileukin diftitox (diphtheria toxin conjugated to IL-2) to deplete Treg but this compound failed to effectively deplete $CD25^+$ cells (data not shown). An alternative approach to deplete Treg may be to treat mice with a low dose of cyclosphosphamide. This treatment selectively removes dividing cells and may efficiently remove Treg that proliferate in TDLN and

tumour tissue (Ghiringhelli, *et al.*2004, Ghiringhelli, *et al.*2005b). This strategy has previously been used to successfully deplete Treg and enhance tumour rejection in mice inoculated with tumour cell lines (North.1982). The efficiency of cyclophosphamide treatment could be further enhanced by providing an agonist to selectively boost Treg proliferation and render Treg more susceptible to cyclophosphamide treatment. The TLR2 ligand presents as one candidate and has been shown to selectively expand $TLR2^+$ Treg (Sutmuller, *et al.*2006).

Examination of how anti-tumour function is enhanced by Treg depletion

Treg infiltration of tumours normally may inhibit mechanisms that are capable of preventing tumour development. These might include the activation and accumulation of tumour specific T cells that are capable of rejecting immortalised cells at an early stage of tumour development. Since Tregs have been shown to suppress both innate and adaptive immune responses to tumours (Betts, *et al.*2006) (see appendix) it is likely that multiple arms of the immune system including both antigen-specific T cells and non-specific inflammatory responses are more effective following Treg depletion. Observations in this study revealed that MC induced tumours attract a broad repertoire of leukocytes besides $CD4^+$ Treg and conventional T cells. Flow cytometric analysis of tumour infiltrates revealed that CD8 T cells, B cells, and neutrophils, NK and NKT cells and $TCR\gamma\delta^+$ T were present within the tumours (data not shown). Previous studies have displayed an anti-tumour function of $TCR\gamma\delta^+$ cells, NKT and NK cells using the MC model (Table 1.1). Further research have shown that Treg suppression extends beyond that of T cells and encompasses cells that direct both innate (Maloy, *et al.*2003, Maloy, *et al.*2005)and adaptive immunity and a more in depth analysis may reveal whether Treg suppress anti-tumour function of these cells and if particular subsets are more susceptible to suppression. In the case of MC induced tumours, Nishikawa *et al.* recently showed that Tregs, induced by immunisation with a SEREX-defined self antigen expressed in tumour cells, inhibited NK and NKT cells capable of inhibiting the development of MCA-induced tumours, thus indicating that these cells are targets of Treg activity and important anti-tumour effector cells (Nishikawa, *et al.*2005c).

Mice depleted of Treg using PC61 and remaining tumour free were investigated to establish if self-reactive immune responses correlated with the absence of a tumour. Depletion of Treg using PC61 appeared to be necessary to trigger self-reactivity, as circulating auto-antibodies were not elevated in naïve mice or tumour bearing mice treated with isotype control antibody GL113. Tumour free mice that were injected with PC61 did have circulating auto-antibodies that may be indicative of autoimmune activity capable of attacking early tumours Mice treated with isotype control antibody that remained tumour free did not develop high levels of auto-antibody. This showed that responses against self-antigen expressed by tumour are not robust enough to elicit systemic autoreactivity on normal tissue expressing the same self-antigens. The absence of evidence of auto-immunity in GL113 treated tumour free mice and failure of autoimmune responses to protect against tumour growth in tumour bearing mice injected with PC61 shows that robust responses to self-antigen may not be necessary or sufficient to prevent tumour. However, it may still be the case that self-reactivity present in PC61 treated mice may make some contribution to the enhanced anti-tumour rejection observed in this group. Depletion of Treg may have triggered a systemic release of inflammatory cytokines thereby promoting and facilitating the function of T cells recognising tumour specific antigens created by MC mediated mutagenesis.

Further experiments were performed to identify inflammatory factors that may contribute to the reduction of tumour incidence following Treg depletion. Previous studies have highlighted a critical role for IFNγ in controlling development of MC-induced tumours (Shankaran, *et al.*2001, Kaplan, *et al.*1998). IFNγ can control tumour growth directly through pro-apoptotic, anti-angiogenic and anti-proliferative effects and indirectly, by facilitating induction of anti-tumour innate and adaptive immune responses (reviewed in (Smyth, *et al.*2006a)). Tumour induction experiments, following partial depletion of Tregs, were therefore repeated in mice deficient of IFN-γ and showed the inhibition of tumour induction following Treg depletion was abrogated by a deficiency of IFN-γ. This indicated that a substantial proportion of the anti-tumour function elicited by the absence of Treg is mediated through IFN-γ. The source and effect of IFN-γ remains to be defined.

IFN-γ in the vicinity of the MC injection site may have multiple roles. Based on previous studies, several possibilities exist. IFN-γ enhances deposition of collagen to restrict further exposure to MC mutagenic activity (Qin, *et al.*2002). IFN-γ is known to inhibit angiogenesis (Blankenstein, *et al.*2003) and alter the processing of self antigen and enhance MHC I peptide presentation (Shankaran, *et al.*2001) and activate TIL (Blankenstein, *et al.*2003). Indeed, it is likely that IFN-γ inhibits tumour development through multiple mechanisms.

Impaired Treg infiltration of the tumour in IFN-$\gamma^{-/-}$ mice

Preliminary experiments performed in this study indicated that tumour rejection following depletion of Treg is IFN-γ dependent implying that Treg either normally suppress IFNγ production or that IFNγ is required for control of tumour growth regardless of whether Treg are present or not. Identification of how IFN-γ mediates the anti-tumour effect was not explored within this study. These experiments also indicated that tumour development was accelerated in PC61 treated IFN-$\gamma^{-/-}$ mice compared to mice injected with isotype control antibody. Tumours that developed in IFN-$\gamma^{-/-}$ mice were analysed to assess the contribution of IFN-γ to Treg infiltration of tumours and to explore why administration of PC61 enhanced tumour development in IFN-$\gamma^{-/-}$ mice.

A similar number of Treg infiltrated the spleens of IFN-$\gamma^{-/-}$ and WT tumour bearing mice, however, Treg infiltration of IFN-$\gamma^{-/-}$ mice tumours was significantly lower than tumours isolated from WT mice. Previous studies have reported a relatively normal Treg function within naïve IFN-$\gamma^{-/-}$ mice; however, the ability of Treg in IFN-$\gamma^{-/-}$ mice to control inflammation appears defective and corresponds to more severe pathology during rheumatoid arthritis (RA) (Kelchtermans, *et al.*2005) and experimental autoimmune encephalitis (EAE) (Wang, *et al.*2006). Inflammation created by invasive tumour growth and associated disruption of surrounding tissue and cell stress may be important for triggering Treg infiltration of developing tumours in WT mice. In light of the impaired Treg infiltration into inflamed tissue during RA and EAE, it is possible that an

impairment of Treg to respond and migrate into inflamed tumours within IFN-$\gamma^{-/-}$ mice accounts for the marked reduction of Treg infiltration of IFN-$\gamma^{-/-}$ tumours compared to tumours that develop in WT mice. In the absence of a reduced Treg infiltration of tumours that develop in IFN-$\gamma^{-/-}$, the benefit of depleting Treg maybe reduced in these mice compared to in WT mice.

An accumulation of data obtained in *in vivo* studies using tumour cell lines support the concept that Treg depletion will have a beneficial effect in cancer immunotherapy. The experiments described in this Chapter support this premise by revealing a role for Tregs in suppressing effective immune surveillance of carcinogen-induced tumours in intact animals. Finding in this chapter have recently being published (Betts *et al* 2007) (appendix).

4. The design of experimental procedures for phenotypic and functional analyses of human $CD4^+CD25^+$ regulatory T cells.

4.1. Introduction

Another main aim of this thesis was to analyse the influence of Tregs on immune responses in patients with colorectal cancer. For this purpose it was first necessary to establish protocols for the reproducible enumeration of Tregs isolated from healthy controls and patients with CRC. Since this study began before FOXP3-specific antibodies were available, early identification of Tregs was performed through analysis of CD25 expression on $CD4^+$ T cells. Baecher-allan *et al* has demonstrated that $CD4^+CD25^+$ regulatory T cells within human PBMC are confined to the $CD4^+CD25^{hi}$ population (Baecher-Allan, *et al.*2001). The group stained PBMC with anti-CD4 and –CD25 specific antibodies and FACS sorted $CD4^+$ lymphocytes into three populations according to expression of CD25, containing $CD4^+CD25^-$, $CD4^+CD25^{int}$ and $CD4^+CD25^{hi}$ PBMC (Baecher-Allan, *et al.*2001). $CD25^{hi}$ PBMC were identified as the population of $CD4^+$ cells with CD25 expression above the maximal CD25 expression on $CD4^-$ PBMC. The suppressive function of sorted populations was examined by incubating each population with sorted $CD4^+CD25^-$ cells and stimulating the cells with anti-CD3 antibody and antigen presenting cells. These experiments revealed that suppressive activity was restricted to $CD4^+CD25^{hi}$ PBMC. The fidelity of the $CD4^+CD25^{hi}$ population was subsequently verified by co-staining PBMC with the constitutive Treg markers CTLA-4 (Jonuleit, *et al.*2001) and CD45RO (Jonuleit, *et al.*2001, Baecher-Allan, *et al.*2001). This study has reproduced the methods described above to compare the frequency of Tregs in PBMC of age matched controls and patients diagnosed with colorectal cancer.

An investigation into the most efficient method for depletion of $CD4^+CD25^{hi}$ cells from PBMC was also conducted. A number of methods were compared to establish which technique removed all $CD4^+CD25^{hi}$ Tregs, whilst removing the minimum proportion of $CD4^+CD25^{int}$ conventional T cells. Once a suitable technique was identified, functional T

cell assays were optimised in order to later determine whether depletion of $CD4^+CD25^{hi}$ cells enhanced antigen specific immune responses.

4.2. Results

4.2.1. Identification of $CD4^+CD25^{hi}$ T cells

The aim of the following procedures was to stain PBMC with antibodies specific for CD4 and CD25. For this purpose, peripheral blood lymphocytes (PBL) were first gated and stained with isotype control antibodies and antibodies specific for CD4 and CD25 (Figure 4.1. A-E). Anti-CD4 and –CD25 antibody efficiently labelled lymphocytes, whilst non-specific binding by CD4 isotype control IgG1κ FITC and CD25 isotype control IgGκ1 APC to PBL was negligible. The frequency of Treg in $CD4^+$ PBMC i.e. percentage of $CD4^+CD25^{hi}$ of total $CD4^+$ PBL was established (Figure 4.2.).

4.2.2. Analysis of constitutive $CD4^+CD25^{hi}$ markers

PBMC, stained with CD4 and CD25-specific antibodies, were also stained with antibodies specific for CTLA-4 and CD45RO, markers known to be expressed by Tregs (Jonuleit, *et al.*2001, Baecher-Allan, *et al.*2001).

$CD4^+CD25^{hi}$ cells express intra-cellular CTLA-4

$CD4^+CD25^{hi}$ Tregs have been previously described to express CTLA-4 (Jonuleit, *et al.*2001).

To observe CTLA-4 expression, non-specific binding by an isotype control antibody was first analysed. Binding with this antibody was insignificant within the $CD4^+CD25^{hi}$ population (Figure 4.3. A), present within the $CD4^+CD25^{int}$ cells (Figure 4.3. C) and relatively high within the $CD4^+CD25^-$ population (Figure 4.3.E), making an exact determination of CTLA-4 expression within the $CD4^+CD25^-$ population (Figure 4.3. F) unreliable. $CD4^+CD25^{hi}$ PBL however, uniformly expressed intra-cellular CTLA-4

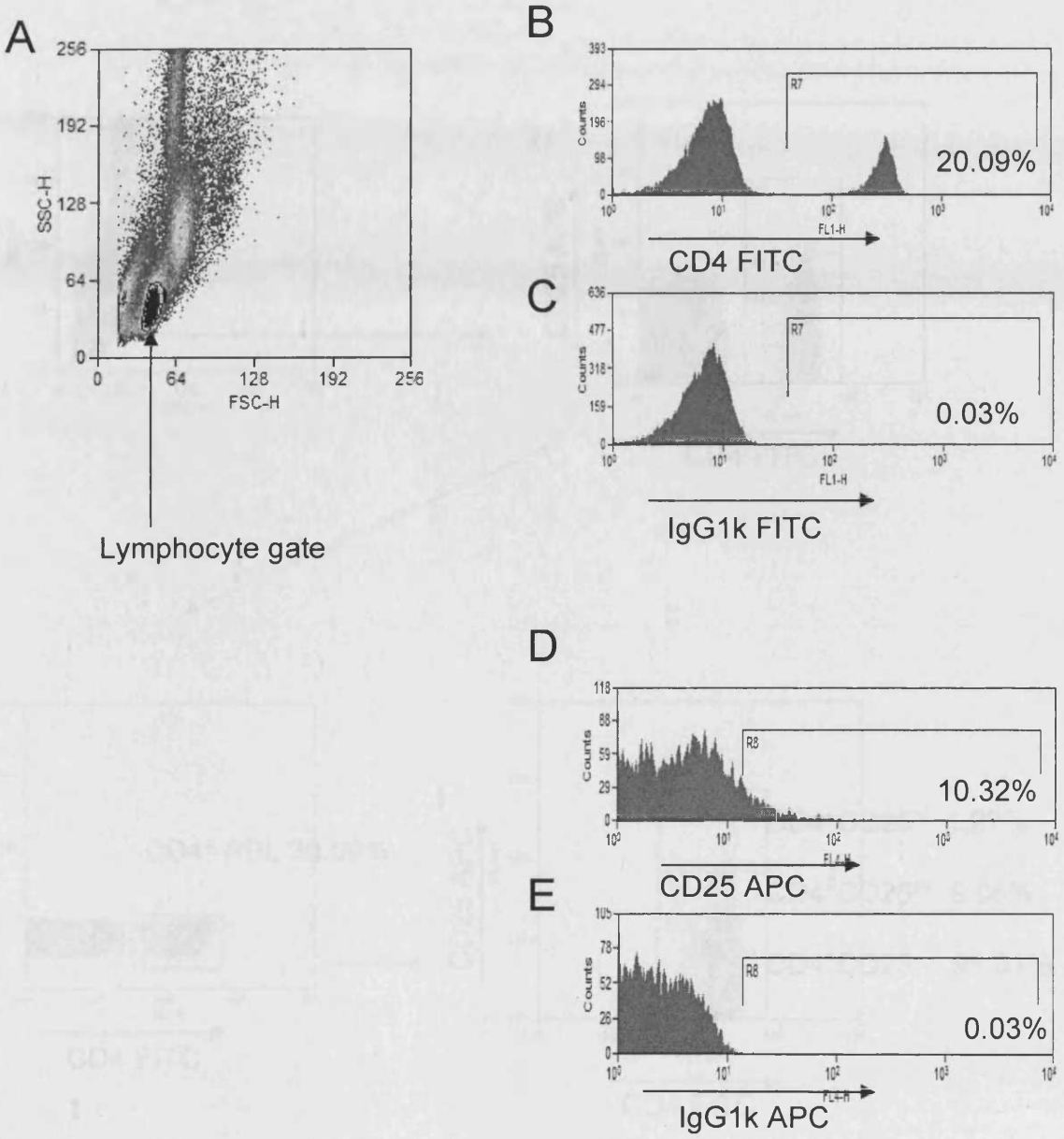


Figure 4.1. Controlling CD4 FITC and CD25 APC specific staining of PBL

PBMC were stained with anti-CD4 FITC, -CD25 APC and CD4 isotype control IgG1κ FITC and CD25 isotype control IgG1κ APC mAbs. PBL were gated (A) and non-specific staining of PBL with CD4 FITC (B), IgGκ1 FITC (C), CD25 APC (D) and IgG1κ APC (E) was analysed.

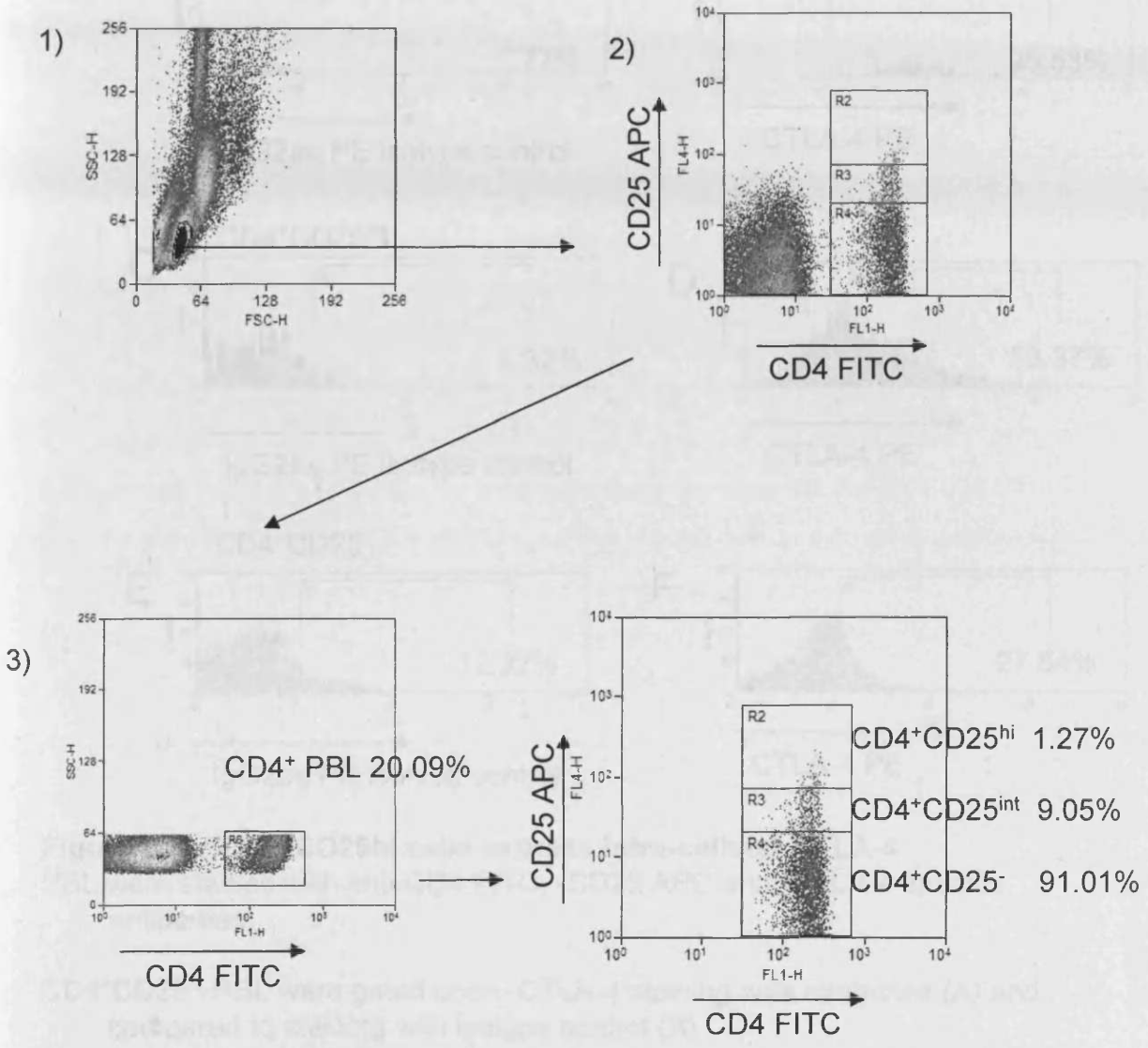


Figure 4.2. Summary of the method to determine the frequency of $CD4^+CD25^{hi}$ cells in $CD4^+$ PBMC

PBMC were stained with anti-CD4 FITC and -CD25 APC mAbs. Peripheral blood lymphocytes (PBL) were defined and gated according to their SSC and FSC (step 1). $CD4^+CD25^{hi}$ cells were identified as those $CD4^+$ cells with CD25 staining intensity greater than $CD4^-$ cells (step 2). This setting was re-calculated for each sample analysed. All $CD4^+$ PBL were gated (step 3) and the frequency of $CD4^+CD25^{hi}$ cells in $CD4^+$ PBL was determined according to the cells CD25 staining intensity, determined in step 2.

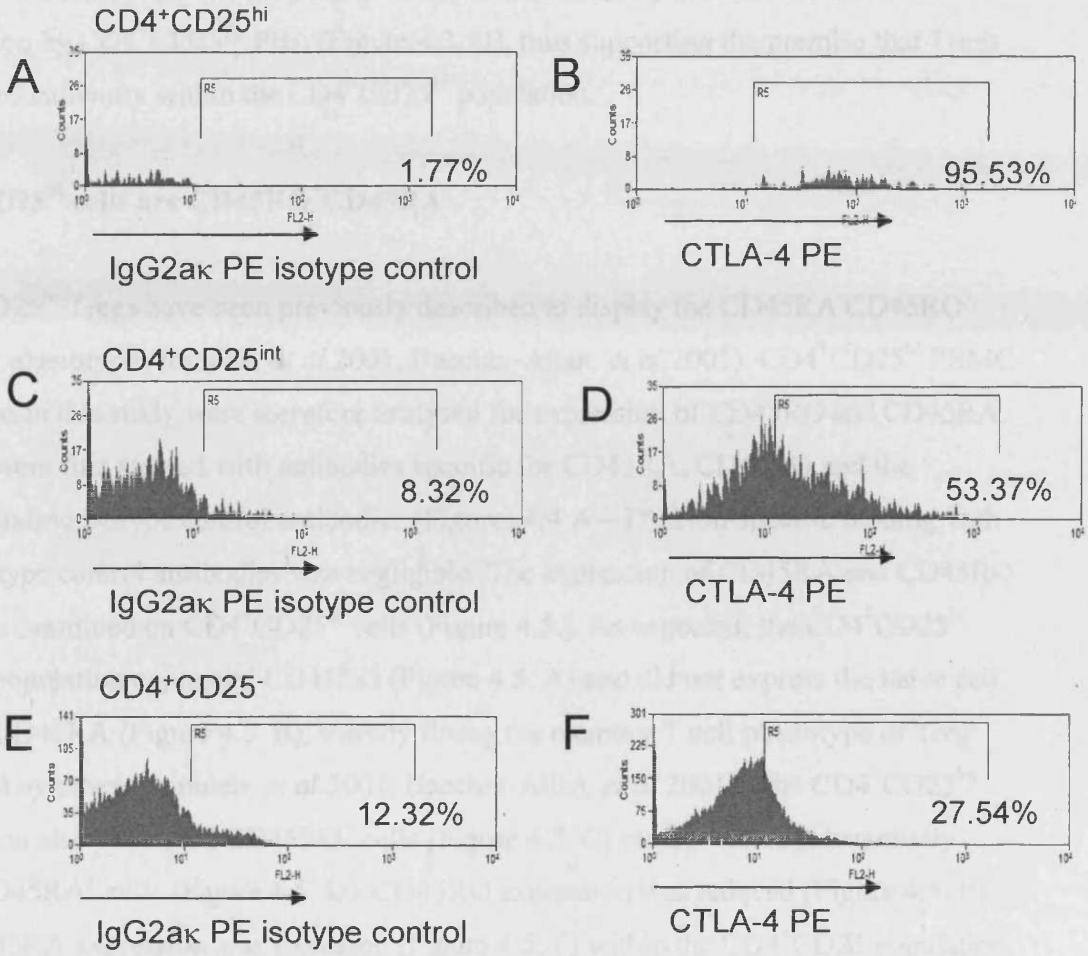


Figure 4.3. CD4+CD25hi cells express intra-cellular CTLA-4

PBL were stained with anti-CD4 FITC, -CD25 APC and -CTLA-4 specific antibodies.

$CD4^+CD25^{hi}$ PBL were gated upon. CTLA-4 staining was examined (A) and compared to staining with isotype control (B)

$CD4^+CD25^{int}$ PBL were gated upon. CTLA-4 staining was examined (C) and compared to staining with isotype control (D)

$CD4^+CD25^-$ PBL were gated upon. CTLA-4 staining was examined (E) and compared to staining with isotype control (F)

(Figure 4.3. B), which contrasts significantly to the markedly reduced CTLA-4 expression by $CD4^+CD25^{int}$ PBL (Figure 4.3. D), thus supporting the premise that Tregs exist predominantly within the $CD4^+CD25^{hi}$ population.

$CD4^+CD25^{hi}$ cells are $CD45RO^+CD45RA^-$

$CD4^+CD25^{hi}$ Tregs have been previously described to display the $CD45RA^-CD45RO^+$ memory phenotype (Jonuleit, *et al.*2001, Baecher-Allan, *et al.*2001). $CD4^+CD25^{hi}$ PBMC identified in this study were therefore analysed for expression of CD45RO and CD45RA. PBMC were first stained with antibodies specific for CD45RA, CD45RO and the corresponding isotype control antibodies (Figures 4.4 A – D). Non-specific binding with both isotype control antibodies was negligible. The expression of CD45RA and CD45RO was then examined on $CD4^+CD25^{hi}$ cells (Figure 4.5.). As expected, the $CD4^+CD25^{hi}$ PBMC population expressed CD45RO (Figure 4.5. A) and did not express the naïve cell marker CD45RA (Figure 4.5. B), thereby fitting the memory T cell phenotype of Treg observed by others (Jonuleit, *et al.*2001, Baecher-Allan, *et al.*2001). The $CD4^+CD25^{int}$ population also contained $CD45RO^+$ cells (Figure 4.5. C) but contained substantially more $CD45RA^+$ cells (Figure 4.5. D). CD45RO expression was reduced (Figure 4.5. E) and CD45RA expression was increased (Figure 4.5. F) within the $CD4^+CD25^-$population

4.2.3. Staining $CD8^+$ T Cells and B Cells

For analyses of patient samples (Chapter 5) staining methods were established for $CD8^+$ T cells and B cells. For this purpose, subjects PBMC were stained with anti-CD8 and -CD19 antibodies and compared to isotype controls (Figure 4.6. A-D). Non-specific staining by isotype control antibodies was insignificant.

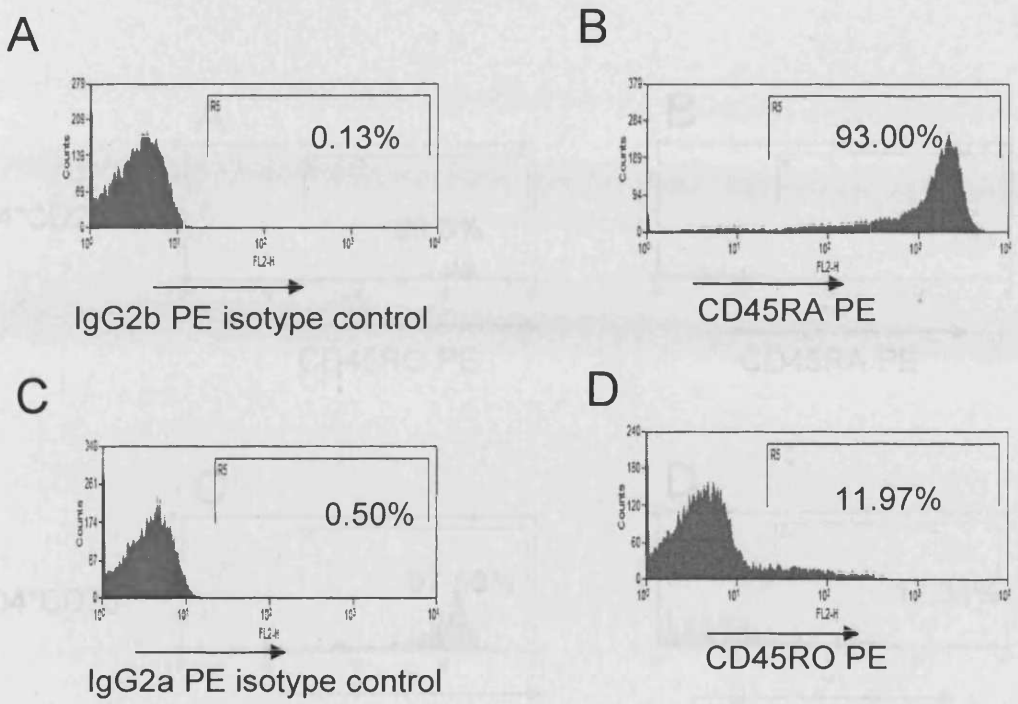


Figure 4.4. Controlling CD45RA and CD45RO staining of PBL
PBMC were stained with anti-CD4 FITC and -CD45RA PE specific antibodies or isotype control antibody. $CD4^+$ PBL were gated (data not shown) and analysed for staining with isotype control (A) or CD45RA (B)

PBMC were stained with anti-CD4 FITC and -CD45RO PE specific antibodies or isotype control antibody. $CD4^+$ PBL were gated and analysed for staining with isotype control (C) or CD45RO (D)

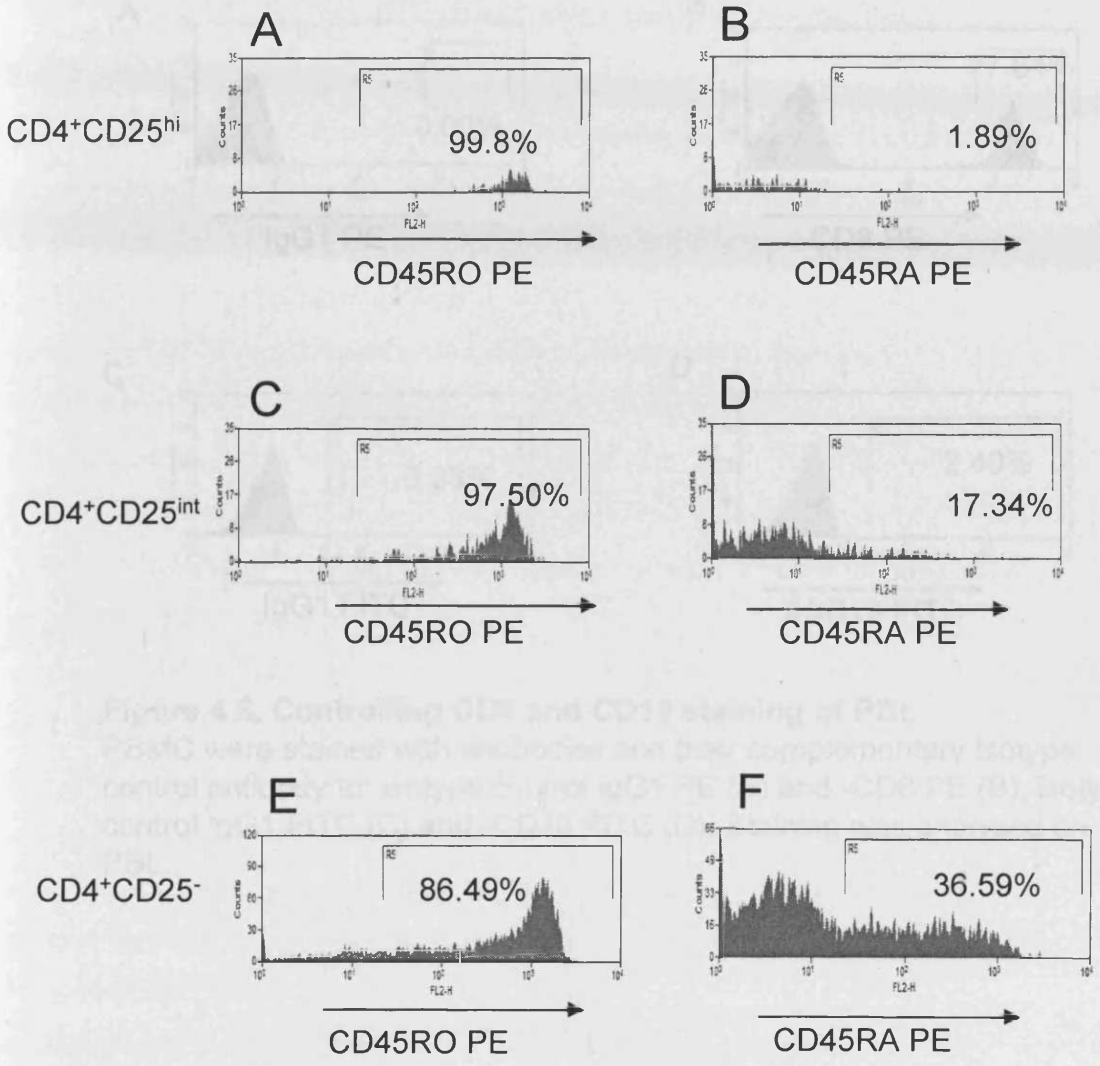


Figure 4.5. CD4$^+$CD25^{hi} cells are CD45RO$^+$CD45RA$^-$
PBL were stained with anti-CD4 FITC, -CD25 APC, -CD45RO PE and -CD45RA PE specific mAbs. CD45RA and CD45RO expression was analysed on the CD4$^+$CD25^{hi} (A-B), CD4$^+$CD25^{int} (C-D) and CD4$^+$CD25$^-$ (E-F) PBL.

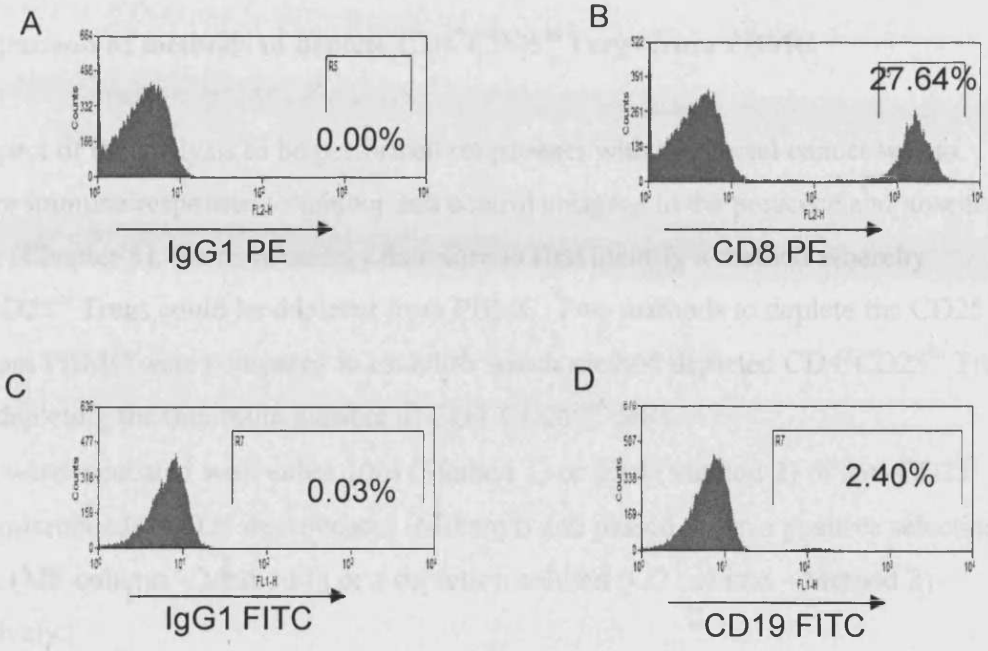


Figure 4.6. Controlling CD8 and CD19 staining of PBL
PBMC were stained with antibodies and their complementary isotype control antibody to: isotype control IgG1 PE (A) and -CD8 PE (B); isotype control IgG1 FITC (C) and -CD19 FITC (D). Staining was analysed on PBL.

4.2.4. Depletion of $CD25^+$ cells from PBMC

A comparison of methods to deplete $CD4^+CD25^{hi}$ Tregs from PBMC

One aspect of the analysis to be performed on patients with colorectal cancer was to compare immune responses to tumour and control antigens in the presence and absence of Treg (Chapter 5). It was necessary therefore to first identify a method whereby $CD4^+CD25^{hi}$ Tregs could be depleted from PBMC. Two methods to deplete the $CD25^+$ cells from PBMC were compared to establish which method depleted $CD4^+CD25^{hi}$ Treg whilst depleting the minimum number of $CD4^+CD25^{int/-}$ cells.

PBMC were incubated with either 10µl (Method 1) or 20µl (Method 2) of anti-CD25 coated microbeads (CD25 microbeads) (Miltenyi) and passed down a positive selection column (MS column – Method 1) or a depletion column (LD column – Method 2) respectively.

Undepleted PBMC and PBMC that were separated using each column were stained with anti-CD4 and –CD25 antibodies and analysed by flow cytometry to establish the efficiency of $CD25^{hi}$ cell depletion using each method (Figure 4.7.). $CD4^+CD25^{hi}$ cells were clearly visible within undepleted PBMC (Figure 4.7. A). The gates used to quantify undepleted $CD4^+$ PBMC according to their expression of CD25 were maintained to observe the frequency of each population in the $CD25^+$ cell depleted populations obtained using each method.

Method 1 removed the entire $CD4^+CD25^{hi}$ population and around 75% of the $CD4^+CD25^{int}$ population (Figure 4.7. B). Method 2 also removed all $CD4^+CD25^{hi}$ cells and almost all of the $CD4^+CD25^{int}$ population (Figure 4.7. C). In order to analyse the cells eluted from the MS and LD column, cells were stained with a CD25-specific antibody known to co-stain CD25 on cells coated with CD25 microbeads. This data shows that the $CD4^+CD25^{hi}$ population was clearly visible within undepleted PBMC (Figure 4.8. A) and that $CD4^+CD25^{hi}$ and $CD4^+CD25^{int}$ cells were enriched within the MS column eluent (Figure 4.8. B). Method 1 was chosen to be used for routine depletion of Treg from patient PBMC samples, on the basis that all $CD4^+CD25^{hi}$ cells were depleted whilst minimising the depletion of $CD4^+CD25^{int}$ cells.

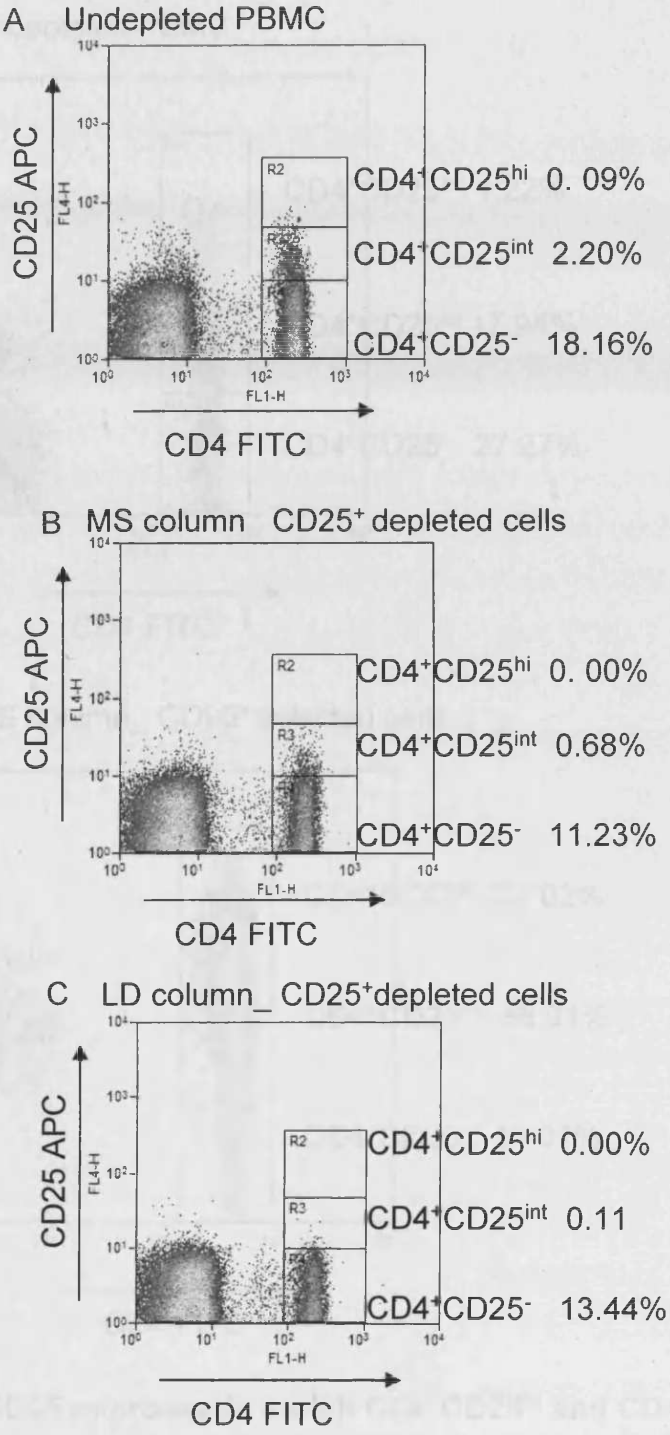


Figure 4.7. Depletion of CD25⁺ cells from PBMC
The efficiency of two methods for depleting $CD25^{hi}$ Tregs from PBMC was investigated. PBMC were incubated with either 10µl (method 1) or 20µl (method 2) of anti-CD25 microbeads per 10^7 cells and passed down a MS or LD column respectively. PBMC were stained with anti-CD4 FITC and –CD25 APC specific mAbs and the percentage of $CD4^+CD25^{hi}$, $CD4^+CD25^{int}$ and $CD4^+CD25^-$ in undepleted PBMC (A) and PBMC passed down an MS (B) or LD column (C) was then determined.

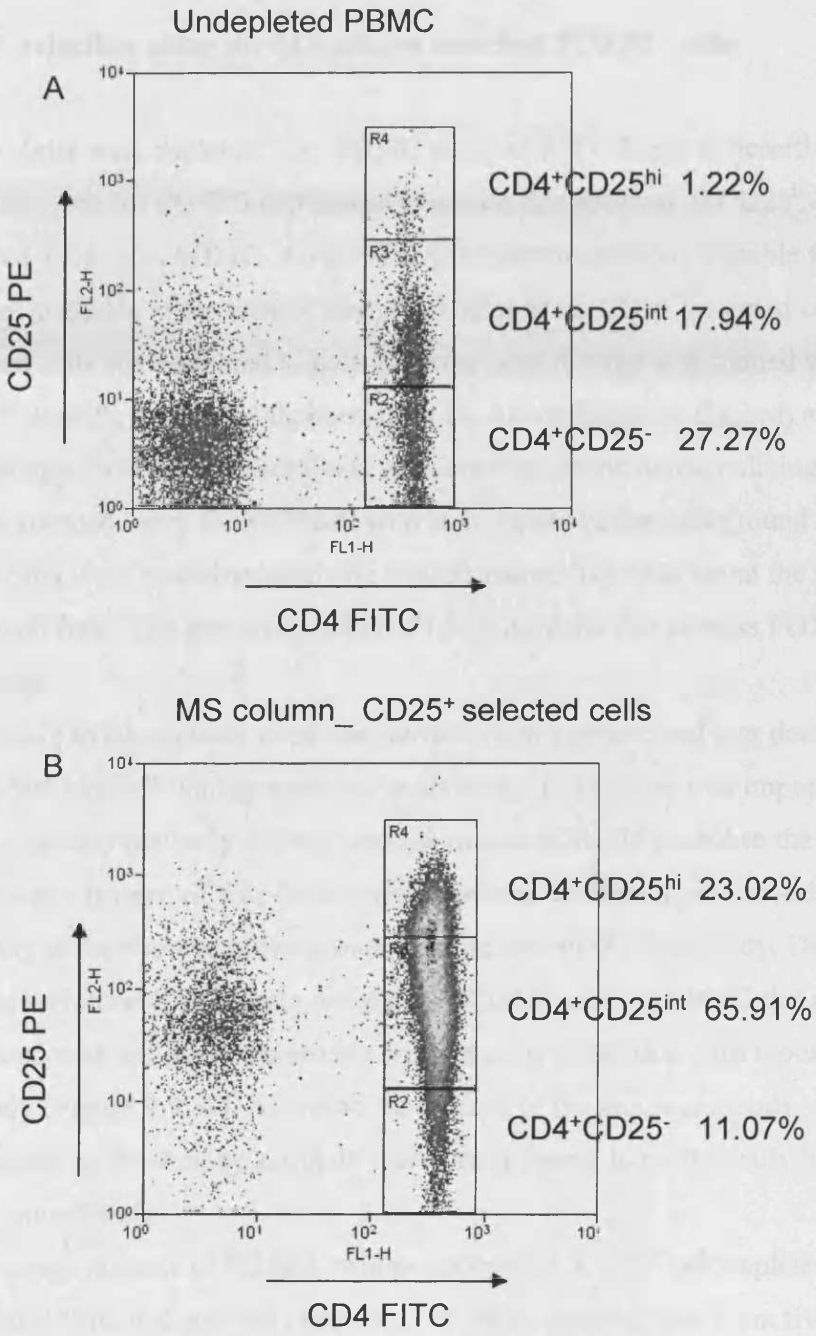


Figure 4.8. CD25 microbeads enrich $CD4^+CD25^{hi}$ and $CD4^+CD25^{int}$ PBMC

PBMC were incubated with 10μl of CD25 microbeads and passed through an MS column. Undepleted (A) and $CD25^+$ selected cells (B) were stained with anti-CD4 FITC and –CD25 PE antibodies.

