Assessing the Role of Regulatory T Cells in the Development and Progression of Tumours

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Summary

A large body of work generated using mouse models indicates that the immune system has the capacity to recognise and eliminate tumour cells. However, it has been suggested that a population of T cells, named $CD4^+Foxp3^+$ regulatory T cells (Treg), hamper effective anti-tumour immunity. A substantial enrichment of Tregs is observed within carcinogen (methylcholanthrene, MCA) induced murine tumours. Furthermore, depletion of Tregs in mice given MCA significantly reduces tumour induction.

To investigate the role of Tregs in anti-tumour immunity a detailed characterisation of tumour-infiltrating lymphocytes (TIL) from MCA induced tumours was performed. These experiments showed that control of tumour growth following Treg cell depletion is dependent on $CD8^+$ cells. The ratio of Tregs to conventional $CD4^+$ T (Tconv) cells and particularly $CD8^+$ T cells altered within the tumour to favour immune suppression. Furthermore, tumour-infiltrating Tregs displayed a phenotype associated with immunosuppression whereas only a small fraction of tumour-infiltrating Tconv and $CD8^+$ T cells appeared activated. Together these findings support the hypothesis that Tregs accumulate within tumours and gain control of T cells preventing anti-tumour immunity.

Evidence exists to suggest that soluble factors subvert anti-tumour immunity by converting tumour-infiltrating Tconv cells into Tregs. To address this issue, the T cell receptor (TCR) repertoires of Tregs and Tconv cells within the MCA induced tumours were analysed. The TCR repertoires of Treg and Tconv cells were found to be largely distinct suggesting that conversion between these two T cell populations does not readily occur. However, it was found that tumour specific proliferation and survival of Treg cells may instead account for their accumulation in tumours. These studies indicate that identification of factors causing preferential proliferation and survival of Treg cells in tumours may enable the inhibition of their suppressive effects. The findings of this thesis are important for the rational design of cancer immunotherapeutic strategies.

Table of Contents

List of Abbreviations

7-AAD	7-aminoactinomycin D
Ab	Antibody
APC	Allophycocyanin
Bcl-2	B-cell lymphoma 2
BrdU	Bromodeoxyuridine
cDNA	Complementary DNA
CDR3	Third complementarity-determining region
CTLA-4	Cytotoxic T-lymphocyte antigen 4
dLN	Draining lymph node
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
GFP	Green fluorescent protein
GITR	Glucocorticoid induced TNFR (tumour necrosis factor receptor)-related
HA	Hyaluronic acid
ICOS	TCR-inducible costimulatory receptor
IFNγ	Interferon-gamma
IL	Interleukin
IPTG	Isopropyl-beta-D-thiogalactoside
LB	Luria-Bertani
LN	Lymph node
MCA	3-methylcholanthrene
MFI	Mean fluorescence intensity
MH	Morisita-Horn
ndLN	Non-draining lymph node
NK	Natural killer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed Death-1
Pe	Phycoerythrin
PeCy7	Phycoerythrin-Cy7
PerCpCy5.5	Peridinin Chlorophyll Protein Complex-Cy5.5
PMA	Phorbol myristate acetate
RBC	Red blood cell
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI	RPMI-1640 media
RT	Reverse transcriptase
SA	Streptavadin
Tconv	$CD4^{+}Foxp3^{-}$ conventional T cells
TCR	T cell receptor

TGFβ	Transforming growth factor-beta
Th	T helper
TNFα	Tumour necrosis factor-alpha
TRBC	T cell receptor beta common
TRBJ	T cell receptor beta joining
TRBV	T cell receptor beta variable
Treg	$CD4^{+}Foxp3^{+}$ regulatory T cells
X-gal	5-bromo-4-chloro-3-indolyl-Beta-D-galactoside

Chapter 1 – Introduction

1.1 Tumour development

1.1.1 Tumourigenesis

The development of cancer in humans is a multistep process that usually occurs over many decades. This process is the result of an accumulation of numerous mutational and epigenetic changes that drives the progressive transformation of normal cells into malignant neoplastic derivatives. It is widely believed that during tumourigenesis most, if not all, types of cancers must attain a catalogue of fundamental alterations termed "acquired capabilities" (Hanahan and Weinberg 2000, Hahn and Weinberg 2002). These are required for the neoplasm to evade programmed cell death (apoptosis), develop angiogenic ability, resist anti-growth signals, generate mitogenic signals, replicate limitlessly, and invade neighbouring tissue / metastasise (Figure 1.1).

1.1.2 Cancer immunosurveillance

The role of the immune system in tumour development has been ardently debated for many decades (reviewed in Dunn *et al.* 2002). In 1909, Paul Erhlich was amongst the first to propose that the host immune response could protect from neoplastic disease. However, it was not until the second half of the 20th century that this hypothesis was extensively investigated. Following the development of inbred strains of mice, and thus the ability to successfully carry out graft transfers between syngeneic mice, it was possible to test whether tumour tissue was more immunogenic than normal tissue. Indeed, it was ultimately shown that mice could be immunised against syngeneic implants of tumours demonstrating the

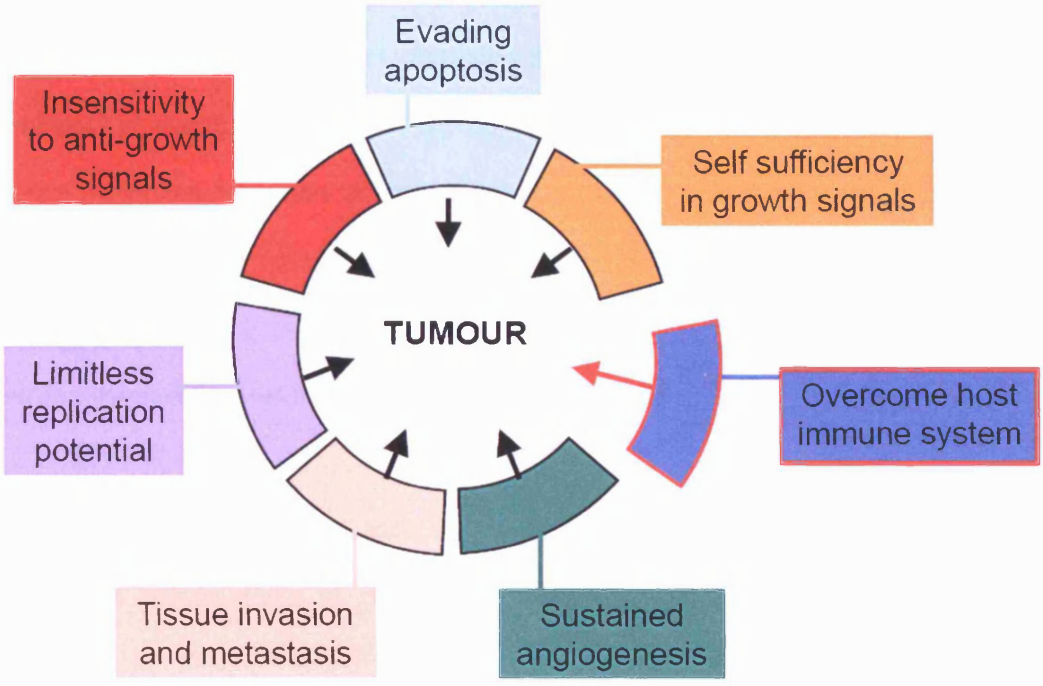


Figure 1.1. Acquired capabilities required for tumour development.
It is currently believed that most if not all tumours have acquired a number of capabilities to become tumourigenic. Six of these acquired capabilities (evading apoptosis, self sufficiency in growth signals, insensitivity to anti-growth signals, limitless replication potential, tissue invasion and metastasis, and sustained angiogenesis) are commonly considered as hallmarks of cancer. An accumulation of evidence now suggests that another capability could be included in this list: the ability to overcome effective anti-tumour immunity. [Adapted from The Hallmarks of Cancer *Cell*, Volume 100, Issue 1, Pages 57-70]