$CD25^+$ selection using the MS column enriched $FOXP3^+$ cells

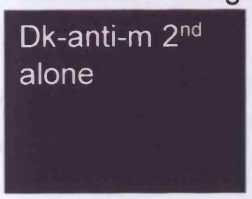
$CD25^+$ cells were depleted from PBMC using an MS column as described above and were analysed for FOXP3 expression to ensure that removal of $CD25^{hi}$ cells effectively depleted Treg from PBMC. An anti-FOXP3 specific antibody suitable for flow cytometry was not available at the time of analysis. Undepleted, $CD25^+$ selected cells and $CD25^+$ depleted cells were adhered to poly-L-lysine coated slides and stained with an anti-FOXP3 specific antibody (obtained from Dr Alison Banham, Oxford) and analysed by microscopy. Five high-power fields were analysed using density slicing software. Density slicing counted every $FOXP3^+$ cell with staining above the background intensity in each field. Cells were co-stained with the nuclear marker DAPI to count the total number of cells each field. The percentage of DAPI positive cells that express FOXP3 was then calculated.

The anti-FOXP3 antibody used was derived from a mouse and was detected using an Alexa 594 labelled donkey anti-mouse antibody. It therefore was important to first check that the donkey antibody did not bind the mouse antibody coated to the CD25 microbeads. Images of five fields were examined for binding of secondary anti-mouse antibody in the absence of the primary mouse anti-FOXP3 antibody. $DAPI^+$ cells were present within each field (data not shown). $CD25^+$ selected PBMC did not stain when incubated with the donkey antibody without prior incubation with mouse-anti-FOXP3 antibody (Figure 4.9. A). Access to the epitope of the mouse antibody which is recognised by the donkey antibody therefore appeared to be sterically blocked by the CD25 microbeads.

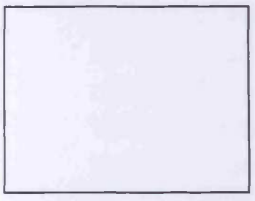
The average number of $FOXP3^+$ within undepleted, $CD25^+$ cell depleted and $CD25^+$ cell selected PBMC is displayed (Table 4.1). A representative field from five fields used to generate the percentage of $FOXP3^+$ cells in each fraction is presented (Figure 4.9 B-D respectively). The two photographs in the left and right upper panels of figures B-D were used to generate density slices of cells. Density slices of images are displayed in the lower panels of images, where each black dot represents one cell with staining above

A. CD25 selected PBMC Alexa 594 secondary control

Fluorescent image



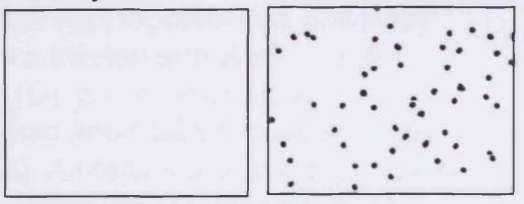
Density slice



C. CD25 depleted PBMC FOXP3

Fluorescent image

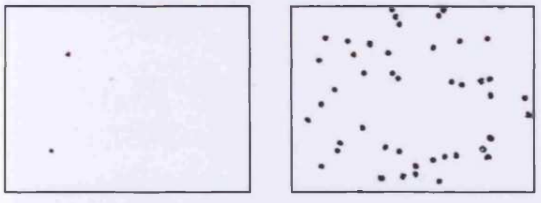
Density slice



B. Undepleted PBMC

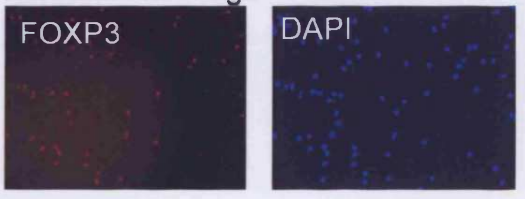
Fluorescent image

Density slice



D. CD25 selected PBMC FOXP3

Fluorescent image



Density slice

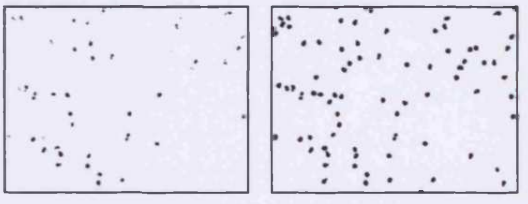


Figure 4.9. Miltenyi CD25 microbeads remove $CD25^{+}FOXP3^{+}$ cells from PBMC.
Purified PBMC were incubated with Miltenyi CD25 microbeads and passed through a Miltenyi MS positive selection column. CD25 selected (A, D), CD25 depleted (B) and undepleted (C) PBMC fractions were adhered to poly-L-lysine coated slides. Cells were stained with mouse anti-FOXP3 antibody, Donkey anti-mouse Alexa 594 and DAPI nuclear marker. The fluorescent intensity of positively stained cells was determined using density slicing software. Images of fluorescent staining are displayed in top panels and density slices are shown in the bottom panels, where each spot represents a positively stained cell. CD25 selected PBMC were stained with donkey-anti-mouse Alexa 594 secondary antibody (Dk-anti-m-2nd) alone to observe if secondary antibody recognised anti-CD25 mouse antibody bound to miltenyi CD25 microbeads (A). All cells stained with the DAPI nuclear marker which allowed the frequency of $FOXP3^{+}$ cells to be determined. Five high powered images were taken of each fraction to provide a the percentage of $FOXP3^{+}$ cells present (Table 4.1). A representative image of FOXP3 staining by undepleted (B), CD25 depleted (C) and CD25 selected PBMC (D) is displayed.

Slide	No. DAPI$^+$ cells	No. FOXP3$^+$ cells	% FOXP3$^+$
Undepleted PBMC count 1	40.0	1.0	2.5
Undepleted PBMC count 2	46.0	2.0	4.3
Undepleted PBMC count 3	41.0	2.0	4.9
Undepleted PBMC count 4	43.0	0.0	0.0
Undepleted PBMC count 5	41.0	1.0	2.4
		Average	**2.83**
CD25$^+$ selected PBMC count 1	77.0	22.0	28.6
CD25 $^+$ selected PBMC count 2	82.0	36.0	43.9
CD25 $^+$ selected PBMC count 3	75.0	34.0	45.3
CD25 $^+$ selected PBMC count 4	66.0	31.0	47.0
CD25 $^+$ selected PBMC count 5	50.0	30.0	60.0
		Average	**44.96**
Depleted PBMC count 1	49.0	1.0	2.0
Depleted PBMC count 2	55.0	0.0	0.0
Depleted PBMC count 3	54.0	1.0	1.9
Depleted PBMC count 4	53.0	0.0	0.0
Depleted PBMC count 5	42.0	0.0	0.0
		Average	**0.78**

Table 4.1. FOXP3$^+$ cell frequency in PBMC following the depletion of CD25$^+$ cells from PBMC
PBMC were incubated with CD25 microbeads and depleted of CD25^{hi} cells using an MS column. PBMC, CD25^{hi} depleted and CD25^{hi} selected PBMC were incubated with anti FOXP3 antibody and visualised by microscopy. Cells were co-stained with the DAPI nuclear marker to determine the total number of cells present. Five high powered images were visualised and used to calculate the percentage of DAPI$^+$ cells that expressed FOXP3. Representative examples of staining are displayed (Figure 4.9). DAPI$^+$FOXP3$^+$ and DAPI$^+$FOXP3$^-$ cells were counted using density slicing software, which highlighted cells that fluoresced above background intensity.

background intensity. The total number of cells in each field was determined by the number of dots identified using the DAPI nuclear stain (lower right panel of B-D). The number of $FOXP3^+$ cells is highlighted in a similar fashion (upper right panel of B-D). Transposable images of DAPI and FOXP3 staining were taken on the same field of cells. Overall, the analysis revealed that approximately 75% of $FOXP3^+$ cells were depleted. The remaining $FOXP3^+$ cells within $CD25^+$ cell depleted PBMC may be due to $CD4^+CD25^{int}$ PBMC that express FOXP3. $FOXP3^+$ cells were substantially enriched within $CD25^+$ cell selected PBMC, whereby approximately 50% of $CD25^+$ selected PBMC were $FOXP3^+$ (Table 4.1)

$CD25^+$ selection using the MS column removed Treg from PBMC

It was important to confirm that the process of positive selecting $CD25^+$ cells from PBMCs effectively removed Tregs. A functional suppression assay was therefore conducted to observe whether Tregs were contained within the cell fraction removed by CD25 microbeads. $CD25^{hi}$ cells were depleted from PBMC using CD25 microbeads in a similar manner to described previously with an additional $CD4^+$ purification step included prior to CD25 depletion. The $CD4^+$ purification step was performed to enable an analysis of the capacity of $CD4^+CD25^{hi}$ depleted T cells (and to avoid contamination of $CD4^-CD25^+$ cells) to suppress $CD4^+CD25^-$ cells and thereby replicate the accepted method to show suppressive function described by others (Baecher-Allan, *et al.*2001). Similar to cell fractions obtained by depleting CD25 cells from whole PBMC, the CD25 microbeads depleted the entire $CD4^+CD25^{hi}$ population and a large proportion of $CD4^+CD25^{int}$ cells to obtain $CD4CD25^{int/-}$ PBMC (Figure 4.10. A-C). CD25 microbeads bound to the cell surface of $CD25^{hi/int}$ cells sterically blocked recognition of anti-CD25 APC antibody (data not shown); however, CD25 microbeads used to deplete $CD25^{hi}$ cell from $CD4^+$ cell preparations were identical to those used to deplete $CD25^{hi}$ cells from whole PBMC. Previous staining using another anti-CD25 antibody that recognised a separate CD25 epitope has shown that the $CD25^{hi}$ fraction obtained by CD25 microbeads did indeed contain an enrichment of $CD25^{hi/int}$ cells (Figure 4.8. B).

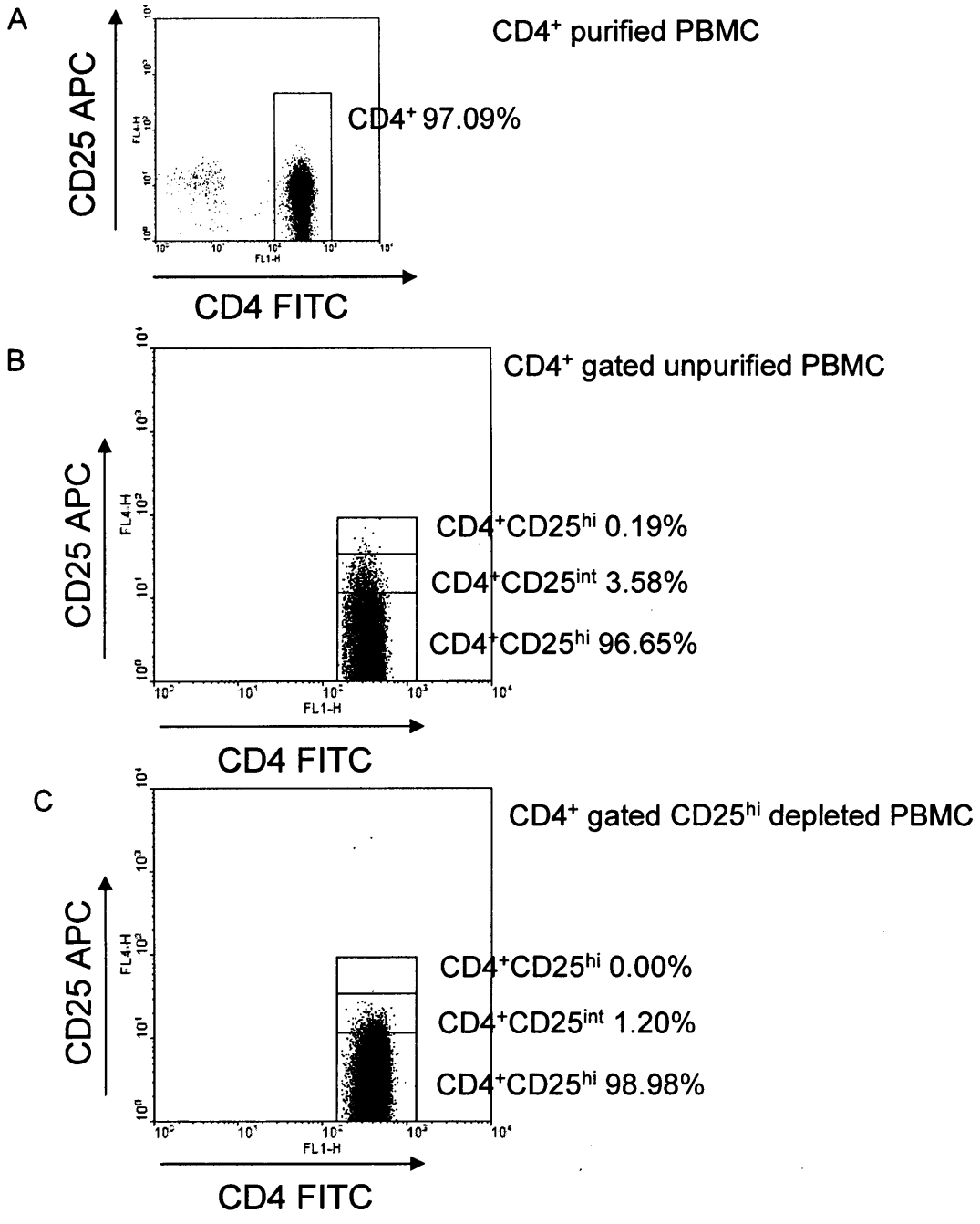


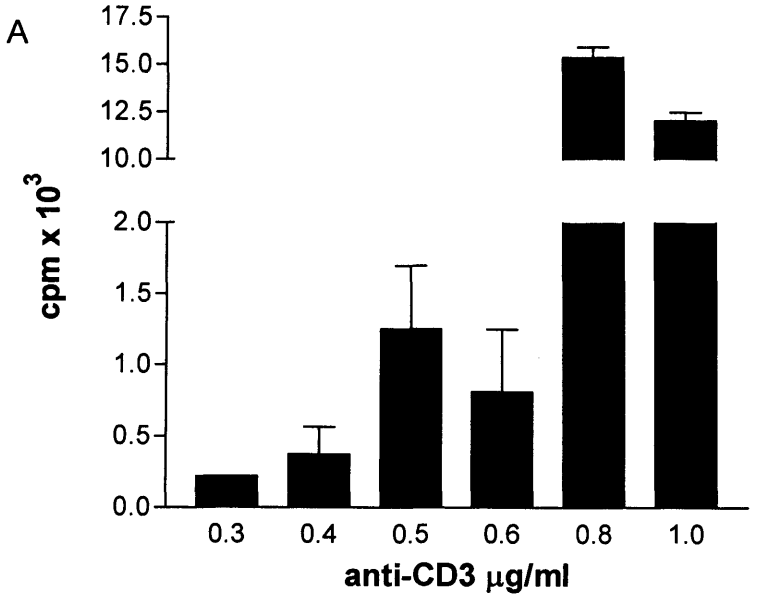
Figure 4.10. CD25 microbeads deplete $CD25^{hi}$ cells from purified $CD4^+$ PBMC

$CD4^+$ PBMC were purified by negative selection using miltenyi magnetic beads and passing cells through a miltenyi LD column to obtain a 97% pure population (A). $CD4^+$ PBMC were subsequently separated into $CD4^+CD25^{int/hi}$ and $CD4^+CD25^{int/-}$ fraction by incubating with CD25 miltenyi microbeads and passing cells through a MS column. $CD4^+$ purified (A), unpurified and $CD4^+CD25^{int/hi}$ depleted PBMC were stained with anti-CD4 FITC and –CD25 APC antibodies. Unpurified (B) and $CD4^+CD25^{hi}$ (C) depleted PBMC gated on $CD4^+$ cells are displayed.

It was therefore established that incubation of PBMC with CD25 microbeads removed a similar population of $CD25^{hi/int}$ cells, regardless of whether a prior $CD4^+$ cell purification step was included.

Experiments were performed to optimise a functional suppression assay to test the suppressive function of the $CD25^{hi/int}$ cell fraction (referred from now on as $CD25^{hi}$) eluted from the MS column. The non-selected population ($CD4^+CD25^{int/-}$ PBMC) plated in wells coated with a range of plate bound anti-CD3 antibody and anti-CD28 antibody (Figure 4.11. A-B). In order to analyse the suppressive capacity of Treg, it was important to avoid stimulating $CD4^+CD25^{int/-}$ cells to an extent that might render them unresponsive to Treg suppression. Stimulation with 0.5μg/ml anti-CD3 antibody and 1μg/ml anti-CD28 antibody was considered to induce a low yet consistent cellular proliferation and was chosen for subsequent suppression assays. $CD4^+CD25^{int/-}$ cells received this polyclonal stimulation and were incubated with various ratios of $CD4^+CD25^{hi}$ cells. $CD4^+CD25^{hi}$ cells demonstrated robust suppression of $CD4^+CD25^{int/-}$ cells by inhibiting approximately 80% of proliferation at a 1:1 ratio and 40% of proliferation at 1:10 ratio, whilst $CD4^+CD25^{hi/int}$ cells were anergic (Figure 4.11 C). These results show that the use of CD25 microbeads and separation outlines above effectively removed a mainly a Treg population from PBMC.

Overall, the method of depleting Treg by passing PBMC though an MS column, prior to incubation with CD25 microbeads was demonstrated as the most effective method to deplete Treg from PBMC. The MS column removed $CD4^+CD25^{hi}$ cells most efficiently and in doing so removed the majority of $FOXP3^+$ cells and those cells that demonstrated robust suppressive capacity. This method was therefore chosen for the routine removal of $CD4^+CD25^{hi}$ cells from PBMC. Depletion of $CD4^+CD25^{hi}$ cells from patient PBMC samples was checked on each occasion by flow cytometry using anti-CD4 and –CD25 specific antibodies.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17.5
15.0
12.5
10.0
2.0
1.5
1.0
0.5
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cpm x 10^3
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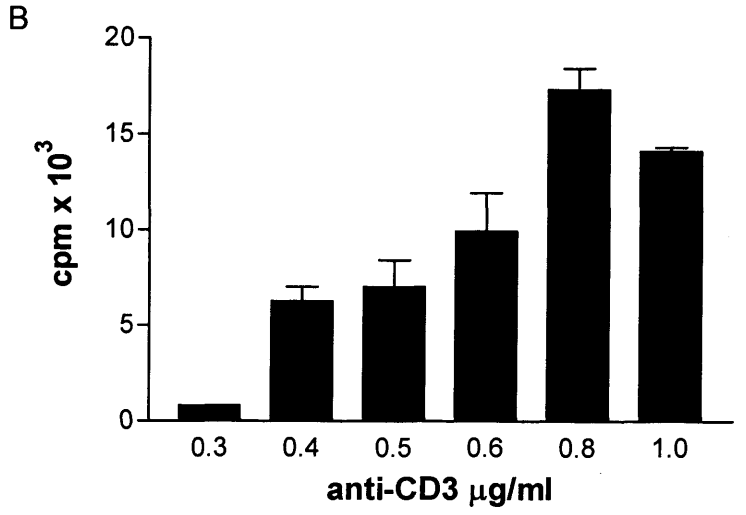
0.4
0.5
0.6
0.8
1.0
anti-CD3 μg/ml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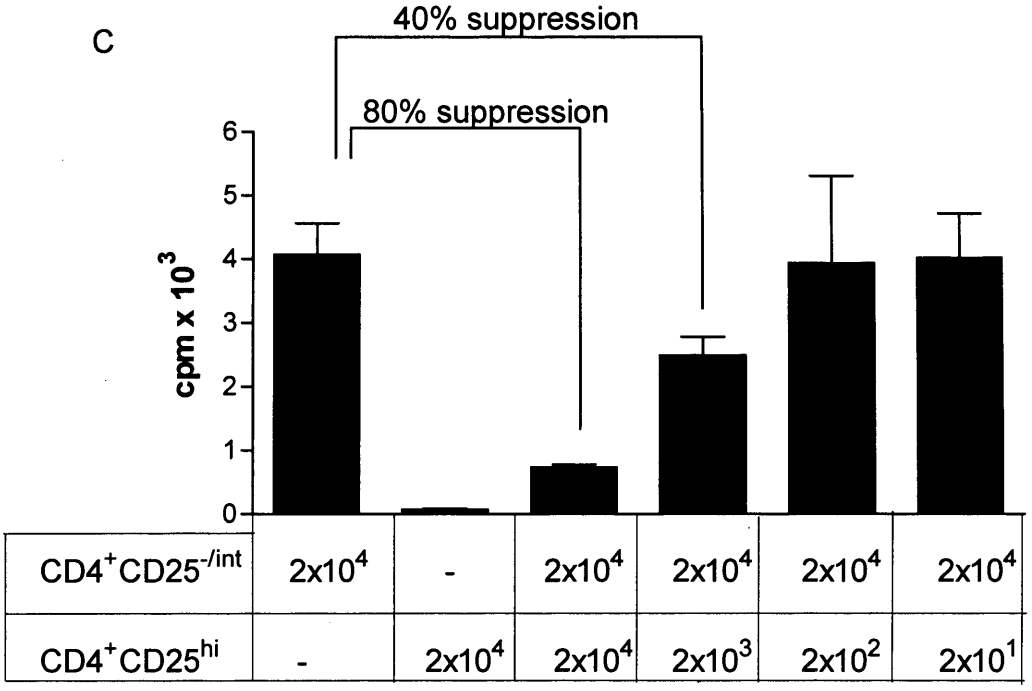


Figure 4.11. Regulatory function is restricted to the $CD4^+CD25^{hi/int}$ cell fraction purified by miltenyi microbeads
$2x10^4$ purified $CD4^+CD25^{int/-}$ cells were incubated with various concentrations of plate bound anti-CD3 antibody and either 0.5 μg/ml (A) or 1.0 μg/ml anti-CD28 antibody (B). $2x10^4$ $CD4^+CD25^-$ cells were subsequently incubated with various ratios of $CD^+CD25^{int/hi}$ cells, 0.5μg/ml plate bound anti-CD3 and 1 μg/ml anti-CD28 antibody (C). Cells were incubated for 3 days before incubating cells for 18 hours with [Methyl-3H]-thymidine.

4.2.5. Optimisation of T cell proliferation assays

The aim of future studies of patients with colorectal cancer was to compare T cell responses to control and tumour antigens using both an *ex-vivo* IFN-γ release ELISpot assay and proliferation assay (Chapter 5).

The method used to perform *ex-vivo* IFN-γ ELISpot assays had already been established within the laboratory (see Materials and Methods), thus the focus of the following work was to optimize T cell proliferation assays. Antigen specific T cell proliferation was analyzed by stimulating PBMC with antigen for six-days before the addition of [Methyl-3H]-thymidine. Incorporation of [Methyl-3H]-thymidine into the DNA of dividing cells reflects the extent of T cell proliferation and is measured as cpm in a beta counter. The objective of the following experiments was to identify a method whereby background proliferation was minimised in order to facilitate identification of antigen-specific responses. For this purpose, PBMC were either left unstimulated (background proliferation) or stimulated with the common recall antigens, purified protein derivative (PPD) and influenza protein haemagglutinin (HA). The recall antigens PPD and HA were chosen, as the majority of the UK population has been exposed to one or either of these antigens via the BCG vaccination program and seasonal influenza infections. The parameters investigated were 1) the duration of pulsing with [Methyl-3H]-thymidine, 2) the use of different concentrations of pooled AB serum and 3) the use of autologous versus pooled AB serum.

Comparison of 6 and 18 hour incubation with [Methyl-3H]-thymidine

PBMC were stimulated for six-days with 1μg/ml HA or PPD protein in 10% pooled AB serum RPMI. [Methyl-3H]-thymidine was added to PBMC during the final 6 or 18 hours of incubation in order to determine whether a lower incubation time with [Methyl-3H]-thymidine would reduce the background counts within unstimulated PBMC. Cells were harvested and cpm were analysed (Figure 4.12.). The PBMC within this experiment mounted robust responses to the PPD and HA recall antigens and no difference was

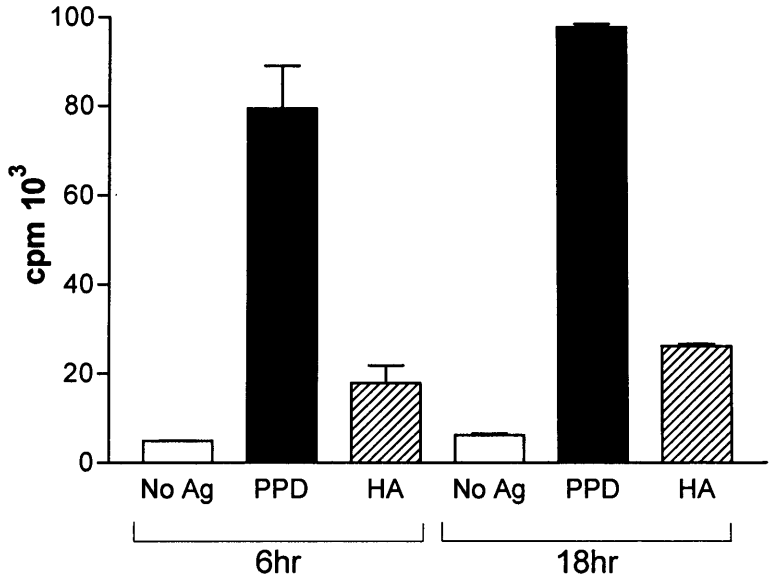


Figure 4.12. Comparison of 6hr v 18hr incubation with tritiated thymidine
$2x10^5$ PBMC were stimulated for six days with 1μg/ml HA or PPD protein. PBMC were incubated in 10% pooled AB serum RPMI. Conditions were analysed in triplicate wells. The data is representative of two experiments.

observed between cpm obtained following 6 hour and 18 hour incubation with [Methyl-3H]-thymidine (Figure 4.12.). However, the cpm of unstimulated cells was relatively high at approximately 8 $x10^3$ cpm, which would be too high to observe subtle antigen specific responses. Thus further optimisation of the assay was required.

Comparison of the use of 5% and 10% pooled AB serum RPMI.

Background proliferation may reflect proliferation of PBMC in response to proteins present within the serum used in the assays. The concentration of serum used in the assays was therefore titrated. PBMC were stimulated for six-days with 1μg/μl HA or PPD protein and incubated in RPMI supplemented with either 5% or 10% pooled AB serum. After six-days, [Methyl-3H]-thymidine was added for a further 18 hours. PBMC that were incubated in RPMI/5% pooled AB serum had reduced background proliferation compared to PBMC that were incubated in RPMI/10% pooled AB serum, whilst the ability of PBMC to respond to PPD and HA antigen was maintained (Figure 4.13.). This shows that antigen specific responses could be measured effectively 5% serum RPMI; however, the background proliferation remained unacceptably high.

Comparison of the use of autologous serum with pooled AB serum

Pooled AB serum provides a readily available source of nutrition to PBMC and should not trigger background proliferation to the same extent as foetal calf serum. The use of pooled AB serum is based on the premise that a particular antigen present within the serum from one individual, that induced background proliferation of PBMC, would be diluted by the serum obtained from other donors; however, in the fore mentioned titrations, the background proliferation using pooled AB serum was not reduced to an extent that would enable low level specific immune responses to be detected. The proliferation of unstimulated cells was therefore tested using autologous serum. PBMC

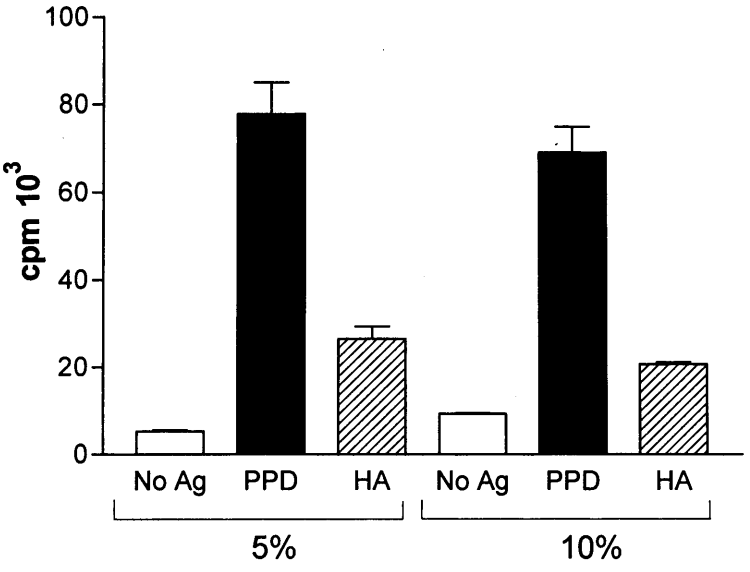


Figure 4.13. Comparison of the use of 5% v 10% pooled AB serum $2x10^5$ PBMC were stimulated for six days with 1μg/ml HA and PPD protein. PBMC were incubated in either 5% or 10% pooled AB serum RPMI. Conditions were analysed in triplicate wells. Data is representative of two experiments.

were stimulated for six-days with 1μg/μl HA or PPD protein and incubated in either 5% pooled AB serum or 5% autologous serum RPMI. [Methyl-3H]-thymidine was added for a further 18 hours. The use of autologous serum reduced the non-specific background of PBMC, whilst the ability of PBMC to respond to PPD and HA antigen was maintained (Figure 4.14.).

All six-day proliferation assays were subsequently performed using 5% autologous serum and PBMC were incubated with Methyl-3H]-thymidine for 18 hour before the assays were harvested.

Titration of recall antigens

Routine use of PPD and HA antigen within proliferation assays of patient PBMC demanded that the quantity of antigen used in assays was restricted to realistic concentrations to ensure stocks were not exhausted, yet was still high enough to induce robust proliferation. Use of one master stock of antigen minimised cost and was also expected to enhance the standardisation of assays between separate patient samples. The procurement of a second batch of antigen might alter antigen responses. Once the non-specific background proliferation in six-day proliferation assays was reduced to an acceptable level, the concentration of PPD and HA protein used in assays was titrated. PBMC were stimulated with increasing concentrations of PPD and HA protein for six-days with antigen in RPMI/5% autologous serum. [Methyl-3H]-thymidine was added for a further 18 hours. 1 μg/ml protein induced robust proliferation and the magnitude of responses to PPD and HA antigens did not increase significantly with increasing concentrations of protein tested (Figure 4.15.). A relatively small increase of proliferation was observed by cells incubated with 2μg/ml HA protein compared to 1μg/ml protein (Figure 4.15). Routine use of all antigens at 1μg/ml was considered to be acceptable and further titration of concentration was not considered to be necessary.

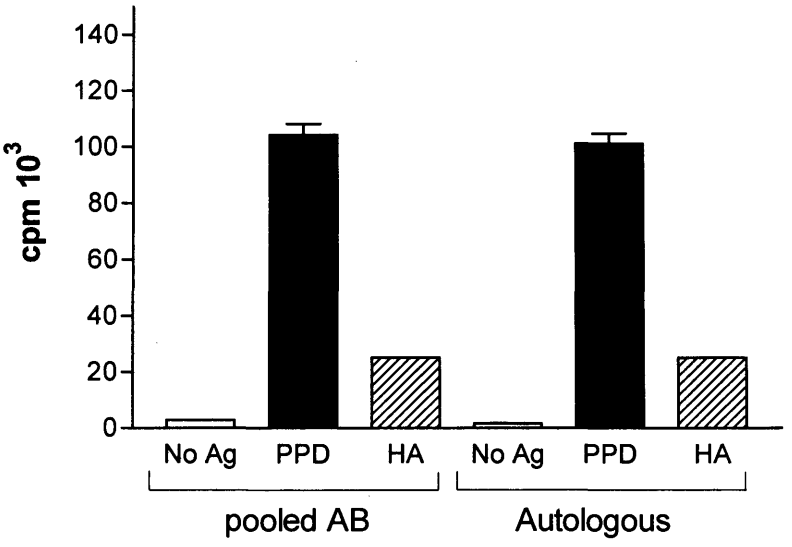


Figure 4.14. Comparison of the use of autologous serum with pooled AB serum

$2x10^5$ PBMC were stimulated for six days with 1μg/ml HA and PPD protein. PBMC were incubated in either RPMI containing 5% autologous or pooled AB serum. Conditions were analysed in triplicate wells. The data is representative of two experiments.

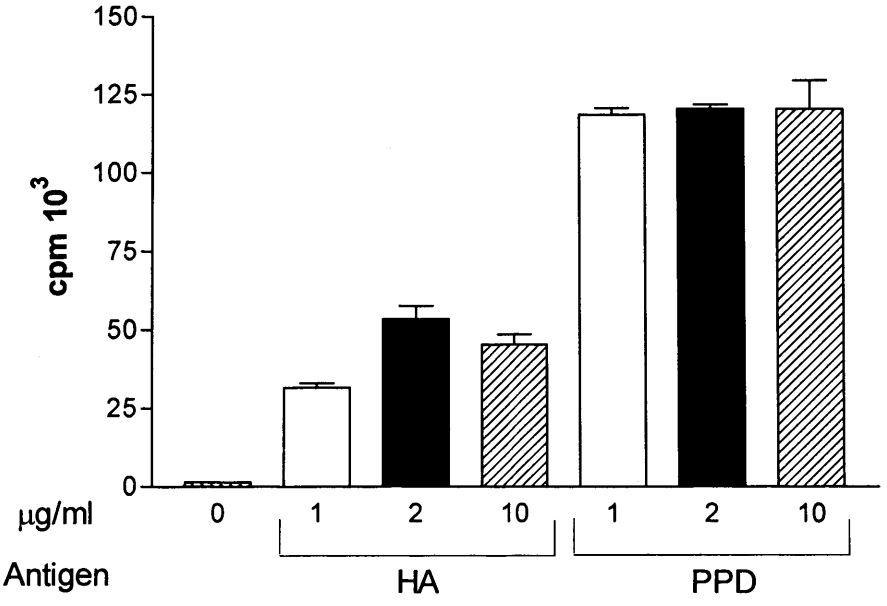


Figure 4.15. Titration of recall antigens
2 x10^5 PBMC were stimulated with increasing concentrations of PPD and HA protein for six days with antigen in 5% autologous serum RPMI. Conditions were analysed in triplicate wells. Data is representative of two experiments.

4.3. Discussion

This chapter describes preliminary work performed to establish techniques to stain PBMC and identify Tregs. This entailed the identification of Tregs as $CD4^+CD25^{hi}$ cells (Baecher-Allan, *et al.*2001) that express CTLA-4 (Jonuleit, *et al.*2001) and CD45RO (Baecher-Allan, *et al.*2001, Jonuleit, *et al.*2001). Subsequently, experiments were performed to identify the optimal technique for removal of $CD4^+CD25^{hi}$ cells and the minimum amount of $CD4^+CD25^{int/-}$ cells. It was considered important to preserve activated $CD4^+CD25^{int}$ cells to avoid the removal of conventional effector T cells that might otherwise obscure an enhancement of responses by the depletion of Treg. This premise was especially important when considering that CRC patients may contain circulating $CD4^+CD25^{int}$ anti-tumour T cells that recognise tumour antigens (Chapter 5). A number of methods to deplete $CD25^{hi}$ cells were considered. FACS sorting was originally used to purify $CD4^+CD25^{hi}$ Treg (Baecher-Allan, *et al.*2001). When considering the relatively large cohort of subjects to be included in this study, FACS sorting samples was deemed to be prohibitively expensive. In addition, FACS sorting is a relatively abrasive method of cell purification and the process may alter the functional characteristics of the cells. Depletion of $CD4^+CD25^{hi}$ cells by labelling PBMC with anti-CD25-specific magnetic beads was tested. Initial depletion experiments were performed using Dynal magnetic beads coated with anti-CD25 specific antibody; however, these beads failed to reproducibly deplete $CD4^+CD25^{hi}$ PBMC and, due to the large size of these beads, it proved difficult to analyse the $CD25^+$ selected cell fraction obtained. Depletion trials were subsequently performed using Miltenyi magnetic beads coated with anti-CD25 specific antibody. This technique required passing cells through an inert column in a magnetic field after incubation with beads in order to remove labelled cells. Two columns, termed MS and LD columns were tested. The MS column was designed to derive a high purity of $CD25^+$ selected cells by positive selection. The LD column (i.e. negative selection) was eliminated as a method since all $CD25^+$ cells were depleted using this column. The MS column however only removed a proportion of the $CD4^+CD25^{int}$ cells but all of the $CD4^+CD25^{hi}$ cells. The $CD4^+CD25^{hi}$ selected fraction that was

depleted from PBMC was confirmed to be markedly enriched with suppressive function and to be almost completely anergic.

FOXP3 is currently the only known unique marker of Treg and is believed to be expressed by all Treg of the naturally occurring $CD4^+CD25^+$ cell lineage (Fontenot, *et al.*2005c, Sakaguchi.2004). The $CD4^+CD25^{hi}$ cells depleted fraction was therefore stained for $FOXP3^+$ cells to analyse the efficiency of Treg depletion using the MACS-based method described above. An anti-FOXP3 specific antibody suitable for flow cytometry was not available at the time of this analysis and therefore cells were stained and observed by histology. This technique indicated that the CD25 microbeads removed approximately 75% of $FOXP3^+$ cells. The fact that the MS column removed the entire $CD4^+CD25^{hi}$ population indicated that the remaining $FOXP3^+$ cells must reside in the $CD4^+CD25^{int}$ population. When considering that previous work has determined that $CD4^+CD25^{int}$ cells do not have suppressive function (Baecher-Allan, *et al.*2001) and a proportion of anti-tumour T cells may have the $CD4^+CD25^{int}$ phenotype, it was deemed appropriate to use the MS column to deplete $CD4^+CD25^{hi}$ cells and retain as many $CD4^+CD25^{int}$ cells as possible.

After methods to identify and deplete Treg had been established, it was important to optimise sensitive functional T cell assays capable of measuring antigen specific immune responses. Since one main aim of the study was to investigate tumour antigen specific immune responses which are expected to be generally low in frequency, it was deemed necessary to perform sensitive assays capable of measuring *ex-vivo* IFN-γ release and six-day proliferation responses, elicited by T_{EM} and T_{CM} memory cells respectively. *Ex-vivo* IFN-γ release assays had been optimised previously and are described to efficiently measure T_{EM} responses (Godkin, *et al.*2002). Measurement of six-day proliferation responses needed to be optimised. Initial experiments used pooled AB serum but high background proliferation elicited by the serum could not be reduced sufficiently to examine antigen specific responses efficiently. Various batches of serum were tested without success (data not shown) and it was therefore deemed necessary to perform experiments using autologous serum. By using autologous serum, six-day proliferation assays were deemed to be as sensitive as possible and were used, alongside *ex-vivo* IFN-γ

release assays, to measure antigen specific responses of healthy controls and CRC patients (chapter 5). An important aim of this study was to establish the extent that Treg inhibited antigen-specific immune responses. Following the experiments described in this chapter, suitable experimental methods were established for subsequent studies of immune responses in patients with CRC. Specifically, these methods were used, as described in detail in the next chapter, to compare the frequency and activity of Treg in groups of healthy controls and CRC patients (chapter 5)

5. Analyses of $CD4^{+}CD25^{hi}$ Treg in PBMC of patients with colorectal carcinoma

5.1 Introduction

This chapter describes analyses of Tregs in human malignancy. Previous research in mice has demonstrated an enrichment of Treg within tumours (Needham, *et al.*2006, Ghiringhelli, *et al.*2005a, Ghiringhelli, *et al.*2005b, Ko, *et al.*2005) and a role for Treg in suppressing anti-tumour immunity (Sutmuller, *et al.*2001, Shimizu, *et al.*1999, Onizuka, *et al.*1999, Golgher, *et al.*2002). It is possible therefore that Tregs also contribute to the progression of human malignancy.

The aim of the work performed in this chapter therefore was to enumerate Tregs in patients with CRC in order to determine whether Treg numbers are elevated in the patient group compared to controls. Studies in mice have unequivocally demonstrated the importance of Treg in suppressing autoreactive T cells. It was therefore of interest to enumerate Treg frequencies in patients with CRC. The frequency of Treg in PBMC obtained from CRC patient and age matched controls were assessed, using the methods described in Chapter 4, in order to test the hypothesis that cancer is associated with an elevation in Treg numbers.

Subsequently experiments were performed to analyse antigen-specific T cell responses in CRC patients. These experiments were carried out to determine 1) whether immune responses to common recall antigens were impaired in CRC patients, 2) whether Tregs suppressed immune responses to these antigens and 3) whether anti-tumour immune responses, detected in CRC patients were subject to suppression by Tregs. These experiments were performed using *ex-vivo* IFN-γ release and six-day proliferation assays as described in Chapter 4.

With respect to the analyses of immune responses to tumour antigens, two antigens, namely 5T4 and carcinoembryonic antigen (CEA) were chosen for study.
CEA is a GPI-anchored 180kDa protein that is extensively glycosylated with approximately 60% of its total molecular weight consisting of carbohydrate moiety

(reviewed in (Thompson, *et al.*1991)). CEA was originally identified as colorectal tumour antigen and was subsequently also found within liver metastasis. More sensitive detection methods have since established that CEA is expressed at low levels by normal colonic mucosa and can be found with secretions of the gastrointestinal tract, including within gastric juice and saliva and within amniotic fluid. Approximately 60% of CRC patients have an elevated level of circulating CEA, whereby a CEA level >10ng/ml is a strong indicator of malignancy in the absence of other epithelial inflammation (healthy subjects have a circulating CEA with 0-2.5ng/ml) (reviewed in (Shively, *et al.*1985)). An elevated CEA level is often used to assess remission of colorectal cancer following resection and a raised CEA level has also been associated with several other malignancies, including breast, lung and pancreatic cancer (Shively, *et al.*1985).

5T4 is a heavily glycosylated 72kDa protein that is normally expressed on placental trophoblast and a variety of malignancies, including CRC, gastric, renal and ovarian cancer (Southall, *et al.*1990, Starzynska, *et al.*1992, Starzynska, *et al.*1994) but has a limited expression on normal tissue. The 5T4 protein has been shown to disrupt cell-cell adhesion and to enhance the motility of malignant cells and is associated with an increased risk of metastasis (Carsberg, *et al.*1996). One study examined 5T4 expression in 72 CRC patients and established that 85% of colorectal tumours express 5T4 and staining was more frequent in advanced disease (spread of disease) but did not correlate with the grade (tumour cell differentiation) (Starzynska, *et al.*1992). A later study using the same 72 subjects grouped patients according to the stage of disease and analysed expression of 5T4 by tumours. This analysis showed 5T4 expression is associated with worse prognosis ($p< 0.001$) and is indicator of return of malignant disease after potentially curative surgery (Starzynska, *et al.*1994). 5T4 staining was observed in either both tumour cells and surrounding stroma (45%) or just in stroma (40%) and in these cases the intensity of stoma 5T4 staining diminished with distance from tumour cells. 5T4 expression was not detected in healthy epithelial tissue (Starzynska, *et al.*1992). These results show that 5T4 expression is restricted to individuals with malignancy and therefore would not be expected to be expressed by healthy individuals.

A number of studies have demonstrated 5T4-specific $CD8^+$ T cell responses of PBMC in healthy controls and CRC patients (Redchenko, *et al.*2006, Smyth, *et al.*2006b). One

study performed *ex-vivo* IFN-γ release ELISpots to observe 5T4 specific activation of $CD4^+$ depleted PBMC incubated with 5T4 peptide pools (Redchenko, *et al.*2006). One healthy control from a cohort of 30 healthy volunteers produced a 5T4 specific response and 5T4 specific $CD8^+$ T cells clones were subsequently generated from this individual. This research was able to identify the HLA restriction (HLA-Cw7) of this response and researchers proceeded to vaccinate HLA-$Cw7^+$ CRC patients with a MVA (modified vaccinia Ankara) encoding the 5T4 protein. This vaccination induced new 5T4 specific $CD8^+$ responses in 4 patients (25%) (Redchenko, *et al.*2006). Other research has observed a higher rate of 5T4 specific responses than previous studies in $CD8^+$ PBMC isolated from healthy controls (6/8) using a more powerful stimulation regime to uncover responses *in vitro* (Smyth, *et al.*2006b). In this study, $CD8^+$ purified PBMC received multiple stimulations with DC infected with adenovirus expressing 5T4 protein. This showed that 5T4 responses mediated by controls were polyclonal and capable of killing tumour cells that expressed 5T4 (Smyth, *et al.*2006b). These results established that healthy individuals have 5T4 specific T cell populations in the absence of 5T4 antigen expression.

Due to the high expression of both CEA and 5T4 in colorectal tumours, these antigens are deemed as suitable candidate antigens for immune recognition in CRC patients, by both conventional and regulatory T cells. Very little is known about T cell responses to these antigens and whether Tregs impinge on these responses.

5.2. Results

5.2.1. Patients with colorectal carcinoma have an elevated percentage of Treg within the $CD4^+$ lymphocyte population in PBMC

The frequency of Treg within $CD4^+$ PBMC and obtained from healthy controls and CRC patients was investigated to examine whether significant differences in Treg frequency could be observed within experimental groups.

Single cell suspensions of PBMC were stained with anti-CD4 FITC -CD25 APC specific antibodies. The percentage of $CD4^+CD25^{hi}$ Tregs within $CD4^+$ PBL was determined as

described in chapter 4. (FACS analysis on a number of patients was kindly performed by Dr Clarke; and in order to ensure consistent numeration of Treg, data analysis of Treg staining by flow cytometry was performed by one operator, Dr Sarah Clarke).

There was no significant difference in the percentage of $CD4^+$ PBL between controls and CRC patients (Figure 5.1. A); however, Treg comprised a significantly higher percentage of $CD4^+$ PBL within PBMC of CRC patients compared to controls ($p= 0.002$) (Figure 5.1. B). Healthy control subjects were chosen to reflect the age of the CRC patient group as closely as possible, however the average age of the CRC group was approximately 10 years older than the control group and contained a number of subjects that were approximately 15-20 years older than the mean age of the control group. It was therefore important to investigate whether the elevated level of Treg within the CRC patient group occurred due to a number of older patients in that group, rather than the presence of malignant disease. A linear regression analysis was performed on pooled data from control and CRC patient subjects. This analysis confirmed that the level of Treg did not correlate to the age of subjects ($r^2 = 0.07$; a r^2 of 1.0 would represent a perfect positive correlation) (Figure 5.1. C). A similar analysis performed separately on control ($r^2=0.025$) and CRC patient ($r^2=0.032$) groups also revealed a lack of correlation of the level of Treg in $CD4^+$ PBMC and the age of subjects (data not shown). Other research has compared the level of Treg within relatively young (<40yr) and old subjects (>70 yr) and demonstrated an increase in Treg with age (Vukmanovic-Stejic, *et al.*2006, Trzonkowski, *et al.*2006); however, the subjects within CRC and age matched control groups examined within this report are all relatively older and together form a more homogeneous age group. This would account for why a correlation of Treg frequency with the age of subjects was not demonstrated in this study; and why the tumour burden

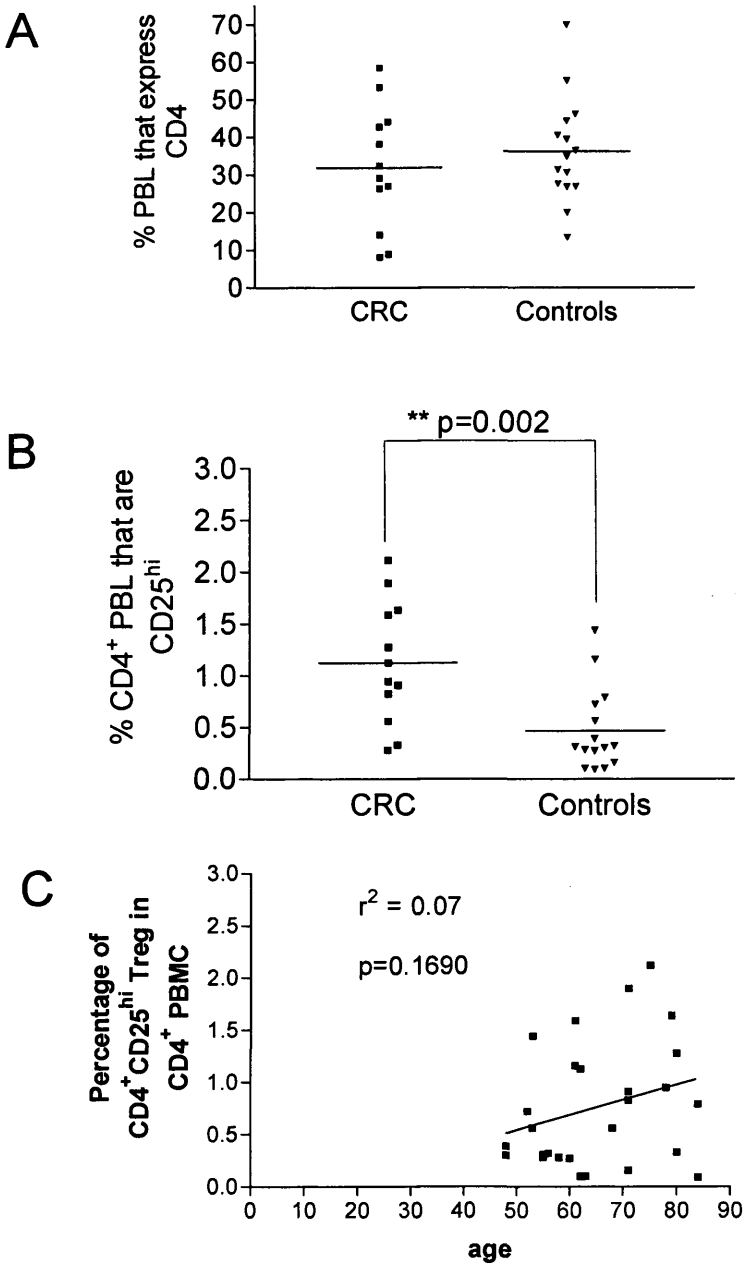


Figure 5.1. Patients with Colon Carcinoma have an elevated percentage of $CD4^+CD25^{hi}$ PBMC

PBMC were purified from CRC patients and age matched healthy controls. Cells were stained with anti-CD4 FITC and -CD25 APC specific antibodies. The percentage of $CD4^+$ (A) and $CD4^+CD25^{hi}$ cells (B) within PBMC was determined as described previously (Figure 4.2). Data was analysed with an unpaired *student's t-test*. The percentage of $CD4^+CD25^{hi}$ Treg within $CD4^+$ PBL of pooled CRC and control subjects was checked for a correlation with the age of subjects using a linear regression analysis (C).

within the CRC group is believed to account for the elevated level of Treg within this group.

5.2.2. Authentication of the Treg phenotype of $CD4^+CD25^{hi}$ gated lymphocytes

The majority of the patients included with this study were recruited before an anti-FOXP3 specific antibody was available to identify Treg and therefore, Tregs were analysed by enumerating $CD4^+CD25^{hi}$ cells in blood samples as described in Chapter 4 (Figure 4.2).

Samples were stained with three antibody mixes to examine the integrity of the $CD4^+CD25^{hi}$ Treg gated population of each subject by examining the expression of constitutive Treg markers CTLA-4 and CD45RO and the naïve cell marker CD45RA. CTLA-4 staining was not performed on a proportion of subjects as they were recruited to the project before the CTLA-4 staining procedure had been set up. However, CTLA-4 staining that was performed enabled the average CTLA-4 expression by the $CD4^+CD25^{hi}$ gated population of each experimental group to be estimated. CD45RO and CD45RA staining was performed on the majority of subjects. This method was described in detail in chapter 4. The $CD4^+CD25^{hi}$ population gated from blood obtained from CRC patients and controls conformed to the $CD45RO^{hi}$ (Figure 5.2 A), $CD45RA^{lo}$ (Figure 5.2 B), CTLA-4^{hi} (Figure 5.2 C) Treg phenotype; and therefore confirmed the integrity of the $CD4^+CD25^{hi}$ as Treg.

5.2.3. Analysis of FOXP3 expression by Flow Cytometry

FOXP3 is currently the most specific marker expressed by Treg (Reviewed in (Kim, *et al.*2006a, Sakaguchi, *et al.*2006)), however, an anti-FOXP3 specific antibody suitable for flow cytometry was not available at the time of the experimental set up of procedures to identify Treg described here. An anti-FOXP3 antibody did become available during the course of this study, following the analysis of the majority of patients analysed in the investigation, and was used to confirm that the $CD4^+CD25^{hi}$ PBMC are in fact, Treg.

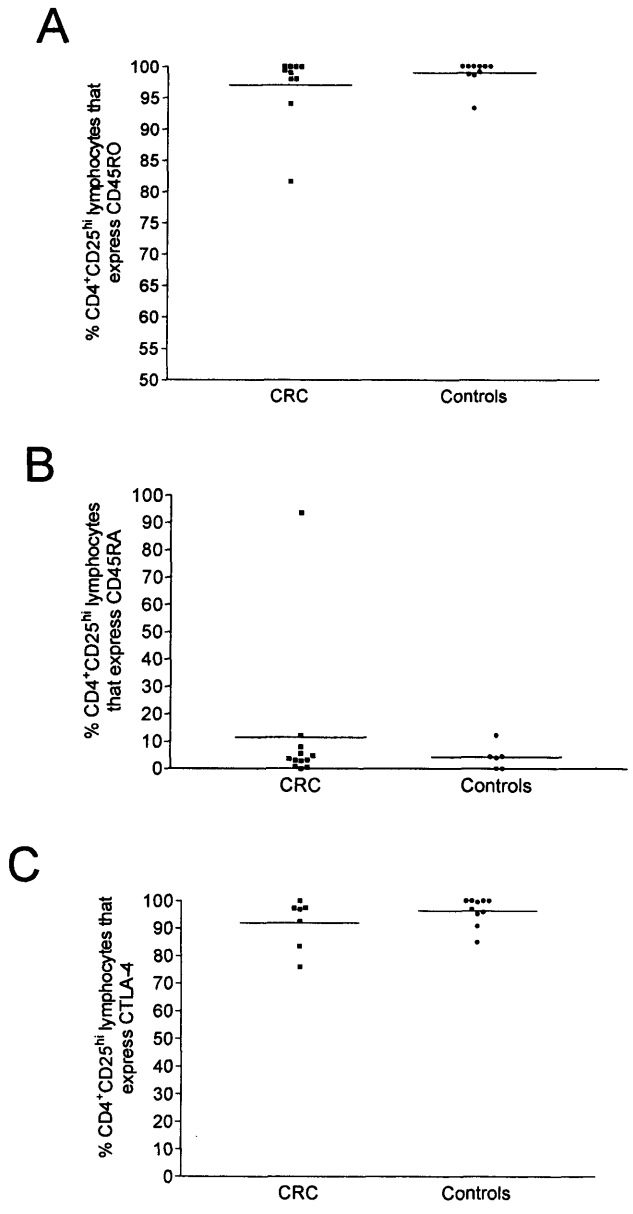


Figure 5.2. Authentication of the Treg phenotype of $CD4^{+}CD25^{hi}$ gated lymphocytes

Single cell suspensions of PBMC were stained with anti-CD4 FITC, -CLTA-4 PE, CD45RA PE and –CD45RO PE specific antibodies. $CD4^{+}CD25^{hi}$ PBL were gated and examined for the expression CD45RO (A), CD45RA (B) and CTLA-4 (C).

$CD4^+CD25^{hi}$ Treg are $FOXP3^+$

PBMC were initially stained with anti-CD4, CD25 and –FOXP3 antibodies or IgG2a FITC isotype control. PBL were distinguished by FSC/SSC (Figure 5.3. A) and all $CD4^+$ lymphocytes were gated (5.3. B). FOXP3 antibody labelled $CD4^+$ lymphocytes (Figure 5.3. C) and the isotype control antibody labelled an insignificant amount of lymphocytes (Figure 5.3.D).

PBMC were stained with anti-CD4, -CD25, -CD3 and –FOXP3 antibodies and $CD4^+CD25^{hi}$, $CD4^+CD25^{int}$ and $CD4^+CD25^-$ cells were gated. PBL were distinguished according to their FSC/SSC (Figure 5.4. A) and $CD3^+$ T cells were gated (Figure 5.4. B). $CD4^+$ T cells were identified (Figure 5.4. C) and CD25 expression was analysed (Figure 5.4. D) in order to determine the FOXP3 expression on $CD4^+CD25^{hi}$ (Figure 5.4. E), CD^+CD25^{int} (Figure 5.4. F) and $CD4^+CD25^-$ gated T cells (Figure 5.4. G). Analysis revealed that over 90% of $CD4^+CD25^{hi}$ T cells expressed FOXP3 and confirmed that the vast majority $CD4^+CD25^{hi}$ cells were Treg. Approximately 30% of $CD4^+CD25^{int}$ cells also expressed FOXP3 (Figure 5.4. F) and less than 5% of $CD4^+CD25^-$ cells expressed FOXP3 (Figure 5.4. G). Thus, although Treg are highly enriched within the $CD4^+CD25^{hi}$ population, some Treg also exist within the $CD4^+CD25^{int}$ and $CD4^+CD25^-$ compartments (Figure 5.4.). The intensity of FOXP3 expression was highest on $CD4^+CD25^{hi}$ cells (Figure 5.4. F) compared to $CD4^+CD25^{int}$ (Figure 5.4. F) and $CD4^+CD25^-$ cells (Figure 5.4. G). The level of FOXP3 expression may be directly proportional to the extent of suppressive function; however, proving this point remains difficult as it is not possible to sort lymphocytes according to FOXP3 expression as the FOXP3 staining process requires the permeablisation of cells. However, regulatory T cell have cells have been identified in the $CD4^+CD25^{hi}$ population in chapter 4.

Examination of CD25 expression by $CD4^+FOXP3^+$ PBMC

Experimental data has strongly suggested that Treg are confined to the $CD4^+CD25^{hi}$ population of cells within human PBMC (Baecher-Allan, *et al.*2001). With the advent of

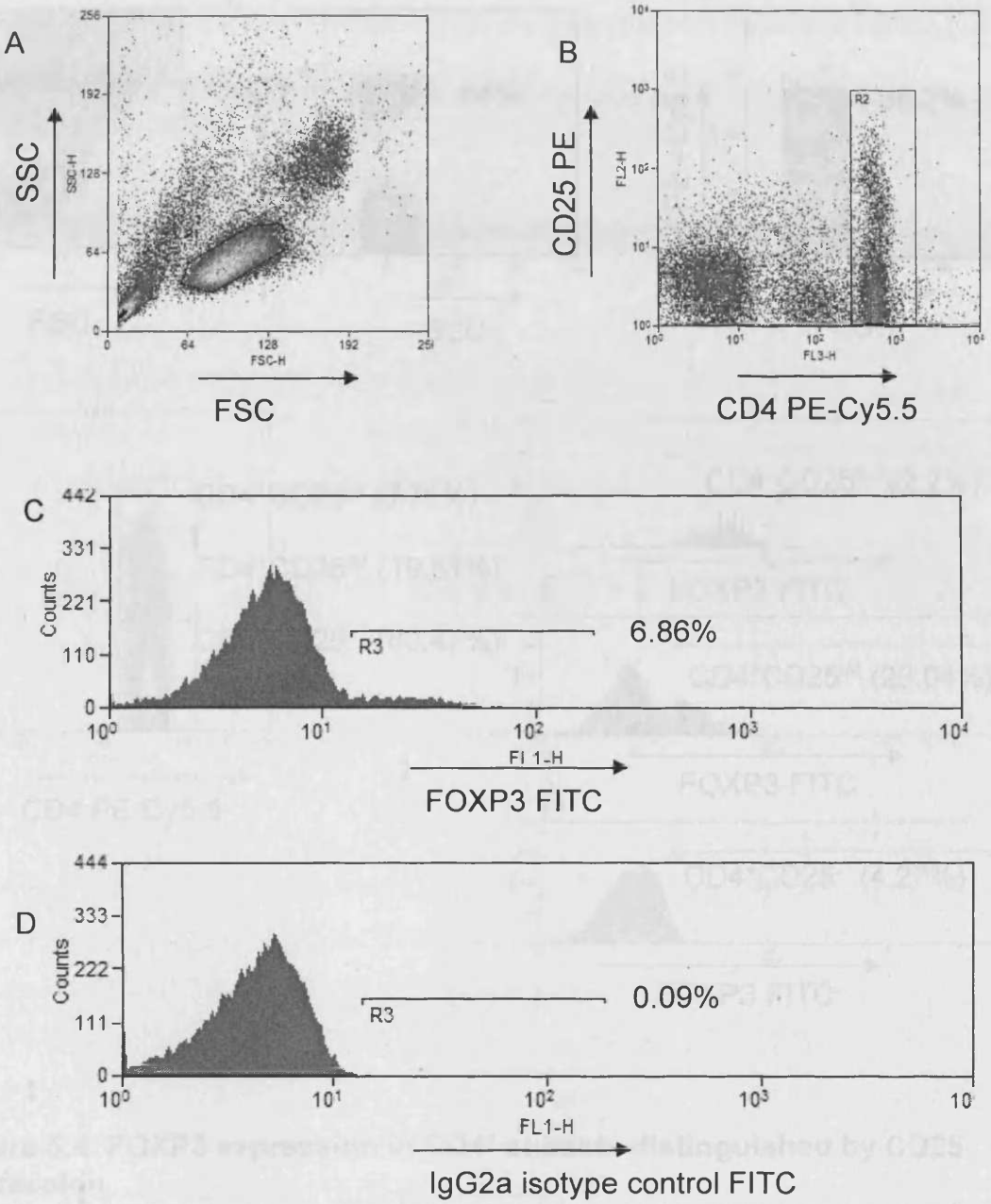


Figure 5.3. Controlling FOXP3 staining of PBL
PBMC were stained with anti-CD4 PE-Cy5.5, -CD25 PE and –FOXP3 FITC or IgG2a FITC isotype control antibody. PBL were gated (A) and the entire $CD4^+$ lymphocyte population was gated (B). $CD4^+$ lymphocytes were examined for staining of anti-FOXP3 (C) or isotype control antibody (D).

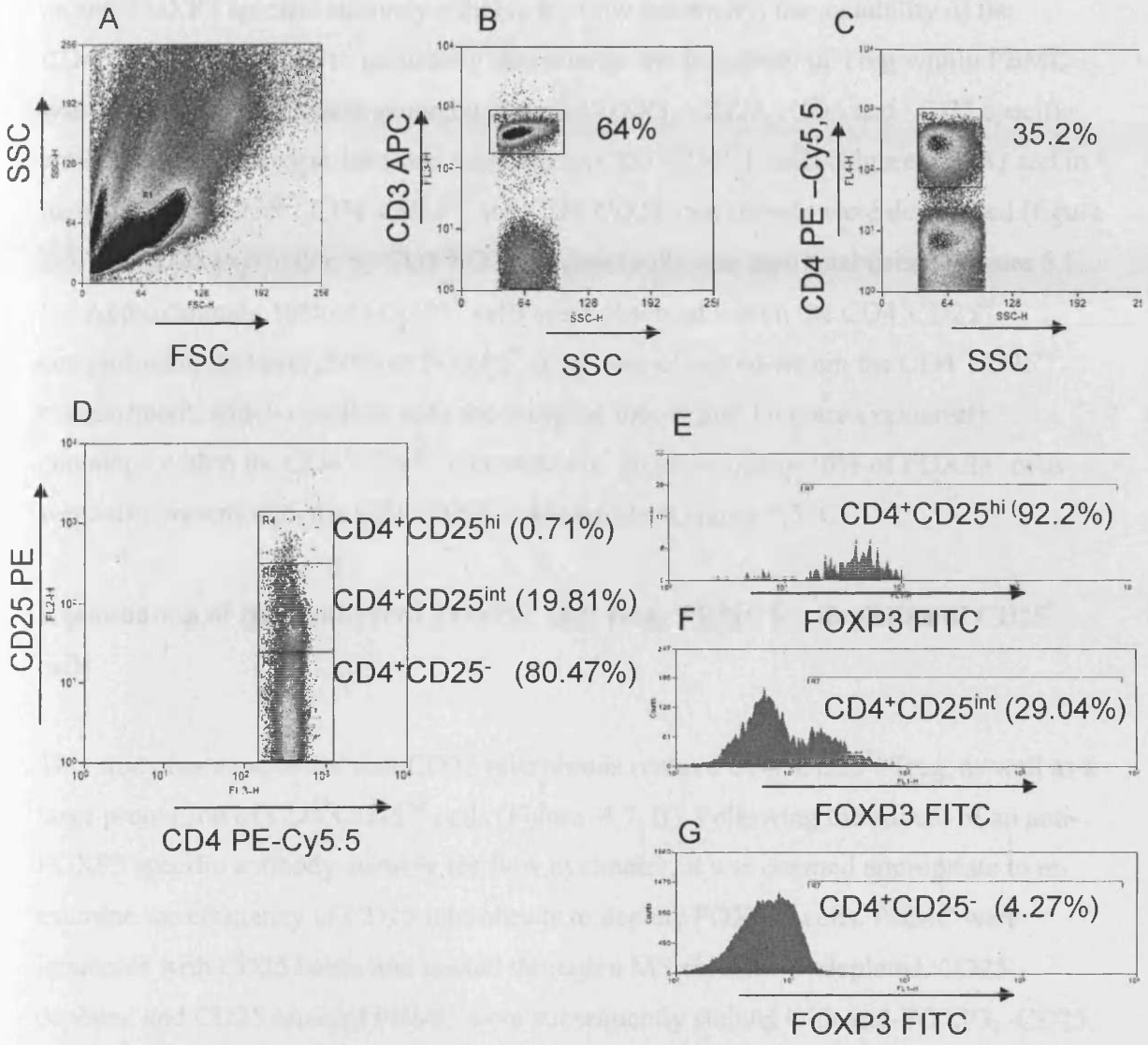


Figure 5.4. FOXP3 expression in CD4⁺ subsets distinguished by CD25 expression

PBMC were stained with anti-FOXP3 FITC, -CD25 PE, -CD4 PE-Cy5.5 and -CD3 APC specific antibodies. PBL were distinguished and gated according to their SSC and FSC (A). $CD3^+$ T cells were gated (B) and $CD4^+CD25^{hi}$, $CD4^+CD25^{int}$, $CD4^+CD25^-$ cells were gated using the upper most CD25 expression on $CD4^-$ cells to distinguish $CD4^+CD25^{hi}$ cells (data not shown). $CD4^+$ T cells were gated (C) in order to present the percentage each $CD4^+$ subset of total $CD4^+$ T cells that expressed FOXP3 (D). FOXP3 expression by $CD4^+CD25^{hi}$ (E), $CD4^+CD25^{int}$ (F) and $CD4^+CD25^-$ (G) cells was established. Data is representative of two experiments.

an anti-FOXP3 specific antibody suitable for flow cytometry, the suitability of the $CD4^+CD25^{hi}$ phenotype to accurately characterise the frequency of Treg within PBMC was re-visited. PBMC were stained with anti-FOXP3, -CD25, -CD4 and –CD3 specific antibodies. FOXP3 expressed was analysed on $CD3^+CD4^+$ T cells (Figure 5.5. A) and in parallel, $CD4^+CD25^{hi}$, $CD4^+CD25^{int}$ and $CD4^+CD25^-$ cell subsets were designated (figure 5.5. B). CD25 expression by $CD4^+FOXP3^+$ gated cells was then established (Figure 5.5. C). Approximately 10% of $FOXP3^+$ cells were observed within the $CD4^+CD25^{hi}$ compartment; however, 80% of $FOXP3^+$ cells were observed within the $CD4^+CD25^{int}$ compartment, which conflicts with the accepted theory that Treg are exclusively contained within the $CD4^+CD25^{hi}$ compartment. Approximately 10% of $FOXP3^+$ cells were also present with the $CD4^+CD25^-$ compartment (Figure 5.5. C).

Examination of the removal of $FOXP3^+$ cells from PBMC via depletion of $CD25^+$ cells

This study has established that CD25 microbeads remove $CD4^+CD25^{hi}$ Treg, as well as a large proportion of $CD4^+CD25^{int}$ cells (Figure. 4.7. B). Following the advent of an anti-FOXP3 specific antibody suitable for flow cytometry, it was deemed appropriate to re-examine the efficiency of CD25 microbeads to deplete $FOXP3^+$ cells. PBMC were incubated with CD25 beads and passed through a MS column. Undepleted, CD25 depleted and CD25 selected PBMC were subsequently stained with anti-FOXP3, -CD25, CD4 and –CD3 specific antibodies. Analysis of undepleted (Figure 5.6. A) and CD25 depleted PBMC (Figure 5.6. B) by flow cytometry revealed that CD25 depletion proceed as normal, whereby the vast majority of $CD4^+CD25^{hi}$ and a large proportion of $CD4^+CD25^{int}$ cells were depleted. FOXP3 expression by $CD4^+$ gated cells within undepleted (Figure 5. 6. C), CD25 depleted (Figure 5.6. D) and CD25 selected PBMC (Figure 5.6. E) was then analysed. This analysis revealed that approximately 10% of $CD4^+$ cells within undepleted PBMC expressed FOXP3 and that CD25 depletion removed approximately half of the $FOXP3^+$ population (Figure 5.6. D). $FOXP3^+$ cells were significantly enriched within the $CD25^+$

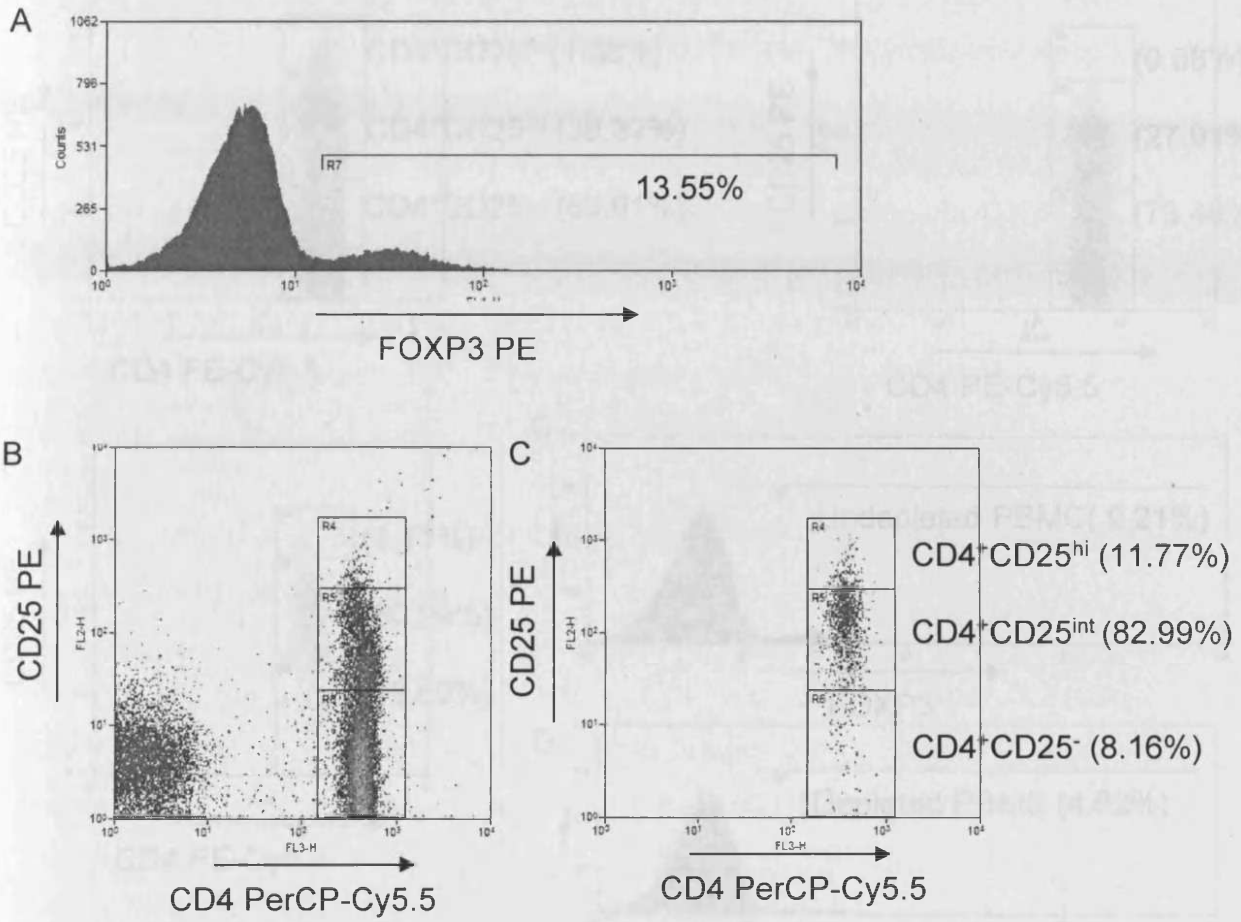


Figure 5.5. Identification of CD25 expression by CD4$^+$FOXP3$^+$ T cells
PBMC were purified and stained with anti-FOXP3 FITC, -CD25 PE, CD4 PerCP-Cy5.5 and –CD3 APC specific antibodies. PBL were gated according to their FSC/SSC; T cells were gated according to their expression of CD3; and CD3$^+$CD4$^+$ T cells were subsequently gated according to their expression of CD4. (data not shown). CD4$^+$FOXP3$^+$ T cells were then gated (A). CD25 expression on CD3$^+$ T cells was analysed in parallel, whereby CD4$^+$CD25^{hi}, CD4$^+$CD25^{int} and CD4$^+$CD25$^-$ cells were compartmentalised using the upper most CD25 expression on CD4- cells to distinguish CD4$^+$CD25^{hi} cells (B). The level of CD25 expression on CD3$^+$CD4$^+$FOXP3$^+$ gated cells was then analysed (C).

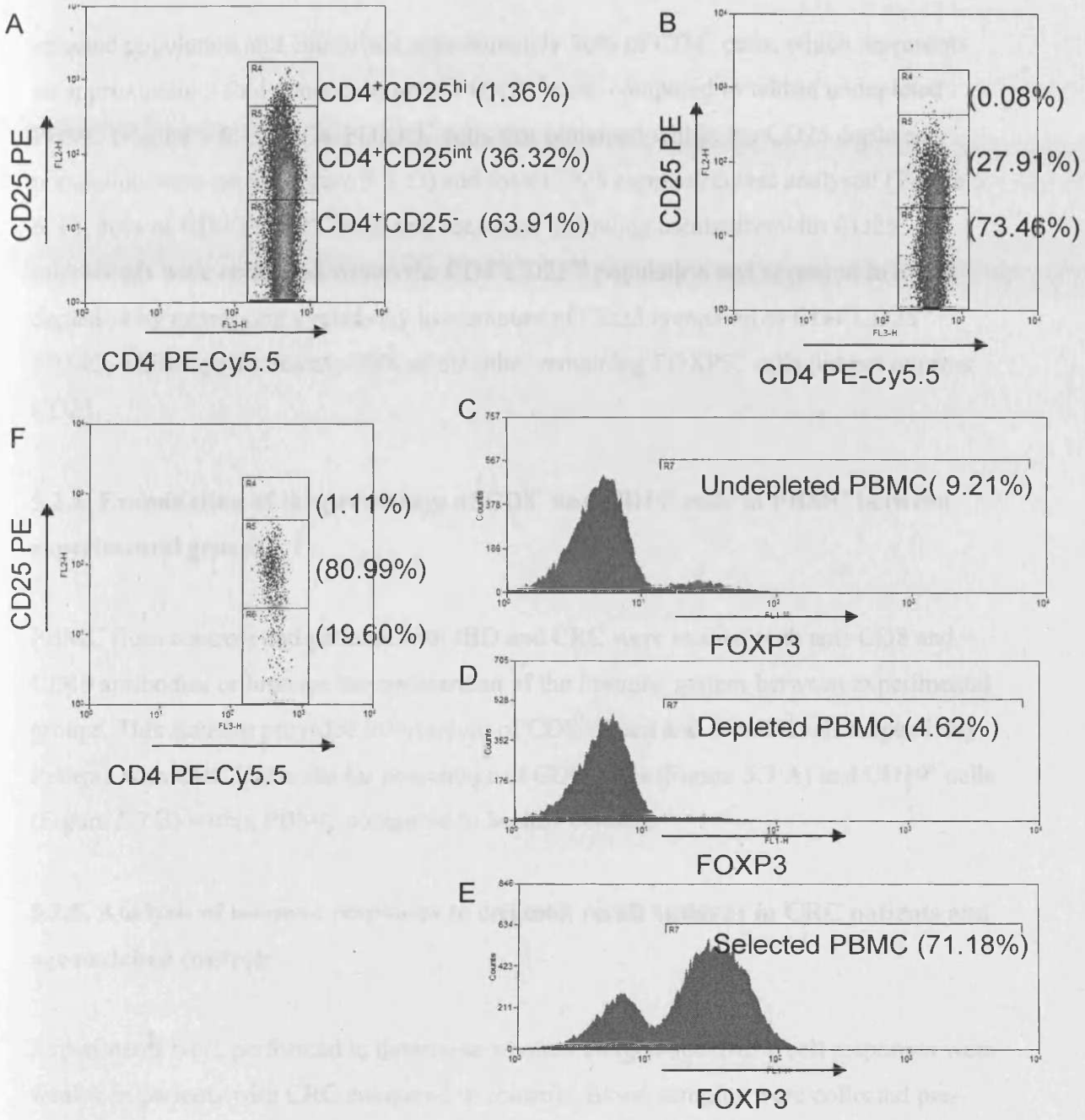


Figure 5.6. CD25 beads deplete approximately half of total FOXP3⁺ cells
PBMC were incubated with CD25 microbeads and passed through an MS column. Undepleted, CD25 depleted and CD25 selected PBMC were subsequently stained with anti-FOXP3, -CD25 PE, CD4 PECy5.5 and –CD3 specific antibodies. $CD3^+$ PBL were gated (data not shown) and the percentage of $CD4^+CD25^{hi}$, $CD4^+CD25^{int}$ and $CD4^+CD25^-$ cells within total undepleted (A) and CD25 depleted (B) $CD4^+$ cells was analysed to ensure CD25 depletion had proceeded as normal. Total $CD4^+$ cells were then gated (data not shown) and the percentage of $CD4^+$ cells that expressed FOXP3 within undepleted (C), CD25 depleted (D) and CD25 selected PBMC was then established (E). The remaining $FOXP3^+$ cells with CD25 depleted PBMC were gated and analysed for expression of CD4 and CD25 (F).

selected population and comprised approximately 70% of $CD4^+$ cells, which represents an approximate 7 fold concentration of $FOXP3^+$ cells compared to within undepleted PBMC (Figure 5.6. E). $CD4^+FOXP3^+$ cells that remained within the CD25 depleted population were gated (Figure 5.6. D) and their CD25 expression was analysed (Figure 5. 6. F). 80% of $CD4^+FOXP3^+$ cells that remained following incubation with CD25 microbeads were contained within the $CD4^+CD25^{int}$ population and appeared to avoid depletion by expressing a relatively low amount of CD25 compared to $CD4^+CD25^{hi}$ PBMC, whilst approximately 20% of the other remaining $FOXP3^+$ cells did not express CD25.

5.2.4. Examination of the percentage of $CD8^+$ and $CD19^+$ cells in PBMC between experimental groups.

PBMC from controls and patients with IBD and CRC were stained with anti-CD8 and – CD19 antibodies to broaden the comparison of the immune system between experimental groups. This staining provided information of $CD8^+$ T cell and B cell levels respectively. Patients with CRC had a similar percentage of $CD8^+$ cells (Figure 5.7 A) and $CD19^+$ cells (Figure 5.7 B) within PBMC compared to healthy controls.

5.2.5. Analysis of immune responses to common recall antigens in CRC patients and age matched controls

Experiments were performed to determine whether antigen-specific T cell responses were weaker in patients with CRC compared to controls. Blood samples were collected pre-operatively from patients undergoing elective bowel resection to remove their tumour and from healthy age matched controls. PBMC were purified and antigen specific responses were measured in *ex-vivo* IFN-γ ELISpot assays and in six-day proliferation assays. These assays measured the status of the effector memory (T_{EM}) and central memory (T_{CM}) compartments respectively (reviewed in discussion).

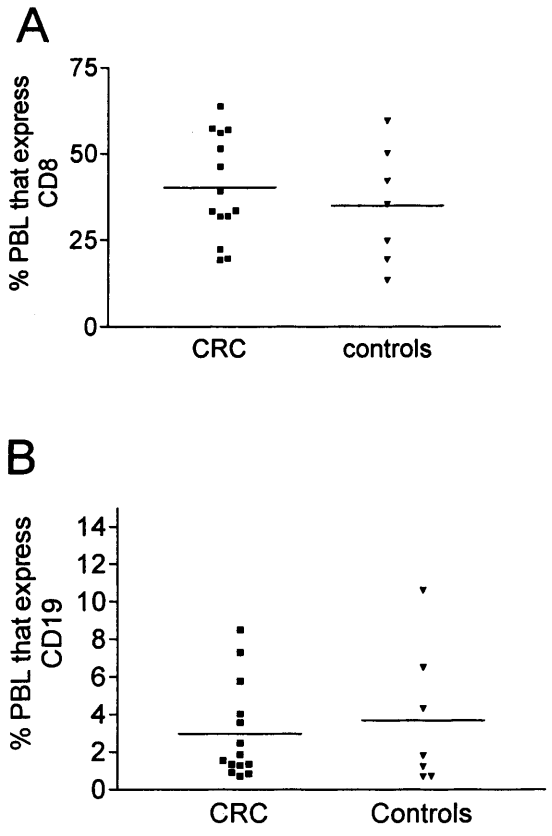


Figure 5.7. CRC patients and controls have similar levels of $CD8^+$ and $CD19^+$ PBMC

Single cell suspensions of PBMC were prepared from CRC patients and and controls. Cells were stained with anti-CD8 PE and -CD19 FITC specific antibodies. The percentage of PBL that expressed CD8 (A) respectively) and CD19 (B) was determined. Data was analysed using an unpaired *student's t-test.*

Measurement of antigen specific *ex-vivo* IFN-γ release

ELISpot assays were carried out by stimulating PBMC for 18hr with 1μg/ml of PPD and HA recall antigens in separate wells. An antigen specific response was designated by a spot count of at least 50% above and ≥5 sfc/10^6 cells incubated in media alone.
All control subjects CRC patients responded to at least one recall antigen *ex-vivo* (Figure 5.8.). As a group, antigen specific IFN-γ release to both PPD and HA recall antigens appeared marginally reduced between PBMC isolated from tumour bearing CRC patients and controls but this was not significantly different (Figure 5.8.). This indicated that effector memory T cells of CRC patients might be slightly suppressed by the presence of malignant disease. *Ex-vivo* IFN-γ release by patients was listed alongside the age and stage of disease (Duke's classification) (Appendix, Tables 3 and4). Weak *ex-vivo* responses to recall antigen did not appear to correlate to the age of patients or the stage of disease; however the number of patients within each subgroup was small thus this analysis should be extended to a larger cohort of patients.

Measurement of antigen specific six-day proliferation

Proliferation assays were performed by stimulating PBMC with 1μg/ml of PPD and HA recall antigen for six-days in separate wells before proliferation was determined by thymidine incorporation. PBMC were also incubated in media alone to calculate spontaneous non-specific proliferation.
Data was analysed by two methods to provide a read out of antigen specific proliferation. Firstly, non-specific proliferation of cells incubated with media alone was subtracted from proliferation of cells incubated with antigen. Antigen specific proliferation within the experimental groups was compared using a student's t-test. Subjects that did not respond to a particular antigen were arbitrarily designated to have a proliferation count of 0.1 x 10^3 cpm in order to visualise data of all subjects in graphical form. The magnitude of proliferation responses by each group was compared by assessing the percentage of individuals that responded either robustly to recall antigen i.e. a cpm ≥ 5 x 10^3 or weakly

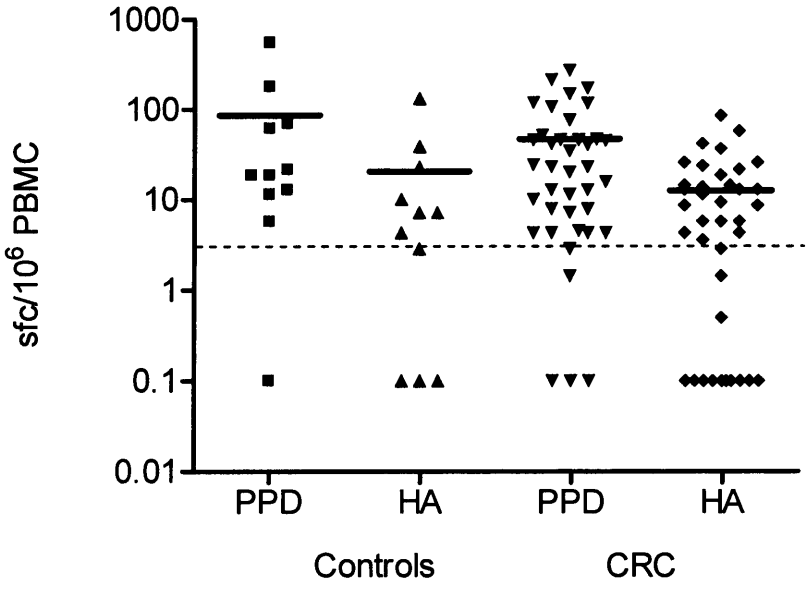


Figure 5.8. CRC patients do not have impaired antigen specific IFN-γ release.
The health of the immune system of CRC patients was established by measuring HA and PPD recall responses and comparing the responses to healthy age matched controls. Responses were measured by detecting *ex-vivo* IFN-γ release by ELIspot. 3.5 x 10^5 undepleted PBMC were incubated in duplicate wells for 18hrs with 1μg/ml of PPD or HA protein and sfc/10^6 PBMC was calculated. A response was at least double and ≥5 sfc/10^6 (indicated by broken line) above the background. Subjects that did not respond to a particular antigen were arbitrarily designated to have 0.1 scf/10^6 cells in order to visualise data of all subjects in graphical form.

i.e. $< 5x10^3$ cpm. A response of $<5\ x10^3$ cpm was chosen to designate weak responses as this figure clearly separated robust from weak responses.
Secondly, the proliferation of cells incubated with antigen was divided by that of cells incubated with media alone to derive a stimulation index (SI). A SI ≥ 3 was considered to be a robust antigen specific response. The percentage of subjects with a SI ≥ 3 was compared between experimental groups.
Data was first analysed by subtracting non-specific background proliferation from cells incubated with antigen. All controls responded to at least one recall antigen. PPD induced robust proliferation in all controls but, as a group, significantly weaker responses were observed by the CRC patient group ($p=0.0016$). Indeed 47% of CRC patients either failed to respond to PPD or mounted weak responses (cpm $<5x10^3$) compared to controls (Figure 5.9. A). CRC patients also displayed a trend of reduced anti-HA responses compared to the control group ($p=0.056$) (Figure 5.9. A). An analysis of the percentage of subjects with HA responses $\geq 5\ x10^3$ cpm revealed that approximately 80% of controls mounted strong anti-HA responses compared to just 30% of CRC patients (Figure 5.9. A).

Six-day proliferation data was also examined by calculating the SI of anti-PPD and -HA responses. In a similar manner to data calculated by subtracting non-specific proliferation from antigen stimulated cells (Figure 5.9. A); a higher percentage of controls demonstrated anti-PPD and –HA responses with SI$\geq$3 compared to patients (Figure 5.9. B). 100% of controls had an anti-PPD response of SI $\geq$3, compared to 76% of patients with an anti-PPD SI$\geq$3; and 78% of controls had an SI $\geq$3 to HA, compared to 46% of patients with an anti-HA response SI$\geq$3 (Figure 5.9. B).