principle that tumours possessed tumour-specific antigens which could induce robust immune responses (reviewed in Old and Boyse 1964, Klein 1966). During the emergence of these and other findings Macfarlane Burnet formally proposed the concept of "immunological surveillance" of cancers, whereby the immune system is able to recognise and eliminate or inactivate potentially dangerous mutant cells that arise in the host (Burnet 1970, 1957). Since then, an extensive body of evidence from mouse studies and clinical observations has developed which strongly supports the notion that a process of cancer immunosurveillance functions to protect the host from the development of cancer.

Much of the evidence to support cancer immunosurveillance has come from studying tumour occurrence in various strains of genetically altered, immune deficient rodents (particularly mice; Dunn *et al.* 2002, 2004, and discussed below). For these studies many different models of tumour occurrence have been used. These include spontaneous tumour development in wild type animals, spontaneous tumour development in animals genetically predisposed to tumour formation (such as $p53^{-/-}$ mice), tumour induction by chemical carcinogens and growth of transplantable tumour cell lines in recipient mice. The most commonly used model of tumour formation for studying cancer immune surveillance is chemical induction with the carcinogen 3-methylcholanthrene (MCA).

1.1.2.1 Methylcholanthrene induced tumour development

The induction of tumours by methylcholanthrene has been extensively used to study carcinogenesis and particularly tumour immunosurveillance, *in vivo* (reviewed in Schreiber and Podack 2009). Typically experimental mice are injected with 50-400μg of methylcholanthrene subcutaneously or intra-muscularly and after 100-200 days the mice

develop palpable fibrosarcomas at the injection site (van den Broek *et al.* 1996, 2000a, Smyth, Crowe and Godfrey 2001, Takeda *et al.* 2002, Qin *et al.* 2002). Methylcholanthrene is a polycyclic aromatic hydrocarbon (PAH) (reviewed in DiGiovanni 1992). PAHs are organic compounds found in environmental pollutants and appear to contribute to cancer in humans (reviewed in Boffetta, Jourenkova and Gustavsson 1997). The process of toxification and detoxification of PAHs such as methylcholanthrene is complex requiring multiple enzymes such as cytochrome P450 for activation (reviewed in DiGiovanni 1992). Resulting metabolites are believed to cause damage to the nucleotide base structure of DNA at random sites within the genome of somatic cells.

In experimental mice the local response following MCA insult resembles that of a wound healing process (Blankenstein and Qin 2003). Extensive cell death and inflammation occurs. Fibroblasts accumulate at the injection site surrounding the MCA fibres. These fibroblasts secrete extracellular matrix, encapsulating the MCA, causing fibrosis and detoxifying the MCA. However, during this effort to protect the host from MCA the fibroblasts are exposed to the genotoxicity of MCA and often become transformed, potentially cancerous cells. Presumably these mutated cells become tumours if and when sufficient oncogenic mutations have occurred. Examples of the mutations noted in MCA induced tumours include mutations in protooncogenes like ras and tumour suppressor genes like p53 (Halevy *et al.* 1991, Watanabe *et al.* 1999).

Using the model of MCA induced tumours to study tumour development offers many advantages over other models. For example, for tumours to establish in experimental mice injected with MCA the mutated cells must first overcome all the biological obstacles necessary for tumour development whereas transplantable cell lines have already established

these "acquired capabilities" prior to injection. However, the obvious advantages of transplantable cell lines, that is, that they are easier and quicker experimentally, make them popular. In contrast monitoring for development of spontaneous tumours can be a slow process, ultimately leading to increased mouse housing costs and less flexibility in experimental set ups. Furthermore, in contrast to spontaneous tumours, with MCA, tumour occurrence is restricted to a particular, easy to monitor site (the injection site).

1.1.2.2 Susceptibility of immuno-compromised mice to tumour development

Many components of the immune system have been implicated in having a role in the development of tumours (reviewed in Dunn *et al.* 2002, 2004). The susceptibility of immuno-compromised (often "knockout") mice to tumour development has particularly helped to identify specific cell types or molecules of the immune system which may be involved in tumour immunity. Cells of the adaptive arm of the immune system appear important, suggesting the recognition of tumour specific antigens. Mice lacking RAG-2 (or RAG-1) are unable to rearrange their antigen-receptor genes and as a result the development of T cells, B cells and NKT cells is arrested leaving $RAG^{-/-}$ mice without mature lymphocytes (Shinkai *et al.* 1992). Experiments performed by Shankaran *et al.*, (2001) showed that $RAG^{-/-}$ mice developed spontaneous tumours more readily than wild type (WT) controls. Furthermore, $RAG^{-/-}$ mice were also found to be highly susceptible to MCA induced tumour development (Shankaran *et al.* 2001, Smyth *et al.* 2001). Similarly, $J\alpha18^{-/-}$ mice lack $V\alpha14J\alpha18$ expressing invariant NKT cells and have been shown to develop MCA-induced sarcomas at two to three times higher frequency than WT controls (Cui *et al.* 1997, Smyth *et al.* 2000a). Innate immune cells, such as NK cells and γδ T cells, may also contribute to tumour immunity in response to stress or danger signals induced in the tumour microenvironment. Depletion of

NK cells with either anti-NK1.1 or anti-asialo-GM1 monoclonal antibodies can increase MCA susceptibility (Smyth *et al.* 2001). Also, TCR$\gamma^{-/-}$ (which lack γδ T cells) are more susceptible to MCA induced tumour formation compared to control mice (Girardi *et al.* 2001, Gao *et al.* 2003). Together these data suggest that lymphocytes are important for protecting mice against chemically induced and spontaneous tumours.

Many of the effector molecules utilised by cells of the immune system appear to mediate tumour suppression. For example, mice deficient of the IFNγ receptor 1 or IFNγ are more prone to MCA-induced sarcomas (Street, Cretney and Smyth 2001, Shankaran *et al.* 2001, Kaplan *et al.* 1998). Likewise, mice lacking IFNγ are also more susceptible to spontaneous lymphomas and lung adenocarcinomas (Street *et al.* 2002). Another important immune factor implicated in tumour immunity is the cytolytic molecule perforin. Mice deficient of perforin were found to be more prone to MCA induced tumour development as well as spontaneous disseminated lymphomas (van den Broek *et al.* 1996, Smyth *et al.* 2000b, Street *et al.* 2001). Finally, there is also evidence to suggest that enhancing immune system activity can reduce tumour development. For example, Noguchi *et al.*, (1996) demonstrated that treatment of mice with the powerful T and NK cell-stimulatory cytokine, IL-12 significantly delayed tumour appearance and ultimately reduced tumour incidence.