In order to verify that a proportion of the CRC patient group was immunosuppressed, six-day proliferation responses were analysed to examine whether these particular patients failed to mount strong responses to both HA and PPD antigens.
Individual responses to both recall antigens were compared and shown as above i.e. both cpm and SIs. Two controls failed to mount strong anti-HA responses but these subjects did respond to PPD antigen, as did all other controls. In contrast, 5/9 CRC patients that

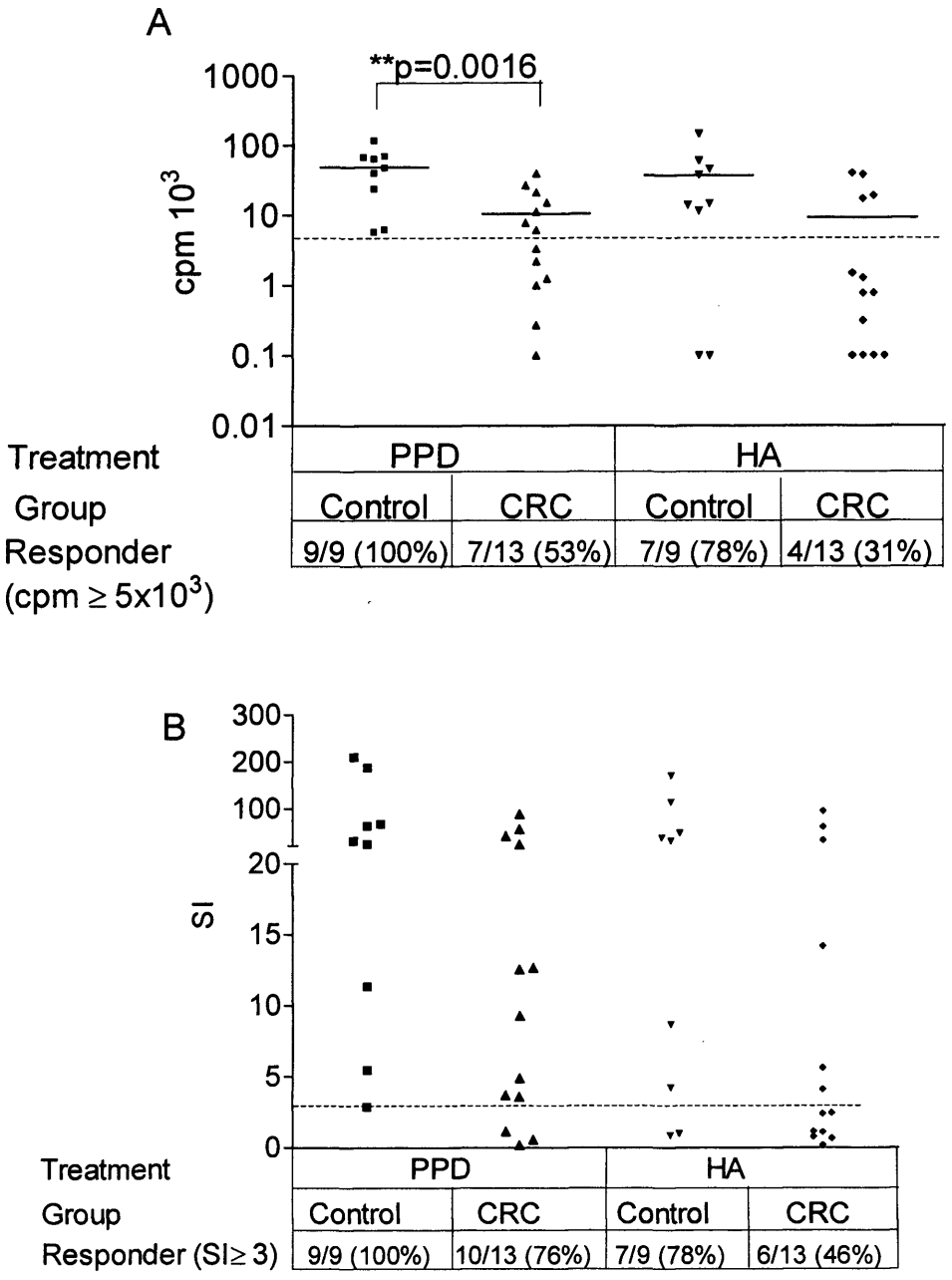


Figure 5.9. Six day proliferation responses to recall antigens in controls and CRC patients

2 x 10^5 PBMC were stimulated with 1μg/ml of either PPD and HA antigen in separate wells for 6 days before proliferation was determined by the incorporation of [Methyl-3H]-thymidine. Antigen specific proliferation was analysed by subtracting non-specific proliferation counts of cells incubated with media from those of antigen stimulated wells (A). Subjects that did not respond to a particular antigen were arbitrarily designated to have a cpm of 0.1 x 10^3 in order to visualise data of all subjects in graphical form. Data was analysed using an unpaired *student's t-test.* Data was also analysed by dividing antigen stimulated counts by non-specific counts to derive SI (B).

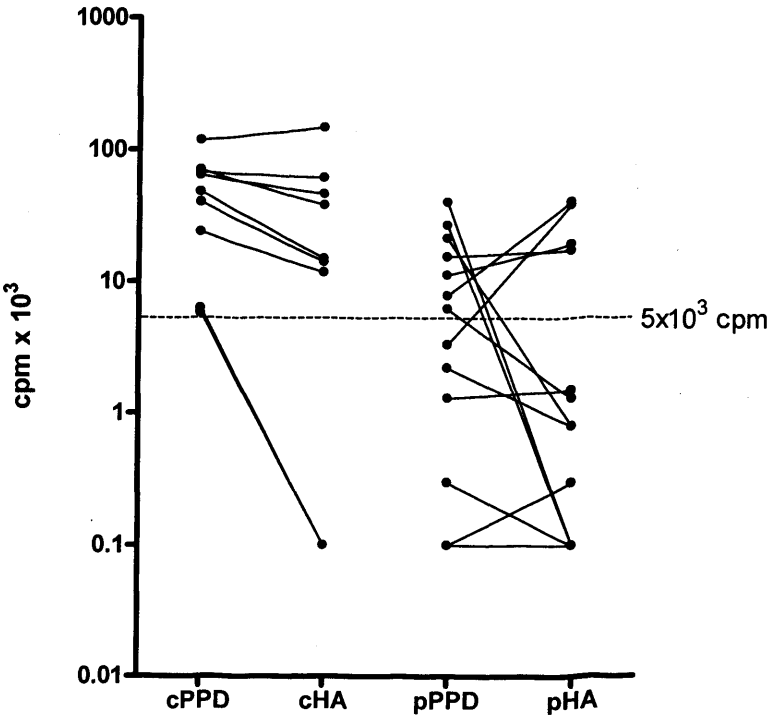


Figure 5.10. Immunosuppressed CRC patients fail to mount both strong anti-PPD and –HA responses
Responses to PPD antigen by CRC patients (pPPD) and controls (cPPD) were linked to their responses to HA antigen (pHA and cHA respectively). Data was analysed by counting the number of subjects with a cpm ≥$5x10^3$.

failed to mount a strong response to HA antigen also failed to mount a strong response to PPD and 5/6 patients that failed to mount a strong anti-PPD response also failed to mount a strong anti-HA response (Figure 5.10.) These five CRC patients failed to mount a strong response to either antigen. All controls responded robustly to at least one recall antigen. Thus a substantial proportion of CRC patients mounted weak six-day proliferation responses to recall antigen compared to controls. This interpretation was also checked by analysing six-day proliferation data and identifying the number of subjects with responses of SI≥3 and revealed a similar pattern of poor proliferation responses within the CRC group (data not shown). Overall this analysis showed that an elevated proportion of the CRC patient group mount weak responses to recall antigens compared to controls. These results indicate that the ability of the immune system to mount robust memory responses is reduced in CRC patients.

It appeared age may contribute to poor proliferation observed by those CRC patients that failed to mount strong responses to either antigen. CRC patients were therefore ordered according to their age and proliferation responses were analysed (Table 5.1. PPD specific (PPDu) and HA specific (HAu) responses by undepleted PBMC). CRC patients analysed had a mean age of 72 (range 55-80), where as the control groups mean age was 56 (range 48-64). Those patients that failed patients that failed to respond to either antigen had a mean age of 81 (range 73-88). Those subjects previously identified as having impaired proliferative responses (Table 5.1.) did not display impaired *ex-vivo* IFN-γ release to recall antigens (data not shown). Overall, these results imply old age is associated with an impairment of six day proliferation responses whilst *ex-vivo* responses are minimally reduced. An analysis of the Duke's classification of patient tumours was also undertaken to identify whether poor proliferative responses corresponded to more advanced tumours. No correlation was identified but an investigation of a larger number of patients may be necessary before solid conclusions can be drawn

In light of the marginally reduced antigen specific *ex-vivo* responses and more striking reduction of antigen specific six-day proliferation responses, the data as a whole implied that CRC patients are partially immunosuppressed. Further experiments using older

Patient No	Duke's stage	Age	PPDu	PPDd	HAu	HAd	
16	A	62	11.2	10.4	19.3	19.3	
8	C	66	40.1	29.6	0.1	0.3	
17	C1	66	26.9	60.1	0.1	0	
12	C	67	3.3	0	38.5	53.4	
11	B	71	15.4	12.2	17.3	11.5	
20	C	73	0.1	0	0.3	0.7	*
19	C	75	7.8	21.5	40.5	46.1	
15	B	76	21.4	35.1	0.8	0	
10	B	80	6.2	29	1.3	0	
40	C1	81	1	0	0.1	0	*
18	B	82	0.3	2.2	0.1	0	*
13	C	83	2.2	3.1	0.8	2.2	*
9	A	88	1.3	6.1	1.5	0.9	*

Table 5.1. The magnitude of six-day proliferation responses of PBMC purified from CRC patients before and after depleting Treg
2 x 10^5 undepleted (u) and Treg depleted (d) PBMC were purified from CRC patients and stimulated in triplicate with 1μg/ml of PPD and HA antigen in separate wells for six days before proliferation was determined by the incorporation of [Methyl-3H]-thymidine. Antigen specific proliferation was analysed by subtracting non-specific proliferation counts of cells incubated with media from those of antigen stimulated wells. Patients that failed to mount strong responses (≥5x10^3 cpm) to both PPD and HA antigen prior to depletion are indicated by *. A patient number was assigned to subjects when a list of *ex-vivo* elispot responses was tabulated (see appendix Table 3).

control subjects that are more closely age matched to CRC patients is required to derive more comparable control data and confirm the results collected so far. It is possible that malignancy and old age combine to reduce proliferation responses. The key experiment is to determine whether proliferation responses by these patients return to normal after resection of their tumour.

Data was next analysed to observe if poor ($\leq 5x10^3$ CPM) six-day proliferation responses mounted by undepleted PBMC previously identified in a number of CRC patients was enhanced following depletion of $CD4^+CD25^{hi}$ cells (Table 5.1.). Responses by undepleted PBMC by low responders and were compared to those mounted by the same patients following depletion of Treg. This revealed that depletion of Treg enhanced six-day proliferation in 1/5 poor responders to PPD antigen (Table 5.1.); however this was an isolated case and overall, depletion of Treg did not enhance proliferation responses of subject that mounted low responses prior to depletion. This data implied that an increased level of Tregs during malignancy did not account for the decline of six-day proliferation responses with the progression of old age in CRC patients.

5.2.6. Responses to recall antigens following the depletion of Treg cells from PBMC.

As described above, PBMC derived from CRC patients contained an elevated percentage of Treg within the $CD4^+$ T cell compartment (Figure 5.2. B). Experiments were performed to analyse if Treg suppressed antigen-specific responses to the recall antigens PPD and HA. Undepleted and Treg depleted PBMC were incubated in parallel in media alone, HA or PPD protein. *Ex-vivo* IFN-γ release ELISpot and six-day proliferation function was examined.

Antigen specific *ex-vivo* IFN-γ release to recall antigens is partially suppressed by Treg

Ex-vivo IFN-γ release ELISpot assays were performed to measure and compare recall responses by undepleted and Treg depleted PBMC to establish the extent that Treg

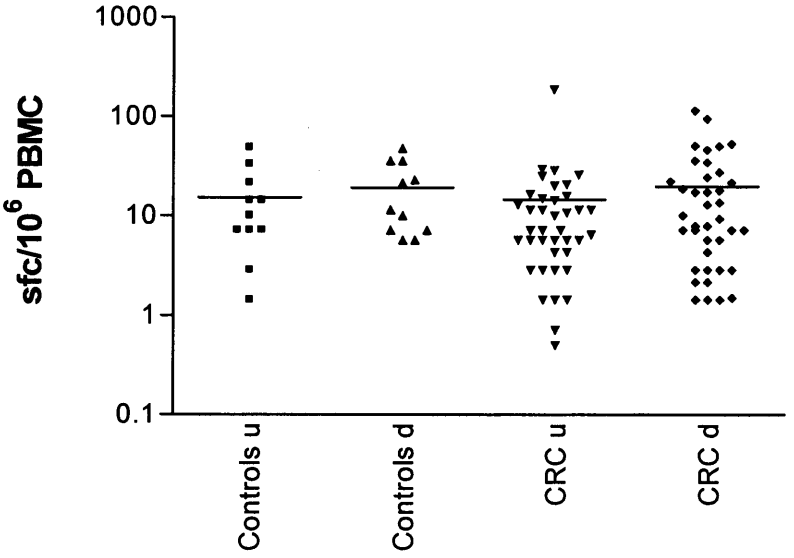


Figure 5.11. Depletion of Treg does not induce non-specific *ex-vivo* IFN-γ release

PBMC were isolated from controls and CRC patients and incubated with CD25 microbeads and passed down an MS column to remove Treg. 3.5 x 10^5 undepleted and Treg depleted PBMC were incubated with media alone and the number of spot forming cells was counted to calculate the amount of non-specific *ex-vivo* IFN-γ release.

inhibited these responses. PBMC were stimulated in parallel with media alone or 1μg/ml of either PPD or HA protein in separate wells and antigen specific sfc/10^6 PBMC was calculated as previously described. A comparison of the amount of non-specific IFN-γ release by undepleted and Treg depleted cells, isolated from controls and CRC patients and incubated with media alone, demonstrated that the process of depleting Treg did not enhance spontaneous IFN-γ release (Figure 5.11.). As before, an antigen specific response was designated by a spot count of at least 50% above and ≥5 sfc/10^6 cells incubated in media alone. The ability of Treg to enhance antigen-specific responses to HA and PPD antigens was designated when depletion of Treg enhanced the number of spots by at least 50%, after subtracting the background number of spots in unstimulated wells. A new response was only considered genuine if depleted PBMC also fulfilled the criteria of being at least 50% and ≥5 spots above those of unstimulated cells. The effect of depleting Treg on the response of each subject was classified to either: enhance an existing response; uncover a new response; have no effect or reduce an existing response; or failed to uncover a response. It was rare for depletion to significantly reduce responses, but in these cases it was deemed most likely that the process of depletion removed activated $CD25^+$ cells that contributed to the response of undepleted PBMC. It was therefore deemed appropriate to group these subjects with those where depletion failed to substantially enhance responses.

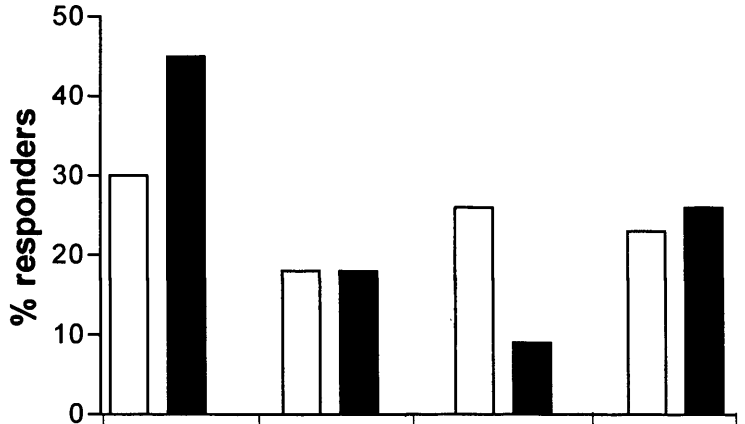
HA responses

Depletion of Treg from PBMC did not have a universal effect on *ex-vivo* HA responses; however, Treg depletion either enhanced (27% CRC patients, 45% controls) or uncovered new (23% CRC patients, 18% controls) *ex-vivo* anti-HA responses in both CRC patient and control groups (Figure 5.12. A). It was therefore considered that Treg inhibited HA specific responses in these individuals.

Approximately 30% of CRC patients and control subjects failed to respond to HA antigen either before or after the removal of Treg. A significant proportion of subjects (26% CRC patients, 9% controls) had existing HA responses that were not boosted by depletion of Treg (Figure 5.12. A). A failure for depletion to enhance these responses may have arisen

HA

A



	R→I	NR→R	R→NC/D	N→N
CRC (39)	12 (30%)	7 (18%)	10 (26%)	10 (26%)
Controls (11)	5 (45%)	2 (18%)	1 (9%)	3 (27%)

KEY

R ⟶ I = A response to antigen pre-depletion is enhanced 50% post-depletion of Treg

NR ⟶ R = No response pre-depletion and a new response is uncovered post-depletion of Treg

R ⟶ NC/D = A response to antigen pre-depletion and either no enhancement or a reduction post depletion of Treg

N ⟶ N = No response to antigen pre-depletion and no response uncovered post-depletion of Treg

B

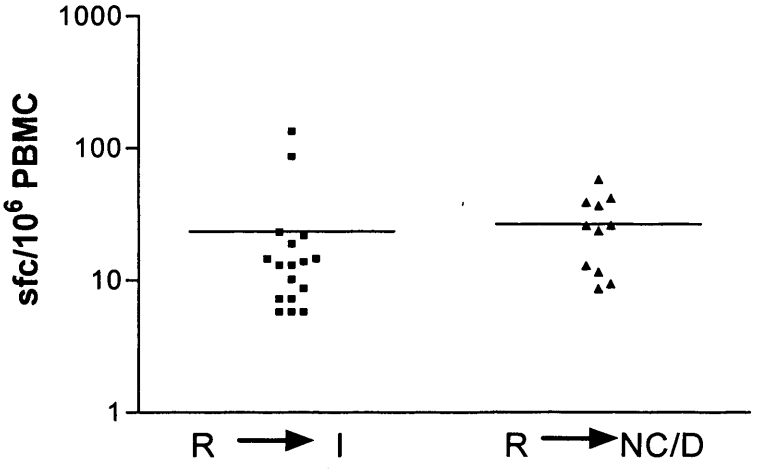


Figure 5.12. *Ex-vivo* IFN-γ release to HA recall antigen following the depletion of $CD25^{hi}$ cells from PBMC.
Ex-vivo IFN-γ release ELIspot assays were performed to measure and compare recall responses by undepleted and $CD25^{+}$ cell depleted PBMC. 3.5 x 10^5 PBMC were incubated in duplicate wells for 18hrs with 1μg/ml of HA protein and sfc/10^6 PBMC was calculated. A response was at least 50% and 5 sfc/10^6 above the no antigen background. The effect of Treg cell depletion on HA responses were classified according to the above criteria and displayed as a percentage of the sample group (A). Subjects with existing responses prior to depletion that were either elevated or remained unchanged by depletion of Treg were identified and grouped. The magnitude of responses produced by undepleted PBMC of these groups was then compared (B). Data was analysed using a student's t-test.

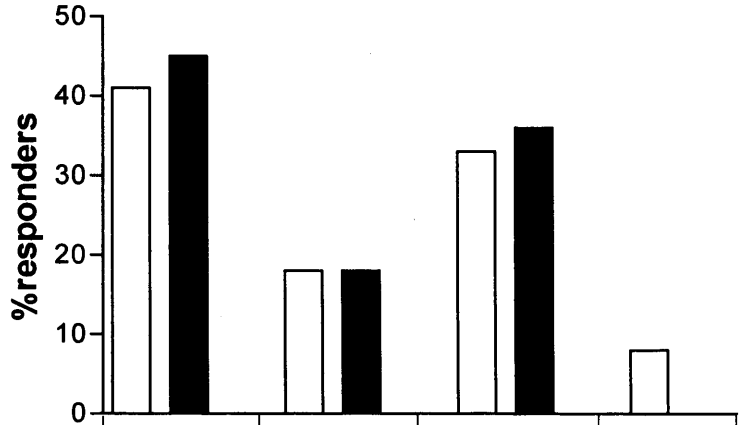
because the existing response by undepleted PBMC was sufficiently strong to completely override suppression by Treg and therefore depleting Treg made no enhancement to responses. To investigate this theory, the magnitude of responses produced by undepleted cells of controls and CRC patients that were enhanced by depletion was compared to those that depletion either failed to enhance or reduced. The number of spots formed by cells incubated with media alone was subtracted from those incubated with antigen. This analysis showed that responses mounted by undepleted PBMC that were enhanced by depletion were marginally weaker than those that were not enhanced by Treg depletion but this difference was not significant (Figure 5.12. B). Interestingly, a higher proportion of controls had HA specific reponses that were suppressed by Treg compared to the CRC patient group (26% CRC patients, 9% controls) (Figure 5.12 A). This finding may indicate that CRC patients have a lower frequency of HA specific Treg.

PPD responses

A small number of subjects (0% controls, 8% CRC patients) failed to respond to PPD either before or after depletion (Figure 5.13. A). Treg did appear to suppress PPD responses in a relatively large proportion of subjects, whereby depletion enhanced an existing response in approximately 40% of CRC patients and controls and initiated a novel response in 18% of CRC patients and controls (Figure 5.13. A). However, a large contingent of subjects (33% of CRC patients; controls 36%) had existing PPD responses that did not appear to be suppressed by Treg and were not further enhanced by depletion of Treg (Figure 5.13. A). A failure for depletion to enhance these responses may have arisen because the existing response by undepleted PBMC was sufficiently strong to completely override suppression by Treg. This theory was previously investigated with anti-HA responses and PPD response data was analysed in an identical fashion. Analysis showed that responses mounted by undepleted PBMC that were enhanced by depletion were marginally weaker than those that were not enhanced by Treg depletion (Figure 5.13. B), although this difference was not significant.

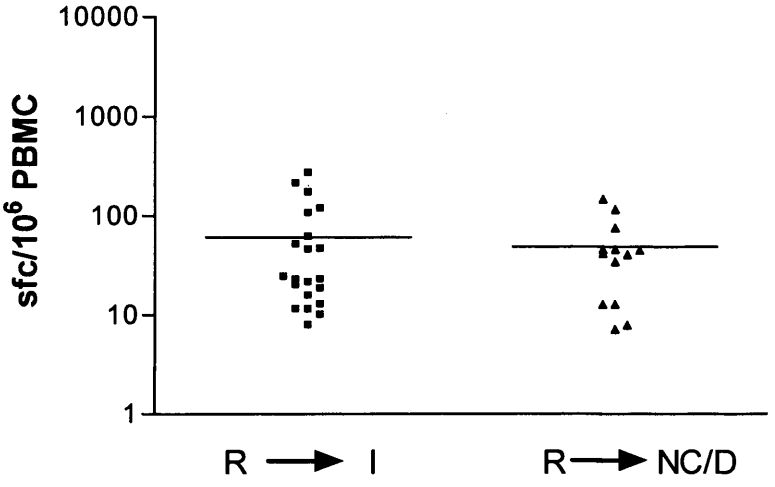
PPD

A



	R→I	NR→R	R→NC/D	N→N
CRC (39)	16 (41%)	7 (18%)	13 (33%)	3 (8%)
Controls (11)	5 (45%)	2 (18%)	4 (36%)	0 (0%)

B



KEY

R → I = A response to antigen pre-depletion is enhanced 50% post-depletion of Treg

NR → R = No response pre-depletion and a new response is uncovered post-depletion of Treg

R → NC/D = A response to antigen pre-depletion and either no enhancement or a reduction post depletion of Treg

N → N = No response to antigen pre-depletion and no response uncovered post-depletion of Treg

Figure 5.13. *Ex-vivo* IFN-γ release to PPD recall antigen following the depletion of $CD25^{hi}$ cells from PBMC.
Ex-vivo IFN-γ release ELIspot assays were performed to measure and compare recall responses by undepleted (u) and $CD25^{+}$ cell depleted (d) PBMC. 3.5 x 10^5 PBMC were incubated in duplicate wells for 18hrs with 1μg/ml of PPD protein and sfc/10^6 PBMC was calculated. A response was at 50% above and 5 sfc/10^6 above the no antigen background. The effect of Treg cell depletion on PPD responses were classified according to the above criteria and displayed as a percentage of the sample group (A). Subjects with existing responses prior to depletion were either elevated or remained unchanged by depletion of Treg were identified and grouped. The magnitude of responses produced by undepleted PBMC of these groups was then compared (B). Data was analysed using a student's t-test.

To summarise, depletion of Treg from PBMC did appear to either enhance existing responses or uncover a new *ex-vivo* HA and PPD specific immune responses in a large proportion (50-65%) of controls and CRC patients. This data indicated that Treg do suppress *ex-vivo* IFN-γ responses to recall antigens. Overall, Treg did not suppress recall responses by CRC subjects more extensively than compared to the control. This suggested that the elevated number of Treg identified within PBMC of CRC patients does not correlate to an enhanced level of suppression to these antigens. Further analysis to compare the frequency of Treg to the effect of depleting Treg on the response to recall antigen in each subject is required.

The effect of Treg depletion on each patients' *ex-vivo* response to HA and PPD was tabulated (Appendix, Table 4) and the effect of Treg depletion on these responses was studied according to the stage of the patients' tumour (Duke's stage, Appendix, Table 4 Part 1) and the age of patients (Appendix Table 4, Part 2). This analyses did not distinguish a higher level of Treg suppression within older patients or those with more advanced disease; however a larger cohort of patients may yet reveal statistically significant differences between groups.

Six-day proliferation responses to recall antigens are partially suppressed by Treg

Proliferation responses to PPD or HA proteins were measured in the presence and absence of Treg and the extent that Treg inhibited responses was analysed. Background proliferation was subtracted from that of cells incubated with antigen and then depletion was concluded to increase the magnitude of a response when the proliferation of depleted cells was double that of undepleted cells. The effect of depleting Treg on the response of each subject was classified to either: enhance an existing response; uncover a new response; have no effect or reduce an existing response; or failed to uncover a response.

HA responses

Anti-HA responses mounted by each subject's undepleted and depleted PBMC were compared to determine the extent that Treg inhibit anti-HA responses. A breakdown of

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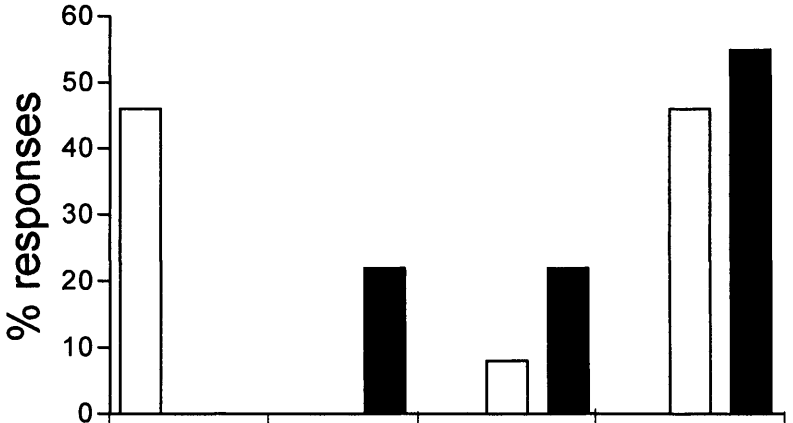


	NR→NR	NR →R	R →I	R→ NC/D
CRC (13)	6 (46%)	0 (0%)	1 (8%)	6 (46%)
Controls (9)	0 (0%)	2 (22%)	2 (22%)	5 (55%)

KEY

NR ⟶ NR = No response to antigen pre-depletion and no response uncovered post-depletion of Treg

NR ⟶ R = No response pre-depletion and a new response is uncovered post-depletion of Treg

R ⟶ I = A response to antigen pre-depletion is enhanced >50% post-depletion of Treg

R ⟶ D/NC = A response to antigen pre-depletion and either no enhancement or a reduction post depletion of Treg

Figure 5.14. The effect of depletion of Treg on six-day anti-HA responses in controls and CRC patients

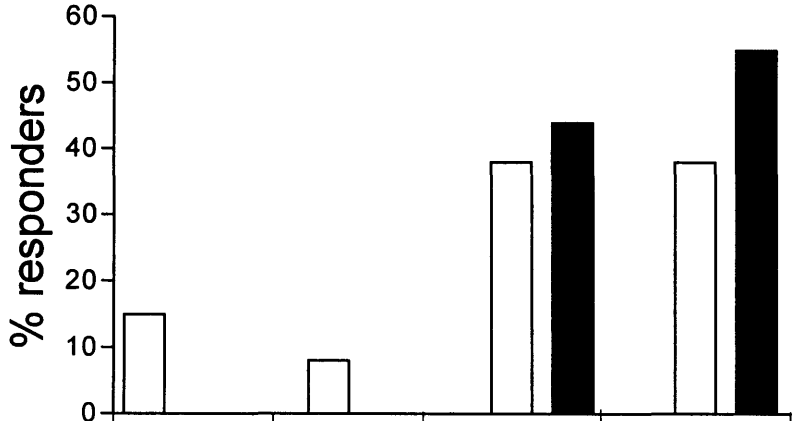
PBMC were obtained from CRC patients and controls. 2 x 10^5 undepleted and $CD25^+$ depleted PBMC were stimulated with 1μg/ml HA protein for six days before proliferation was determined by the incorporation of [Methyl-3H]-thymidine. The effect of $CD25^+$ cell depletion on HA responses was classified according to the above criteria and displayed as a percentage of the patient group (C).

responses mounted by individual subjects showed a variable effect of Treg depletion on anti-HA responses (Figure 5.14.). Treg depletion enhanced an existing response in two controls and uncovered a new anti-HA response in two other controls. In contrast, depletion of Treg failed to unmask any new anti-HA responses in CRC patients but enhanced an existing anti-HA response by one CRC patient. Overall, it appeared that Treg did impart suppression to HA specific responses in approximately 40% of controls (22% uncovered a new response by depletion; 22% response enhanced by depletion); but inhibited only 8% of CRC patients (Figure 5.14.). Analyses of more subjects is required before firm conclusions can be drawn; but it may be that since CRC patients have higher levels of Treg, possibly over a long period of time, anti-HA six-day proliferation responses in CRC patients have been suppressed to a level that is not recovered by short term depletion of Treg.

PPD responses

Anti-PPD responses mounted by each subject's undepleted and depleted PBMC were compared to determine the extent that Treg inhibit anti-PPD responses. A breakdown of responses mounted by individual subjects showed a variable effect of Treg depletion on anti-PPD responses (Figure 5.15.). Treg depletion revealed a new anti-PPD response by one CRC patient and enhanced existing responses by five others; however, depletion either had no effect or decreased responses mounted by the remaining patients (Figure 5.15). Depletion of Treg also had a variable effect on anti-PPD responses mounted by controls, whereby responses were depleted in five controls and enhanced in four controls (Figure 5.15). Overall, Treg inhibited anti-PPD responses in 44% subjects, whereby depletion either enhanced or induced new responses. In contrast to anti-HA responses, the level of Treg inhibition was comparable between CRC and control groups.

Overall, Treg were shown to suppress six-day proliferation responses in a large proportion of controls and CRC patients. The frequency of suppression between subjects



	NR→NR	NR →R	R →I	R→ NC/D
CRC (13)	2 (15%)	1 (8%)	5 (38%)	5 (38%)
Controls (9)	0 (0%)	0 (0%)	4 (44%)	5 (55%)

KEY

NR → NR = No response to antigen pre-depletion and no response uncovered post-depletion of Treg

NR → R = No response pre-depletion and a new response is uncovered post-depletion of Treg

R → I = A response to antigen pre-depletion is enhanced >50% post-depletion of Treg

R → NC/D = A response to antigen pre-depletion and no enhancement/decrease post depletion of Treg

Figure 5.15. The effect of depletion of Treg on six-day anti-PPD responses in controls and CRC patients

PBMC were obtained from CRC patients and controls. 2 x 10^5 undepleted and $CD25^+$ depleted PBMC were stimulated with 1μg/ml PPD protein for six days before proliferation was determined by the incorporation of [Methyl-3H]-thymidine. Antigen specific proliferation was analysed by subtracting non-specific proliferation counts of cells incubated with media from those of antigen stimulated wells. The effect of $CD25^+$ cell depletion on PPD responses was classified according to the above criteria and displayed as a percentage of the patient group.

appeared to vary with the type of stimulus. Suppression of the relatively strong PPD stimulus was observed in 44% of controls and CRC patients but the level of Treg suppression seemed to be reduced when HA specific six-day proliferation responses were observed. However this seemed to be because a higher proportion of CRC patients did not proliferate at all to HA stimulus as described previously (Figure 5.9.).
The ability of depleting Treg to enhance *ex-vivo* and six-day responses to PPD and HA antigens was compared for each subject (data not shown). This analysis demonstrated that an elevation of HA specific *ex-vivo* IFN-γ production often did not correspond to an enhancement of the HA specific six-day response and vice versa. The same was true for PPD specific responses. This is discussed later.

5.2.7. Responses to the tumour antigens 5T4 and CEA following the depletion of Tregs from PBMC.

Following the demonstration that patients with CRC contain more Tregs than healthy controls (Figure 5.1. B), a central aim of this project was to examine whether Tregs impinged on the ability of the immune system to mount immune responses to tumour antigens. This question was addressed by examining anti-tumour immune responses in PBMC of patients in the presence and absence of Tregs. Undepleted and PBMC depleted Tregs were incubated with the tumour antigens 5T4 (Starzynska, *et al.*1992, Starzynska, *et al.*1994) and CEA (reviewed in (Thompson, *et al.*1991)), known to be expressed in the majority of CRC tumours and tumour antigen specific responses were analysed by *ex-vivo* IFN-γ ELISpot and six-day proliferation assays.

Ex-vivo IFN-γ release following stimulation with the tumour antigens 5T4 and CEA

Ex-vivo IFN-γ release ELISpot assays were performed to measure antigen specific immune responses to the tumours antigens 5T4 and CEA in undepleted and Treg-depleted PBMC. Cells from healthy controls and patients with CRC were stimulated with 1μg/ml of 5T4 and CEA protein in separate wells and sfc/10^6 PBMC was calculated. As expected, antigen specific immune responses to tumour proteins were not as robust as

PPD or HA specific immune responses, however, the high sensitivity of the ELISpot IFN-γ release assay did detect 5T4 and CEA specific immune responses. An example of an elispot that measured a positive anti-5T4 response is shown (Figure 5.16). Tumour antigen specific *ex-vivo* responses mounted by CRC patients and controls were classified according to the extent that Treg suppressed (Treg depletion enhanced) these responses (Table 5.2).

5T4

Total 5T4 specific responses (including new responses, responses partially suppressed and those not suppressed by Treg) were observed at a slighty higher frequency in CRC patients (15/39, 38%) compared to healthy controls (3/11, 27%) (Table 5.2). Depletion of Treg uncovered new responses to the tumour antigen 5T4 and were therefore judged to normally suppress these responses. This observation was largely restricted to PBMC isolated from CRC patients (23% (9/39); however, one control subject (9%, 1/11) did develop a new 5T4 response (Figure 5.17.A). Experiments that blocked presentation of HLA-DR and –DQ presentation strongly suggested that these responses were mediated by $CD4^+$ T cells (data not shown, kindly provided by Dr Sarah Clarke). These new 5T4 responses formed 60% (9/15) of total 5T4 responses mounted by CRC patients (Table 5.2.). The number of sfc/10^6 PBMC of each of the nine CRC patients that presented with a new 5T4 response following depletion of Treg (patients 1-9) is displayed (Figure 5.17 B). New responses accounted for a smaller proportion (33%, 1/3) of total responses mounted by controls. Partial suppression of 5T4 total responses (enhancement of an existing response following depletion) was observed at a similar frequency in CRC patients (3/15 (20%) and controls (1/3, 33%) (Table 5.2, Figure 5.17. A). This was also the case for responses that not suppressed by Treg (Table 5.2). The majority of subjects failed to respond to 5T4 either before or after depletion (62% (24/39) CRC patients, 73% (8/11) controls) (Figure 5.17.A).

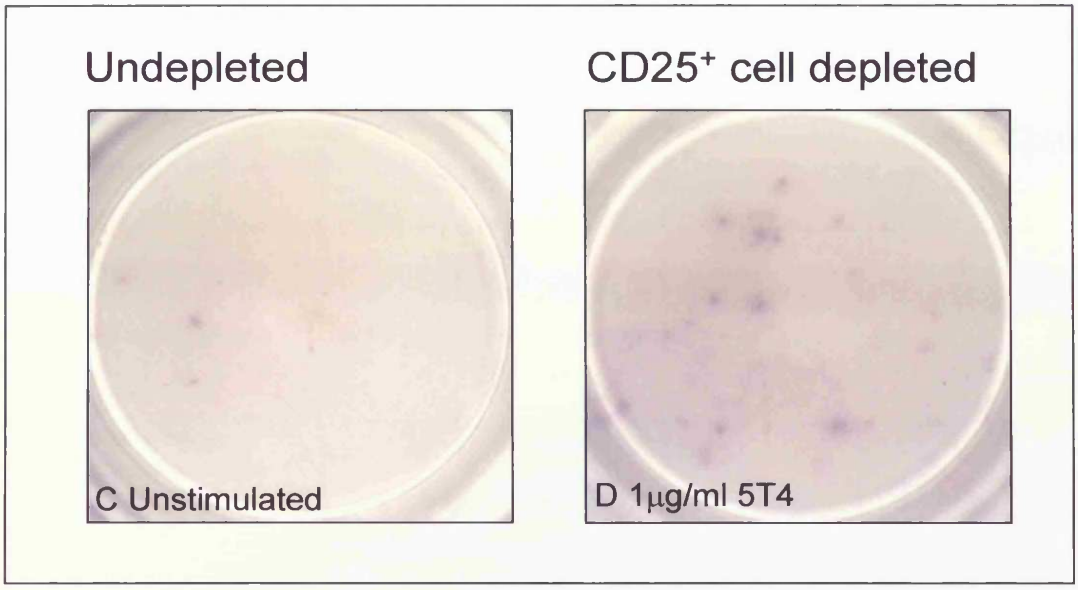
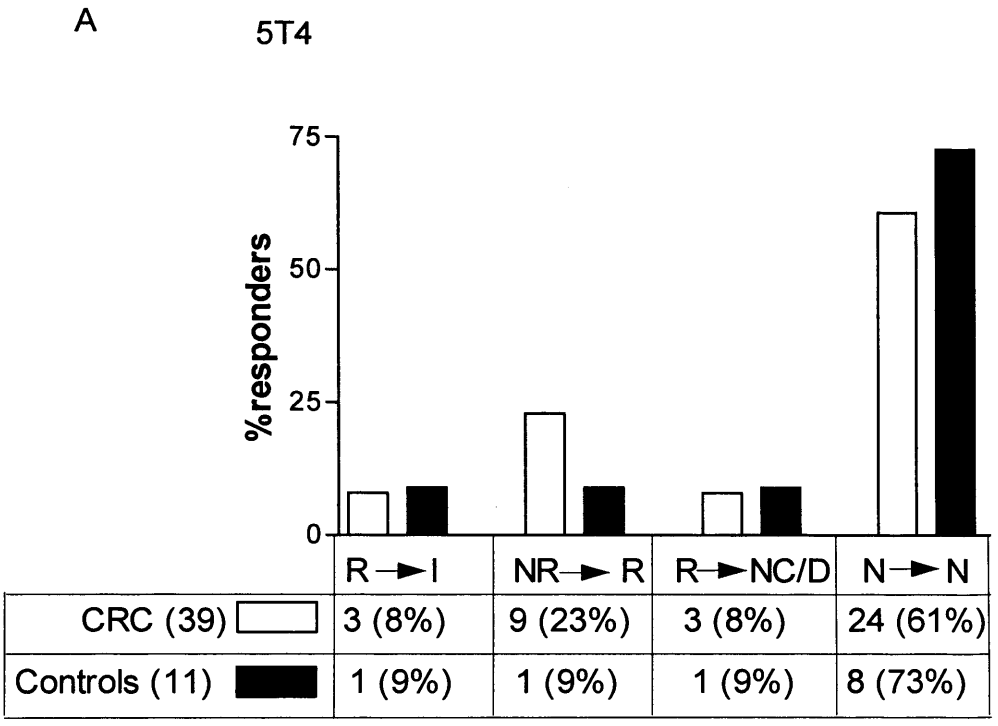


Figure 5.16. Depletion of CD25$^+$ cells enhances *ex-vivo* IFN-γ responses to 5T4 protein
3.5x10^5 undepleted and CD25$^+$ cell depleted PBMC were resuspended in 5% FCS RPMI with and without 1μg/ml 5T4 protein. IFN-γ release was measured after 18hrs. Antigen responses were measured in quadruplicate wells. Representative staining of wells incubated in either media alone (A) or 5T4 (B) is displayed.

Table 5.2. Suppression of tumour antigen specific *ex-vivo* responses

Suppression of tumour-specific responses by Treg	5T4		CEA	
	CRC	Control	CRC	Control
% of responders where tumour-specific responses were completely suppressed by Treg	9/15 (60%)	1/3 (33%)	8/17 (47%)	3/6 (50%)
% of responders where tumour-specific responses were partially suppressed by Treg	3/15 (20%)	1/3 (33%)	3/17 (18%)	3/6 (50%)
% of responders where tumour-specific responses were not suppressed by Treg	3/15 (20%)	1/3 (33%)	6/17 (35%)	0/6 (0%)
Total responses by all subjects	15/39 (38%)	3/11 27%	17/39 (44%)	6/11 (55%)

A



	R→I	NR→R	R→NC/D	N→N
CRC (39)	3 (8%)	9 (23%)	3 (8%)	24 (61%)
Controls (11)	1 (9%)	1 (9%)	1 (9%)	8 (73%)

KEY

R ⟶ I = A response to antigen pre-depletion is enhanced 1 fold post-depletion of Treg

NR ⟶ R = No response pre-depletion and a new response is uncovered post-depletion of Treg

R ⟶ NC/D = A response to antigen pre-depletion and either no enhancement or a reduction post depletion of Treg

N ⟶ N = No response to antigen pre-depletion and no response uncovered post-depletion of Treg

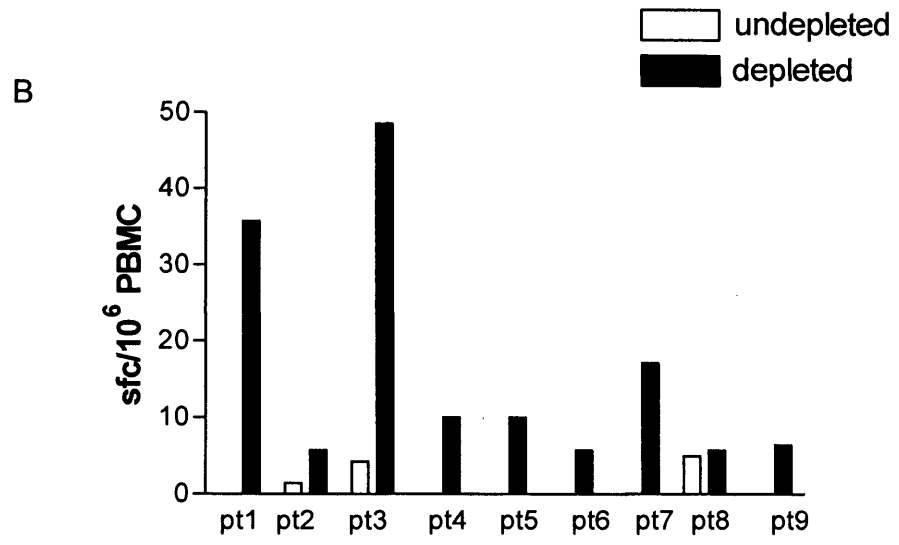


Figure 5.17. 5T4 specific *ex-vivo* IFN-γ release 5T4 following the depletion of CD25⁺ cells from PBMC.
Ex-vivo IFN-γ release ELIspot assays were performed to measure and compare recall responses by undepleted and $CD25^+$ cell depleted PBMC. 3.5×10^5 PBMC were incubated in quadruplicate wells for 18hrs with 1μg/ml of 5T4 protein and $sfc/10^6$ PBMC was calculated. A response was at least 50% above and 5 $sfc/10^6$ above the no antigen background. The effect of $CD25^+$ cell depletion on 5T4 responses were classified according to the above criteria and displayed as a percentage of the patient group (A). The $sfc/10^6$ PBMC of each 5T4 responder is displayed (B). Data was analysed using an unpaired student's t-test

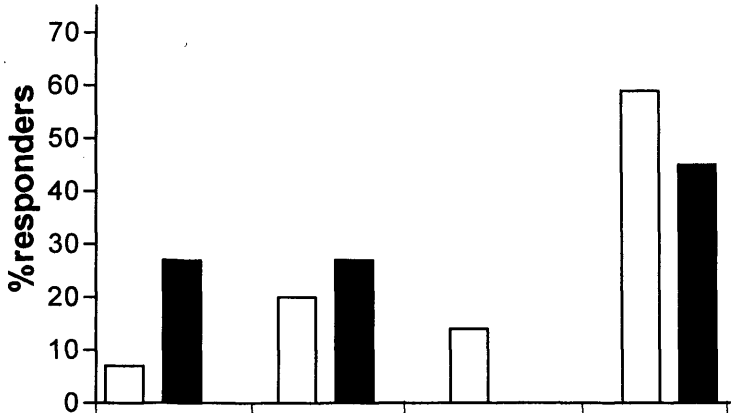
These results demonstrated that *ex-vivo* 5T4 specific responses are marginally more frequent in CRC patients (15/39, 38%) compared to normal controls (3/11, 27%). *Ex-vivo* release assays were performed to detect memory T cells that are primed to release IFN-γ when exposed to antigen (discussed later). It is interesting that 5T4 specific *ex-vivo* memory responses could be detected in controls that are not thought to express 5T4 but *ex-vivo* $CD8^+$ 5T4 peptide specific responses have also been detected in healthy controls by others (Redchenko, *et al.*2006). As expected, *ex-vivo* 5T4 responses occurred at a lower frequency in controls and CRC patients compared to the frequency of PPD and HA *ex-vivo* responses. 5T4 specific T cells might have been stimulated to enter the memory lineage through activation with antigens that are cross-reactive with 5T4. The fact that CRC patients have an increased frequency of responses that are completely suppressed by Treg (9/15, 60% of total responses) compared to control subjects (1/3, 33%) might arise because CRC patients have a raised level of 5T4 specific Treg. An expansion of 5T4 specific Treg might occur in patients if these cells encounter 5T4 antigen in the presence of a tolerising tumour environment.

It was possible that an expansion of 5T4 specific Treg in CRC patients contributed to the increased frequency that 5T4 specific response were completely suppressed. A preliminary comparison of 14 CRC patients (5/14 that developed a new 5T4 response after depletion) was performed to analyse if patients that responded to 5T4 after depletion had an elevated level of Treg in PBMC. An elevated level of Treg might have been expected in order to suppress anti-5T4 immune responses that correlated with the survival of 5T4 expressing tumour tissue in these patients. However, a comparison of Treg level failed to identify an elevated level of Treg in PBMC isolated from patients that responded to 5T4 after depletion and those that did not failed to respond. (data not shown).

CEA

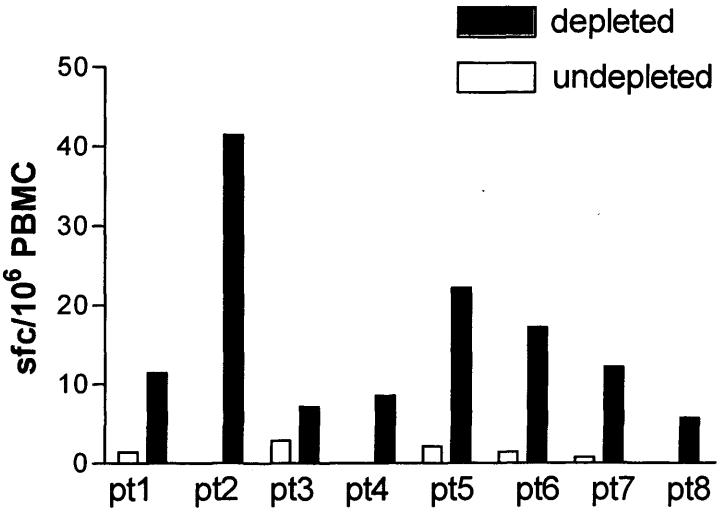
CRC patients and controls mounted a similar frequency of total CEA specific responses (including new responses, those partially suppressed and those not suppressed by Treg) (Table 5.2.). The frequency that these responses were completely suppressed by Treg (depletion of Treg uncovered new responses) was similar in CRC patients (8/17, 47%)

A CEA



	R→I	NR→R	R→NC/D	N→N
CRC (39)	3 (8%)	8 (21%)	6 (15%)	22 (56%)
Controls (11)	3 (27%)	3 (27%)	0 (0%)	5 (45%)

B



KEY

R ⟶ I = A response to antigen pre-depletion is enhanced 1 fold post-depletion of Treg

NR ⟶ R = No response pre-depletion and a new response is uncovered post-depletion of Treg

R ⟶ NC/D = A response to antigen pre-depletion and either no enhancement or a reduction post depletion of Treg

N ⟶ N = No response to antigen pre-depletion and no response uncovered post-depletion of Treg

Figure 5.18. *Ex-vivo* IFN-γ release by the tumour antigen CEA following the depletion of $CD25^+$ cells from PBMC.
Ex-vivo IFN-γ release ELIspot assays were performed to measure and compare recall responses by undepleted and $CD25^+$ cell depleted PBMC. 3.5 x 10^5 PBMC were incubated in quadruplicate wells for 18hrs with 1μg/ml of CEA protein and sfc/10^6 PBMC was calculated. A response was at least 50% and 5 sfc/10^6 above the no antigen background. The effect of $CD25^+$ cell depletion on CEA responses were classified according to the above criteria displayed as a percentage of the patient group (A). The sfc/10^6 PBMC of each CEA responder is displayed (B).

and controls (3/6, 50%) (Table 5.2.). The number of sfc/10^6 PBMC of each of the eight CRC patients that displayed a new CEA response following depletion of Treg (patients 1-8) is displayed (Figure 5. 18. B). In contrast, the frequency that Treg partially suppressed CEA specific responses (depletion of Treg enhanced an existing response) was reduced in CRC patients (3/17, 18%) compared to controls (3/6, 50%); whilst existing responses that were not suppressed (enhanced by depletion of Treg) by Treg occurred in CRC patients (6/17, 35%) but not controls (Table 5.2, Figure 5.18. A). Taken together, CEA specific responses were either completely or partially suppressed in a larger frequency of controls (6/6, 100%) compared to in CRC patients (11/17, 65%). Overall it appears that CEA specific responses occur at a similar frequency in controls and CRC patients but are suppressed more frequently in controls. This indicates that controls have a higher frequency of CEA specific Treg than CRC patients. The pattern of suppression of 5T4 and CEA specific responses by Treg was therefore different. Controls and CRC patients both had similar overall frequency of these responses but Treg appeared to suppress 5T4 responses more often in CRC patients but suppressed CEA responses more often in controls.

The effect of depleting Treg on 5T4 and CEA specific responses by each subject was compared to determine if removal of Treg enhanced multiple responses to tumour antigens (data not shown). CRC patients were analysed first and this analysis demonstrated that depletion of Treg could enhance or induce new responses to multiple tumour antigens. Treg depletion enhanced an existing response to either CEA or 5T4 in 14/44 patients; 4/14 patients had new responses to both 5T4 and CEA. Depletion of Treg was also capable of enhancing existing responses to both tumour antigens (1/5 patients that responded to at least one antigen). Depletion of Treg was also capable of enhancing responses to both tumour antigens in controls.

Six-day proliferation responses to the tumour antigens 5T4 and CEA

Six-day proliferation assays were performed to measure antigen specific immune responses to the tumour antigens 5T4 and CEA in the presence and absence of Tregs. Undepleted and Treg depleted PBMC were stimulated with 1μg/ml 5T4 and CEA protein, in separate wells, for six-days before proliferation was determined by the incorporation of [methyl-3H]-thymidine. As expected, responses to tumour antigens were significantly weaker than those to recall antigens (data not shown). For this reason, the criteria used to identify proliferation responses to recall antigens were not considered appropriate for analysing responses to tumour antigens. Data was instead analysed by comparing cpm of proliferation responses, after subtracting background cpm of experimental groups. The effect of depleting Treg on responses was categorised to either: enhance an existing response; inhibit or have no effect on an existing response; uncover a new response or; or to fail to uncover a response.

An overview of all responses by undepleted and Treg depleted PBMC showed that the process of Treg depletion inhibited six-day proliferation responses to tumour antigens in a relatively large proportion of subjects. For this reason it was deemed necessary to separately present cases where Treg depletion suppressed responses.

5T4

Data was analysed by subtracting non-specific background proliferation from cells incubated with 5T4. Six-day anti-5T4 proliferation responses were a log fold smaller than anti-PPD and HA responses (Data not shown).

The pattern of six-day anti-5T4 responses appeared different to 5T4 responses mounted *ex-vivo* in two ways. Firstly, substantially more subjects (23% (3/13) of CRC patients and 60% (5/9) of controls) mounted a six-day anti-5T4 response prior to depletion of Treg (Figure 5.19.). Secondly, depletion of Treg either had no effect on or reduced existing 5T4 responses in both controls and CRC patients (Figure 5.19.). This difference may be accounted for because a larger proportion of 5T4 specific cells resided in the $CD4^{+}CD25^{int}$ population and were removed in the process of depleting Treg. However, in this case it remains to be explained why Treg depletion uncovered 5T4 specific *ex-vivo* IFN-γ release.

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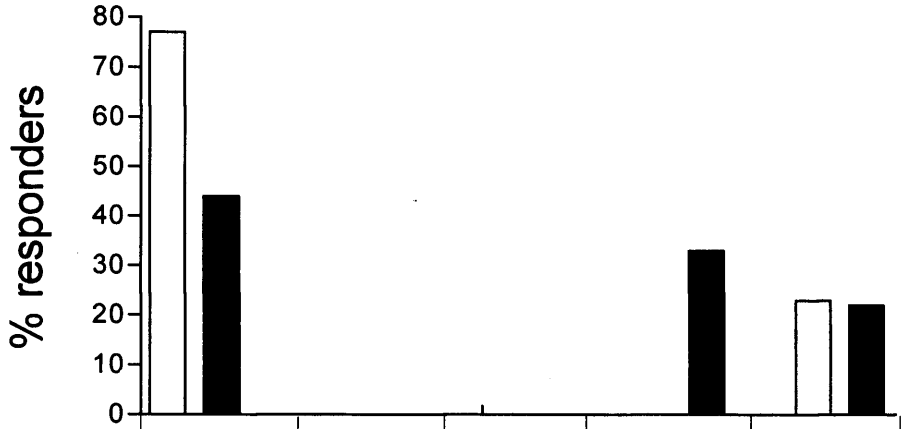


	NR→NR	NR→R	R→I	R→NC	R→D
CRC (13)	10 (77%)	0 (0%)	0 (0%)	0 (0%)	3 (23%)
Controls (9)	4 (44%)	0 (0%)	0 (0%)	3 (33%)	2 (22%)

KEY

NR ⟶ NR = No response to antigen pre-depletion and no response uncovered post-depletion of Treg

NR ⟶ R = No response pre-depletion and a new response is uncovered post-depletion of Treg

R ⟶ I = A response to antigen pre-depletion is enhanced >50% post-depletion of Treg

R ⟶ NC = A response to antigen pre-depletion and no enhancement post depletion of Treg

R ⟶ D = A response to antigen pre-depletion that is decreased post depletion of Treg

Figure 5.19. Depletion of Treg from CRC and control PBMC does not enhance proliferation of six-day anti-5T4 responses

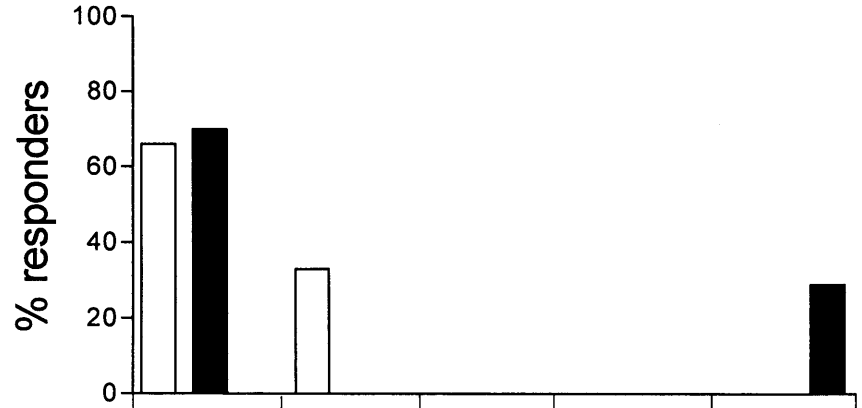
PBMC were obtained from CRC patients and controls. 2 x 10^5 undepleted and $CD25^+$ depleted PBMC were stimulated with 1μg/ml 5T4 protein for six days before proliferation was determined by the incorporation of [Methyl-3H]-thymidine. The effect of $CD25^+$ cell depletion on 5T4 responses was classified according to the above criteria and displayed as a percentage of the patient group (A).

CEA

Data was analysed by subtracting non-specific background proliferation from cells incubated with CEA. In a similar manner seen with six-day anti-5T4 responses, anti-CEA responses were substantially weaker than those against PPD and HA recall antigens (Data not shown). Substantially less subjects mounted six-day proliferation responses compared to those that mounted *ex-vivo* IFN-γ release responses. Approximately 70% of controls and CRC patients failed to respond to CEA (Figure 5.20. A). Tregs inhibited small responses to CEA in two CRC patients (depletion uncovered new responses) whilst depletion removed existing responses in two controls (Figure 5.20. A). Overall, anti-CEA responses were poor. In fact the proliferation of cells that did not respond following incubation with CEA was predominantly below that of cells incubated in media alone (demonstrated by subjects with a SI <1) (Figure 5.20. B). The reason for the suppression of proliferation identified with cells incubated with CEA remains to be determined definitively; however studies performed by others indicate that CEA may mediate inhibition of leukocytes and inhibit proliferation by binding CEA-related cell adhesion molecule 1 (CEACAM-1). CEACAM-1 is expressed on the cell surface by a wide variety of cell types, including T cells (reviewed in (Gray-Owen, *et al.*2006)). The molecule has a number of splice variants with restricted expression patterns. A number of these splice variants contain inhibitory motifs and are expressed on lymphocytes between 24-72 hours following activation (reviewed in (Gray-Owen, *et al.*2006)). Previous research has observed that soluble CEA could inhibit NK function (Stern, *et al.*2005), yet an inhibition of T cell function appeared to require a source of cell bound CEA (Kammerer, *et al.*1996). The reason for the conflicting reports of the ability of soluble verses cell bound CEA to suppress lymphocyte function is currently unclear.

To summarise the results of the proliferation assays, undepleted and Treg depleted PBMC mounted substantially weaker responses to tumour antigens 5T4 and CEA compared to HA and PPD. Six-day proliferation responses to CEA were poor and depletion of Treg did not substantially alter this situation. Further experimentation using

A



	NR→NR	NR→R	R→I	R→NC	R→D
CRC (6)	4 (66%)	2 (33%)	0 (0%)	0 (0%)	0 (0%)
Controls (7)	5 (70%)	0 (0%)	0 (0%)	0 (0%)	2 (29%)

KEY

NR ⟶ NR = No response to antigen pre-depletion and no response uncovered post-depletion of Treg

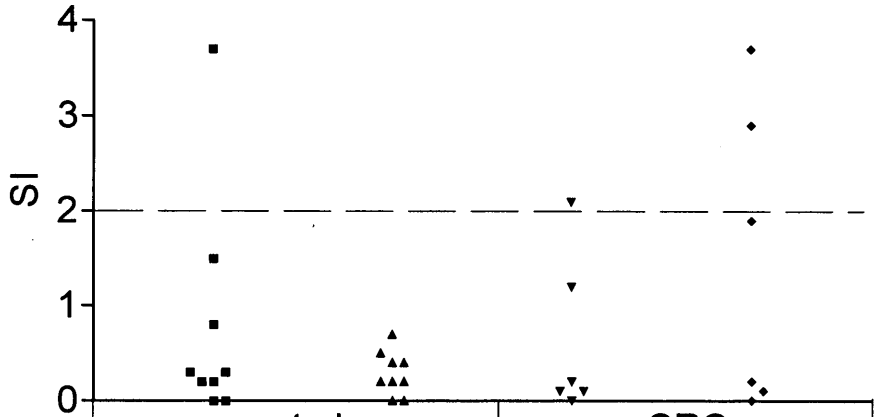
NR ⟶ R = No response pre-depletion and a new response is uncovered post-depletion of Treg

R ⟶ I = A response to antigen pre-depletion is enhanced >50% post-depletion of Treg

R ⟶ NC = A response to antigen pre-depletion and no enhancement post depletion of Treg

R ⟶ D = A response to antigen pre-depletion that is decreased post depletion of Treg

B



Group	control		CRC	
Treatment	CEAu	CEAd	CEAu	CEAd
Responder (SI≥ 2)	1/9 (11%)	0/9 (0%)	1/6 (16%)	2/6 (33%)

Figure 5.20. Depletion of Treg from CRC and control PBMC does not enhance proliferation of six-day anti-CEA responses
PBMC were obtained from CRC patients and controls. 2 x 10^5 undepleted (CEAu) and $CD25^+$ depleted (CEAd). PBMC were stimulated with 1μg/ml CEA protein for six days before proliferation was determined by the incorporation of [Methyl-3H]-thymidine. The effect of $CD25^+$ cell depletion on CEA responses was classified according to the above criteria and displayed as a percentage of the patient group (A). Data was also analysed by producing a SI of responses (B).

an antibody to block CEACAM-1 association with CEA may reveal CEA responses that are normally suppressed by Treg.

In contrast to *ex-vivo* IFN-γ responses, six-day proliferation responses to 5T4 were more frequent than those mounted *ex-vivo* and were not restricted to CRC patients. Depletion of Treg did not enhance existing or elicit new 5T4 proliferation responses.

The ability of Treg depletion to enhance 5T4 specific *ex-vivo* and six-day responses was compared for each subject (data not shown). In a similar situation as with recall antigens, analysis demonstrated that an elevation of 5T4 specific *ex-vivo* IFN-γ production by Treg depletion did not correspond to an enhancement of the 5T4 specific six-day response by depletion of Treg. In fact, all CRC patients that demonstrated a new 5T4 specific *ex-vivo* response and were included in six-day proliferation assay failed to mount a 5T4 specific response proliferation response. The reason for the inconsistent effect of depleting Treg in *ex-vivo* and six-day assays is not clear and is discussed in more detail below.

5.3. Discussion

The experiments described in this chapter set out to establish if Treg levels are elevated during colorectal malignancy and to examine the extent that Tregs inhibit immune responses in patients with CRC. These experiments were performed on the premise that Treg are primarily located within the $CD4^+CD25^{hi}$ lymphocyte population. This conclusion was based on functional assays to show that suppressive function was unique to $CD4^+CD25^{hi}$ sorted cells and absent from $CD4^+CD25^{int}$ and $CD4^+CD25^-$ cells (Baecher-Allan, *et al.*2001).

Analysis of FOXP3 expression by flow cytometry, using a recently available anti-FOXP3 antibody, established that FOXP3 expression is not restricted to $CD4^+CD25^{hi}$ cells but is also expressed by approximately 30% of $CD4^+CD25^{int}$ cells. Previous research using murine conventional $CD4^+CD25^-$ T cells has shown that forced expression of FOXP3 in these cells induces the suppressive Treg phenotype (Hori, *et al.*2003); and polyclonal stimulation of $CD4^+CD25^-$ T cells, to initiate an activated $CD4^+CD25^+$ phenotype, did not induce FOXP3 expression. These results initially indicated that $FOXP3^+$ expression is

directly associated with suppressive function. The association between FOXP3 expression and suppressive function is not as clear in human T cells as *de novo* FOXP3 expression has been demonstrated in conventional $CD4^+FOXP3^-$ cells following activation *in vitro* and subsequent analysis showed these newly created $FOXP3^+$ were not necessarily suppressive (Wang, *et al.*2007). It is therefore difficult to establish if FOXP3 expression by $CD4^+CD25^{int/-}$ cells confers suppressive function. Preliminary data presented in this study has demonstrated that $CD4^+CD25^{hi}$ cells express the higher levels of FOXP3 compared to $CD4^+CD25^{int}$ cells. A higher level of FOXP3 expression may confer suppressive function, whilst lower FOXP3 expression by $CD4^+CD25^{int}$ cells may not be enough to initiate the regulatory phenotype. This theory is compatible with the observation that suppressive function is restricted to $CD4^+CD25^{hi}$ T cells and absent from $CD4^+CD25^{int}$ sorted T cells (Baecher-Allan, *et al.*2001). However, as only approximately 30% of $CD4^+CD25^{int}$ T cells express FOXP3, the suppressive function of these cells may be diluted by $CD4^+CD25^{int}FOXP3^-$ cells. A more detailed analysis of the suppressive function of $CD4^+CD25^{int}$,$FOXP3^+$, that appear to express lower levels of FOXP3 compared to $CD4^+CD25^{hi}$ cells, is restricted as the FOXP3 staining protocol kills cells. This study has compared the level of Treg between patients and controls using the best available techniques at the time of analysis; however, further research is clearly needed to establish if additional Treg reside outside the $CD4^+CD25^{hi}$ compartment.

T cell function during malignancy

The precise reason for the elevation of Treg during malignancy is unclear. The tumour antigens 5T4 (Southall, *et al.*1990) and CEA (reviewed in (Thompson, *et al.*1991)) are expressed during normal foetal development and may have a role to control maternal immune recognition of the partially allogenic foetus; and indeed CEA has been shown to suppress T cell responses through an interaction with CEACAM-1 (reviewed in (Gray-Owen, *et al.*2006)) (described later). It is possible that 5T4, CEA and a large cohort of additional tumour antigens are endowed with an innate capability to suppress immune responses and may even initiate proliferation of Treg or the conversion of conventional T cells to the Treg phenotype. Murine studies have described an important role of TGF-β

and myeloid DC that present tumour antigens that drive the proliferation of Treg and suppression of anti-tumour immunity (described earlier in chapter 1). The environment within healthy intestinal tissue is considered to be highly tolergenic and is thought to be necessary to prevent destructive inflammation triggered by gut bacteria. It may be the case that tumours derived from bowel tissue are programmed to inherit these suppressive traits from healthy intestinal tissue and promote escape from immune recognition. Studies examining cytokine production by PBMCs isolated from CRC patients indicate that CRC patients may be systemically immunosuppressed due to factors released by colorectal tumour tissue (reviewed in (Evans, *et al.*2006)).

The theory that malignant disease may directly initiate immune suppression prompted an investigation of whether CRC patients had suppressed T cell responses compared to age matched controls. Initially, *ex-vivo* IFN-γ release and six-day proliferation assays by undepleted PBMC in response to the recall antigens HA and PPD were compared between healthy controls and patients with CRC. Data presented in this chapter revealed a marginal difference of PPD and HA specific *ex-vivo* IFN-γ release between group control and CRC patient groups but the CRC patient group did demonstrate reduced HA and PPD specific responses measured in six day proliferation assays. A close analysis of this data revealed that the old age of some patients with CRC correlated with low responses. A similar correlation between the progression of old age and magnitude of proliferation was not identified in controls, albeit the controls were younger. Further analysis with a cohort of older controls is necessary but it is possible that factors associated with old age and malignancy may combine to trigger the induction of immunosuppression in the elderly. Previous studies have reported impaired immune responses and an altered repertoire of T cells in elderly subjects. One study used deuterated glucose to label PBMC and track the level of cell proliferation and death. Elderly (mean age 76) and young (mean age 26) subjects PBMC were labeled *in vivo* and isolated for analysis by flow cytometry (Wallace, *et al.*2004). These experiments demonstrated that elderly subjects had approximately double the level of circulating $CD8^+CD45RO^+$ T cells and a lower level of $CD8^+CD45RA^+$ apoptosis. Expansion of $CD8^+$ T cells with impaired function in the elderly is thought to be largely due to a failure to clear viruses such as CMV and EBV and the subsequent chronic exposure to persistent

viral antigens (Wallace, *et al.*2004). Elderly subjects also demonstrate a defective $CD4^+$ T cell population (reviewed in (Kovaiou, *et al.*2006)). A defective capacity to form immunological synapse with antigen loaded antigen presenting cell is thought to correspond with a reduced level of IL-2 secretion and subsequent impairment of $CD4^+$ T cell activation. Addition of exogenous IL-2 is able to restore full responses *in vitro*. Memory populations formed during youth remain normal but new stimuli such as flu vaccination fails to prime long term memory and hints that elderly subjects have a selective impairment in forming new memory populations during exposure to novel antigens. Other research has demonstrated that healthy elderly subjects (age 65-99) $CD8^+$ T cell *ex-vivo* IFN-γ and perforin production is impaired; whilst $CD4^+CD25^-$ IL-2 production is also reduced compared to healthy young subjects (age 19-40) (Trzonkowski, *et al.*2006). In these experiments Treg were elevated in elderly subjects and their removal restored T cell function.

It is possible to speculate why six-day proliferation responses appear to be reduced in elderly CRC subjects whilst *ex-vivo* IFN-γ release only appears to be marginally reduced. Proliferation and *ex-vivo* IFN-γ release assays are thought to measure the responses of different memory T cell subsets, effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) respectively (reviewed in (Sallusto, *et al.*2004)). T_{EM} cells maybe distinguished from T_{CM} cells by high expression of CD62L and CCR7 (Sallusto, *et al.*1999). T_{EM} cells primarily reside in tissue and are primed to exert an immediate effector function but unable to undergo significant proliferation. Conversely, T_{CM} cells primarily reside within secondary LNs and are able to proliferate but require a period of reactivation before developing effector function. The finding that a proportion of CRC patients exhibited lower responses in six-day proliferation assays but not *ex-vivo* IFN-γ release assays implies that the activity of T_{CM} but not T_{EM} cells are impaired in this group. However, it is also possible that the observation of selective impairment of T_{CM} function reflects the different methods used to analyse T_{CM} and T_{EM} cells; whereby in elderly subjects the capacity to proliferate is lost before the capacity to release IFN-γ. Research of chronic LCMV infection in mice has indicated that T cells sequentially lose components of normal T cell function. During persistent exposure to cognate LCMV

antigen, T cells undergo various stages of exhaustion sequentially losing the ability to secrete IL-2, which is an important stimulus of T cell proliferation, then TNF-α and finally IFN-γ, before ultimately undergoing apoptosis (Wherry, *et al.*2003).

Treg mediated suppression of T cell function

A key aim of this project was to assess the extent that Treg suppress responses to tumour antigens. Treg mediated inhibition of these responses was measured by comparing responses to tumour antigens either in the presence or absence of Treg. Treg inhibition of responses to recall antigens were measured alongside that of tumour antigens in order to gauge the extent that Treg also suppress responses to non-self antigens and establish a comparison to put Treg suppression of responses to tumour antigen into context. Responses to the recall antigens PPD and HA were analysed as the majority of the UK population have been exposed to these antigens and it would be expected that CRC patients and control subjects would have a similar exposure to these antigens. Results presented in this chapter have shown Treg do suppress responses to recall antigens. Suppression of these responses was not universal and the extent of suppression varied between a partial to complete suppression; however, the level of suppression appeared similar between CRC patients and controls. This overview implies that controls and CRC patients have populations of Treg that suppress responses to recall antigens to an extent that is similar between subject groups. Treg specific to particular recall antigens may have been expanded following prior exposure to these antigens by vaccination.

Treg suppression of tumour antigen specific responses was also examined in this report and suggested that patients have a higher frequency of 5T4 specific Treg and controls have a higher frequency of CEA specific Treg. The reason for this is unclear but could perhaps be related to the different expression profile of these antigens in controls and patients. 5T4 expression in adults is largely restricted to tumour tissue (Starzynska, *et al.*1994, Starzynska, *et al.*1992) and potential 5T4 specific effector T cell expansion is presumably restricted to when 5T4 specific Treg may also be expanded. T cells in control subjects are not expected to be exposed to 5T4 antigen *in vivo* and therefore might be

present at a lower frequency in controls compared to CRC patients. A key future experiment is to analyse if CRC patients whose 5T4 specific responses were uncovered by depletion of Treg expressed 5T4 on their tumours.

In contrast to 5T4, CEA antigen is expressed at low levels by healthy controls (reviewed in (Thompson, *et al.*1991)) and in this case CEA Treg might be expanded in controls and subsequently suppress CEA specific responses mounted by controls. However, CRC patients also express CEA that could also expand CEA specific Tregs and would therefore be expected to suppress patient's CEA specific responses as frequently as in controls. However, this does not appear to be the case and might be explained because CEA is expressed to a much greater extent in CRC patient tumours and alter the expansion of CEA specific Treg and effector T cells in favour of a larger effector T cell population.

Healthy controls mounted a high frequency of 5T4 specific proliferation responses (5/9, 57%) compared to *ex-vivo* responses (3/11, 27%). Controls subjects also mounted a higher frequency of six-day 5T4 responses than CRC patients (3/13, 23%). The reason for this difference is unclear but might occur because control subjects have a larger subset of naïve 5T4 specific conventional T cells, which require six-day stimulation to be identified.

CEA suppressed six-day proliferation

CEA proteins imparted an innate and significant suppression of six-day proliferation, whereby CEA containing wells suppressed proliferation to below that observed by cells incubated in media alone. The six-day proliferation assay was subsequently deemed as inappropriate to measure CEA specific proliferation. CEA may inhibit proliferation by binding to CEACAM-1 molecules expressed by a variety of mononuclear cells. CEACAM-1 is expressed at a very low amount on resting $CD4^+$ cells but becomes upregulated within 24-72 hours following T cell activation (reviewed in (Gray-Owen, *et al.*2006)). Splice variants of CEACAM-1 contain an inhibitory ITIM motif which recruits the SHP-1 protein and suppresses T cell activation following TCR stimulus (Chen, *et al.*2004). Various studies have reported that CEA may bind CEACAM-1 expressed by T

cells (Kammerer, *et al.*1996) and NK cells (Stern, *et al.*2005) and inhibit cell activation. There is a degree of uncertainty over how CEA is thought to operate, as inhibition of anti-tumour NK activity was reported to occur through soluble CEA (Stern, *et al.*2005), yet others have implicated that CEA mediated suppression required cell-bound CEA to suppress T cells activation (Kammerer, *et al.*1996). It would be of interest to determine whether the inhibitory effect of CEA on T cell proliferation can be reversed using CEACAM-1-specific blocking antibodies.

The extent of immunosuppression imparted by CEA has major implications to anti-tumour mediated immune responses. Previous research has established that TIL do mount anti-tumour immune responses and the extent of infiltration has been correlated an improved disease free survival of patients undergoing elective surgery to remove colorectal tumours (Galon, *et al.*2006). According to previous observations (reviewed in (Gray-Owen, *et al.*2006)), activated T cells begin to upregulate suppressive splice variants of CEACAM-1 with 24-72 hours after activation. In this case, activated TIL will come into close cell-cell contact with tumours cells that will often express high levels of CEA that will ligate CEACAM-1 and inhibit TIL. The inhibitory activity of CEA ligation of CEACAM-1 is innate and is in theory able to inhibit TIL regardless of the antigen specificity. CEA is also found in the circulation of CRC patients (reviewed in (Shively, *et al.*1985)) and may induce some systemic immunosuppression. It is possible that surgical debulking of tumour and removal of the major source of CEA in CRC patients may reduce the ligation of CEACAM-1 and thereby improve anti-tumour immune responses to residual tumour by enhance a diverse array of tumour specific T cells. Optimal strategies to improve anti-tumour function and patient survival may therefore involve debulking of the tumour by surgery to reduce innate suppression mediated by the tumour, alongside depletion of Treg and vaccination with tumour antigens to boost anti-tumour immunity.

The increased frequency of Treg in CRC patients and immune responses revealed after depletion of Treg described in this chapter has recently being published (Clarke *et al* 2007) (appendix).

6.0. Discussion

6.1. Treg inhibit immunity to spontaneous carcinogen induced tumour development

This study used methylcholanthrene, a carcinogen that has been widely used to identify a role for a series of immune cell types and signalling molecules in suppressing tumour development. The study revealed several interesting findings. A significant enrichment of $CD4^+FOXP3^+$ Tregs in tumours developing *in vivo* was identified, where approximately half of total $CD4^+$ TIL were $FOXP3^+$. Moreover, injection of mice with the CD25-specific antibody, PC61, resulted in a transient depletion of a proportion of the Tregs prior to MC injection. This investigation revealed that a significant proportion of Treg escape PC61 mediated depletion indicating that previous tumour induction experiments performed using PC61 (Shimizu, *et al.*1999, Onizuka, *et al.*1999, Golgher, *et al.*2002, Sutmuller, *et al.*2001) overestimated the extent of Treg depletion using this antibody. When these experiments were performed, $CD4^+CD25^+$ cells were thought to encompass the majority of the Treg population in mice and accordingly that PC61 depleted the majority of Treg. Work in this thesis demonstrates however that a significant proportion of Treg do not express CD25 and therefore escape depletion with PC61. Recent investigations have reported that $CD25^-$ Treg are capable of suppression (Fontenot, *et al.*2005c) and it appears likely that a significant proportion of Treg that escape depletion are capable of suppressing anti-tumour immune responses. This may have led to an underestimation of the extent that anti-tumour immunity would be enhanced by the absence of Treg.

Despite the incomplete depletion of Tregs, use of PC61 significantly reduced MC mediated tumour development. Similar findings were reported in a separate study using Balb/c mice (Tawara, *et al.*2002) and interesting parallels can be drawn between this work and a study performed twenty years previously by Prehn and Kojima (Prehn, *et al.*1986). Prehn and Kojima, working on the premise that "the presence of autoimmunity implies an increased anti-tumour immunity" found that 3-day thymectomy inhibited development of tumours using a high dose of MC. Based on the observation that thymic production of $CD25^+$ T cells, capable of suppressing the activity of autoreactive T cells,

begins only on day 3 after birth in mice (Asano, *et al.*1996), it appears that inhibition of tumour development by day-3 thymectomy and by injection of the CD25-specific antibody, PC61, occurs by ablation of Treg activity.

Ambrosino and colleagues recently described treatment of Erbb2 transgenic mice with the CD25-specific antibody, PC61 (Ambrosino, *et al.*2006). These animals develop multiple mammary carcinomas as a result of overexpression of the Erbb2 oncogene. PC61-treated mice demonstrated reduced carcinoma multiplicity and a concomitant increase in immune responses to p185, the protein product of Errb2 (Ambrosino, *et al.*2006).

Previous studies demonstrated that vaccination of self-antigen expanded Treg and subsequently inhibited NK/NKT cell anti-tumour function and exacerbated MC mediated tumourigenesis (Nishikawa, *et al.*2005a). The key question arising from this thesis relates to the type of effector cells promoting tumour rejection after depletion of Tregs. Since Tregs have been shown to suppress both innate and adaptive immune responses to tumours (reviewed in (Betts, *et al.*2006)), it is likely that multiple arms of the immune system including both antigen-specific T cells and non-specific inflammatory responses are more effective following Treg depletion. Future experimentation should be performed to compare anti-tumour function in the presence and absence of Treg in the tumour environment and define which cell types and signalling molecules are inhibited by Treg during early tumour development. Such studies should allow the nature of the anti-tumour responses induced in Treg depleted, MC-injected, mice to be defined.

In the case of MC-induced tumours, Nishikawa *et al.* recently showed that Tregs, induced by immunisation with a SEREX-defined self antigen expressed in tumour cells, inhibited NK and NKT cells capable of inhibiting the development of MC-induced tumours, thus indicating that these cells are targets of Treg activity and important anti-tumour effector cells (Nishikawa, *et al.*2005c). Whether self-reactive responses contribute to anti-tumour immunity in this model requires further investigation.

Overall, experiments conducted in this report show that although PC61 has proved a useful tool to appreciate the role of Treg in malignant progression, new methods under development may improve the study the role of Treg in tumour immunity. Until now, the

intracellular location of FOXP3 has restricted effective depletion of Treg. New mice strains that co-express pertussis toxin in $FOXP3^+$ cells enable complete elimination of Treg (Kim, *et al.*2007), whilst siRNA technology capable of suppressing FOXP3 expression is under development. These technologies may not offer therapeutic potential but should nevertheless result in an improved understanding of Treg interactions within the tumour environment.

6.2. Treg suppress IFN-γ dependent anti-tumour function

IFN-γ is a pluripotent molecule and is likely to influence tumour growth through multiple mechanisms (Boehm, *et al.*1997). Previous research has documented that IFN-γ mediates an innate inhibition of MC tumourigenesis by promoting the deposition of a collagenous capsule that envelops MC particles and inhibits further mutagenesis (Qin, *et al.*2002) and also inhibits angiogenesis necessary for tumour growth. Other research has shown an acceleration of spontaneous tumour development in $p53^{-/-}$ mice when IFN-γ signalling is abrogated (Kaplan 1998). IFN-γ also contributes to adaptive immune responses by enhancing MHC presentation and T cell function (Boehm, *et al.*1997). IFN-γ signalling and lymphocytes have been shown to collaborate to reduce MC induced tumourigenesis (Shankaran, *et al.*2001). The relative contribution of each of these mechanisms still needs to be delineated; however the role of IFN-γ in propagating T cell mediated anti-tumour activity may be important. Results described in this report indicate that tumour development is reduced in the absence of Tregs and that this effect is lost in the absence of IFNγ. It is possible that IFN-γ is essential for enhancing the activity of tumour - specific T cells following Treg depletion. Alternatively, IFNγ may be the key anti-tumour cytokine produced by tumour-specific T cell responses uncovered as a result of Treg depletion. However, due to the multiplicity of IFN-γ function, it is possible that important anti-tumour functions that operated independently of Treg function were abrogated by IFN-γ deficiency. Future work may be performed to explore these possibilities.

6.3. IFN-γ contributes to Treg infiltration of tumours

Although IFN-γ clearly makes an important contribution to the control of tumour development, IFN-γ has also been shown to be necessary to mediate normal Treg function and control pathological inflammation in models of RA (Wang, *et al.*2006) and EAE (Kelchtermans, *et al.*2005). Crucially, IFN-γ has been shown to be capable of converting conventional T cells into Treg (Wang, *et al.*2006). This study has shown a markedly reduced Treg infiltration of tumours that developed in IFN-$\gamma^{-/-}$ mice compared to WT mice and indicates a role of IFN-γ to coordinate Treg migration into tumours and expand Treg TIL and in light of previous data (Wang, *et al.*2006), IFN-γ may play an important role in WT mice, converting conventional $CD4^+$ TIL into Treg within MC induced tumours. IFN-γ mediated conversion of Treg may contribute to the similarity of the TCR repertoire of Treg of conventional TIL observed in MC induced tumours (data not shown) and also account for the reduction of Treg in the absence of IFN-γ. This possibility could be tested by comparing TCRvβ usage by Treg and conventional T cells in TIL from WT and IFN-$\gamma^{-/-}$ mice.

The theory of immunosurveillance suggests that anti-tumour immune responses may effectively eliminate a proportion of the tumour but that escape variants frequently emerge and eventually form the bulk of the tumour, now unreceptive to the immune system and able to grow progressively. In light of this theory, it is interesting to speculate that a reduced Treg infiltration of tumours that develop in IFN-$\gamma^{-/-}$ mice is also the result of the tumours being exposed to a weaker anti-tumour immune response. In this case, anti-tumour cells have reduced function and therefore the drive to produce tumour escape variants capable of releasing soluble factors that attract Treg or promote their expansion is reduced.

Although Treg infiltration of MC induced tumours was reduced in the absence of IFN-γ, tumours that arose in IFN-$\gamma^{-/-}$ were still relatively enriched with Treg and demonstrated that although IFN-γ may play some role in enriching Treg in tumours, other factors clearly contribute to Treg infiltration.

In summary, these results show that Treg inhibit mechanisms that normally reduce tumour development. Future work should focus on identification of these mechanisms and those involved in promoting enrichment of Tregs within tumours.

6.4. CRC patients have an elevated frequency of Treg

Treg in PBMC

This study has shown that CRC patients have an elevated level of Treg, designated as $CD4^+CD25^{hi}$, within $CD4^+$ PBMC. A number of studies published during the course of this project have established similar findings within a wide range of human malignancies (reviewed in (Betts, *et al.*2006)). Early research identified Treg as $CD4^+CD25^+$ and therefore appeared to overestimate the frequency of Treg within cohorts of healthy controls and cancer patients. Assessment of Treg frequency improved as the field of T cell immunology progressed to use more stringent methods to distinguish Treg. Advancements came with the identification of Treg as $CD4^+CD25^{hi}$ and the association of molecules constitutively expressed by Treg such as CTLA-4, GITR and CD45RO (reviewed in (Betts, *et al.*2006)). The recent identification of FOXP3 as a unique marker of Treg has been used to confirm the Treg phenotype of $CD4^+CD25^{hi}$ cells. Despite different criteria used to distinguish Treg, later studies have maintained the observation that Treg with PBMC, LN and TIL and are elevated in patients with malignancy compared to healthy controls (Table 6.1.-6.3). The majority of studies (9/13) have demonstrated a statistically significant increase in Treg frequencies in the PBMC of cancer patients compared to healthy controls (Wang, *et al.*2005, Gray, *et al.*2003, Wolf, *et al.*2003, Sasada, *et al.*2003, Beyer, *et al.*2005, Ichihara, *et al.*2003, Liyanage, *et al.*2002, Schaefer, *et al.*2005, Chen, *et al.*2005b) whilst a minority did not (4/13) (Unitt, *et al.*2005, Woo, *et al.*2002, Viguier, *et al.*2004, Marshall, *et al.*2004). Neither the criteria used to identify Treg nor the particular type of malignancy could account why Treg were not elevated in certain studies but the fact that Treg may behave differently in some malignancies can not be dismissed.