1.1.3 Evidence for cancer immunosurveillance in humans.

There is also evidence to support the existence of an immunosurveillance process in humans (reviewed in Dunn *et al.* 2002, 2004). Three main lines of evidence exist: immuno-compromised humans are more susceptible to cancer; there is a strong correlation between

the infiltration of immune cells and patient prognosis; cancer patients can develop spontaneous immune responses to tumours.

1.1.3.1 Susceptibility of immuno-compromised humans to cancer

Studies show that individuals with congenital or acquired immune deficiencies are more susceptible to tumours of viral origin such as Kaposi's sarcoma, non-Hodgkin's lymphoma and cancers of the anal and urogenital tract (Gatti and Good 1971, Penn 2000, Boshoff and Weiss 2002). However, a true indicator of a tumour immunosurveillance process in humans would be to observe an increased incidence of tumours of non-viral origin in these individuals. Unfortunately, this is confounded by the fact that these people are far more susceptible to lethal viruses and pathogens as well as tumours of viral origin thus not allowing the opportunity for the development of spontaneous tumours. Nevertheless, in support of the immunosurveillance notion, a number of studies have shown that transplant recipients on immunosuppressive drugs display a higher incidence of non viral cancers compared to age matched controls (Birkeland *et al.* 1995, Penn 1996, Pham *et al.* 1995). It is worth noting that not all studies have found this to be the case but an overall examination of epidemiological data does suggest an increase in (non viral) tumour incidence in patients receiving immunosuppression. The reasons for the increased incidence appear to be two fold. Firstly, these patients have an increased susceptibility to developing tumours *de novo*, presumably as a result of reduced recognition or elimination of aberrant cells. However, it has also been observed in a number of cases that the transplantation of an organ, accompanied by an immunosuppressive regime, has allowed for the outgrowth of an occult tumour previously kept dormant by the donor's intact immune system. A recent example reported the transfer of

a fatal melanoma transferred in a donated kidney 16 years after the donor had melanoma surgery (MacKie, Reid and Junor 2003).

1.1.3.2 Correlation between the presence of tumour infiltrating immune cells and patient prognosis

Another indicator that the immune response influences the behaviour of human tumours is the strong correlation between the presence of immune cells infiltrating tumours and increased patient survival (reviewed in Dunn *et al.* 2004). The infiltration of effector T cells has been associated with a better prognosis in patients with melanoma (Clark *et al.* 1989, Clemente *et al.* 1996, Mihm, Clemente and Cascinelli 1996), ovarian carcinoma (Zhang *et al.* 2003), colorectal cancer (Naito *et al.* 1998, Ohtani 2007) and oesophageal carcinoma (Schumacher *et al.* 2001). Similarly the presence of NK cells correlated with the survival of patients with gastric carcinoma (Ishigami *et al.* 2000), squamous cell lung carcinoma (Villegas *et al.* 2002) and colorectal cancer (Coca *et al.* 1997). One of the most convincing studies to date, demonstrated that the type, density and location of immune cells in colorectal tumours was a better predictor of patient prognosis than the histopathological methods current used for staging cancers (Galon *et al.* 2006).

1.1.3.3 Spontaneous development of immune responses to tumours

Another factor that suggests the immune system can influence tumour growth in humans is the spontaneous development of immune responses to tumours in patients. A large range of immunogenic human tumour antigens has now been identified. Exemplifying this, the human cancer immunone database (CIDB) has been established for cataloguing these human tumour antigens and now contains well over 1000 entries. These antigens have been identified by the

characterisation of tumour specific antibodies or T cells isolated from cancer patients. The serological expression cloning technique (SEREX) developed by Pfreundschuh and colleagues (Sahin *et al.* 1995) has significantly helped to expand the list of antigens recognised by the humoural immune response in humans. A second example of spontaneous immune responses to tumour antigens in humans is that seen in paraneoplastic neurologic disorder/degeneration (PND). This autoimmune disorder is thought to be caused by the formation of an antitumour immune response that cross reacts with cells of the nervous system (reviewed in Albert and Darnell 2004). Most commonly associated with tumours of the breast, lung and ovary, PND patients harbour high titers of antibodies against neuronal antigens that are present both in the neuronal cells affected and the accompanying tumour. Interestingly, patients with PND often present with neurodegenerative symptoms and at the time are unaware they have cancer, yet with time are found to harbour small tumours. Thus it appears that an immune response to neuronal antigens expressed by the tumour cells recognise and suppress the growth of the malignancy. This phenomenon becomes apparent in patients with PND when the immune response also recognises neuronal cells expressing the same antigens leading to autoimmunity and neurological symptoms. A number of recent papers have demonstrated that PND patients possess $CD8^+$ CTLs specific for neuronal antigens such as CDR2 and HuD, and that these T cells can kill target cells *in vitro* (Santomasso *et al.* 2007, Roberts *et al.* 2009). However, it is worth noting that although patients with PND generally have a more benign cancer course than patients without PND, only 10% of PND patients appear tumour free thus the anti-tumour immune response in these patients is, at best, incomplete.

Finally, there is also evidence showing that cells of the innate immune arm can specifically recognise tumours in human patients. One primary example of this involves the proteins

MICA/B (MHC class I chain-related proteins A and B) which have been found to be expressed on many, but not all, human tumours (Groh *et al.* 1999, Vetter *et al.* 2002, Jinushi *et al.* 2003). These proteins act as ligands for two receptors expressed on cells of the innate immune system: NKG2D on NK cells and most human γδ T cells and the T cell receptor on Vδ1 γδ T cells. It has been demonstrated, *in vitro*, that tumour cells expressing NKG2D ligands can be killed by NK cells and γδ T cells and that this killing required the activity of the NKG2D receptor and/or the γδ TCR. Two independent studies also showed there is a strong correlation between the expression of MICA/B on tumours and a) tumour infiltration by Vδ1 γδ T cells (Salih, Rammensee and Steinle 2002) and b) NKG2D on tumour infiltrating lymphocytes (Groh *et al.* 1999).

In summary, it is clear that many patients can develop immune responses that recognise the tumours they bear. These spontaneous immune responses of the innate and adaptive immune system observed in cancer patients shed light on the possible mechanisms used by the immune system to recognise tumours and support the idea of immunosurveillance in humans. However, in cancer patients the immune responses observed are clearly unsuccessful as the process of immune surveillance has failed to eliminate the tumour. Thus, the existence of cancer immunosurveillance in humans remains likely but unproven.

1.1.4 Cancer immunoediting

Despite evidence that a plethora of immune effector functions contribute to tumour immunosurveillance, tumours do arise in immunocompetent "normal" hosts. This clinical reality may initially seem paradoxical to the immunosurveillance theory. However, it has been suggested that the immune system actually has a dual effect in tumour development.

Not only does it eliminate aberrant, potentially cancerous cells but the same immune pressure may ultimately promote the outgrowth of poorly immunogenic tumour cell variants. This hypothesis has been termed cancer immunoediting (reviewed in Dunn *et al.* 2002, 2004, Smyth, Dunn and Schreiber 2006).