Table 6.1. Frequency of Treg in patient PBMC with malignant disease

Criteria	Groups	Treg % of $CD4^+$ PBMC	Significance	Ref.
$CD4^+CD25^+$	control	8.9 +/- 3.5	(n=16)	(Ichihara, *et al.*2003)
	gastric	18.2 +/- 4.1	p <0.05 (n=20)	
	oesophageal	26.5 +/- 4.9	p <0.05 (n=10)	
$CD4^+CD25^+$	control	12 (2 -18)	Data not shown (n=7)	(Woo, *et al.*2002)
	lung	33 (10 -60)	(n=9)	
$CD4^+CD25^+$	control	9 (2 –17)	(n=35)	(Liyanage, *et al.*2002)
	breast	17 (3 –30)	p<0.01 (n=35)	
	pancreas	13 (4 –35)	p<0.01 (n=30)	
$CD4^+CD25^+$	control	12.5 +/- 3.9	(n=4)	(Marshall, *et al.*2004)
	Hodgkin's Lymphoma	13.5 +/- 4.2	p =0.71 (n=9)	
$CD4^+CD25^+$	control	26.5 +/- 4.7	(n=10)	(Sasada, *et al.*2003)
	gastric	43.7 +/- 12.7	p =$4x10^{-9}$ (n=55)	
	colorectal carcinoma	39.7 +/- 11.3	p =$9x10^{-7}$ (n=48)	
	pancreatic	44.0 +/- 9.0	p =$9x10^{-6}$ (n=13)	
	oesophageal	47.0 +/-9.8	p =$1x10^{-7}$ (n=17)	
	liver carcinoma	42.1 +/- 14.7	p =$9x10^{-4}$ (n=16)	
$CD4^+CD25^{hi}$	control	9.1	(n=14)	(Gray, *et al.*2003)
	melanoma	14.7	p=0.007 (n=20)	
$CD4^+CD25^{hi}$	control	2 +/- 0.5	(n=15)	(Wang, *et al.*2005b)
	Acute myeloid leukaemia	4.8 +/- 1.8	p <0.0001 (n=36)	
$CD4^+CD25^{hi}$	control	4.7 +/- 0.5	(n=34)	(Wolf, *et al.*2003)
	epithelial cancer	12.5 +/- 0.9	p <0.001 (n=42)	
$CD4^+CD25^{hi}$	control	9.2 (1.6-30.2)	(n= 48)	(Unitt, *et al.*2005)
	hepatocellular carcinoma	7.2 (1.2-23.3)	p =0.895 (n= 25)	
$CD4^+CD25^+$ $FOXP3^+$	control	4.5 +/- 1.1	(n=42)	(Beyer, *et al.*2005)
	B cell chronic lymphocytic leukaemia	10.4 +/- 4.4	p <0.001 (n=73)	
$CD4^+CD25^+$ $FOXP3^+$	control	5.8 +/- 1.7	(n=data not shown)	(Viguier, *et al.*2004)
	melanoma	4.9 +/- 1.9	p <0.37 (n=13)	
$CD4^+CD25^{hi}$ $FOXP3^+$	control	5.4 +/- 2.7	(n=17)	(Schaefer, *et al.*2005)
	head / neck carcinoma	10.1 +/- 4.7	RG p <0.04 (n=24) AD p <0.0008	
$CD4^+CD25^+$ $FOXP3^+$	control	11.5 +/- 0.9	(n=14)	(Chen, *et al.*2005b)
	lung malignant perfusion	9 +/- 0.6	p <0.001 (n=15)	
	lung non-malignant lavage	9.9+/- 0.8	p <0.001 (n=13)	

AD = Active disease; RG = Regressed disease

Table 6.2 Frequency of Treg in LN of patients with malignant disease

Criteria	Groups	Treg % of $CD4^+$ LN cells	Significance	Ref.
$CD4^+CD25^+$	pancreas / breast lymph node	20 (n=20)		(Liyanage, *et al.*2002)
$CD4^+CD25^+$	Hodgkin's Lymphoma	35.8 +/-16.6 (n=6)		(Marshall, *et al.*2004)
$CD4^+CD25^+$	cervical	N1TDLN 4.2 +/- 1.4	(n=13) not significant	(Fattorossi, *et al.*2004)
		N2TDLN 3.8 +/- 1.0		
	endometrial	N1TDLN 2.5 +/- 0.9	(n=26)	
		N2TDLN 1.8 +/- 0.9	p<0.2	
$CD4^+CD25^{hi}$	control mesenteric lymph node	1.2 +/- 0.3	(n=44)	(Kawaida, *et al.*2005)
	early stage gastric	N1 LN 3.3 +/- 0.3	(n=31)	
		N2 LN 2.4 +/- 0.3	(n=13) (p<0.05) higher in N1 vs N2 in early disease	
	late stage gastric	N1 LN 3.3 +/- 0.2	(n=13) p<0.01 higher than mesenteric lymph node	
		N2 LN 3.3 +/- 0.2	(n=13) p<0.02 higher in N1 and N2 during advanced disease compared to mesenteric lymph node	
$CD4^+CD25^+$	tumour free control lymph node	6 (2-9)	(n=6)	(Yang, *et al.*2006)
$FOXP3^+$	Non Hodgkin's lymphoma lymph node	17 (6-43)	(n=10) p<0.001	
$CD4^+CD25^+$	tumour free control lymph node	6.2 +/- 4.2	(n=16)	(Viguier, *et al.*2004)
$FOXP3^+$	melanoma	11.06 +/- 1.7	(n=13) p<0.0006	
$CD4^+CD25^+$	control lymph node from non-cancer patient	8	(n=8) p<0.01 control have MORE Treg	(Curiel, *et al.*2004)
	ovarian carcinoma stage 1	5	(n=6)	
	stage 2	5	(n=5)	
	stage 3/4	1	(n=25) p<0.01 early stage have MORE Treg than late	

Table 6.3. Frequency of Treg in TIL of patients with malignant disease

Criteria	Groups	Treg % of TIL	Reference
$CD4^+CD25^+$	gastric early	(n=7) (% of $CD3^+$ TIL) 4.8 +/-2.1	(Ichihara, *et al.*2003)
	late	(n=8) (%of $CD3^+$ TIL) 19.8 +/- 4.5 p<0.05	
$CD4^+CD25^+$	lung	(n=9) (% of $CD4^+$ TIL) 33 (20-40)	(Woo, *et al.*2002)
$CD4^+CD25^+$	pancreas/breast	(n=20) (% of $CD4^+$ TIL) 22	(Liyanage, *et al.*2002)
$CD4^+CD25^+$	uninvolved tissue	(n=6) (% of $CD4^+$ TIL) 2.4 (1.5-5.6) p=0.014	(Unitt, *et al.*2005)
	hepatocellular carcinoma	(n=12) (% of $CD4^+$ TIL) 8.7 (1.4-13.8)	
$CD3^+CD4^+$ $FOXP3^+$	head / neck carcinoma	(n=84) $CD3^+CD4^+FOXP3^+$ infiltrate present	(Badoual, *et al.*2006)
$CD4^+CD25^+$	breast carcinoma	(n=47) 60% (50% of $CD4^+CD25^+$ are $FOXP3^+$)	(Leong, *et al.*2006)
$CD4^+CD25^+$	adult T-cell leukaemia / lymphoma	(n=25) (% of malignant T cells) 32% $FOXP3^{hi}$	(Roncador, *et al.*2005)
$CD25^+FOXP3^+$	ovarian carcinoma	(n=117) low $CD8^+$: FOXP3 ratio indicates poor prognosis	(Sato, *et al.*2005)
$FOXP3^+$	ovarian carcinoma	(n=99) $FOXP3^+$ RT-PCR for defining prognosis	(Wolf, *et al.*2005)
$CD4^+CD25^+$ $FOXP3^+$	non-malignant ovarian tissue	(n=4) none detected	(Curiel, *et al.*2004)
	ovarian carcinoma	(n=104) 23 +/- 11	
	stage 1	(n=12) 12	
	stage 2	(n=25) 25	
	stage 3/4	(n=30) 30 p<0.001 more Treg in stage 2-4 compared to stage 1	
$CD4^+CD25^{hi}$	liver carcinoma	(n=5) 6.55 +/- 3.5	(Ormandy, *et al.*2005)

The work presented in this thesis demonstrates that Treg are elevated within PBMC during CRC thus contributing to the theory that malignancy is associated with an elevation of Treg. The majority of this study was performed before the availability of anti-human FOXP3 antibodies suitable for flow cytometry and Treg were therefore distinguished as $CD4^+CD25^{hi}$ and co-expression of CD45RO and CTLA-4 on these cells was used to confirm the Treg phenotype. Preliminary investigations performed late-on in this study used the recently available anti-human FOXP3 antibody to confirm the integrity of $CD4^+CD25^{hi}$ cells; however, it now appears that approximately 80% of Treg ($FOXP3^+$ cells) reside outside the $CD4^+CD25^{hi}$ population. Recent research has examined the fidelity of FOXP3 expression in humans and demonstrated activated effector T cells are capable of transiently expressing FOXP3 without exhibiting suppressive function (Wang, *et al.*2007). $CD4^+CD25^{hi}$ cells examined in this thesis expressed the highest levels of FOXP3 which may be a requirement for suppressive capacity. This raises the possibility that $FOXP3^+$ cells outside the $CD4^+CD25^{hi}$ population are not Treg. Further research is necessary to firstly confirm whether $FOXP3^+$ cells that reside in the $CD4^+CD25^{int}$ and $CD4^+CD25^-$ population are suppressive; and secondly, if $CD4^+CD25^{int}FOXP3^+$ and $CD4^+CD25^-FOXP3^+$ cells do prove to be suppressive, to confirm the growing consensus that patients with malignant disease have elevated levels of Treg. Previous investigations performed by sorting $CD4^+$ T cells according to the expression of CD25 identified that suppressive function was restricted to $CD4^+CD25^{hi}$ PBMC absent and from $CD4^+CD25^{int}$ and $CD4^+CD25^-$ cells (Baecher-Allan, *et al.*2001); but given that only 30% of $CD4^+CD25^{int}$ cells express FOXP3, potential Tregs present in the $CD4^+CD25^{int}$ population are already considerably diluted by conventional T cells before suppressive function was tested.

It is possible that induction of FOXP3 expression is part of the normal proliferation cycle of all T cells and recent research has demonstrated that Treg in humans have a high rate of proliferation, short telomeres and a turnover which implies that the $CD4^+CD25^{hi}$ Treg population need to be constantly replenished (Vukmanovic-Stejic, *et al.*2006). Researchers then investigated human blood T cell populations that use dominant TCRvβ chains promoted during chronic CMV infection and demonstrated clonal TCR homology between $CD4^+FOXP3^-$ and $CD4^+FOXP3^+$ expanded cells (Vukmanovic-Stejic, *et*

*al.*2006). The authors of this report concluded that peripheral Treg production is derived from rapidly dividing, highly differentiated memory T cells. The aforementioned report and data presented in this thesis present the possibility that T cells upregulate FOXP3 expression following activation and accumulate higher levels of FOXP3 and CD25 during chronic stimulation to obtain full regulatory function. These results correlate with the indistinguishable TCRvβ usage of conventional T cell and Treg TIL in MC induced tumours in mice (data not shown) and suggest the high level of Treg infiltration in these tumours may be derived by chronic stimulation of terminally differentiated memory T cells within TIL.

Treg in LNs

Obtaining information regarding lymph nodes from healthy people is difficult, although many studies have enumerated Tregs in tumour draining lymph nodes (TDLN) (Table 2). Increased frequencies of Tregs are seen in the tumour draining lymph nodes (compared to the non-draining lymph nodes in some studies of certain cancers including endometrial cancer, stomach cancer, melanoma and non Hodgkin's lymphoma (Table 6.2) (Kawaida, *et al.*2005, Yang, *et al.*2006, Viguier, *et al.*2004, Fattorossi, *et al.*2004). A recent study however, performed on lymph nodes from patients with early and late stage ovarian cancer actually reported a statistically significant decrease in the numbers of Tregs in lymph node compared to frequencies observed in patients without cancer (Curiel, *et al.*2004). In this case, the authors postulated that since in the same patients, the frequency of Tregs in the tumours correlated with the stage of the cancer, that the Tregs were fewer in frequency in the lymph nodes due to migration of the cells into the tumour. This possibility highlights the pitfalls of confining studies to one compartment such as the PBMC and/or lymph node, which does not take into account of the entire pattern of Treg migration in relation to the tumour.

Tregs in tumour tissue

This thesis has not investigated Treg infiltration of TIL, however, a particularly informative study examined TIL within a large cohort of CRC patients to establish the prognostic value of TIL (Galon, *et al.*2006). An examination of tumour biopsies isolated from 415 CRC patients-established that a high density of infiltration by T cells inversely correlated with recurrence of disease after surgery. Histological staging has served as the best indicator of prognosis, whereby patients with early stage of disease have a reduced level of disease reoccurrence than patients with advanced disease. An inverse correlation between the extent of T cell infiltration and level of reoccurrence persisted from patients with early stage disease through to advanced disease and actually served as more effective predicator or prognosis than histological staging of disease (Galon, *et al.*2006). These observations support the premise that immunosurveillance continues to target malignant cells in advanced disease despite alterations to transformed cells that enable disease progression. A selection of patients was identified with early stage disease with a low T cell infiltration that had a higher reoccurrence of disease than would be predicted by histological staging of their disease. The level of TIL in tumour could therefore identify those patients within high risk group of reoccurrence that would benefit from further adjuvant therapy after surgery (Galon, *et al.*2006).

Several studies have now identified Treg cells within TIL in a range of human malignancies (Table 6.3). It seems likely that if the Tregs act locally at the site of the tumour, then they would need to be present at a certain frequency in order to prevent effective anti-tumour immune responses. An indication of the pathogenic role of Treg in the progression of tumours is the increased Treg frequency observed in some tumours as the disease advances (Beyer, *et al.*2005, Curiel, *et al.*2004, Ichihara, *et al.*2003) (Table 6.3).

Three studies of patients with ovarian cancer have revealed a relationship between prognosis and TILs (Curiel, *et al.*2004, Sato, *et al.*2005, Wolf, *et al.*2005). The first study reported a statistically significant correlation between frequencies of tumour infiltrating Tregs and survival (Curiel, *et al.*2004). Whilst one interpretation of these data is that Tregs promote disease progression, it is also possible that the extent of Treg infiltration is a consequence rather than a cause of advanced disease. A second report did not find a significant association between the frequency of tumour-infiltrating Treg cells and poorer

survival but did find that a favorable prognosis correlated with the frequency of intraepithelial $CD8^+$ T cells and more significantly with a high ratio of $CD8^+$ T cells to $CD4^+Foxp3^+$ T cells (Sato, *et al.*2005). Further research quantified Foxp3 by RT-PCR in 99 samples from ovarian cancer patients and found that the level of Foxp3 in a tumour sample was predictive of overall survival (Wolf, *et al.*2005).

Two other studies have also reported that Treg frequencies are indicative of disease progression. One study grouped patients with gastric cancer according to the percentage of $CD4^+CD25^+$ PBMC in the total $CD4^+$ PBMC population: low <40%, medium 40-50% and high >50% (Sasada, *et al.*2003). Those classified as "high" demonstrated reduced survival compared to those classifed as low ($p=0.007$). Other investigations examined Foxp3 expression in T cell lymphomas (Roncador, *et al.*2005). Expression was detected only in adult T cell lymphomas (ATLL, 17/25 samples tested were Foxp3 positive) and although not statistically significant, a trend was observed between the extent of Foxp3 expression and survival. The presence of Foxp3 positive cells in combination with cells expressing cytotoxic granules was however, found to be associated with an unfavourable outcome in a study of patients with Hodgkin's lymphoma (Alvaro, *et al.*2005).A study of patients with head and neck cancer found that high levels of $CD4^+CD69^+$ T cells were associated with better locoregional control and survival (Badoual, *et al.*2006). Surprisingly, high levels of Foxp3 positive cells were also associated with better locoregional control, a finding that also appears to be at odds with the studies previously mentioned in this Section. The authors of this study postulate that the Tregs may in fact serve to dampen down inflammatory processes responsible for promoting tumour progression. The presence of Foxp3 positive cells in combination with cells expressing cytotoxic granules was found to be associated with an unfavourable outcome in a study of patients with Hodgkin's lymphoma (Alvaro, *et al.*2005). A similar situation has been described in mouse models of colon cancer. In addition to TILs, Tregs have been identified in ascites and pleural effusions (Table 6.4). Comparisons of the frequencies of Tregs (assessed for expression of Foxp3) within malignant and non-malignant ascites/pleural effusions observed an increased frequency of Tregs within the malignant group (Curiel, *et al.*2004, Chen, *et al.*2005b).

6.5. Identification of anti-tumour responses by depletion of Treg

Experiments were performed to establish if Treg inhibit ex-*vivo* IFN-γ release and six-day proliferation to the tumour antigens 5T4 and CEA by PBMC derived from CRC patients and controls. Preliminary data has shown the process of depleting $CD4^+CD25^{hi}$ cells (and, incidentally, a large proportion of $CD4^+CD25^{int}$ cells) described in this project removed approximately 50% of $FOXP3^+$ cells. As discussed above, further investigation is required to confirm that $FOXP3^+$ cells present outside of the $CD4^+CD25^{hi}$ population are suppressive. In light of the fact that 50% of $FOXP3^+$ cell remain in "Treg depleted" preps, the process of depleting $CD25^{hi}$ cells in this report may underestimate the frequency and magnitude of potential anti-tumour responses that are analysed following the "depletion" of Treg from PBMC.

Despite the incomplete depletion of all potential Treg, this study has documented the enhancement of anti-tumour responses following depletion of $CD4^+CD25^{hi}$ Treg. *Ex-vivo* IFN-γ specific 5T4 and CEA responses were enhanced in a substantial proportion of patients, whilst anti-CEA were also enhanced in a number of controls. Previous studies have demonstrated 5T4-specific $CD8^+$ T cell responses (described in Chapter 5). Data collected during this project has demonstrated that new *ex-vivo* IFN-γ release responses specific for 5T4 protein uncovered by the depletion of Treg were $CD4^+$ T cell restricted (Experiments were performed by Dr Sarah Clark). These results indicate that a significant proportion of CRC patients have $CD4^+$ effector memory T cells specific to 5T4 protein and that the majority of these responses are completely suppressed by Treg. 5T4 specific $CD4^+$ T cell responses identified in ELISpot assays could potentially coordinate immune responses to $5T4^+$ tumours, including $CD8^+$ responses observed by others (Redchenko, *et al.*2006, Smyth, *et al.*2006b) and induce regression of malignant disease in CRC patients. A recent report documented results of a phase I/II trial that vaccinated CRC patients with attenuated vaccinia virus vector encoding 5T4 protein (TroVax) (Harrop, *et al.*2006). Patients received injections with TroVax and the majority of patients developed humoural responses (14/17) and underwent seroconversion, with one patients' tumour showing signs of necrosis. TroVax vaccination also induced proliferation responses to

5T4 proteins (8/17) (Harrop, *et al.*2006). These were most likely mounted by $CD4^+$ T cells, given that external proteins are processed though the MHCII pathway. Although the number of patients in the TroVax clinical trial and this thesis are relatively small, a similar frequency of 5T4-specific proliferative responses were observed in both studies.

6.6. Manipulation of Tregs for Therapeutic Purposes

The aforementioned studies and reports from other studies have identified that Treg suppresses effective anti-tumour immunity in mice and are elevated in malignant disease in patients. These studies suggest that depletion of Treg may be performed to enhance anti-tumour immunity and contribute toward the treatment of patients with malignant disease. One possible treatment strategy identified in murine studies is depletion of Treg using CD25-specific antibodies; however a potential restriction with this treatment is the simultaneous depletion of conventional $CD25^+$ effector T cells, whose loss may compromise the beneficial effect of depleting the Tregs. Another effective approach tested in rodent models uses cyclophosphamide. Administration of cyclophosphamide in combination with tumour cell vaccination led to regression of established tumours in rats (Ghiringhelli, *et al.*2004). More recently, a combination of cyclophosphamide and tumour-derived exosomes has also been shown to promote rejection of established tumours in mice (Taieb, *et al.*2006).

Another strategy for treatment of established tumours is adoptive immunotherapy of T lymphocytes. Adoptive transfer of T cells specific for a melanocyte antigen was shown to result in regression of melanoma following their transfer into irradiated mice. Notably, co-transfer of $CD4^+CD25^+$ T cells abrogated this effect (Antony, *et al.*2005). Studies where lymphocytes have been transferred into patients with melanoma have identified T cell survival as an important correlate of anti-tumour activity (reviewed in (Dudley, *et al.*2003)), thus depletion of Tregs prior to transfer as well as lymphoablation (providing space for expansion and survival of transferred cells) may significantly improve the future success of adoptive immunotherapy.

Tregs constitutively express the IL-2 receptor α-chain CD25. However, CD25 is also

expressed on activated effector T cells. The usefulness of CD25 as a target for Treg depletion is therefore problematic since agents targeting CD25, e.g. CD25-specific antibodies that have a long half-life *in vivo*, would also target useful anti-tumour immune responses. Denileukin diftitox, trade name Ontak (Attia, *et al.*2005), is a recombinant protein, which has a short half-life (60 minutes) yet is cytocidal to cells expressing the high affinity CD25 receptor. It is a fusion protein consisting of part of the IL-2 chain (Ala_1 - Thr_{133}) fused with diptheria toxin fragments A and B (Met_1-Thr_{387}) and has recently been introduced for treating patients with cutaneous T cell lymphoma with promising results and acceptable toxicity. Dannull *et al.* recently reported that a single dose of denileukin diftitox selectively depletes Tregs (measured by loss of $CD4^+CD25^{hi}$ T cells *in vivo*, loss of suppressor activity), and enhanced immune responses following vaccination with dendritic cells transfected with tumour RNA in patients with renal cell carcinoma (n = 6) compared to those observed in patients (n = 4) receiving only vaccination (Dannull, *et al.*2005). Despite these promising findings, a seperate report indicated however, that treatment of cancer patients (12 with metastatic melanoma and 1 with metastatic renal cell carcinoma) with denileukin diftitox had no effect on reducing Treg number or function in the PBL of the patients (Attia, *et al.*2005). Different treatment regimes were used in these studies and further work is needed to understand these conflicting results.

As well as the use of agents that target the IL-2 receptor, results obtained in mice indicate that low doses of immunosuppressive agents such as cyclophosphamide reduce Treg numbers and function, thereby allowing anti-tumour responses to flourish. It will be of interest therefore to determine whether cyclophosphamide similarly represents a useful method of inactivating Tregs and promoting immune responses to tumours in cancer patients. Treatment of patients with fludaribine also holds some promise. Both the frequency and suppressor activity of Treg cells was significantly reduced in CLL patients after receiving fludaribine treatment demonstrating that Treg cells are more sensitive to fludaribine than other T cell populations (Beyer, *et al.*2005). Tumour regression was also observed in melanoma patients adoptively transferred with tumour-specific T cells following fludaribine-containing chemotherapy (Dudley, *et al.*2002). Other possibilities for inactivating Treg cells include targeting cell surface molecules known to be expressed

by Tregs (GITR, B7-H1) or cytokines/chemokines known to be involved in Treg survival, expansion and migration (TGFβ, IL-2, CCL22).
Investigations within this report indicate that Treg suppress 5T4 specific responses. Accordingly, 5T4 specific responses triggered by TroVax vaccination may be enhanced by co-depletion of Treg, possibly by administering TroVax with ONTAK. A similar approach appears unlikely to be possible with the CEA protein as it is more widely expressed in normal tissue and the boosting of CEA specific responses may induce autoimmunity; however, induction of autoimmune disease may be deemed to be acceptable if these responses induce regression of tumours.

6.7. Potential problems associated with manipulation of Tregs

Knowledge of Treg cells has highlighted several potential pitfalls when considering immunotherapeutic strategies that may be used for patients with cancer. A recent study showed that administration of IL-2, used for *treatment* of patients with melanoma, resulted in expansion of Treg numbers and an increase in Treg activity (Ahmadzadeh, *et al.*2006). There is also evidence, obtained in a mouse model, that immunisation with SEREX-defined self-antigens results in activation of Treg cells exhibiting potent suppressive activity and FOXP3 expression elevated 5-10 fold above that found in Treg cells isolated from naïve animals (Nishikawa, *et al.*2005b). Significantly, vaccination with these autoantigens inhibited immune recognition of tumours, demonstrated by increased metastatic nodules following i.v. inoculation of tumour cells (Nishikawa, *et al.*2003) and by an increase in the frequency and rate of tumour induction following injection of mice with methylcholanthrene (Nishikawa, *et al.*2005c). An inhibition of NK mediated cytotoxicity and a reduction in iNKT cell numbers was central to the suppression of tumour immunity by Tregs elicited by SEREX-defined antigens (Nishikawa, *et al.*2003).

Contrary to injection with the SEREX-defined autoantigens alone, co-immunisation with a peptide epitope recognised by $CD8^+$ T cells and expressed by the tumour cells, significantly enhanced tumour rejection (Nishikawa, *et al.*2001). Results of the study indicated that IFNγ, secreted by the $CD8^+$ T cells inhibited Treg activity.

Interestingly, tumour rejection by the $CD8^+$ T cells was abolished by depletion of $CD4^+$ T cells and the authors demonstrated that $CD4^+CD25^-$ Th1 cells recognising a SEREX defined autoantigen were primed and which were effective only when Tregs were inhibited. This study highlights the importance of epitope mapping in order to precisely define the antigens recognised by Tregs and in particular, determining whether Tregs and conventional $CD4^+$ T cells recognise the same or distinct peptide epitopes. This information is clearly key to the design of vaccines that maximise activation of conventional effector T cell responses whilst minimising activation of Tregs.

There is general agreement that the suppressive effect of Tregs is not antigen-specific and in the case of tumours would impinge on immune responses to all tumour antigens, including self-antigens. Some studies have observed autoimmune depigmentation in melanoma-immune mice following depletion/inactivation of Tregs (Jones, *et al.*2002, Sutmuller, *et al.*2001). Several studies have shown that simply depleting $CD25^+$ T cells does not, at least in the mouse strains tested, induce autoreactivity thereby demonstrating that an appropriate environment for antigen presentation is required to initiate an immune response even in a Treg-depleted host. This point was recently illustrated in a study by Wei *et al.* who found that mice mounting an immune response to tumour cells injected with thyroglobulin in the absence of $CD25^+$ T cells, were more prone to developing thyroglobulin-specific autoreactivity than those that did not respond to the tumour indicating that where depletion of Tregs leads to an active anti-tumour response, the potential for inducing autoreactivity against concurrently presented antigens is increased (Wei, *et al.*2005). It has also been observed that cancer patients showing signs of tumour regression after receiving CTLA4-specific antibodies suffered autoimmune side effects such as dermatitis, enterocolitis, hepatitis and hypophysitis (Phan, *et al.*2003). The effect of administering CTLA4-specific antibodies may be more far-reaching however than the targeting of Treg cells since CTLA4-specific antibodies may promote rejection of tumours through abrogating Treg activity whilst simultaneously directly boosting effector T cell activity.

Finally a large body of evidence indicates that inflammation can promote tumour growth (Balkwill, *et al.*2004). Since the results of some recent studies indicate that the inhibitory effects of Tregs extend to antigen-non-specific inflammatory responses, it is possible that Tregs, by suppressing inflammatory responses, actually hinder the progression of some tumours. Evidence to support this possibility is accumulating; adoptive transfer of $CD4^+CD25^+$ T cells was shown to prevent development of *Helicobacter hepaticus*-associated tumours in infected Rag – knockout mice (Erdman, *et al.*2003). Similarly, adoptive transfer of Tregs hindered development of intestinal adenomas in ApcMin/+ mice, an effect that correlated with a decrease in pro-inflammatory cytokines and Cox2 expression (Erdman, *et al.*2005). In agreement with this finding, Sharma and colleagues, have shown that inhibition of Cox2 led to a decrease in Treg activity with a concomitant increase in production of pro-inflammatory cytokines and *in vivo* growth of Lewis Lung carcinoma in mice (Sharma, *et al.*2005). Collectively, these data imply that Tregs can suppress the activity of different arms of the immune system, which in turn promote or impede tumour growth.

6.8. Conclusions

Future strategies to treat malignant disease will be aided by identification of T cell responses to tumour antigens and methods to enhance these responses to an extent that initiates clinical regression of tumours. Overall, there is overwhelming evidence, including results presented in this thesis, to support the hypothesis that Tregs can inhibit immune responses to tumours and promote cancer progression. It may therefore prove useful, in future clinical trials, to combine Treg depletion with delivery of anti-tumour vaccines or the adoptive transfer of anti-tumour T cells; depletion of Treg may enhance the capability of these treatments to effectively clear tumour cells. Results in this study have indicated that cancer patients may have populations of Treg that recognise and promote suppression of responses to particular tumour antigens. Previous research has also shown that vaccination of tumour antigens may expand populations of Treg, particularly if these are self-antigens (Nishikawa, *et al.*2005a). It is therefore important to discriminate between tumour antigens recognised by conventional effector T cells and

those recognised by Treg in order to avoid selectively expanding the corresponding Treg population. It may be the case that particularly in large primary tumours, effector T cells convert to a regulatory phenotype when exposed to the intra-tumour tolerising environment. In these circumstances, Treg and effector T cells may recognise the same epitopes and co-depletion of Treg would be important avoid their expansion by vaccination.

Although Treg do appear to confer a large degree of suppression of anti-tumour immunity, other mechanisms are also involved in reducing effective tumour-specific immune responses (reviewed in (Smyth, *et al.*2006a)). These include a reduced presentation of tumour antigens; due to down-regulation of tumour MHCI (reviewed in (Algarra, *et al.*2000; Marincola, *et al.*2000)) and molecules associated with MHCI antigen presentation and processing (reviewed in (Seliger, *et al.*2000)), including β2-microglobulin, low-molecular weight protein (LMP) and transporter associated with antigen processing (TAP). Tumour cells may also lose sensitivity to IFNs (Dunn, *et al.*2005b; Kaplan, *et al.* 1998; Wong, *et al.*1997) further reducing antigen presentation. Tumour cells have been described to secrete soluble ligands of inhibitory NK receptors (Groh, *et al.*2002); and are in fact capable of producing a range of soluble factors, including TGF-β, IL-10, vascular endothelial growth factor (VEGF) and indoleamine 2,3-dioxygenase (IDO) that all have suppressive effects on the immune system (reviewed in (Kim, *et al.*2006b))

Data presented in this study has suggested that the age may correlate with a weaker immune system and an enhanced susceptibility to malignancy. In addition, preliminary work in this report suggests that CEA secretion represents a mechanism used by tumours to evade anti-tumour immunity. Blocking the interactions of T cells and secreted factors from tumours might therefore be necessary to optimise the anti-tumour capacity of a given immune response.

Overall, it appears likely that the generation of effective immune responses capable of tumour rejection is going to require a multi-pronged approach aimed not only at providing optimal stimulation of effector cells but also at disabling the myriad of immunsuppressive strategies implemented by tumour cells to escape the attention of the immune system.

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d-1/3	MC d 0	d 21/23	d 41/43
G	400ug	G	G
P	400ug	G	G
P	400ug	P	G
P	400ug	P	P
G	400ug	P	G
G	400ug	G	P

Appendix Table 1 Schedule of multiple rounds of antibody injections
Mice were injected with two 500μg doses of PC61 (P) or isotype control GL113 (G) antibody prior to the administration of MC. Groups of mice then received various combinations of further injections of either PC61 or GL113 mAbs

Treatment	Surviving mice	% Surviving mice
GGG	1/20	5
PGG	4/10	40
PPG	1/8	12.5
PPP	4/10	40
GPG	0/10	0
GGP	1/10	10

Appendix Table 2. Multiple rounds of $CD25^+$ cell depletion does not enhance immunosurveillance.
Tumour incidence in each treatment group is summarised (Table 2).

Patient	Age	Duke	PPD		HA		
			undepleted	CD25^{hi} depleted	undepleted	CD25^{hi} depleted	
1	72	C	41.43	32.86	-1.43	-1.43	
2	70	C1	118.57	537.14	25.71	35.71	
3	83	C1	45.71	12.86	-1.43	1.43	
4	79	D	2.86	2.86	14.29	51.43	
5	80	C1	7.14	5.71	41.43	28.57	
6	65	B	75.71	74.29	21.43	78.57	
7	87	C	4.29	37.14	5.71	14.29	
8	66	C	11.36	22.85	-1.43	8.57	P
9	88	A	4.29	62.86	2.86	-32.86	P
10	80	B	148.57	45.71	-1.43	0.00	P
11	71	B	15.71	44.29	25.71	4.29	P
12	67	C	45.71	32.86	1.43	62.86	P
13	83	C	4.29	50.00	5.71	38.57	P
14	82	B	12.86	10.00	12.86	5.71	
15	76	B	4.50	26.50	0.50	-1.00	P
16	62	A	-1.43	71.43	14.29	25.71	P
17	66	C1	22.86	94.29	12.86	21.43	P
18	82	B	-15.74	187.12	-54.28	70.00	P
19	75	C	20.00	82.86	57.14	74.29	P
20	73	C	7.86	102.85	23.57	5.71	P
21	60	A	45.71	125.71	8.57	155.71	
22	63	A	22.86	40.95	18.57	185.24	
23	50	A	106.43	297.85	-3.57	6.43	
24	61	C1	51.43	172.85	-10.01	2.85	
25	73	C	10.00	110.00	12.86	25.71	
26	60	C1	212.85	391.42	4.29	7.14	
27	47	B	12.85	15.71	11.42	7.13	
28	73	C1	46.43	157.14	85.00	155.71	
29	57	C	24.29	181.43	0.00	34.29	
30	56	B	116.43	106.43	13.57	23.57	
31	62	B	172.85	751.40	-3.14	14.28	
32	46	B	270.00	449.99	8.57	8.57	
33	66	B	40.71	36.43	3.57	3.57	
34	70	B	7.86	-2.14	9.29	5.00	
35	68	B	34.29	43.57	4.29	-2.14	
36	63	C	-7.86	5.00	-15.01	6.42	
37	57	A	45.00	2.14	36.43	6.43	
38	72	B	1.43	-14.29	-2.86	71.43	
39	82	B	4.29	7.86	5.71	27.86	

Appendix Table 3 ***Ex-vivo*** **IFN-γ release following exposure to recall antigen.** ELISpot assays were carried out by stimulating PBMC for 18hr with 1μg/ml of PPD and HA antigens in separate wells. An antigen specific response was designated by a spot count of at least 50% above background and ≥5 sfc/10^6 cells incubated in media alone. The ability of Treg to enhance antigen-specific responses to HA and PPD antigens was designated when depletion of Treg enhanced the number of spots by at least 50%, after subtracting the background number of spots in unstimulated wells. A new response was only considered genuine if depleted PBMC also fulfilled the criteria of being at least 50% above background and ≥5 spots above those of unstimulated cells. The effect of depleting Treg on the response of each subject either: enhanced an existing response (blue cells); uncovered a new response (green cells); had no effect or reduced an existing response (purple cells); or failed to uncover a response (yellow cells). Those patients PBMC that were also examined by six-day proliferation assay are identified by P.

Appendix Table 4. Part 1

PPD

Response	Duke's A	Duke's B	Duke's C	Duke's D
NR->NR	0 (0%)	2 (13%)	0 (0%)	1 (100%)
NR->R	2 (33%)	2 (13%)	3 (18%)	0 (0%)
R-> I	3 (50%)	3 (20%)	10 (59%)	0 (0%)
R->NC/D	1 (19%)	8 (54%)	4 (24%)	0 (0%)
Total	n=6	n=15	n=17	n=1

HA

Response	Duke's A	Duke's B	Duke's C	Duke's D
NR->NR	2 (33%)	4 (27%)	4 (24%)	
NR->R	3 (50%)	3 (20%)	4 (24%)	
R-> I	3 (50%)	3 (20%)	5 (24%)	1 (100%)
R->NC/D	1 (17%)	5 (33%)	5 (29%)	
Total	n=6	n=15	n=17	n=1

Appendix Table 4. Part 2

PPD			
Response	n=	mean age	age range
NR->NR	n=3	77.7	72.82
NR->R	n=7	77.3	62-88
R-> I	n=16	64.1	46-75
R->NC/D	n=13	68.7	47-83
HA			
Response	n=	mean age	age range
NR->NR	n=10	71.0	50-88
NR->R	n=7	66.0	57-82
R-> I	12	70.8	56-87
R->NC/D	n=10	67.0	46-82

Table 4. The effect of Treg depletion on *ex-vivo* IFN-γ release following exposure to recall antigen. A breakdown of responses within each Duke's stage.
The effect of depletion of Treg on *ex-vivo* IFN-γ release to recall antigens PPD and HA was determined as previously described (Appendix, Table 3). The effect of depleting Treg on the response of each patient was classified to either: enhance an existing response (R->I); uncover a new response (NR->R)); have no effect or reduce an existing response (R->NC/D); or failed to uncover a response (NR->NR). A summary of the effect of Treg depletion according to the Duke's stage of patients' tumour (appendix Table 4.1) and age of the patient (appendix Table 4.2) is displayed.

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Regulating the immune response to tumours ☆

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Abstract

Naturally occurring regulatory T cells (Tregs) have been shown to suppress immune responses to self-antigens, thereby limiting autoimmunity. In the case of tumours, where immune responses to self-antigens are beneficial and lead to elimination of the tumour, such suppressive activity is actually detrimental to the host. Manipulation of Tregs holds great promise for the immunotherapy of cancer. Several studies performed using rodent models and indicate that Tregs cells inhibit effective anti-tumour immune responses and that their removal promotes tumour rejection. The increasing number of studies of Tregs in patients with cancer also point to a role for these cells in promoting disease progression. This review summarises the findings of these studies and addresses the advantages and potential pitfalls of manipulating Treg activity for the treatment of cancer.

Keywords: Tumour immunity; T regulatory cells

Contents

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1. Introduction

The immune system has evolved to protect the host from infection with pathogens. For this purpose, a series of immune cell surface receptors act as sensors that recognise and bind "pathogen associated molecular patterns" (PAMPs), such as double-stranded RNA or components of microbial cell walls. These interactions trigger cell activation and cytokine production resulting in an ordered sequence of events that through the concerted action of a number of different effector cells results in control of the infection (reviewed in [1,2]). Although recognition of PAMPs is a highly effective means of immune activation, the adaptive arm of the immune system has also the capacity to recognise a plethora of antigenic epitopes using the B and T cell antigen receptors. While these receptors allow recognition of foreign antigens, thereby contributing to recognition and elimination of invading pathogens, the antigen receptors are also capable of recognising self-antigens and therefore retain the potential to mediate destruction of normal host tissues and organs. Despite processes of clonal deletion in the bone marrow and thymus, autoreactive B and T cells can still be demonstrated in healthy individuals, indicating that other mechanisms, operating in the periphery, must exist to limit activation of these cells. The notion that active processes of immune regulation operate to control unwanted immune responses has been the subject of several studies and much controversy and debate for at least 30 years. The last 10 years, however, have witnessed a renaissance in the field of active mechanisms of immune regulation, and much of this renewed interest centres on a population of T cells, currently described as $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) (reviewed in [3,4]). The view that these cells keep the immune system in check, limiting the potential for autoreactivity and autoimmune disease, is becoming increasingly accepted. The poor immunogenicity of tumour cells and the fact that the vast majority of antigens expressed by tumours are also expressed on normal cells, led to the hypothesis that Tregs must also limit immune responses to tumours. Indeed, studies using a variety of rodent models and more recently studies of human cancer imply that Tregs have a major impact on tumour development. These studies and the possibility that Tregs can be manipulated for immunotherapeutic purposes will form the focus of this review.

2. Characteristics of Tregs

The core features of Tregs include (1) constitutive expression of CTLA4 and GITR, (2) expression of the transcription factor, Foxp3, (3) an inability to proliferate in response to TCR-driven signals *in vitro*, (4) dependence on IL-2 for expansion and function [5] and (5) the ability to suppress the proliferation of other T cells *in vitro* and *in vivo* (reviewed in [4]). Recent studies of mice expressing fluorescent reporter-Foxp3 fusion proteins have indicated that mouse Tregs develop in the thymus and that Foxp3 expression does indeed define a regulatory T cell lineage [6,7]. The features listed above are common to both mouse and human Tregs, but some differences do exist between the species. Whilst the majority of $CD4^+CD25^+$ T cells found in naïve mice express Foxp3 and express the functional and phenotypic characteristics of Treg cells, the equivalent cells in humans lie largely within the $CD4^+$ T cell population that express high levels of CD25 ($CD4^+CD25^{hi}$ cells) [8]. In addition, several reports indicate that unlike mouse $CD4^+$ T cells, human $CD4^+CD25^-$ T cells, though lacking Foxp3 expression initially, can upregulate Foxp3 expression upon repeated stimulation with antigen *in vitro*, and possibly therefore *in vivo* [9]. The phenotypic and functional characteristics of these cells

are indistinguishable from freshly isolated Treg cells. Lastly, two isoforms of Foxp3 have been identified in human T cells whereas only one of these is represented in mouse cells [10]. The functional significance of the second isoform, and indeed, whether a single T cell expresses both isoforms concurrently, is not yet known.

3. Influence of Tregs on immune responses to tumours

3.1. Rodent studies

Robert North et al. provided evidence for suppressor T cell involvement in anti-tumour immune responses in the 1980s [11]. The results of these studies pointed to a role for a population of $CD4^+$ T cells in inhibiting tumour immunity, an activity that could be abrogated by treating mice with the immunosuppressive agent, cyclophosphamide [12,13]. Many parallel exist between these and current studies of Tregs, whose depletion can also promote immune-mediated control of tumour growth, concomitant tumour immunity and whose activity is abrogated by cyclophosphamide treatment [14–19].

3.2. Human studies

3.2.1. Markers used to identify and enumerate Tregs

Analysis of mouse Tregs has proven to be relatively straightforward since $CD4^+CD25^+$ T cells in mice are predominantly Treg cells, an assertion recently confirmed by single-cell analysis of Foxp3 expression. This is a consequence of clean housing conditions and limited exposure to pathogens. In humans, intermittent exposure to environmental pathogens results in conventional T cell activation and consequently expression of CD25 on $CD4^+$ T cells, making identification of the Treg population difficult. Prior to the advent of Foxp3-specific antibodies, a cohort of markers were used to define human Tregs in order to derive as accurately as possible an assessment of their frequency during health and disease. As a consequence, many studies, including studies of patients with cancer have used different criteria for the identification and enumeration of Treg cells, making direct comparisons of Treg frequencies between studies difficult.

Most early studies of human malignancy defined Tregs using a platform of CD4 and CD25 co-expression and some excluded recently activated cells that expressed CD69 [20–23], lacked CD62L [24–26], exhibited a blastoid phenotype [21], or expressed Ki67 [21,26]; others corroborated the $CD4^+CD25^+$ Treg phenotype by analysing expression CD45RO and CTLA-4 [20–22,26–31]. As new data demonstrated the distinction in level of CD25 expression between mouse and human Treg, later studies proceeded to define Tregs using $CD4^+CD25^{hi}$ co-expression and often included expression of the constitutive Treg markers, CTLA-4, GITR, CD45RO and TGF-βR1 [21,30]. The discovery of the transcription factor Foxp3 and its seminal role in Treg function has greatly improved the ability to study Treg cells (reviewed in [32]). While new reagents have enabled easy detection of Foxp3, due to its nuclear localisation, direct detection is not possible on live cells, meaning that while Foxp3 is a good marker for Treg cells, we still have no way to clearly isolate and perform functional studies with Foxp3+ Treg cells. Initially, Foxp3 was detected by RT-PCR, which allowed investigators to confirm that Foxp3 expression, and therefore, that Treg activity is found predominantly within the $CD4^+CD25^{hi}$ population, thereby validating previous Treg analyses that were based on CD25 staining [24,33]. The recent introduction of anti-Foxp3 antibodies to directly immunolabel individual cells has simplified the accurate assessment of Treg frequency [25,26,34,35] and again confirms that Foxp3 expression is found predominantly, although not exclusively, within the $CD4^+CD25^{hi}$ T cell population [24,31,36,37]. The phenotype of a human Treg cell is now generally accepted as $CD4^+CD25^{hi}CTLA4^+$ $GITR^+Foxp3^+CD45RO^+CD45RA^-CD69^-Ki\text{-}67^-$.

3.2.2. Frequency of Tregs in PBMCs of cancer patients

In light of evidence showing that Treg cells suppress anti-tumour immune responses in mice, preliminary studies in humans using the markers described above in conjunction with functional Treg assays investigated the frequency of these cells in cohorts of healthy controls and patients. The hypothesis under study is that more Tregs will be found in patients with cancer than healthy controls. Despite the lack of an available definitive Treg marker during early studies, many of the findings made in the first studies have been corroborated in later studies that used the Treg marker, Foxp3. Overall, whilst refinement of the methodologies has led to a re-evaluation of Treg frequencies in both healthy controls and patients

with malignancy, the conclusion that Treg frequency is increased in malignant disease has been verified. The results of a number of studies, looking at a spectrum of different cancers, are summarised in Table 1. Most studies (9/12) demonstrate a statistically significant increase in putative Treg frequencies in the PBMC of cancer patients compared to healthy controls [20–24,27,29,37,38] whilst a minority did not (3/12) [26,28,33,39].

3.2.3. Treg frequency in lymph nodes of cancer patients

Hiura et al. reported that Treg cells capable of suppressing immune responses to tumours were enriched in the tumour draining lymph nodes of mice compared to distal lymph nodes [40]. Obtaining information regarding lymph nodes from healthy people is difficult, although many studies have enumerated Tregs in tumour draining lymph nodes (TDLN) (Table 2). Increased frequencies of Tregs are seen in the tumour draining lymph nodes compared to non-draining lymph nodes in some studies of certain cancers including endometrial cancer, stomach cancer, melanoma and non-Hodgkin's lymphoma (Table 2) [30,31,33,41]. A recent study, however, performed on lymph nodes from patients with early and late stage ovarian cancer actually reported a statistically signif-

Table 1
Treg as % of total $CD4^+$ PBMC

Criteria	Groups	Treg % of $CD4^+$ PBMC	Significance	Reference
$CD4^+CD25^+$	Control	8.9±3.5	(n=16)	[27]
	Gastric	18.2±4.1	$p<0.05$ (n=20)	
	Oesophageal	26.5±4.9	$p<0.05$ (n=10)	
$CD4^+CD25^+$	Control	12 (2–18)	Data not shown (n=7)	[28]
	Lung	33 (10–60)	(n=9)	
$CD4^+CD25^+$	Control	9 (2–17)	(n=35)	[29]
	Breast	17 (3–30)	$p<0.01$ (n=35)	
	Pancreas	13 (4–35)	$p<0.01$ (n=30)	
$CD4^+CD25^+$	Control	12.5±3.9	(n=4)	[39]
	Hodgkin's Lymphoma	13.5±4.2	$p=0.71$ (n=9)	
$CD4^+CD25^+$	Control	26.5±4.7	(n=10)	[23]
	Gastric	43.7±12.7	$p=4\times10^{-9}$ (n=55)	
	Colorectal carcinoma	39.7±11.3	$p=9\times10^{-7}$ (n=48)	
	Pancreatic	44.0±9.0	$p=9\times10^{-6}$ (n=13)	
	Oesophageal	47.0±9.8	$p=1\times10^{-7}$ (n=17)	
	Liver carcinoma	42.1±14.7	$p=9\times10^{-4}$ (n=16)	
$CD4^+CD25^{hi}$	Control	9.1	(n=14)	[20]
	Melanoma	14.7	$p=0.007$ (n=20)	
$CD4^+CD25^{hi}$	Control	2±0.5	(n=15)	[21]
	Acute myeloid leukaemia	4.8±1.8	$p<0.0001$ (n=36)	
$CD4^+CD25^{hi}$	Control	4.7±0.5	(n=34)	[22]
	Epithelial cancer	12.5±0.9	$p<0.001$ (n=42)	
$CD4^+CD25^{hi}$	Control	9.2 (1.6–30.2)	(n=48)	[26]
	Hepatocellular carcinoma	7.2 (1.2–23.3)	$p=0.895$ (n=25)	
$CD4^+CD25^+$ $FOXP3^+$	Control	4.5±1.1	(n=42)	[24]
	B cell chronic lymphocytic leukaemia	10.4±4.4	$p<0.001$ (n=73)	
$CD4^+CD25^+$ $FOXP3^+$	Control	5.8±1.7	(n=data not shown)	[33]
	Melanoma	4.9±1.9	$p<0.37$ (n=13)	
$CD4^+CD25^{hi}$ $FOXP3^+$	Control	5.4±2.7	(n=17)	[37]
	Head/neck carcinoma	10.1±4.7	RG $p<0.04$ (n=24) AD $p<0.0008$	
$CD4^+CD25^+$ $FOXP3^+$	Control	11.5±0.9	(n=14)	[38]
	Lung malignant perfusion	9±0.6	$p<0.001$ (n=15)	
	Lung non-malignant lavage	9.9±0.8	$p<0.001$ (n=13)	

AD=Active disease.
RG=Regressed disease.