Evidence to support this extended theory comes from observations that tumours derived from immuno-compromised mice are more likely to be rejected when transferred to immunocompetent hosts. This suggests that the immune system can influence the immunogenicity of developing tumours. For example, Shankaran *et al.*, (2001) found that MCA tumours established in the absence of T cells are more immunogenic that those originating in an intact immune system. Specifically they found that tumours developed from RAG-$2^{-/-}$ mice and WT mice have the same ability to establish themselves and showed similar growth kinetics when transplanted into RAG-$2^{-/-}$ recipients. Conversely, the majority (8 of 20) of RAG-$2^{-/-}$ tumours were rejected when transplanted into immuno-competent WT mice. Similar findings have been made using tumours developed in nude, SCID, NKT cell deficient and perforin knockout mice (Svane *et al.* 1996, Engel *et al.* 1997, Smyth *et al.* 2000a, Street *et al.* 2002). Thus tumours derived in hosts with an intact immune system are less immunogenic than tumours derived from mice without a complete immune system. Together this suggests that the immune environment in which a tumour grows sculpts the developing tumour, favouring the outgrowth of less immunogenic forms.

Cancer immunoediting is proposed to occur in three sequential phases – elimination, equilibrium and escape (Figure 1.2). The elimination phase represents the immune surveillance of tumours described above. However, cells that manage to evade elimination enter a period of equilibrium. This is a period of dynamic interaction between the host

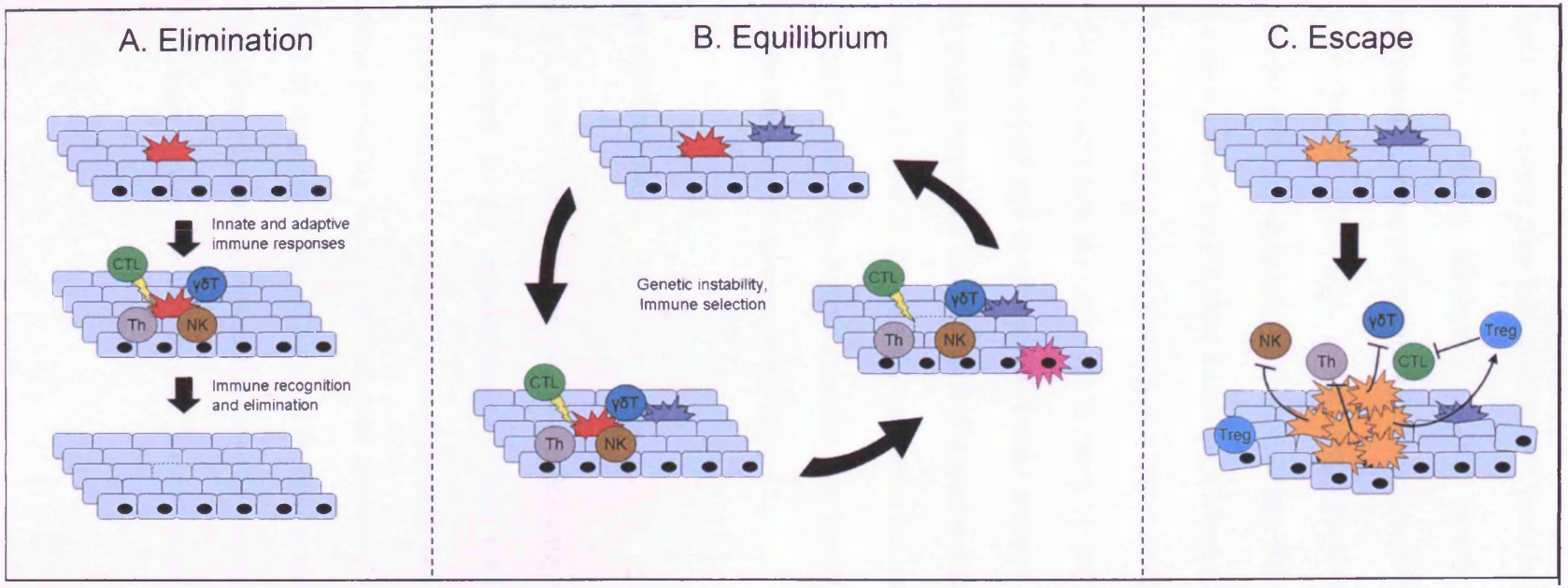


Figure 1.2. The 3 E's of cancer immunoediting.
Cancer immunoediting is proposed to occur in three phases. The first phase (A) is analogous to classical cancer immunosurveillance whereby occult cells are recognised and eliminated by innate and adaptive immune responses. A period of equilibrium (B) may also be entered through cycles of genetic instability and immune selection where cancer cell growth and immune deletion are at equilibrium. This period, which may last many years in humans, could ultimately result in deletion of all pre-cancerous cells or the escape of variants which have managed to avoid detection and/or elimination by the immune system. The escape phase (C) describes the uncontrolled growth of cancer cells leading to a palpable tumour. Th = T helper cell; CTL = cytotoxic T cell; NK = NK cell; γδT = gammadelta T cell; Treg= regulatory T cell; flashes represent aberrant cells. Adapted from Tumour microenvironments, the immune system and cancer survival. Strausberg, 2005. *Genome Biology*, 6(3) p211.

immune system and the tumour, resulting in an apparent dormancy where tumour expansion is kept in check. This phase may last up to several years in humans and can lead to three possible outcomes: eventual elimination of the tumour, permanent maintenance in equilibrium, or tumour escape, where tumour cell variants emerge from the equilibrium phase and present as clinically detectable, progressively growing tumours. Perhaps the most compelling evidence for the existence of an equilibrium phase in tumour development was provided in a recent report from Robert Schreiber's laboratory (Koebel *et al.* 2007). Using chemically induced (MCA) murine tumours, this report demonstrated that tumour cells can exist in an occult state where they are kept in check by the immune system and that these cells can become edited and spontaneously escape immune control. In immunocompetent hosts tumour escape represents the outgrowth of a tumour that is able to either resist immune detection, resist elimination by the immune system, or induce immune suppression and thus tolerance of the tumour. Thus overcoming the host immune defences may represent an additional barrier for a tumour to overcome (Figure 1.1).

1.1.5 Escape mechanisms

Tumour escape is the process wherein the immunologically sculpted tumour expands in an uncontrolled manner in the immunocompetent host (Dunn *et al.* 2002). During the equilibrium phase, through a process akin to Darwinian selection, tumour variants emerge with mutations providing them with increased resistance to immune attack. Ultimately, enough genetic or epigenetic changes occur in the tumour cells leading to variants that have acquired insensitively to immune detection and/or elimination. Since both innate and adaptive immunity have been shown to be important for tumour control, it is likely that escape variants

have acquired mechanisms to overcome both arms of the immune system. For this, individual tumour cells may have obtained multiple mechanisms for immune evasion.

Several mechanisms of immune escape have been demonstrated in tumours. At the tumour cell level, alterations in the recognition of tumour cells have been shown. Tumour cells can lose the expression of antigen or downregulate MHC molecules which present antigen to immune cells (reviewed in Campoli and Ferrone 2008). It is known that tumour cells can shed NKG2D ligands (such as MICA) releasing soluble ligands which may attenuate the function of NKG2D on host immune cells (Groh *et al.* 2002). Furthermore, Kaplan *et al.*, (1998) demonstrated that insensitivity to IFNγ can affect tumour immunogenicity in mice and that certain types of human tumours are insensitive to IFNγ. IFNγ insensitivity may prevent both immune detection and/or destruction. Evasion of immune destruction may also occur through generation of defects in death receptor signalling, for example TRAIL (Takeda *et al.* 2001). Another example of evasion of immune destruction is the expression of anti-apoptotic factors, a major capability demonstrated in many tumours (reviewed in Lowe and Lin 2000, Kim, Emi and Tanabe 2006a).

Tumour immune escape may also occur through the generation of immunosuppressive cytokines. The tumour microenvironment is thought to be an immunosuppressed environment. This is demonstrated by early experiments performed in Robert North's group displaying concomitant immunity (Berendt and North 1980, North and Bursuker 1984). This is the apparently paradoxical immune status where tumour rejection at a secondary tumour site coincides with tumour growth at the primary site. These experiments suggest that an immunosuppressive environment is established at the primary site which prevents the immunity present within the mice as clearly displayed by the rejection of the tumour at the

secondary site. This immunosuppressive environment may be established by the expression of immunosuppressive cytokines, such as IL-10 and TGFβ, from tumour cells or other cells within the tumour environment. An example of another cell type found within the tumour environment which may produce immunosuppressive cytokines is regulatory T cells (Sakaguchi *et al.* 2001). These cells are found in numerous types of murine and human tumours and were also shown by Robert North's group to cause the loss of concomitant immunity to tumours (North and Bursuker 1984). These cells may inhibit immune responses to tumours by the production of immunosuppressive cytokines such as IL-10 and TGFβ but have also been shown to have other mechanisms of immune suppressive activity (these will be discussed in detail below).

1.1.6 Cancer and inflammation

There is also evidence to show that the immune system not only has anti-tumour effects but to the contrary it may promote tumour development. Another pioneer in medical science, Rudolf Virchow, is attributed with being the first to suggest that inflammation supports cancer (reviewed in Balkwill and Mantovani 2001). In 1863, Virchow noted that neoplastic tissues are often infiltrated by leukocytes and suggested that this infiltrate reflected the origin of cancer at sites of inflammation. There is now increasing evidence to suggest that inflammation can indeed promote tumour development. Indeed, there is sufficient evidence for it to be proposed that an inflammatory environment represents an alternative 7th hallmark of cancer (Colotta *et al.* 2009). Proinflammatory cytokines such as IL-1, IL-6 and in particular TNFα have been shown to be present in many tumours and are associated with tumour progression. These and many other cytokines/chemokines are induced by hypoxia, a major physiological difference between normal and malignant tissue. The inflammatory

infiltrate in tumours can comprise primarily of macrophage secreting factors promoting tumour cell growth/survival, angiogenesis and metastasis. Furthermore, tumour associated dendritic cells are usually of an immature phenotype with defective ability to stimulate T cells. Interestingly, inhibition of the inflammatory cytokine TNFα with the drug etanercept showed possible clinical activity in a clinical trial with ovarian carcinoma patients (Madhusudan *et al.* 2005) and the use of non-steroidal anti-inflammatory drugs (NSAIDS) has been shown to reduce cancer risk (Thun *et al.* 1993, Langman *et al.* 2000, Steinbach *et al.* 2000).

1.2. Regulatory T cells

1.2.1 Preventing autoimmunity

The immune system has evolved to protect the host by combating invading pathogens. Cells of the adaptive immune system, namely T and B cells, contribute to this process and are able to recognise a plethora of antigens. During development in the thymus (T cells) and bone marrow (B cells) lymphocytes that recognise self antigens (autoreactive) are eliminated by a process called clonal deletion (Janeway *et al.* 2001). However, this process is not completely efficient and self-reactive lymphocytes can be detected in the periphery of normal individuals. Despite this, in healthy individuals these potentially hazardous lymphocytes appear to be maintained in an inactive state. It is now recognised that regulatory T cells (Treg) are a cardinal cell type involved in preventing autoimmunity. However, their establishment to such distinction has been onerous (reviewed in Germain 2008).

1.2.2 Historical perspective of Tregs

In the early 1970s evidence began to emerge showing that T cells were not only able to elicit positive effects on immune responses but were also capable of limiting immune responses (Baker *et al.* 1970, Okumura and Tada 1971, Droege 1971, Gershon and Kondo 1971). These negatively acting cells were dubbed suppressor T cells and were suggested to be a specialised subset of T cells (Vadas *et al.* 1976, Cantor, Shen and Boyse 1976, Okumura *et al.* 1977). Suppressor T cells were intensely studied by researchers during the 1970s and early 1980s becoming an accepted part of immunology. However, as techniques in molecular biology improved, a succession of flawed and negative studies on suppressor T cells led to a complete lack of confidence in the suppressor T cell field. Publication of papers in the area declined and funding for research into suppressor T cells was severely restricted ultimately leading to a loss of momentum in suppressor T cell research.

The renaissance in the suppressor T cell field took off following the publication of work from Shimon Sachaguchi's lab in 1995 which identified CD25 as a marker for these cells (Sakaguchi *et al.* 1995). This article demonstrated that the inoculation of athymic nude mice with spleen and lymph node cell suspensions depleted of $CD25^+$ cells caused severe multi-organ autoimmunity within 2 weeks and even death in 30% of the mice within 3 weeks. These data added to recent findings suggesting that a subset of T cells with certain activation/memory cell markers could protect from autoimmune disease in mice (Powrie and Mason 1990, Powrie *et al.* 1993). Further studies supported the existence of suppressor T cells, now more commonly known as regulatory T cells, by showing that $CD4^+CD25^+$ cells could limit TCR induced expansion of CD4 and CD8 T cells *in vitro* (Thornton and Shevach 1998). These experiments were promptly repeated using human $CD4^+CD25^+$ cells providing

evidence for regulatory T cell activity within the human immune system (Stephens *et al.* 2001, Ng *et al.* 2001, Taams *et al.* 2001).

A further advancement in the field of regulatory T cells was the discovery that the transcription factor Foxp3 played a critical role in the development and/or function of Tregs (Hori, Nomura and Sakaguchi 2003, Fontenot, Gavin and Rudensky 2003) and that Foxp3 expression could be a useful marker for distinguishing these cells (Fontenot *et al.* 2005c; discussed below). Perhaps the most persuasive evidence that $Foxp3^+$ cells were important for maintaining immune homeostasis was the dramatic autoimmune phenotype observed from the lack of functional Foxp3 in scurfy mice and humans with IPEX (Wildin *et al.* 2001, Bennett *et al.* 2001, Brunkow *et al.* 2001, Khattri *et al.* 2003).