Table 2
Treg as % of total $CD4^+$ LN infiltrating cells

Criteria	Groups	Treg % of $CD4^+$ LN cells	Significance	Reference
$CD4^+CD25^+$	Pancreas/breast lymph node	20 (n=20)		[29]
$CD4^+CD25^+$	Hodgkin's Lymphoma	35.8±16.6 (n=6)		[39]
$CD4^+CD25^+$	Cervical	N1TDLN 4.2±1.4 N2TDLN 3.8±1.0	(n=13) not significant	[41]
	Endometrial	N1TDLN 2.5±0.9 N2TDLN 1.8±0.9	(n=26) p<0.2	
$CD4^+CD25^{hi}$	Control mesenteric lymph node	1.2±0.3	(n=44)	[30]
	Early stage gastric	N1 LN 3.3±0.3 N2 LN 2.4±0.3	(n=31) (n=13) (p<0.05) higher in N1 vs N2 in early disease	
	Late stage gastric	N1 LN 3.3±0.2 N2 LN 3.3±0.2	(n=13) p<0.01 higher than mesenteric lymph node (n=13) p<0.02 higher in N1 and N2 during advanced disease compared to mesenteric lymph node	
$CD4^+CD25^+$ $FOXP3^+$	Tumour-free control lymph node	6 (2–9)	(n=6)	[31]
	Non-Hodgkin's lymphoma lymph node	17 (6–43)	(n=10) p<0.001	
$CD4^+CD25^{+/hi}$ $FOXP3^+$	Tumour-free control lymph node	6.2±4.2	(n=16)	[33]
	Melanoma	11.06±1.7	(n=13) p<0.0006	
$CD4^+CD25^+$	Control lymph node from non-cancer patient	8	(n=8) p<0.01 control have MORE Treg	[25]
	Ovarian carcinoma stage 1	5	(n=6)	
	Ovarian carcinoma stage 2	5	(n=5)	
	Ovarian carcinoma stage 3/4	1	(n=25) p<0.01 early stage have MORE Treg than late	

icant decrease in the numbers of Tregs in lymph node compared to frequencies observed in patients without cancer [25]. In this case, the authors postulated that since in the same patients, the frequency of Tregs in the tumours correlated with the stage of the cancer, that the Tregs were fewer in frequency in the lymph nodes due to migration of the cells into the tumour (see below). This possibility highlights the pitfalls of confining studies to one compartment such as the PBMC and/or lymph node, which does not take into account the entire pattern of Treg migration in relation to the tumour.

3.2.4. *Treg frequencies in tumours*

Several studies have now identified Treg cells within tumour infiltrating lymphocytes (TIL) (Table 3). Several of these have included assays to measure suppressor activity but it is nevertheless difficult to quantify the impact of these cells on anti-tumour immune responses and the question of how the Treg cells function within the environment of the tumour remains unanswered. It seems likely that if the Tregs act locally at the site of the tumour, then they would need to be present at a certain frequency in order to prevent effective anti-tumour immune responses. An indication of the pathogenic role of Treg in the progression of tumours is the increased Treg frequency observed in some tumours as the disease advances [24,25,27] (Table 3).

Three studies of patients with ovarian cancer have revealed a relationship between prognosis and TILs [25,35,42]. The first study reported a statistically significant correlation between frequencies of tumour infiltrating Tregs and survival [25]. Whilst one interpretation of these data is that Tregs promote disease progression, it is also possible that the extent of Treg infiltration is a consequence rather than a cause of advanced disease. A second report did not find a significant association between the frequency of tumour-infiltrating Treg cells and poorer survival but did find that a favourable prognosis correlated with the frequency of intraepithelial $CD8^+$ T cells and more significantly with a high ratio of $CD8^+$ T cells to $CD4^+Foxp3^+$ T cells [35]. Finally, a study by Wolf et al. quantitated Foxp3 by

Table 3
Treg as % of TIL

Criteria	Groups	Treg % of TIL	Reference
$CD4^+CD25^+$	Gastric early	(n=7) (% of $CD3^+$ TIL) 4.8±2.1	[27]
	Gastric late	(n=8) (%of $CD3^+$ TIL) 19.8±4.5 $p<0.05$	
$CD4^+CD25^+$	Lung	(n=9) (% of $CD4^+$ TIL) 33 (20–40)	[28]
$CD4^+CD25^+$	Pancreas/breast	(n=20) (% of $CD4^+$ TIL) 22	[29]
$CD4^+CD25^+$	Uninvolved tissue	(n=6) (% of $CD4^+$ TIL) 2.4 (1.5–5.6) p=0.014	[26]
	Hepatocellular carcinoma	(n=12) (% of $CD4^+$ TIL) 8.7 (1.4–13.8)	
$CD3^+CD4^+$ $FOXP3^+$	Head/neck carcinoma	(n=84) $CD3^+CD4^+FOXP3^+$ infiltrate present	[34]
$CD4^+CD25^+$	Breast carcinoma	(n=47) 60% (50% of $CD4^+CD25^+$ are $FOXP3^+$)	[36]
$CD4^+CD25^+$	Adult T-cell leukaemia/lymphoma	(n=25) (% of malignant T cells) 32% $FOXP3^{hi}$	[43]
$CD25^+FOXP3^+$	Ovarian carcinoma	(n=117) low $CD8^+$: FOXP3 ratio indicates poor prognosis	[35]
$FOXP3^+$	Ovarian carcinoma	(n=99) $FOXP3^+$ RT-PCR for defining prognosis	[42]
$CD4^+CD25^+$ $FOXP3^+$	Non-malignant ovarian tissue	(n=4) none detected	[25]
	Ovarian carcinoma	(n=104) 23±11	
	Stage 1	(n=12) 12	
	Stage 2	(n=25) 25	
	Stage 3/4	(n=30) 30 $p<0.001$ more Treg in stage 2–4 compared to stage 1	
$CD4^+CD25^{hi}$	Liver carcinoma	(n=5) 6.55±3.5	[53]

RT-PCR in 99 samples from ovarian cancer patients and found that the level of Foxp3 in a tumour sample was predictive of overall survival [42].

Two other studies have also reported that Treg frequencies are indicative of disease progression. Sasada et al. grouped patients with gastric cancer according to the percentage of $CD4^+CD25^+$ PBMC in the total $CD4^+$ PBMC population: low <40%, medium 40–50% and high >50% [23]. Those classified as "high" demonstrated reduced survival compared to those classified as low (p=0.007). Roncador et al. examined Foxp3 expression in T cell lymphomas [43]. Expression was detected only in adult T cell lymphomas (ATLL, 17/25 samples tested were Foxp3 positive) and although not statistically significant, a trend was observed between the extent of Foxp3 expression and survival.

The presence of Foxp3 positive cells in combination with cells expressing cytotoxic granules was, however, found to be associated with an unfavourable outcome in a study of patients with Hodgkin's lymphoma [44]. A study of patients with head and neck cancer found that high levels of $CD4^+CD69^+$ T cells were associated with better locoregional control and survival [34]. Surprisingly, high levels of Foxp3 positive cells were also associated with better locoregional control, a finding that also appears to be at odds with the studies previously mentioned in this section. The authors of this study postulate that the Tregs may in fact serve to dampen down inflammatory processes responsible for promoting tumour progression. The presence of Foxp3 positive cells in combination with cells expressing cytotoxic granules was found to be associated with an unfavourable outcome in a study of patients with Hodgkin's lymphoma [44].

In addition to TILs, Tregs have been identified in ascites and pleural effusions (Table 4). Comparisons of the frequencies of Tregs (assessed for expression of

Table 4
Treg as % total $CD4^+$ ascites

Criteria	Groups	% Treg	Significance	Reference
$CD4^+CD25^+$	Control non-malignant pleural lavage (n=15)	10.7±0.6	$p<0.001$	[38]
$FOXP3^+$	Malignant pleural effusion (n=13)	18.9±1.7		
$CD4^+CD25^+$	Non-malignant ascites n=10	3 (0.7–5)	$p<0.01$	[25]
$FOXP3^+$	Ovarian malignant ascites (n=45)	12		
$CD4^+CD25^{hi}$	Liver carcinoma (n=3)	7.2±2.7		[53]

Foxp3) within malignant and non-malignant ascites/plural effusions observed an increased frequency of Tregs within the malignant group [25,38].

3.2.5. *Tumours attract Treg cells and promote their expansion*

The studies, summarised above, have reported high frequencies of Treg cells within TILs. It seems plausible that the cells exert a suppressive effect on effector T cells present in the tumour environment. There is evidence emerging that the tumours not only attract Tregs to the site of the cancer, but also produce cytokines encouraging the expansion of Tregs locally. Two studies, one of ovarian cancer and the other of non-Hodgkin's lymphoma, have found that tumour cells produce the chemokine CCL22 that serves to attract $CCR4^+$ Treg into the tumour tissue [25,31]. In the study of ovarian cancer, the authors found CCL22 production by ovarian tumour cells and macrophages within malignant ascites, but not by PBMC or normal ovarian tissue [25]. In addition, this study utilised a NOD/SCID mouse model to demonstrate that administration of CCL22 blocking antibodies *in vivo* inhibited Treg migration into transplanted primary human ovarian tumour cells.

There is some data suggesting that TGFβ promotes the proliferation and activity of Treg cells [45,46]. Recently, a study using rodent tumour models found that exposure of immature myeloid DCs to tumour cells or a soluble factor produced by tumour cells resulted in TGFβ production that stimulated expansion of Treg activity *in vivo* [47]. Dissection of this pathway should identify tumour-derived factors that promote proliferation of Tregs thereby revealing new targets for inhibition of Treg activity.

3.3. *Suppression of anti-tumour immune responses by Tregs*

Patients with melanoma that express NY-ESO-1, a germ cell protein often expressed by cancer cells but not normal somatic tissue, have been shown to have circulating anti-NY-ESO-1 antibodies and associated $CD45RO^+CD4^+$ Th1 responses. Patients with tumours lacking NY-ESO-1 expression are seronegative and do not exhibit NY-ESO-1-specific $CD4^+$ T cell responses. Depletion of $CD4^+CD25^+$ cells reveal NY-ESO-1-specific $CD4^+CD45RA^+$ T cell responses in healthy controls, seronegative patients and patients with pre-existing NY-ESO-1-specific $CD4^+CD45RO^+$ T cell responses [48]. These findings imply that cognate T cells are not equally sensitive to suppression by Treg cells. It was recently shown that NY-ESO-1-specific T cells could be induced in seronegative patients by vaccination with NY-ESO-1 peptide in incomplete Freunds adjuvant (IFA) [49]. These T cell responses, detectable in the presence of Tregs, were of low avidity and could not recognise native NY-ESO-1 protein expressed by the tumour cells. In contrast, NY-ESO-1-specific T cells, observed only after depletion of Tregs, recognised the NY-ESO-1 antigen with high avidity. These data indicate that T cells of the same specificity but unequal avidity exhibit different sensitivity to suppression by Treg cells. Only those high-avidity T cells, sensitive to suppression by Treg cells may be capable of tumour rejection (Fig. 1).

The study by Curiel et al. indicated that Tregs purified from ovarian cancer patients inhibit Her2-specific responses (proliferation, cytotoxicity, IL-2 and IFNγ production) [25]. In addition, adoptive transfer of the $CD3^+CD25^-$ cells purified from the cancer patients into NOD/SCID mice significantly slowed the growth of a transplanted primary ovarian tumour, whilst transfer of Tregs promoted tumour growth and abrogated the effect of the transferred $CD3^+CD25^-$ cells. Both of these studies demonstrate that effector T cells can be generated to tumour antigens and at least some of these T cells are susceptible to suppression by Tregs. Ultimately, the contribution that these "suppressed" effector cells make to tumour rejection will only be shown by depletion of Tregs *in vivo* in cancer patients. Possibilities for depletion of Tregs will be discussed later in this review.

Suppression of immune responses by Tregs appears not to be confined to suppression of antigen-specific T cells. Tregs have been shown to suppress T cell-independent inflammatory responses induced by infection of mice with *Helicobacter hepaticus* [50]. Indeed, where inflammation is linked to cancer progression, Tregs have been shown to limit cancer progression through suppressing inflammatory processes. It is not yet clear how Tregs serve to dampen down both innate and specific immune responses; there is increasing evidence to support a role for Tregs in inhibiting NK cell activity since recent studies have shown that Tregs inhibit NK cells in both mice and men. A study of GIST

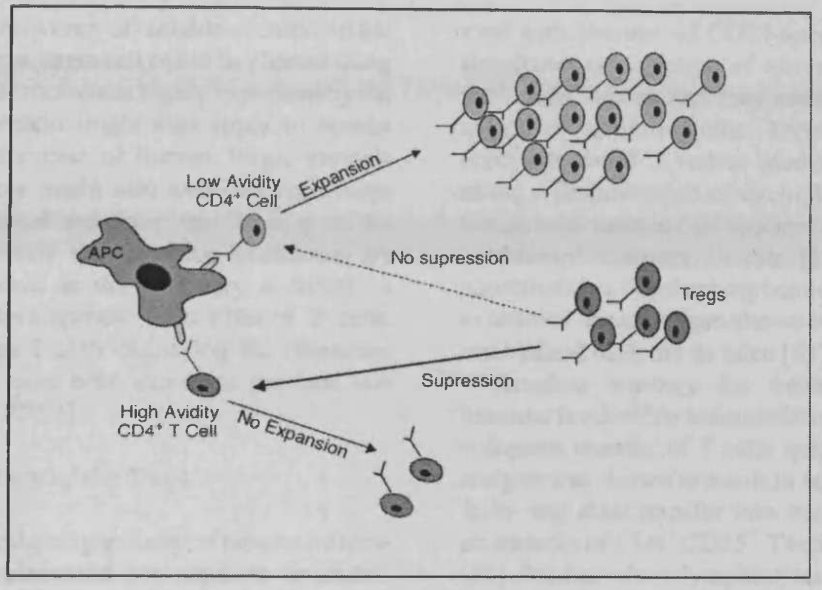

Fig. 1. Antigen presenting cells present tumour antigens to naïve $CD4^+$ T cells. $CD4^+$ that recognise the tumour antigen with high avidity are suppressed by Tregs. However, $CD4^+$ T cells that recognise the tumour antigen with lower avidity escape regulation and expand; thus, only low-avidity $CD4^+$ T cells responses to tumour antigen develop. Vaccination after depletion of Treg cells may therefore uncover high-avidity T cell responses, effective at controlling tumour growth.

(gastrointestinal stromal tumour-bearing) patients who had undergone treatment with a c-kit tyrosine kinase inhibitor (Gleevec STI571) highlighted an inverse relationship between IFNγ production by NK cells and the number of Treg circulating in the blood [51]. *In vitro* analysis indicated that Tregs suppressed cytolytic activity, IFNγ production and NKG2D expression on NK cells in a manner that was dependent on membrane bound TGFβ. The authors went on to show that NK cells recovered from tumour draining lymph nodes exhibited low cytolytic activity but that this was enhanced following Treg depletion. Similar conclusions were drawn from parallel experiments performed in mice, which were later supported by the findings of Smyth et al. [52].

3.4. Functional analysis of Tregs in malignancy

Two prominent functional characteristics of Tregs are their anergic nature and ability to suppress the activity of conventional effector T cells *in vitro*. These fundamental functional characteristics have been used to set the criteria by which potential Treg cells are identified. Many of the tumour studies discussed here have performed conventional Treg assays using polyclonal T cell stimuli and suppression of proliferation and/or cytokine secretion by conventional $CD4^+CD25^-$ T cells or unpurified PBMC as a read-out, to validate that the T cells under study do indeed behave as Treg cells should [20–31,33,38,39,53]. The mechanism of suppression by Tregs is still unclear, and it seems highly likely that more than one mechanism is employed. The requirement for cell–cell contact ranges from absolute to partial in the aforementioned studies; some attenuation of suppression has been observed upon addition of anti-CTLA-4 antibodies [20,33,38,39] or anti-B7-H1 [31] antibodies to the suppression assays. Many groups reported that the putative Treg population produced IL-10 [22,23,29,33,53] and/or TGFβ [28,29,33] but *in vitro* suppression could not always be inhibited by inclusion of neutralising antibodies specific for these cytokines [28,53]. Similar *in vitro* studies performed using mouse T cells have established that Treg mediated suppression is dependent on cell–cell contact with their target [54,55], whilst a more prominent role for soluble immunosuppressive factors is indicated *in vivo* (reviewed in [56]), giving rise to the concept that initial cell–cell interactions mediate the amplification of

suppression using networks of soluble factors. Initial Treg interaction with a target cell could be elicited using one or several surface molecules highly expressed by the cells. A similar scenario might also apply to human Tregs although in the case of human Tregs, intrinsic functional differences might also exist between those that are thymus-derived and those that develop in the periphery. It is possible that cytokine production by Tregs derived *de novo* in the periphery is merely a footprint of their development from effector T cells. Indeed, some human T cells displaying the characteristics of Treg cells have been shown to produce low levels of IFN-γ [22,23,53].

3.5. *Antigen specificity of the Tregs*

Identifying the antigen specificity of tumour infiltrating Tregs is a fundamental pre-requisite to understanding how the cells influence development of anti-tumour immunity. Tregs ($CD4^+CD25^+GITR^+FOXP3^+$), recognising peptides derived from ARCT-1 [57] and LAGE-1 [58], have been cloned from the TIL of a melanoma patient. Activation of these clones by their cognate antigens, ARTC-1 and LAGE-1, led to secretion of different combination of cytokines: IL-10 and IFN-γ or IL-10, IFN-γ and IL-4. Surprisingly, suppression by these Tregs *in vitro* was cell-contact dependent and could not be abrogated by IL-10 and/or TGF-β-specific neutralising antibodies. Whether the production of immunosuppressive cytokines is important for the function of Tregs *in vivo* or, as mentioned above, a footprint of the pathway to Treg production from effector T cell is unknown.

4. Manipulation of Tregs for therapeutic purposes

Many mouse studies have shown that depleting $CD25^+$ cells promotes immune responses induced by tumour cells or antigen-pulsed dendritic cells as well as immune-mediated rejection of tumour cell-lines [19,59,60]. More impressively, intra-tumoural injection of CD4-specific antibodies promoted regression of established tumours [61]. In a separate study using a different tumour cell line, intra-tumour depletion of $CD25^+$ cells was shown to inhibit tumour growth and the delay of tumour growth was extended by multiple rounds of depletion [62]. A potential problem associated with the use of CD25-specific antibodies is the simultaneous depletion of conventional $CD25^+$ effector T cells, whose loss may compromise the beneficial effect of depleting the Tregs. Another effective approach tested in rodent models uses cyclophosphamide. Administration of cyclophosphamide in combination with tumour cell vaccination led to regression of established tumours in rats [17]. More recently, a combination of cyclophosphamide and tumour-derived exosomes has also been shown to promote rejection of established tumours in mice [63].

Another strategy for treatment of established tumours is adoptive immunotherapy of T lymphocytes. Adoptive transfer of T cells specific for a melanocyte antigen was shown to result in regression of melanoma following their transfer into irradiated mice. Notably, co-transfer of $CD4^+CD25^+$ T cells abrogated this effect [64]. Studies where lymphocytes have been transferred into patients with melanoma have identified T cell survival as an important correlate of anti-tumour activity (reviewed in [65]); thus, depletion of Tregs prior to transfer as well as lymphoablation (providing space for expansion and survival of transferred cells) may significantly improve the future success of adoptive immunotherapy.

The usefulness of CD25 as a target for Treg depletion is problematic since agents targeting CD25, e.g., CD25-specific antibodies that have a long half-life *in vivo*, would also target useful anti-tumour immune responses. Denileukin diftitox, trade name Ontak [66], is a recombinant protein, which has a short half-life (60 min) yet is cytocidal to cells expressing the high-affinity CD25 receptor. It is a fusion protein consisting of part of the IL-2 chain (Ala_1–Thr_{133}) fused with diptheria toxin fragments A and B (Met_1–Thr_{387}) and has recently been introduced for treating patients with cutaneous T cell lymphoma with promising results and acceptable toxicity. Preliminary data presented at a 2005 Keystone meeting described a reduction in $CD4^+CD25^+Foxp3^+$ T cells in the peripheral blood of a group of 8 cancer patients, which was accompanied by and increase in numbers of proliferating and IFNγ-producing cells in the peripheral blood of the patients. Dannull et al. recently reported that a single dose of denileukin diftitox selectively depletes Tregs (measured by loss of $CD4^+CD25^{hi}$ T cells *in vivo*, loss of suppressor activity) and results in enhanced immune responses following vaccination

with dendritic cells transfected with tumour RNA in patients with renal cell carcinoma ($n=6$) compared to those observed in patients ($n=4$) receiving only vaccination [67]. Despite these promising findings, a recent report indicated, however, that treatment of cancer patients (12 with metastatic melanoma and 1 with metastatic renal cell carcinoma) with denileukin diftitox had no effect on reducing Treg number or function in the PBL of the patients [68]. Different treatment regimes were used in these studies and further work is needed to understand these conflicting results.

As well as the use of agents that target the IL-2 receptor, results obtained in mice indicate that low doses of immunosuppressive agents such as cyclophosphamide reduce Treg numbers and function, thereby allowing anti-tumour responses to flourish. It will be of interest therefore to determine whether cyclophosphamide similarly represents a useful method of inactivating Tregs and promoting immune responses to tumours in cancer patients. Treatment of patients with fludaribine also holds some promise. Both the frequency and suppressor activity of Treg cells was significantly reduced in CLL patients after receiving fludaribine treatment demonstrating that Treg cells are more sensitive to fldaribine than other T cell populations [24]. Tumour regression was also observed in melanoma patients adoptively transferred with tumour-specific T cells following fludaribine-containing chemotherapy [69]. Other possibilities for inactivating Treg cells include targeting cell surface molecules known to be expressed by Tregs (GITR, B7-H1) or cytokines/chemokines known to be involved in Treg survival, expansion and migration (TGF-β, IL-2, CCL22).

5. Potential problems associated with manipulation of Tregs

Knowledge of Treg cells has highlighted several potential pitfalls when considering immunotherapeutic strategies that may be used for patients with cancer. A recent study showed that administration of IL-2, used for *treatment* of patients with melanoma, resulted in expansion of Treg numbers and an increase in Treg activity [70]. There is also evidence, obtained in a mouse model, that immunisation with SEREX-defined self-antigens results in activation of Treg cells exhibiting potent suppressive activity and FOXP3 expression elevated 5–10-fold above that found in Treg cells isolated from naïve animals [71]. Significantly, vaccination with these autoantigens inhibited immune recognition of tumours, demonstrated by increased metastatic nodules following i.v. inoculation of tumour cells [72] and by an increase in the frequency and rate of tumour induction following injection of mice with methylcholanthrene [73]. An inhibition of NK mediated cytotoxicity and a reduction in iNKT cell numbers was central to the suppression of tumour immunity by Tregs elicited by SEREX-defined antigens [72].

Contrary to injection with the SEREX-defined autoantigens alone, co-immunisation with a peptide epitope recognised by $CD8^+$ T cells and expressed by the tumour cells significantly enhanced tumour rejection [74]. Results of the study indicated that IFNγ, secreted by the $CD8^+$ T cells, inhibited Treg activity. Interestingly, tumour rejection by the $CD8^+$ T cells was abolished by depletion of $CD4^+$ T cells and the authors demonstrated that $CD4^+CD25^-$ Th1 cells recognising a SEREX defined autoantigen were primed and which were effective only when Tregs were inhibited. This study highlights the importance of epitope mapping in order to precisely define the antigens recognised by Tregs and in particular, determining whether Tregs and conventional $CD4^+$ T cells recognise the same or distinct peptide epitopes. This information is clearly the key to the design of vaccines that maximise activation of conventional effector T cell responses whilst minimising activation of Tregs.

There is general agreement that the suppressive effect of Tregs is not antigen-specific and in the case of tumours would impinge on immune responses to all tumour antigens, including self-antigens. Some studies have observed autoimmune depigmentation in melanoma-immune mice following depletion/inactivation of Tregs [16,19]. Several studies have shown that simply depleting $CD25^+$ T cells does not, at least in the mouse strains tested, induce autoreactivity thereby demonstrating that an appropriate environment for antigen presentation is required to initiate an immune response even in a Treg-depleted host. This point was recently illustrated in a study by Wei et al. who found that mice mounting an immune response to tumour cells injected with thyroglobulin in the absence of $CD25^+$ T cells were more prone to developing thyroglobulin-specific autoreactivity than those that did not respond to the tumour indicating that where depletion

of Tregs leads to an active anti-tumour response, the potential for inducing autoreactivity against concurrently presented antigens is increased [75]. It has also been observed that cancer patients showing signs of tumour regression after receiving CTLA4-specific antibodies suffered autoimmune side effects such as dermatitis, enterocolitis, hepatitis and hypophysitis [76]. The effect of administering CTLA4-specific antibodies may be more far-reaching, however, than the targeting of Treg cells since CTLA4-specific antibodies may promote rejection of tumours through abrogating Treg activity whilst simultaneously directly boosting effector T cell activity.

Finally, a large body of evidence indicates that inflammation can promote tumour growth [77]. Since the results of some recent studies indicate that the inhibitory effects of Tregs extend to antigen–non-specific inflammatory responses, it is possible that Tregs, by suppressing inflammatory responses, actually hinder the progression of some tumours. Evidence to support this possibility is accumulating; adoptive transfer of $CD4^+CD25^+$ T cells was shown to prevent development of *H. hepaticus*-associated tumours in infected Rag–knockout mice [78]. Similarly, adoptive transfer of Tregs hindered development of intestinal adenomas in ApcMin/+ mice, an effect that correlated with a decrease in pro-inflammatory cytokines and Cox2 expression [79]. In agreement with this finding, Sharma et al. have shown that inhibition of Cox2 led to a decrease in Treg activity with a concomitant increase in production of pro-inflammatory cytokines and *in vivo* growth of Lewis Lung carcinoma in mice [80]. Collectively, these data imply that Tregs can suppress the activity of different arms of the immune system, which in turn promote or impede tumour growth.

6. Conclusion

Overall, there is overwhelming evidence to support the hypothesis that Tregs can inhibit immune responses to tumours and promote cancer progression. In order to exploit this finding for the purposes of cancer immunotherapy, it will be necessary to gain a more complete understanding of the specificity of Treg cells and also of the types of immune responses that they are capable of suppressing. With this information to hand, it will become possible to consider more carefully the signals the immune system receives in the absence of Tregs in order to ensure that immunotherapeutic strategies involving depletion of Tregs uncover only immune responses which impede rather than promote tumour growth.

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$CD4^+CD25^+FOXP3^+$ Regulatory T Cells Suppress Anti-Tumor Immune Responses in Patients with Colorectal Cancer

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Background. **A wealth of evidence obtained using mouse models indicates that $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) maintain peripheral tolerance to self-antigens and also inhibit anti-tumor immune responses. To date there is limited information about $CD4^+$ T cell responses in patients with colorectal cancer (CRC). We set out to measure T cell responses to a tumor-associated antigen and examine whether Treg impinge on those anti-tumor immune responses in CRC patients.**

Methodology and Principal Findings. **Treg were identified and characterized as $CD4^+CD25^+FOXP3^+$ using flow cytometry. An increased frequency of Treg was demonstrated in both peripheral blood and mesenteric lymph nodes of patients with colorectal cancer (CRC) compared with either healthy controls or patients with inflammatory bowel disease (IBD). Depletion of Treg from peripheral blood mononuclear cells (PBMC) of CRC patients unmasked $CD4^+$ T cell responses, as observed by IFNγ release, to the tumor associated antigen 5T4, whereas no effect was observed in a healthy age-matched control group.**

Conclusions/Significance. **Collectively, these data demonstrate that Treg capable of inhibiting tumor associated antigen-specific immune responses are enriched in patients with CRC. These results support a rationale for manipulating Treg to enhance cancer immunotherapy.**

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INTRODUCTION

Colorectal cancer (CRC) is the fourth most commonly diagnosed malignant disease with an estimated 1 million new cases and over 500 000 deaths each year worldwide [1]. The current management of patients with CRC revolves around excision of the primary tumor and the local lymph nodes and the selective use of 5-fluorouracil (5FU; inhibits thymidylate synthase) based chemotherapy [2]. Recent advances in preoperative imaging, surgical technique and the use of neo-adjuvant chemotherapy have improved outcomes, but colorectal cancer remains the second leading cause of death from cancer in Western countries with 40–50% of patients who undergo a potentially curative operation, undergoing relapse or death from metastatic disease.

Clinico-pathological staging of the disease strongly correlates with prognosis. Interestingly, improved clinical outcome is also associated with the presence of tumor-infiltrating lymphocytes (TILs), particularly if the lymphocytes invade the glandular elements of the tumor [3,4]. This suggests that anti-tumor immune responses can impinge on the growth of the primary tumor, and may have an important role in controlling metastatic disease. Indirect evidence for the importance of the immune response in cancer patients is the epidemiological association in some populations between an increased incidence of tumors and immunosuppression post organ transplantation [5].

Much interest has recently arisen in the role of a naturally occurring population of $CD4^+CD25^+$ regulatory T cells (Treg), characterized by expression of the forked-head transcription factor FOXP3, and increased levels of the surface markers CD45RO, CTLA-4, and GITR [6,7,8]. Treg have been shown to control self-antigen specific responses in the periphery, and hence may play a role in controlling anti-tumor immune responses. Several groups including our own, have demonstrated that depletion of Treg promotes the generation of anti-tumor immune responses and tumor rejection in murine models (reviewed in [9]). There is evidence to suggest that these protective responses target both tumor-specific antigens as well as shared tumor antigens [10,11].

More recently, several studies have observed an increased frequency of $CD4^+CD25^+$ T cells in the peripheral blood of patients with various types of cancer, although the majority of these studies did not confirm that these were Treg by staining for FOXP3 (reviewed in [9]). The presence of $FOXP3^+$ Treg within the TILs of ovarian cancer has, however, been identified as an independent risk factor for poorer prognosis. Furthermore, after purification from tumor ascites, these Treg were shown to suppress Her2-specific T cell responses *in vitro* [12].

The human onco-fetal antigen, 5T4, is a 72kDa leucine-rich membrane glycoprotein which is expressed at high levels on the placenta and also on a wide range of human carcinomas including

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colorectal, gastric, renal and ovarian but rarely on normal tissues [13,14,15,16,17,18]. In this study we tested the hypothesis that Treg develop in CRC patients that suppress 5T4-specific immune responses. Samples from patients with CRC or IBD, and healthy controls were examined to address three questions: is the frequency of Treg ($CD25^{hi}CD4^{+}FOXP3^{+}$ T cells) increased in blood or lymph nodes from patients with CRC; can anti-tumor T cell responses to 5T4 be identified in CRC patients; and do Treg impinge on these anti-tumor immune responses?

METHODS

Sample groups

CRC patients were identified from multi-disciplinary team meetings with the first presentation of an adenocarcinoma and no reported distant metastases on cross sectional imaging (TNM stage I–III; Duke's stage A–C). IBD patients (ulcerative colitis or Crohn's disease) attending clinic or undergoing elective surgical resection were recruited to the study. Local ethical committee approval was granted for the study by the Bro Taf Local Research Ethics Committee.

Antigens

Purified protein derivative (PPD) (Statens Serum Institut, Denmark), hemaglutinin (HA) was a kind gift from Dr John Skehel (NIMR, Mill Hill), 5T4 was produced as described previously [19] (Oxford Biomedica). All antigens were used at a final concentration of 1 μg/ml.

Purification of lymphocytes

30–50 ml of blood was drawn from patients or controls and heparinized (10 U/ml). PBMCs were extracted by centrifugation over Lymphoprep (Axis-Shield, Oslo, Norway). The cells were washed twice in RPMI before further use. Lymph nodes were dissected from fresh specimens within 30 minutes of surgery, washed in RPMI, mashed through a sterile cell strainer and collected by centrifugation.

Flow cytometric analysis of lymphocytes from blood and lymph nodes

Samples were stained with fluorescently-labeled antibodies to CD4 (clone RPA-T4), CD25 (clone M-A251), CD45RO (clone UCHL1) and CTLA4 (clone BNI3) (BD Pharmingen), CD4 (clone S3.5) (Caltag) and CD25 (clone 4E3) (Miltenyi Biotec). All staining was performed in phosphate buffered saline (PBS), 2.5% FCS, 5 mM EDTA and intracellular staining was performed using the cytofix, cytoperm kit (BD Pharmingen). FOXP3 staining was performed using the FITC anti-human FOXP3 staining kit (clone PCH101) (71-5776 eBioscience). Lymphocytes were gated on forward and side scatter profiles. All flow cytometry was performed on a BD FacsCalibur and analyzed using the Summit v3.1 analysis program (build 839 Dakocytomation, Fort Collins, Colorado).

$CD4^{+}CD25^{hi}$ add-back experiments

Purified PBMC were enriched for $CD4^{+}$ cells and then separated into $CD4^{+}CD25^{hi}$ and $CD4^{+}CD25^{-}$ fractions using magnetic beads (Miltenyi $CD4^{+}CD25^{+}$ regulatory isolation kit). Flow cytometry of each fraction showed that the stringency of $CD25^{+}$ cell selection by the Miltenyi $CD4^{+}CD25^{+}$ regulatory isolation kit and Miltenyi anti-CD25 beads was comparable. A proliferation assay was set up using $2x10^{4}$ $CD4^{+}CD25^{-}$ cells, activated with 0.5 μg/ml plate-bound anti-CD3 (clone OKT3) (eBioscience) and 1 μg/ml soluble anti-CD28 (clone 28.2) (BD Pharmingen) antibodies in RPMI 1640 supplemented with 10% heat-treated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. $CD4^{+}CD25^{-}$ cells were activated alone or in the presence of $CD4^{+}CD25^{hi}$ at cells varying ratios of 1:1, 1:0.1, 1:0.01 and 1:0.001 $CD4^{+}CD25^{-}$:$CD4^{+}CD25^{hi}$ cells. Proliferation was measured on day 3 by the addition of 0.037 MBq/well (1 μCI) of [*methyl*-3H]-thymidine (Amersham Biosciences) for the final 18 hours of culture. Cells were harvested onto printed filtermat A filters (Wallac, Turku, Finland supplier Perkin Elmer) using a TomTek cell harvester. Once filters were dry, meltilex A melt-on scintillator sheets (Wallac) were added and filters were read using a Trilux 1450 microbeta liquid scintillation and luminescence counter (Wallac).

IFN-γ ELISPOT assays

Antibodies were obtained from Mabtech (Natka, Sweden) and the ELISPOT was developed using the alkaline phosphatase substrate kit from Bio-Rad (Hercules, California). The effect of Treg depletion on antigen-specific IFN-γ production was assessed in parallel assays. $CD25^{hi}$ cells were depleted using magnetic separation with MACs CD25 microbeads (Miltenyi Biotec, Germany). Briefly, 1.5×10^{7} PBMC were incubated with 15 μl of anti-CD25 coated beads in total reaction volume of 150 μl of buffer (1×PBS, 0.5% BSA, 5 mM EDTA) at 4°C for 15 min. Excess beads were washed off and the PBMC were run down an MS column. The column was washed with 3×1 ml washes in buffer. The efficacy of depletion was confirmed by flow cytometry. Assays were set up using either 3.5×10^{5} undepleted or $CD25^{hi}$-depleted cells in RPMI 1640 supplemented with 5% heat-treated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate per well of a polymer-backed 96-well filtration plate (MAIP-S-4510) (Millipore, Moslheim, France) in a total reaction volume of 100 μl/well. The concentrations of antibodies used and washing steps were according to the manufacturer's instructions, all antibody incubations were with 50 μl/well. The method has been previously described [20]. PPD, HA and 5T4 were tested in duplicate wells, having been added to a final concentration of 1 μg/ml, and compared with control wells with no protein. The plate was incubated at 37°C, 5% CO_2 for 18 h. Activated T cells were enumerated at the single-cell level by counting the number of spots per well using an automated ELISPOT reader (Cadama). Responders were identified as having at least 5 spot-forming cells (sfc) per 10^{6} PBMC, after subtraction of the background, and an increase of at least 50% above background.

Statistical analysis

Flow cytometric data was analyzed using the unpaired student's *t*-test (GraphPad Prism version 2.0 (GraphPad software). The proportion of cancer patients and healthy controls mounting a response to 5T4 was compared using a closed form method for comparing differences and calculating confidence intervals [21].

RESULTS

Defining the human Treg population

Using the strict gating parameters reported recently [8], Treg were identified as CD4 positive cells with brighter CD25 staining than that of the CD4-negative population (Fig 1a). The frequency of Treg was calculated as the percentage of $CD4^{+}CD25^{hi}$ cells in the $CD4^{+}$ population, and was in the range of 0.09–1.44% of $CD4^{+}$ cells in PBMC of healthy age-matched controls. In keeping with previous reports, the vast majority of $CD4^{+}CD25^{hi}$ cells were CTLA4-positive and expressed CD45RO (>97%) [6,7,8]. The

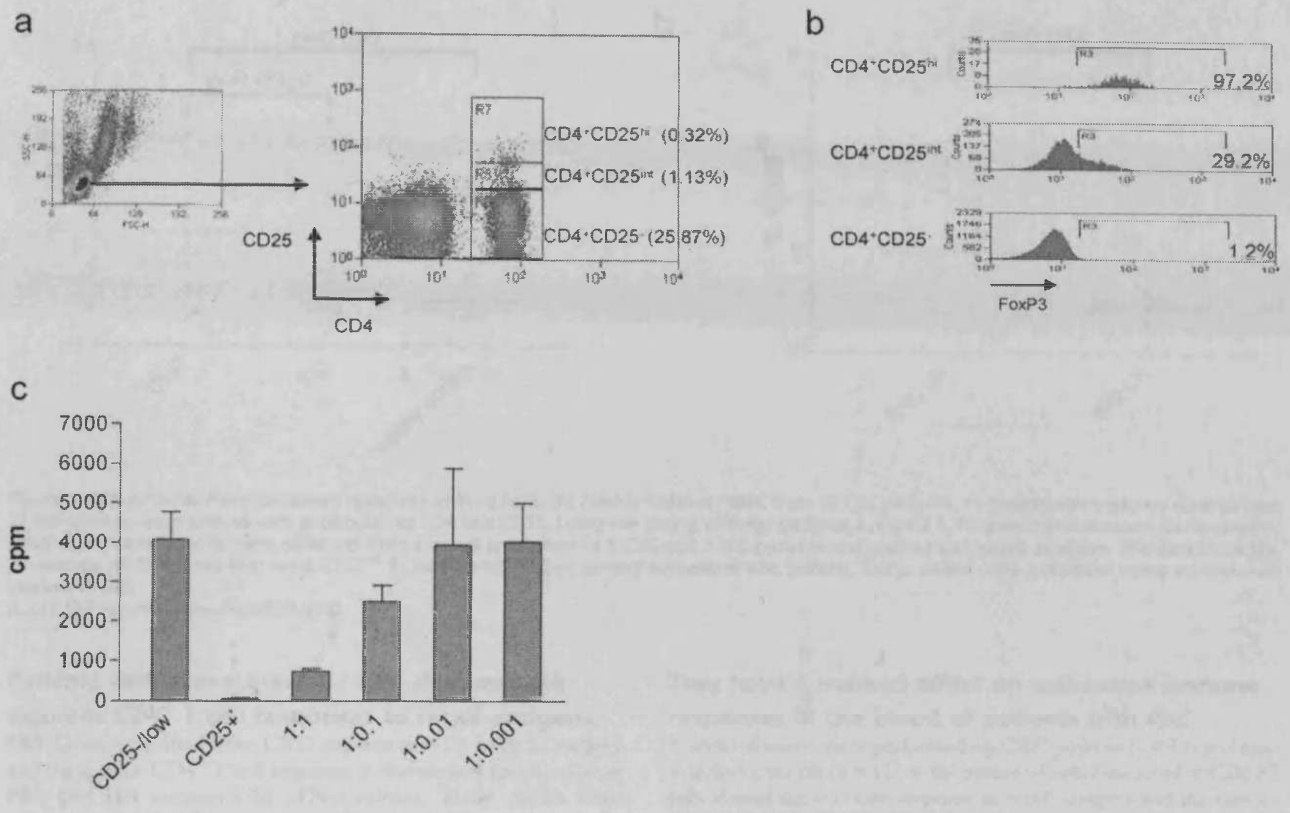

Figure 1. Characterization of the human Treg population. (A) $CD4^+CD25^-$, $CD4^+CD25^{int}$ and $CD4^+CD25^{hi}$ cells were identified as shown. $CD4^+CD25^{hi}$ cells are those where CD25 expression was higher than that on the $CD4^-$ population. (B) FOXP3 expression within each population was assessed by intracellular staining. (C) Freshly isolated $CD4^+CD25^-$ T cells (2×10^4 cells/well) were cultured alone (CD25-/low) or with $CD4^+CD25^{hi}$ T cells at various ratios, and stimulated with plate-bound anti-CD3 and soluble anti-CD28. Proliferation was assessed by (^{3}H)-thymidine incorporation. The results represent the average of triplicate wells with standard deviation, from a representative assay.
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$CD25^{hi}$ population was further analyzed by the expression of FOXP3. As shown in Figure 1b, over 95% of $CD4^+CD25^{hi}$ cells expressed FOXP3, compared with approximately 30% of $CD4^+CD25^{int}$ cells. This pattern of staining was the same for both healthy controls and CRC patients. FOXP3 expression was negligible in $CD4^+CD25^-$ cells (Figure 1b).

To confirm the regulatory phenotype of this population of $CD4^+CD25^{hi}$ cells, their ability to suppress the activation of $CD4^+CD25^-$ cells was assessed using a co-culture *in vitro* proliferation assay. $CD4^+CD25^-$ and $CD4^+CD25^{hi}$ cells were separated from PBMC, as described in methods. The addition of $CD4^+CD25^{hi}$ cells to polyclonally stimulated $CD4^+$ T cells clearly suppressed the proliferation of $CD4^+CD25^-$ T cells and, as reported by others, this suppression was dose-dependent [8,22,23] (Figure 1c).

CRC patients have increased numbers of Treg in peripheral blood

The Treg were phenotypically defined as outlined above. Figure 2a shows the frequencies of Treg in the PBMCs of CRC patients (n = 12, age range 58–80 years) compared with age-matched healthy controls (n = 11, age range 48–93 years) and patients with inflammatory bowel disease (n = 22, age range 19–80 years). In all groups, the frequency of Treg was less than 2% of $CD4^+$ T cells. However, the mean frequency in CRC patients (1.13%) was significantly increased compared with the control groups (IBD patients 0.56%, healthy controls 0.46%). There was no significant difference in the frequency of Treg in healthy controls and IBD patients.

CRC patients have increased numbers of Treg in mesenteric lymph nodes

Obtaining control lymph nodes from subjects with no abdominal disease was not possible. IBD patients offer a colonic disease control group to the CRC patients, the latter also often demonstrating areas of inflammation around the tumor. Furthermore, the results from PBMCs did not demonstrate a significant alteration in the frequencies of Treg in patients with IBD compared with healthy controls. Hence, we considered it reasonable to use mesenteric lymph nodes obtained from IBD patients undergoing surgery as a control group in which to examine Treg in secondary lymphoid tissue.