1.2.3 Generation of Tregs

$CD4^+CD25^+Foxp3^+$ Tregs make up about 5-10% of the CD4 T cell population in naïve mice and around 1% of all $CD4^+$ T cells in normal humans. It is widely thought that the majority of Tregs are generated in the thymus as part of normal T cell development as a consequence of recognition of self antigen. These thymically generated Tregs are termed naturally occurring Treg (nTreg). Tregs may also be generated in the periphery as a consequence of activation of mature precursor T cells (reviewed in Bluestone and Abbas 2003). Distinguishing these cells from nTregs, these peripherally generated Tregs have been termed adaptive or induced Tregs.

1.2.3.1 Thymic production of nTregs

Evidence from studies in mice suggests that nTregs emerge from the thymus as a functionally mature and distinct T cell subpopulation (reviewed in Sakaguchi 2004). Firstly, it has been

shown that both nTregs in the periphery and $CD4^+CD25^+CD8^-$ thymocytes express Foxp3 (Hori *et al.* 2003, Fontenot *et al.* 2003). Secondly, a lack of Foxp3 abrogates both populations (Fontenot *et al.* 2003). Furthermore, both peripheral $CD4^+CD25^+$ Tregs and $CD4^+CD25^+CD8^-$ thymocytes are phenotypically and functionally the same. That is, they constitutively express CTLA-4 (Annunziato *et al.* 2002) and GITR (Shimizu *et al.* 2002), are anergic *in vitro* and have similar suppressive activities *in vitro* (Itoh *et al.* 1999).

It also appears, at least in rodents, that the thymic production of nTregs is temporally distinct to other T cells. Experiments initially suggesting this date back to the late 1960s when Nishizuka and Sakakura (1969) noticed that thymectomy of neonatal mice 3 days after birth resulted in the development of autoimmunity, in particular destruction of the ovaries. Later studies suggested that this was the result of a lack of suppressor (regulatory) T cells (Asano *et al.* 1996). More recently, a study using mice expressing the fluorescent marker GFP in $Foxp3^+$ cells supported these early findings (Fontenot *et al.* 2005a). The study showed that the percentage of $CD4^+CD8^-$ thymocytes expressing Foxp3 slowly rose from <0.1% at 12 hours post birth to ~4% 21 days after birth. However, in concurrence with the day 3 thymectomy data, the increase is not steady and a significant increase in the percentage of $CD4^+CD8^-$ thymocytes expressing Foxp3 occurs between days 3 and 4 post birth.

The mechanism that leads to the development Tregs in the thymus is not well understood. In particular, the role of the T cell receptor (TCR) specificity in directing thymic precursors to become conventional T cells (Tconv) or Tregs remain unclear. Many studies, often using TCR transgenic mice, have suggested that the thymic development of Treg requires unique TCR interactions with self-peptide MHC complexes expressed on thymic stromal cells (Itoh *et al.* 1999, Jordan *et al.* 2001, Kawahata *et al.* 2002, Bensinger *et al.* 2001). Together these

studies indicate that, compared to the thymic selection of conventional T cells, the development of Tregs is facilitated by relatively high TCR avidity interactions with self-peptide MHC complexes. Nonetheless this avidity must not be so high as to lead to their deletion. These findings also form the basis of an opinion that Tregs predominantly recognise self antigens (discussed below).

Interactions via accessory molecules may also be important in the thymic development of Tregs. For example, mice deficient of CD28, B7, CD40, or mice treated with CTLA-4-Ig contain fewer Tregs (Salomon *et al.* 2000, Kumanogoh *et al.* 2001). These accessory molecules may contribute by increasing the avidity of the interaction between the thymic precursor and stromal cell. These interactions may also be important for the peripheral maintenance of Treg.

1.2.3.2 Peripherally induced, adaptive Tregs

In mice, Tregs can also be generated from mature T cell populations in the periphery (*in vivo*) under certain conditions of antigenic stimulation (Kretschmer *et al.* 2005, Apostolou and von Boehmer 2004, Thorstenson and Khoruts 2001) or following the adoptive transfer of non-regulatory T cells (Curotto de Lafaille *et al.* 2004). It is also possible to induce the up-regulation of Foxp3 and a suppressive phenotype *ex vivo* in mouse and human T cells by activation of mature T cells usually in the presence of immunosuppressive cytokines such as IL-10 and TGFβ (Levings, Sangregorio and Roncarolo 2001, Yamagiwa *et al.* 2001, Barrat *et al.* 2002, Chen *et al.* 2003, Walker *et al.* 2003b, Fantini *et al.* 2004). These Tregs have been termed induced or adaptive Tregs (reviewed in Curotto de Lafaille and Lafaille 2009). Like all T cells, adaptive Tregs are proposed to initially originate in the thymus but are derived

from classical T cell subsets or from natural Tregs. Specifically, the distinction between adaptive Tregs and naturally occurring Tregs is the requirement of adaptive Tregs for further differentiation of a T cell subset post thymic development into a Treg phenotype as a consequence of exposure to antigen in a particular immunological context (Bluestone and Abbas 2003, Curotto de Lafaille and Lafaille 2009).

Despite the evidence that Treg induction can occur *in vitro* and to some extent *in vivo*, the relevance of Treg induction in the periphery under normal physiological conditions remains unclear. Perhaps the best example of Treg induction is in the establishment of oral tolerance. Both Coombes *et al.*, (2007) and Sun *et al.*, (2007) showed that tolerance to a harmless non-self antigen (OVA) involved the induction of Tregs in gut-associated lymphoid tissue (GALT). Further evidence for induction of Tregs in the periphery comes from the induction of tolerance to transplants (Cobbold *et al.* 2004) and gut and respiratory mucosa (Curotto de Lafaille *et al.* 2008). Many of the findings have lead to the suggestion that adaptive Tregs may possess a TCR repertoire that is directed primarily towards foreign antigens (Bluestone and Abbas 2003, Curotto de Lafaille and Lafaille 2009). However, it is not specifically known what factors may drive the differentiation of mature T cells into Tregs versus T effectors in the periphery. Due to the apparent differences in the differentiation requirements, the TCR repertoires and the occurrence of natural and adaptive Tregs, it has been proposed that the physiological roles of adaptive and naturally occurring Tregs may be different and dependant on the immunological context (Bluestone and Abbas 2003, Curotto de Lafaille and Lafaille 2009). It has been proposed that, naturally occurring Tregs, which have suppressive activity from inception, function to suppress autoreactive T cells typically in non-inflammatory settings. Conversely, adaptive Tregs arise in an inflammatory context (such as

that caused by infection or inflammatory bowel disease) in order to limit self damaging inflammatory reactions.

Evidence to support the peripheral generation of Tregs in humans came from a recent report by Vukmanovic-Stejic and colleagues (2006). They showed that Tregs have a high rate of turnover *in vivo*, and are susceptible to apoptosis and senescence yet do not diminish throughout life. These data suggest that it is unlikely Tregs are exclusively generated as a separate lineage from the thymus which is subject to involution at puberty. Instead their studies suggested that antigen specific Tregs were continuously derived from the memory $CD4^+$ T cell pool. However, a report since then has shown that the TCR repertoires of Treg and Tconv are generally non-overlapping and that only a minority of Treg cells can develop from $FoxP3^-CD4^+$ non-Treg cells *in vivo* (Miyara *et al.* 2009). Thus the existence of natural and adaptive Tregs in humans (and mice) is plausible but the *in vivo* relevance of peripheral conversion has not yet been demonstrated.

1.2.4 Treg markers

A deficiency of reliable markers for identifying Tregs has been a hindrance on the analysis of Treg since their discovery. Indeed, a wholly reliable surface marker for Tregs still remains sought after. In 1995 Sakaguchi's lab published a landmark paper which identified that a small subset of $CD4^+$ T cells that co-expressed CD25 were important for maintaining immunological tolerance (Sakaguchi *et al.* 1995). In these studies it was shown that depletion of $CD4^+CD25^+$ cells from the T cell pool resulted in widespread autoimmunity when the remaining $CD4^+CD25^-$ cells were transferred into recipient mice. These observations were soon supported by the *in vitro* demonstration that $CD4^+CD25^+$ cells were able to suppress T

cell responses (Thornton and Shevach 1998). Soon after, human $CD4^+CD25^+$ T cells were found to have similar regulatory properties (Stephens *et al.* 2001, Ng *et al.* 2001, Taams *et al.* 2001). However, the use of CD25 as a marker for Tregs is imperfect because it is also transiently expressed on activated non-regulatory cells. Of particular nuisance to researchers, CD25 is expressed on activated $CD4^+$ T cells but it is also found on other cell types following activation including $CD8^+$ T cells and B cells (Muraguchi *et al.* 1985, Lowenthal *et al.* 1985).

As mentioned earlier, at present the best marker for regulatory T cells, especially in mice, is the transcription factor Foxp3 (Forkhead box protein 3). The importance of this transcription factor for maintaining immune homeostasis is clearly demonstrated in mice and humans lacking functional Foxp3. A mutation in the Foxp3 gene in Scurfy mice causes them to develop severe autoimmunity due to a resultant deficiency in Treg (Brunkow *et al.* 2001, Khattri *et al.* 2003). Similarly, humans with the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome carry a mutation in Foxp3 which results in a lack of Tregs and severe multiorgan autoimmunity (Bennett *et al.* 2001, Wildin *et al.* 2001). It was also shown that ectopic expression of Foxp3 in non-regulatory T cells could confer a suppressive phenotype (Hori *et al.* 2003, Fontenot *et al.* 2003). Collectively, these data and others (Gavin *et al.* 2007, Kim, Rasmussen and Rudensky 2007, Lin *et al.* 2007), clearly show that Foxp3 expression is important for the development and maintenance (Williams and Rudensky 2007) of Tregs in mice and in humans. However, despite premature suggestions on the contrary (Fontenot *et al.* 2005c), it is now recognised that Foxp3 expression alone is not sufficient for commitment to the Treg lineage. In humans it has been found that Foxp3 is up-regulated, albeit transiently, in activated T cells and may also be expressed by tumour cells (Ebert *et al.* 2008, Morgan *et al.* 2005, Karanikas *et al.* 2008). In mice Foxp3 does hitherto appear to be Treg specific. However, recent studies using extensive

microarray analysis have identified an expression signature for naturally occurring Tregs (Sugimoto *et al.* 2006, Hill *et al.* 2007). When this was compared to Treg-like cells generated under certain conditions they showed that Foxp3 expression alone does not induce a full Treg genotype (Hill *et al.* 2007). This supports reports that Foxp3 is necessary for Treg effector function but not lineage commitment (Lin *et al.* 2007).

The correlation between CD25 and Foxp3 expression in T cells is not uniform. In normal mice around 10% of $Foxp3^+$ Treg cells do not express CD25. Similarly, around 10% of $CD25^+$ T cells do not express Foxp3. It is believed that these $CD4^+CD25^+Foxp3^-$ cells are activated T cells and not Tregs. The correlation of expression of CD25 and Foxp3 in humans is more complicated (Roncador *et al.* 2005). CD25 expression appears to fall into three levels; high, intermediate and low/no expression (Baecher-Allan *et al.* 2001, 2005). Almost all $CD4^+CD25^{hi}$ T cells express Foxp3, whereas variable percentages of $CD4^+CD25^{int}$ T cells express Foxp3, usually at lower levels also. Typically, Foxp3 expression is not found in $CD4^+CD25^{lo/-}$ human cells.

Despite its benefits over CD25 as a marker for Tregs a major drawback of Foxp3 as a marker for Tregs is its intracellular location. This makes it inaccessible for antibody staining of live, unfixed cells. Thus cell sorting, depletion or selection of Tregs based on Foxp3 expression is not possible with live T cells from humans or unmanipulated mice. This means that CD25 is still quite commonly used to identify Tregs, especially in humans. Recent findings from Shimon Sakaguchi's lab (Miyara *et al.* 2009) suggest that three functionally distinct populations of $CD4^+Foxp3^+$ cells exist in humans and that these populations can be distinguished based on their expression levels of CD45RA and CD25. Those that were $CD45RA^+CD25^{int}$ expressed low levels of Foxp3 and were considered resting Tregs.

$CD45RA^{-}CD25^{int}$ cells also expressed low Foxp3 levels and were found to be cytokine secreting but non-suppressive. Finally, a subset of cells expressing $CD45RA^{-}CD25^{hi}$ were found to express high Foxp3 levels and were found to be terminally differentiated activated Tregs. Interestingly, the proportions of these subpopulations were found to vary under physiological and disease conditions.

1.2.5 Mechanisms of suppression mediated by Treg

The mechanisms by which Treg suppress immune responses are not yet fully understood. Tregs have been shown to suppress using many different mechanisms (reviewed in Vignali, Collison and Workman 2008). A table summarising the various markers and molecules expressed by Tregs is given below (Table 1.1). These can be categorised into four functional groups: suppression by secreting inhibitory cytokines, cytolysis of target cells, suppression by metabolic disruption, and suppression by targeting dendritic cells (Figure 1.3). Variations in Treg requirements during different model systems as well as between *in vitro* and *in vivo* requirements have led to an unclear picture for the way in which Tregs suppress. Indeed, in most *in vivo* models detailed information on the interactions leading to Treg mediated suppression are poorly defined. That is, the precise anatomical location of suppression, the target cells suppressed, and the mechanisms of suppression collectively.

1.2.5.1 Inhibitory cytokines

Inhibitory cytokines such as IL-10 and TGFβ have been associated with Treg activity in many studies. These cytokines have also been the focus of much attention for their role in generating Tregs extrathymically (described above). The general immunosuppressive effects of IL-10 and TGFβ are well known and generally undisputed. However, their full role in Treg

Marker / molecule	Purported function	Expression in Treg cells	Expression in other T cells
CD25	Receptor for IL-2, may act as IL-2 sink on Tregs	Expressed by >80% of Tregs	Also expressed on activated non-regulatory T cells
Foxp3	Transcription factor controlling many Treg functions and phenotypic markers	Expressed by all Tregs	Also shown to be expressed in activated T cells (humans only)
CD45RA	Marker of naïve T cells	Demarcates a subpopulation of resting Tregs (>50%)	Also expressed by non-regulatory T cells
IL-10	Immunosuppressive cytokine expressed by some Tregs	Expressed by <50% of Tregs	Also expressed by other $Foxp3^-$ T cells
TGFβ	Immunosuppressive cytokine expressed by some Tregs	Expressed by <50% Tregs	Also expressed by other $Foxp3^-$ T cells
GranzymeB	Cytolytic molecule expressed by some Tregs	Expressed by <50% Tregs	Also expressed by cytolytic $CD8^+$ T cells
CD39/CD73	Ectonucleotidases involved in the generation of extracellular adenosine	Expressed by >50% Tregs	Also expressed on non-regulatory T cells and other cells
CTLA-4	Suppression of DC maturation and/or function	Expressed by >50% Tregs	Also expressed by activated T cells
LAG-3	Suppression of DC maturation and/or function	Expressed by <20% Tregs	Also expressed by activated T cells
GITR	Member of TNF receptor family	Expressed by >80% Tregs	Also expressed by activated T cells

Table1.1 Summary of Treg cell markers and their function
Treg cells have been shown to express a number of different markers and molecules which contribute to their function and/or delineation. A summary of many of these markers/molecules is given in the table above. For each marker/molecule details of the purported function, expression in Tregs cells (under normal circumstances) and expression in other T cells is given.