Fresh lymph nodes were obtained from the surgically resected specimens and prepared as outlined in methods. The frequencies of Treg in mesenteric lymph nodes obtained from CRC patients (n = 8), and IBD patients (n = 7) were compared (Figure 2b). CRC patients again showed a significant increase in the frequency of Treg, reflecting the increase already described in peripheral blood. The lymph node-derived $CD25^{hi}$ Treg had the same phenotype to those found in blood (data not shown). No difference was observed in the overall proportion of $CD4^+$ or $CD8^+$ T cells (data not shown).

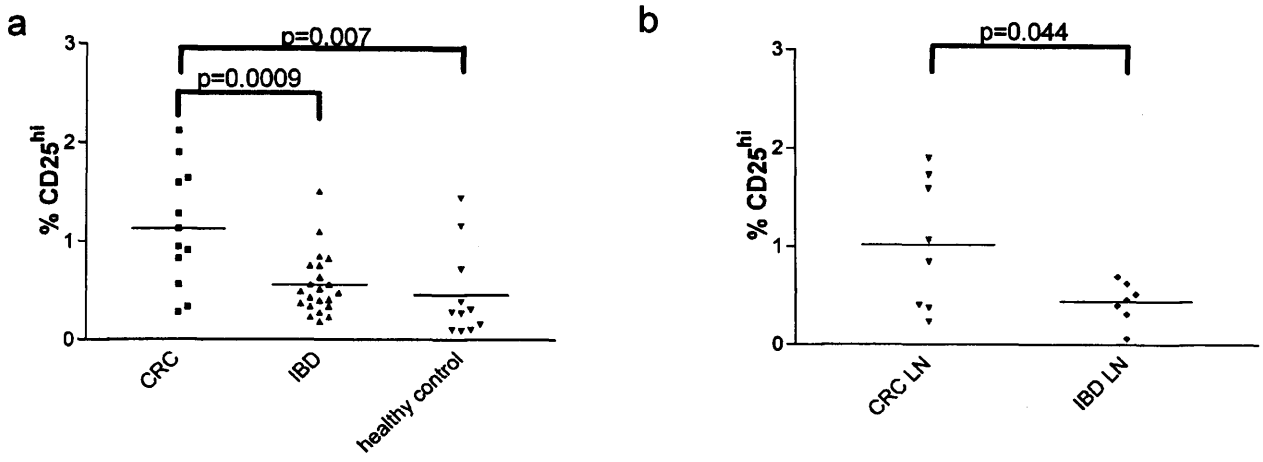

Figure 2. CRC patients have increased numbers of Treg cells. (A) Freshly isolated PBMC from 12 CRC patients, 11 healthy age-matched controls and 22 IBD patients were stained with antibodies to CD4 and CD25. Using the gating strategy outlined in Figure 1, Treg were enumerated. (B) Mesenteric lymph node samples (LN) were collected from surgical specimens of 8 CRC and 7 IBD patients and stained and gated as above. The data show the percentage of $CD4^+$ cells that were $CD25^{hi}$ in each sample. Each symbol represents one patient. The p values were calculated using an unpaired student's *t*-test.

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Patients with non-metastatic CRC demonstrate vigorous $CD4^+$ T cell responses to recall antigens

PBMCs were purified from CRC patients (n = 14) prior to surgery and the specific $CD4^+$ T cell response to the control recall antigens PPD and HA measured by IFN-γ release. These results were compared with healthy age-matched controls (n = 11) (Figure 3). CD4 and CD8 depletion experiments confirmed that these were $CD4^+$ T cell responses (data not shown). There was no evidence that the patients were immunosuppressed; all subjects responded to at least one antigen. Cell proliferation was also assessed in parallel experiments as a measure of T cell activation, and again no evidence of immunosuppression was found pre-operatively (data not shown).

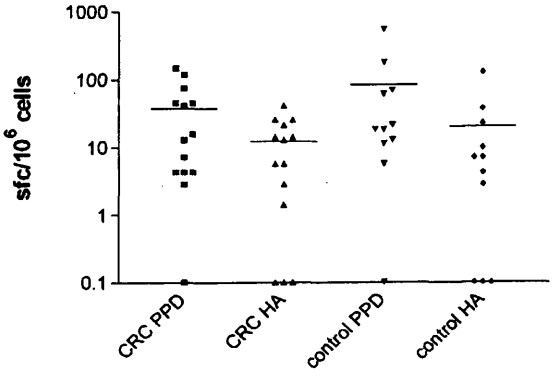

Figure 3. CRC patients are able to mount $CD4^+$ T cell responses to the recall antigens PPD and HA. Whole PBMC from 11 age-matched controls and 14 CRC patients were assessed for responses to the antigens PPD and HA, measured by IFN-γ ELISPOT. Purified PBMC were added at 3.5×10^5 cells/well, and incubated for 18 hours in the presence of either 1 μg/ml PPD, 1 μg/ml HA or with no antigen. Wells were set up in duplicate. Fewer than 5 spot forming cells/10^6 PBMC was considered negative.

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Treg have a marked effect on anti-tumor immune responses in the blood of patients with CRC

A series of assays were performed on CRC patients (n = 14) and age-matched controls (n = 11) to determine whether removal of $CD25^{hi}$ cells altered the immune response to recall antigens and the tumor-associated antigen 5T4. We optimized an isolation system designed specifically to deplete only the $CD25^{hi}$ cells from our samples as outlined in methods (Figure 4a). Freshly-isolated PBMCs were used in *ex vivo* IFN-γ ELISPOT assays as outlined above to determine responses to the recall antigens and the tumor antigen 5T4, before and after depletion of Treg. On the whole, depletion of Treg uncovered some responses to recall antigens in CRC patients and controls (Figure 4b). However, in the case of the tumor associated antigen 5T4, there was a striking difference between controls and CRC patients. A 5T4-specific *ex vivo* response was found in 1/11 of the healthy controls and depletion of Treg enhanced this response. Whilst depletion of Treg augmented the 5T4-specific response in 1/14 of the CRC patients, depletion of this population led to the *unmasking* of a 5T4 response in 5/14 of the patients tested (95% Confidence Interval for paired differences is −0.83 to −0.22 revealing a significant difference in 5T4 responses between CRC patients and healthy controls). These data suggest that Treg are suppressing anti-5T4 immune responses specifically in patients with colorectal cancer. These six patients who demonstrated enhanced 5T4 responses had similar Treg frequencies in peripheral blood (range 0.82–2.35% mean 1.14%) to the frequencies measured in the entire CRC population (mean 1.13%). While IFN-γ-release, measured directly *ex vivo*, enabled anti-5T4 responses to be detected, we did not find vigorous proliferative responses to 5T4 in either CRC patients or controls. However, with several rounds of re-stimulation and the addition of IL-2, we have grown out HLA-DR-restricted 5T4-specific lines (data not shown).

DISCUSSION

We compared the frequency of carefully defined $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) in CRC patients with healthy age-matched controls and IBD patients. CRC

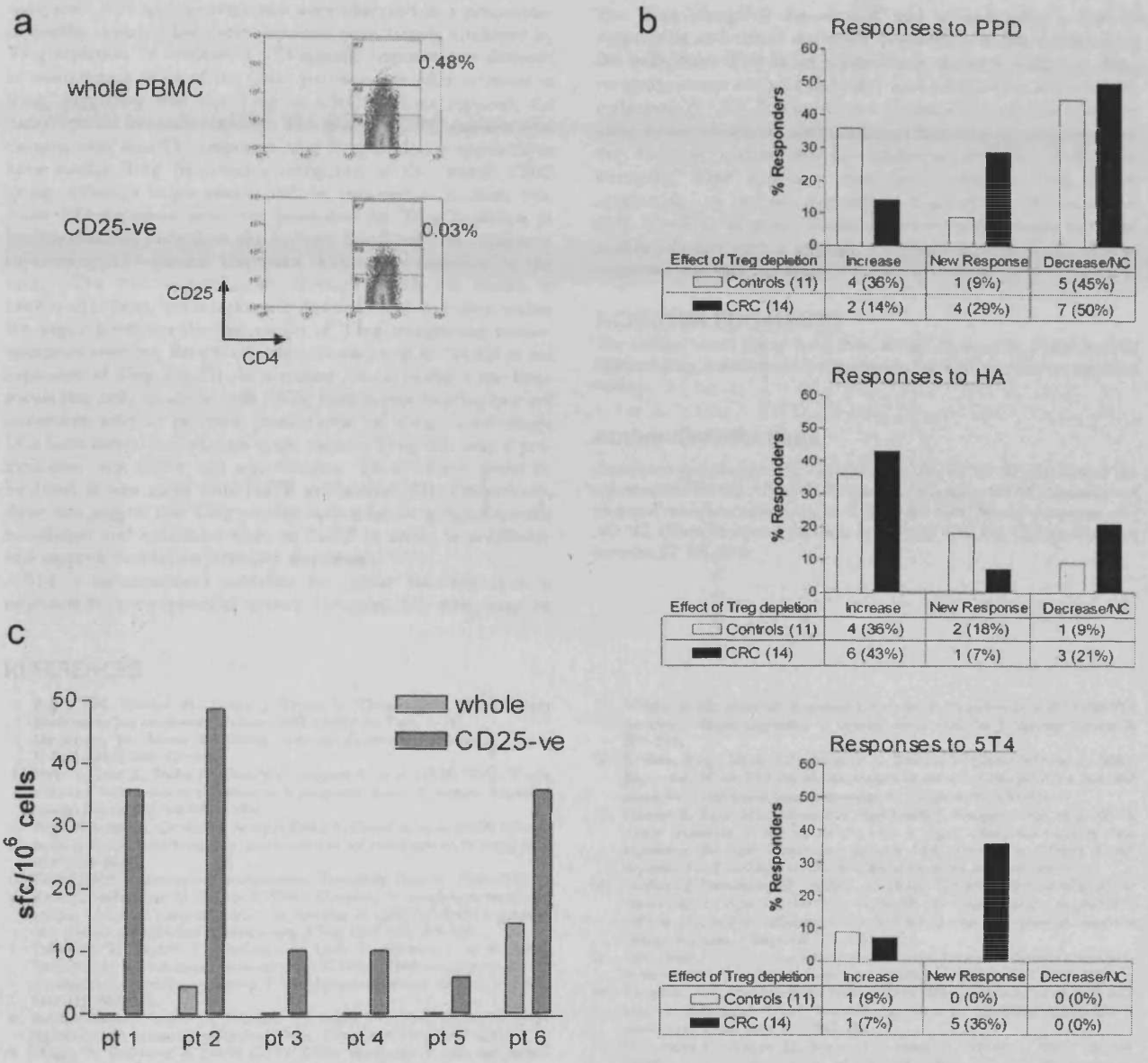

Figure 4. Depletion of Treg unmasks anti-tumor CD4$^+$ T cell responses in CRC patients but not healthy controls. (A) PBMC were purified and two samples prepared: whole PBMC and PBMC depleted of CD25^{hi} cells. (B) Whole PBMC and CD25^{hi}-depleted PBMC from 11 age-matched controls (open bars) and 14 CRC patients (solid bars) were set up in parallel in an IFN-γ ELISPOT assay (3.5×10^5 cells/well) with PPD (top graph), HA (middle graph) or 5T4 (lower graph). The number of responders is shown for each antigen. In each case, the percentage on the left is the frequency of responders that increased after Treg depletion (Increase), the percentage in the middle is the frequency of non-responders that had a response after depletion (New Response) and on the right the frequency of responders that showed a decrease or no change in response after Treg depletion (Decrease/NC). The 95% Confidence Interval for paired differences of proportions between patients and controls is -0.83 to -0.22 revealing a significant difference for 5T4 responses. (C) The 6 CRC patients with responses to 5T4 are shown in more detail. The bars represent the spot-forming cells/10^6 PBMC after subtraction of background spots, whole PBMC (light grey) and CD25^{hi}-depleted PBMC (dark grey). Responses were observed only in CD25^{hi}-depleted PBMC of patients 1–5, whilst a response was observed in both PBMC populations from patient 6. In all assays, wells were set up in duplicate.
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patients have an increased frequency of Treg and there is evidence that these Treg target anti-tumor immune responses.

We assessed whether enhanced numbers of Treg in CRC patients correlated with general immunosuppression. A combination of IFN-γ ELISPOT assays and proliferation assays to monitor responses to the recall antigens PPD and HA, demonstrated equally robust responses in CRC patients and healthy controls, indicating that immune responses to non-tumor antigens are unimpaired in CRC patients.

The pattern of immune reactivity was strikingly different between CRC patients and healthy controls when immune responses to the tumor-associated onco-fetal antigen, 5T4, were

compared. 5T4-specific responses were observed in a proportion of healthy controls, but these responses were largely unaltered by Treg depletion. In contrast, a 5T4-specific response was detected in more than a third of the CRC patients only after removal of Treg, suggesting that the Treg in CRC patients suppress the tumor-specific immune response. This group of CRC patients who demonstrated anti-5T4 responses after Treg depletion appeared to have similar Treg frequencies compared to the overall CRC group, although larger studies will be required to confirm this. Since 5T4-responses were not unmasked by Treg depletion in healthy controls, these data also indicate that Treg cells capable of suppressing 5T4-specific responses develop in response to the tumor. The precise mechanism through which this occurs is unclear at present, but it is possible that the cytokine milieu within the tumor promotes the generation of Treg recognizing tumor-associated antigens. Recent reports indicate a role for TGFβ in the expansion of Treg [24,25]. In a rodent tumor model it has been shown that only dendritic cells (DCs) from tumor-bearing but not tumor-free animals promote proliferation of Treg. Interestingly DCs from tumor-free animals could expand Treg, but only if pre-incubated with tumor cell supernatants. These effects could be inhibited *in vitro* using anti-TGFβ antibodies [26]. Collectively, these data suggest that Treg require both a tumor antigen-specific stimulation and cytokines, such as TGFβ in order to proliferate and suppress anti-tumor immune responses.

5T4 is an attractive candidate for tumor vaccines as it is expressed in many epithelial tumors. However, this study suggests that Treg recognize the antigen and possibly play a role in suppressing anti-tumor responses, presumably to the detriment of the individual. Two other studies have demonstrated that Treg recognize tumor antigens (LAGE-1 and ARTC-1) in subjects with melanoma [27,28]. It is important to ensure that vaccine strategies using tumor-associated antigens do not boost the regulatory arm of the immune system thereby inhibiting potential anti-tumor immunity. One approach may be to deplete Treg before vaccination, as recently demonstrated for renal cell carcinoma [29]. It will be of great interest therefore to determine in future studies whether such a strategy will result in measurable clinical responses in CRC patients.

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Author Contributions

Conceived and designed the experiments: AG AG SC AP. Performed the experiments: SC GB AP KW TE. Analyzed the data: AG SC. Contributed reagents/materials/analysis tools: RH JT BR GW. Wrote the paper: AG AG SC. Other: Collected the data: SC GB AP KW TE. Collected human samples: JT BR GW.

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The impact of regulatory T cells on carcinogen-induced sarcogenesis

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Burnet proposed in the 1950's that the immune system is engaged in identifying and destroying abnormal cancerous cells. This process, termed immune surveillance, has been at the centre of intense debate for decades. Results using immunodeficient mice lend support to the immune surveillance hypothesis. We surmised that immune surveillance would be hampered by the inhibitory effect of naturally occurring FoxP3$^+$ regulatory T cells, a population of T cells shown to be present at an increased frequency in a variety of human tumours. The carcinogen, methylcholanthrene was injected subcutaneously into mice and the steady development of fibrosarcomas was observed over approximately 200 days. These fibrosarcomas were strikingly infiltrated with FoxP3$^+$ regulatory T cells implying that these cells impinge upon immune-mediated rejection of the tumour. This was confirmed by partial ablation of FoxP3$^+$ regulatory T-cell activity, which resulted in a marked reduction in tumour incidence. The reduction of tumour incidence was ablated in mice that lacked interferon gamma. These data offer strong support for the concept of immune surveillance and indicate that this process is limited by the inhibitory effect of FoxP3$^+$ regulatory T cells.
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Translational Therapeutics

Burnet hypothesised in the 1950's that the adaptive immune system could control and eliminate developing tumours, a process later termed tumour immune surveillance (Burnet, 1957). Although this theory subsequently fell from popularity, a large body of recent work from different groups has demonstrated an increase in both spontaneous and carcinogen-induced tumours in immunocompromised mice including those lacking T cells, natural killer cells (NK) and natural killer T cells (NKT), type 1 and type 2 interferons, perforin and the IFNγ receptor (IFNγR); (reviewed by Smyth *et al*, 2006). These experiments have largely been interpreted as evidence to support the tumour immune surveillance concept and demonstrate the many arms of the immune system involved in tumour destruction.

CD4$^+$ FOXP3$^+$CD25$^+$ regulatory T cells (Tregs) comprise approximately 10% of the CD4$^+$ T-cell population in mice. In humans, the regulatory CD4$^+$ T-cell pool is restricted to 1–2% of the CD4$^+$ population that are described as CD25^{hi} (Baecher-Allan *et al*, 2001). These cells are thought to be essential for maintaining tolerance to self-antigens recognised by autoreactive T cells that escape deletion in the thymus (Sakaguchi, 2005). An absence of functional Tregs has been associated with several autoimmune diseases in both animal models (Brunkow *et al*, 2001) and humans (Bennett *et al*, 2001). It was hypothesised that the same Tregs would have a derogatory impact on antitumour immunity by inhibiting the activity of T cells that recognise tumour antigens (Shimizu *et al*, 1999). Indeed, several groups, including our own, have shown that depletion of Tregs promotes rejection of tumour cells lines injected into mice (Onizuka *et al*, 1999; Shimizu *et al* 1999; Sutmuller *et al*, 2001; Golgher *et al*, 2002). In addition, evidence from a number of laboratories indicates that higher frequencies of Tregs are observed in the blood of patients with cancer compared to healthy subjects (reviewed in Betts *et al*, 2006). Results of a study of patients with ovarian cancer indicated that the frequency of tumour infiltrating Tregs correlated with the extent of disease thereby implying that Tregs are involved in the pathogenesis of cancer (Curiel *et al*, 2004; Wolf *et al*, 2005). Furthermore, we have also recently found that Tregs appear to control antitumour immune responses in patients with colorectal cancer (Clarke *et al*, 2006). Despite these observational studies in humans, it is still unclear whether the frequency and activity of Tregs increases in response to the tumour or whether tumours are more likely to develop in individuals with higher frequencies of Tregs, thereby raising the question of whether Tregs play a role in tumour immune surveillance. This question cannot be addressed by inoculation of tumour cell lines as these cells are already endowed with the necessary mutational and epigenetic changes required to rapidly produce palpable tumours. Use of these tumours does not allow for significant interactions between the immune system and cells in the process of transformation. Models that use carcinogens do include these early interactions and therefore may be considered more relevant for deconstructing the relationship between the immune system and developing tumours. In addition, the immune system is thought to not only influence tumour

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outgrowth at the early stages of tumour development but also, through a sustained interaction with the growing tumour, to continuously modify and diminish the immunogenicity of the tumour (Shankaran *et al*, 2001). Thus, cell lines subjected to prolonged periods of *in vitro* culture are almost certainly more immunogenic than tumour cells *in vivo*. With this in mind, it is reasonable to hypothesise that the impact of Tregs on the immune response to tumours developing *in vivo* may differ to their impact on the immune response to tumour cell lines. Injection of the carcinogen methylcholanthrene (MCA) is an established tumour induction model that has been used to examine the role of a series of cell types and signalling molecules that suppress tumour development. Thus, using MCA, we have examined how altered frequencies of Tregs impinge on the development of *de novo* tumours. Specifically, we determined whether (1) Tregs are present in MCA-induced tumours, (2) Tregs influence *de novo* tumour development, (3) IFNγ is required for control of tumour growth in Treg depleted mice and (4) depletion of Tregs promotes autoimmunity in MCA-treated mice. The implications of our findings are discussed in the context of tumour immune surveillance.

MATERIALS AND METHODS

Mice

Six- to twelve-week-old female wild-type (WT) and IFNγ-deficient ($IFN\gamma^{-/-}$) mice on a C57BL/6 (B6, H-2^b) background were sourced on site. $IFN\gamma^{-/-}$ mice were originally purchased from the Jackson Laboratory (Bar harbour, ME , USA) and kindly donated by Professor N. Topley. Mice were housed in filter top cages and supplied with sterile food and bedding. Experiments were performed in compliance with UK Home Office regulations.

Methylcholanthrene injections

A total of 400 μg MCA (Sigma-Aldrich, Gillingham, Dorset, UK) suspended in 100 μl olive oil was injected subcutaneously (s.c.) into the hind leg of the mice. Mice were monitored weekly for tumour development.

Depletion of $CD25^+$ cells

Mice were injected intraperitoneally (i.p.) with 0.5 mg of the CD25-specific monoclonal antibody (mAb), PC61 (Lowenthal *et al*, 1985) or the isotype control mAb, GL113 at the indicated time points in 100 μl PBS before MCA administration.

Flow cytometry

Single-cell suspensions were prepared by filtering mashed tissues through 70 μm nylon strainers (BD falcon, Franklin Lakes, NJ, USA). Cells were stained with anti-CD4-fluorescein isothiocyanate (FITC), anti-CD4-PE Alexa 610, anti-CD25-biotin (7D4)/SA-PerCP-Cy5.5, anti-TCRαβ Allophycocyanin (APC), anti-FcγIII/II receptor (BD Franklin Lakes, NJ, USA) and anti-FOXP3-R-Phycoerythrin (PE) using the ebioscience staining kit (ebioscience, San Diego, CA, USA, staining kit) mAbs.

Suppression assay

$CD4^+$ T cells were purified from single-cell suspensions of splenocytes by magnetic associated cell sorting (MACS) (Miltenyi Biotec, Auburn, CA, USA). $CD4^+CD25^+$ cells were separated from $CD4^+CD25^-$ cells by MACS. $CD4^+CD25^-$ cells (2×10^4) were stimulated with 1 μm anti-CD3 mAb (Leinco, St Louis, MO, USA) and 1×10^5 mitomycin (Sigma-Aldrich, Gillingham, Dorset, UK) treated $CD4^-$ splenocytes and incubated with titrated numbers of $CD4^+CD25^+$ cells.

Immunohistochemistry

Cryostat-frozen sections (10 μm) were dried and fixed with cold acetone for 15 min on ice. Sections were permeabilised with 0.2% Tween 20, 1% BSA for 30 min before a 1-h incubation with rabbit anti-mouse foxp3 polyclonal antibodies (Ab) (Roncador *et al*, 2005) and rat anti-mouse mAb (L3T4, BD San Jose, CA, USA) or rat anti-mouse CD45R/B220 mAb (RA3-6B2, BD Pharmingen San Jose, CA, USA) or rat anti-mouse CD8 mAb (53-6.7, BD Pharmingen, San Jose, CA, USA). Sections were subsequently incubated with biotinylated swine anti-rabbit polyclonal Ab (DakoCytomation, Carpinteria, CA, USA) followed by Alexa594-conjugated streptavidin and Alexa488-conjugated goat anti-rat IgG (Invitrogen, Carlsbad, CA, USA). Sections were mounted (Vecta Shield, Burlingame, CA, USA) and examined with using a DM LB2 microscope (Leica, Hicksville, NY, USA) and analysed using the Openlab software package (Improvision, Coventry, Warwickshire, UK).

Autoantibody detection

Serum was collected from mice and analysed for circulating anti-double-stranded DNA (dsDNA) IgM autoantibody according to the manufacturers' protocol (Alpha Diagnostic, San Antonio, TX, USA).

Statistical analysis

The extent of Treg infiltration in different organs was compared using a paired *t*-test. Comparisons of tumour induction between experimental groups were made using a log-ranked test.

RESULTS

Treg infiltration of MCA-induced tumours

In preliminary experiments, we found approximately 70–80% of mice develop fibrosarcomas by day 200 after injection of a high dose (400 μg) of MCA. We first sought to determine whether Treg infiltrate MCA-induced tumours. Methylcholanthrene-induced tumours were removed from mice and examined histologically for Tregs by staining sections with Abs specific for FOXP3 and CD4. As shown on Figure 1A, tumours were indeed infiltrated with $CD4^+FOXP3^+$ Tregs, which were in close proximity to $CD4^+FOXP3^-$ conventional T cells (Figure 1A) and $CD8^+$ T cells (Figure 1B). The majority of infiltrating lymphocytes were observed at the tumour margin in the interface with healthy leg muscle (data not shown). These data suggest an ongoing immune response to the developing tumour, which may be controlled, to the detriment of the host, by Tregs.

To evaluate further the proportion of Tregs within the tumour infiltrating lymphocytes (TILs), TILs were stained with CD4-, CD25- and FOXP3-specific Abs and analysed by flow cytometry. Supporting the histological observations, approximately half of the $CD4^+$ T cells within the TIL expressed FOXP3 (Figure 1C). Approximately 85% of these cells expressed CD25 (Figure 1D), a finding that is in agreement with a previous study, which indicated that, depending on anatomical location, between 20 and 40% of $CD4^+FOXP3^+$ cells, do not express CD25 (Fontenot *et al*, 2005). Comparisons were made of the Treg frequencies in spleen, blood, pooled tumour-draining lymph nodes (TDLN) and non-TDLNs (NTDLN). These data established that Tregs were markedly enriched in TIL compared to the lymphoid tissues, suggesting that they are exerting local control of antitumour immune responses (Figure 1E). A significantly larger proportion of Tregs

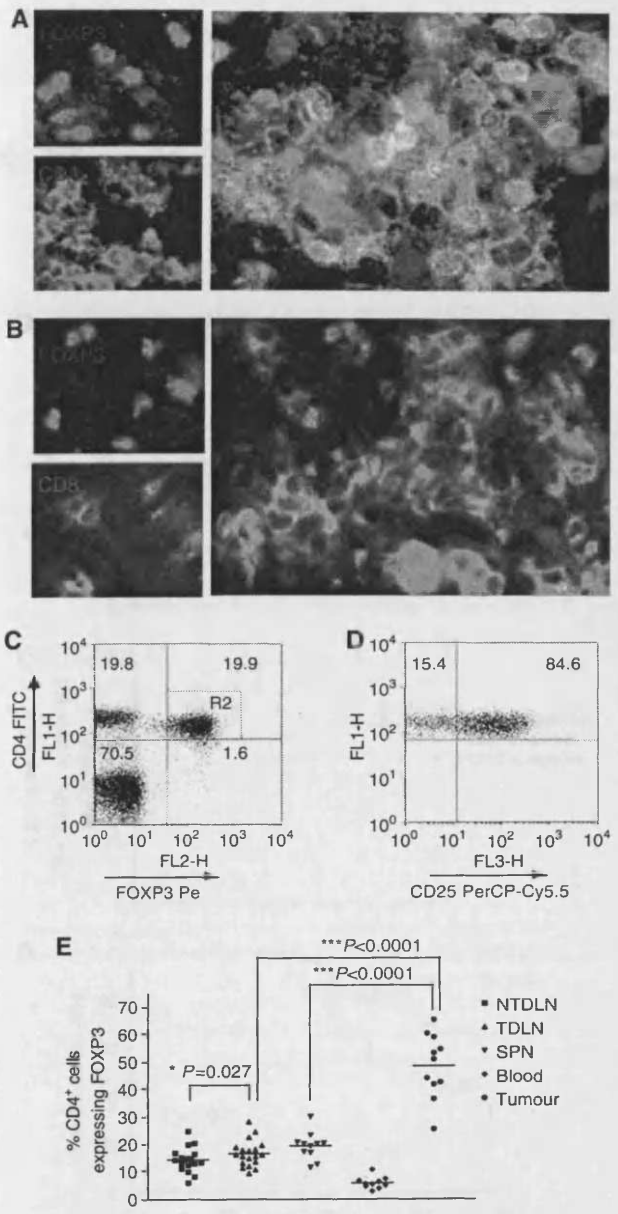

Figure 1 Methylcholanthrene-induced tumours exhibit a significant enrichment of $CD4^+FOXP3^+$ Tregs. Sections of MCA-induced tumours were stained with anti-FOXP3-specific Ab and either anti-CD4 (**A**) or –CD8 (**B**) specific mAb. Single-cell suspensions of TILs, NTDLN, TDLN, spleen and blood were stained with anti-CD4-, anti-CD25- and anti-FOXP3-specific mAbs and analysed by flow cytometry. A representative FACS plot of TIL staining is shown and $CD4^+FOXP3^+$ cells were gated (R2) (**C**). This gate was used to examine CD25 expression as shown in the right panel (**D**). The percentage of $CD4^+$ cells expressing FOXP3 in each compartment is presented (**E**). Data were analysed using a paired student's *t*-test.

was also found within the $CD4^+$ T cells from pooled TDLN mice compared to pooled NTDLN (Figure 1E). Collectively, these data clearly indicate that Tregs expand in response to the developing tumour and are, as described previously in studies using tumour cell lines (Ghiringhelli *et al*, 2005), enriched in areas of tumour-specific immune activity.

Influence of Tregs on development of MCA-induced tumours

The markedly high infiltrate of Tregs within the fibrosarcomas led us to explore whether depletion of the Tregs would protect against the development of MCA-induced tumours.

Depletion of Tregs using the mAb PC61 Depletion of Tregs was carried out using the CD25-specific, depleting mAb, PC61. Phenotypic analyses of PC61-treated mice revealed that administration of PC61 significantly reduced the number and percentage of $CD4^+FOXP3^+$ Tregs in spleens of treated mice (Figure 2A, B). Thus, treatment with PC61 does not completely remove Tregs for, as we outlined above, a proportion of $CD4^+FOXP3^+$ cells express low or no cell surface CD25 (Fontenot *et al*, 2005). $CD4^+CD25^+$ T cells purified from spleens 3 and 6 weeks following administration of PC61 were purified and found to exhibit, on a per cell basis, suppressive capacity comparable to that observed in mice receiving the control mAb, GL113 (Figure 2C).

Tumour incidence in PC61-treated mice Mice treated as described above with either PC61 or control mAbs, were injected 1 day later with MCA mixed in olive oil. Tumour growth was monitored over 200 days. Figure 2D shows pooled data collected from three separate experiments with similar results, including one experiment performed in an animal house at a separate institution. Twice as many mice in the PC61-treated group remained tumour free (18/30) compared to the group treated with control Abs (9/30). These data indicate that even a partial reduction in Treg numbers results in a highly significant reduction in overall tumour incidence and delayed tumour development compared to mice receiving the control mAb, GL113 ($P=0.001$) (Figure 2D). Next, we determined whether additional injections of PC61 would further reduce MCA-induced tumour incidence. Mice were injected with PC61 before MCA administration as described above. Some mice subsequently received further doses of PC61 or isotype control mAb on days 22/24 and 41/43 post-MCA administration. No difference in tumour incidence was observed between the two groups (data not shown) indicating that further injections did not increase the incidence of tumour-free mice. The effect was not due to neutralisation of the rat mAb *in vivo*, as we observed that whereas mouse-anti-rat Abs are induced following administration of PC61, these Abs do not neutralise circulating PC61 or block the PC61 binding site (data not shown). This failure to enhance tumour rejection may however be attributable to codepletion of activated nonregulatory T cells.

Tumour incidence in IFN$\gamma^{-/-}$, PC61-treated mice IFNγ plays an important role in controlling development of MCA-induced tumours (Kaplan *et al*, 1998; Shankaran *et al*, 2001). In agreement with these previous studies, we found that whereas only 70% of WT mice had developed tumours by day 200 after injection with 400 μg of MCA, a similar dose induced tumours in all IFN$\gamma^{-/-}$ mice by day 160 (compare Figure 2D to Figure 3A). To gain an insight into the mechanism of tumour rejection in Treg-depleted animals, we determined whether treatment of IFN$\gamma^{-/-}$ mice with CD25-specific mAbs offered protection against the development of MCA-induced tumours. Depletion of Tregs in these mice did not offer any protection against tumour development. In fact, tumours appeared significantly earlier in IFN$\gamma^{-/-}$ mice treated with CD25-specific mAbs compared to mice receiving the control mAbs (Figure 3A). Interestingly, a much smaller percentage (approximately 40% reduction) of Tregs was observed in tumours excised from IFN$\gamma^{-/-}$ mice compared to those from WT mice (Figure 3B). Similarly, no enrichment of Tregs was observed in the TDLN of IFN$\gamma^{-/-}$ mice (data not shown). These data imply that expansion and/or migration of Tregs in response to a developing tumour is impaired in the absence of IFNγ.

Translational Therapeutics

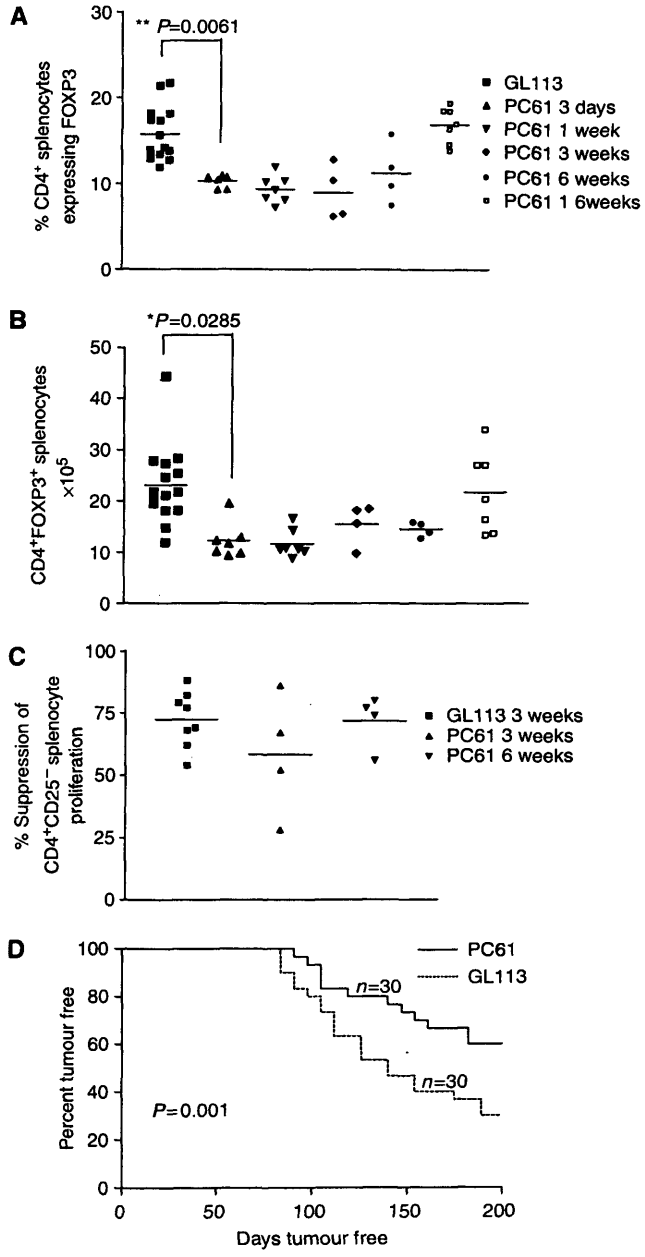

Figure 2 Incidence of MCA-induced tumours is significantly reduced in Treg-depleted mice. The ability of anti-CD25-specific Ab PC61 to deplete $CD4^+FOXP3^+$ Tregs was assessed by analysing the Treg infiltration of spleens following treatment with the mAb PC61 or the isotype control, GL113. Spleens were harvested at 3 days, 1 week, 3 weeks, 6 weeks and 16 weeks after injection and the percentage of $CD4^+$ cells that express FOXP3 (**A**), and the total number of $CD4^+FOXP3^+$ splenocytes (**B**) were measured. The suppressive capacity of Tregs following PC61 injection was measured in an *in vitro* suppression assay (**C**). Data were analysed using an unpaired student's *t*-test. Mice were treated with 1 mg of either PC61 or isotype control GL113 mAb before the injection of MCA. The rate of tumour induction, representing the cumulative data from three experiments, is presented (**D**). Log-rank statistical analysis was used to determine the difference in tumour induction between experimental groups.

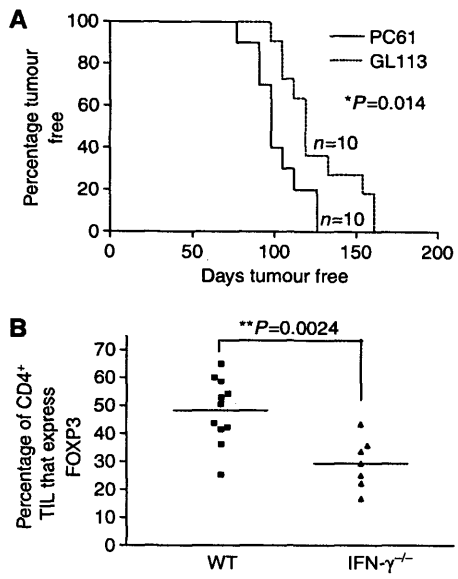

Figure 3 IFNγ deficiency abrogates the ability of Treg depletion to reduce tumour development. $IFN\gamma^{-/-}$ and WT mice were treated with 1 mg of either PC61 or isotype control GL113 mAb before the injection of MCA and tumour development was monitored (**A**). Log-rank statistical analysis was used to determine the difference in tumour induction between experimental groups. Single-cell suspensions were prepared from tumours excised from WT and $IFN\gamma^{-/-}$ tumour bearing mice and stained with anti-CD4 FITC, anti-CD25 PerCP-Cy5.5 and anti-FOXP3 PE-specific mAbs. The percentage of $CD4^+$ TIL that expressed FOXP3 in $IFN\gamma^{-/-}$ and WT derived tumours was compared and analysed using an unpaired student's *t*-test (**B**).

Circulating dsDNA specific autoantibodies in Treg-depleted mice

As a reduction in Treg numbers has been associated with the development of autoimmunity, circulating autoantibodies were analysed in mice that remained tumour free following Treg depletion. Mice treated with PC61 had a significantly elevated concentration of circulating dsDNA-specific IgM autoantibodies compared to mice that were not injected with PC61; however, there was no difference in dsDNA-specific autoantibody levels in mice treated with PC61 and that remained tumour free compared to tumour-bearing mice that received PC61 (Figure 4).

DISCUSSION

The aim of the work described here was to directly investigate whether Tregs impinge on immune surveillance of developing tumours *in vivo*. In this study, we used MCA, a carcinogen that has been widely used to identify a role of a series of immune cell types and signalling molecules that suppress tumour development. The study revealed several interesting findings. We first found a significant enrichment of $CD4^+FOXP3^+$ Tregs in tumours developing *in vivo*, where approximately half of total $CD4^+$ TIL were $FOXP3^+$. Moreover, injection of mice with the CD25-specific mAb, PC61, resulted in a transient depletion of a proportion of the Tregs before MCA injection and a significantly reduced tumour incidence. We believe that interesting parallels can be drawn between this work and a study performed 20 years previously by Prehn and Kojima (1986). Working on the premise that 'the presence of autoimmunity implies an increased antitumour immunity', Prehn and Kojima (1986)found that 3-day thymectomy inhibited development of tumours using a high dose of MCA.

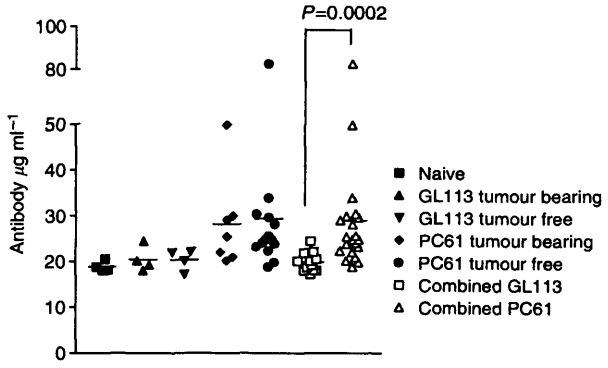

Figure 4 Autoantibody levels are increased in Treg-depleted mice. Serum from naïve mice and mice injected with MCA after treatment with PC61 or GL113 was collected and tested for dsDNA-specific IgM autoantibody and analysed with an unpaired student's *t*-test.

Based on the observation that thymic production of $CD25^+$ T cells, capable of suppressing the activity of autoreactive T cells, begins only on day 3 after birth in mice (Asano *et al*, 1996). It appears that inhibition of tumour development by day-3 thymectomy and by injection of the CD25-specific mAb, PC61, occurs by ablation of Treg activity. Ambrosino *et al* (2006) recently described treatment of Erbb2 transgenic mice with the CD25-specific mAb, PC61. These animals develop multiple mammary carcinomas as a result of overexpression of the Erbb2 oncogene. PC61-treated mice demonstrated reduced carcinoma multiplicity and a concomitant increase in immune responses to p185, the protein product of Errb2 (Ambrosino *et al*, 2006). In our study, we found that circulating dsDNA-specific IgM Abs were elevated in Treg-depleted, MCA-injected mice compared to control mice implying that Treg depletion drives autoreactivity. Collectively, these studies indicate that Tregs inhibit immune surveillance and support the premise that the involvement of Tregs in this process is a consequence of their role in limiting self-reactive immune responses.

The key question arising from our data relates to the type of effector cells promoting tumour rejection after depletion of Tregs. As Tregs have been shown to suppress both innate and adaptive immune responses to tumours (reviewed by Betts *et al*, 2006), it is likely that multiple arms of the immune system including both antigen-specific T cells and nonspecific inflammatory responses are more effective following Treg depletion. In the case of MCA-induced tumours, Nishikawa *et al* (2005) recently showed that Tregs, induced by immunisation with a SEREX-defined self-antigen expressed in tumour cells, inhibited NK and NKT cells capable of inhibiting the development of MCA-induced tumours, thus indicating that these cells are targets of Treg activity and important antitumour effector cells (Nishikawa *et al*, 2005). Ongoing studies in our laboratory are seeking to define precisely the nature of the antitumour responses induced in Treg-depleted, MCA-injected mice.

Previous studies have highlighted a critical role for IFNγ in controlling the development of MCA-induced tumours (Kaplan *et al*, 1998; Shankaran *et al*, 2001). IFNγ can control tumour growth directly through proapoptotic, antiangiogenic and anti-proliferative effects and indirectly, by facilitating induction of antitumour innate and adaptive immune responses (reviewed by Smyth *et al*, 2006). Our study shows that the effect of depleting Tregs is lost in mice lacking IFNγ. This may be because immune responses uncovered by Treg depletion mediate tumour rejection through production of IFNγ and/or that immune responses uncovered by Treg depletion cannot compensate for the lack of IFNγ, which is critical for tumour control even in the absence of Tregs. Interestingly and in contrast to our observations in wild-type mice, tumours appeared slightly yet significantly earlier in $IFN\gamma^{-/-}$ mice receiving CD25-specific mAbs compared to those that received control mAbs. This finding suggests a fundamental difference in the response of wild-type and $IFN\gamma^{-/-}$ mice to MCA injection. It is possible that CD25-specific mAbs deplete important tumour-induced effector cells in $IFN\gamma^{-/-}$ mice. Alternatively, the impact of Treg activity on tumour development may differ between $IFN\gamma^{-/-}$ and wild-type mice. Tregs have been shown in some experimental models to *suppress* tumour progression by inhibiting inflammatory responses that would otherwise promote tumour development (Erdman *et al*, 2003). Such inflammatory responses might be induced in response to MCA injection in $IFN\gamma^{-/-}$ mice, thus the overall influence of Tregs would be to suppress tumour progression through limiting these responses. We did however observe that $IFN\gamma^{-/-}$ mice, when challenged with MCA, demonstrate a reduction of the percentage of $CD4^+FOXP3^+$ Tregs within tumours and TDLN. As mentioned earlier, this may reflect a role for IFNγ in expansion/migration of Treg cells or an overall increase in the expansion of effector T cells in the $IFN\gamma^{-/-}$ animals. Interestingly, the function of Tregs has previously been shown to be impaired in these mice (Kelchtermans *et al*, 2005). Furthermore, Wang *et al* (2006) recently showed that IFNγ is important for the conversion of $CD4^+CD25^-FOXP3^-$ cells to $CD4^+CD25^+FOXP3^+$ Tregs, thus it is possible that those $CD4^+FOXP3^+$ Tregs in MCA-induced tumours are derived from $CD4^+CD25^-FOXP3^-$ cells by a process, which is IFNγ dependent.

An accumulation of data obtained in studies of patients with cancer does support the concept that Treg depletion will have a beneficial effect in cancer immunotherapy. Overall, the results of this study support this premise by revealing a role for Tregs in suppressing effective immune surveillance of carcinogen-induced tumours in intact animals. A more cautionary note, implied by the findings of this study is that the nature of the ongoing immune response to the tumour may alter the outcome of Treg depletion, in some cases favouring tumour progression rather than tumour control. Further studies are clearly required to clarify this issue.

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