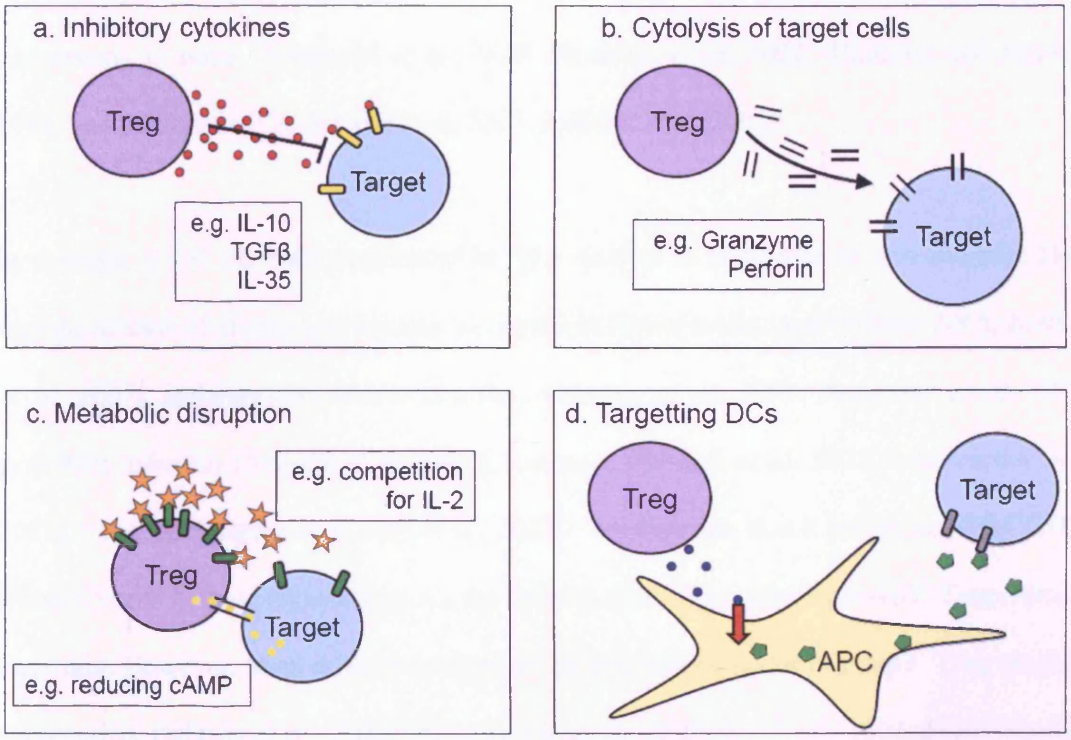


Figure 1.3. Mechanisms of suppression mediated by Treg.
The mechanisms of suppression utilised by Tregs can be divided into four functional groups: suppression by inhibitory cytokines; cytolysis of target cells; suppression by metabolic disruption; and suppression by targeting dendritic cells.

biology in vivo is still unresolved. Experiments in vitro suggest that the roles of soluble suppressive factors, such as IL-10 and TGFβ, in Treg mediated suppression may be limited. Indeed, transwell studies where Tregs physically separated from T effectors were unable to suppress, have led to the popular view that Tregs inhibit via a cell contact dependent mechanism (Takahashi *et al.* 1998, Thornton and Shevach 1998). Furthermore, a number of *in vitro* experiments have specifically found IL-10 and/or TGFβ to be dispensable for Treg suppression in mice (Takahashi *et al.* 1998, Piccirillo *et al.* 2002, Thornton and Shevach 1998) and in humans (Dieckmann *et al.* 2001, Jonuleit *et al.* 2001).

In contrast, IL-10 has been implicated in Treg activity in numerous *in vivo* models. These include models of allergy and asthma (reviewed in Hawrylowicz and O'Garra 2005, Joetham *et al.* 2007), inflammatory bowel disease (Asseman *et al.* 1999, Annacker *et al.* 2003), persistent infection (Belkaid *et al.* 2002), tolerance (Erhardt *et al.* 2007, Schumacher *et al.* 2007), and carcinogenesis (Loser *et al.* 2007). Whether the IL-10 producing $CD4^+CD25^+$ ($Foxp3^+$) cells in these experiments are the same as naturally occurring $Foxp3^+$ Tregs remains uncertain. However, in an attempt to directly test the role of IL-10 in $Foxp3^+$ Treg mediated suppression, Rubtsov *et al.*, (2008) generated mice in which IL-10 was ablated specifically in $Foxp3^+$ Tregs. Interestingly, this did not result in systemic autoimmunity but did enhance pathology in the colon of older mice and in the lungs and skin of mice with induced hypersensitivity. This suggests that IL-10 derived from $Foxp3^+$ Treg is necessary for immune homeostasis but may be restricted to the control of inflammatory responses at environmental interfaces caused by pathogens or environmental insults.

Significant data also exists to suggest that TGFβ produced by Tregs is important *in vivo*. Using a mouse model of colitis it was shown that T cells that cannot respond to TGFβ escape

control by Tregs (Fahlén *et al.* 2005). Similarly, TGFβ deficient Foxp3$^+$ Treg cells could not prevent the development of colitis, demonstrating an essential role for Treg derived TGFβ in inhibiting colitis (Li, Wan and Flavell 2007). The production of TGFβ by Treg was also recently implicated in a model of allergy and asthma (Joetham *et al.* 2007). Furthermore, tumour infiltrating Tregs from patients with head and neck squamous cell carcinoma were found to produce TGFβ (and IL-10), suggesting these molecules are involved in Treg activity during cancer (Strauss *et al.* 2007). Interestingly, an increasing body of evidence is emerging suggesting that membrane bound TGFβ plays a role in Treg mediated suppression (Nakamura, Kitani and Strober 2001, Ostroukhova *et al.* 2006, Xia *et al.* 2007, Green *et al.* 2003). Moreover, this method of suppression is consistent with the observation that Tregs inhibit via cell to cell contact. Taken together, current data suggests that soluble and/or membrane bound TGFβ is important for the function of regulatory T cells.

Recently, another immunosuppressive cytokine, IL-35, was shown to be required for the maximal suppressive activity of Treg in mice (Collison *et al.* 2007). Unlike IL-10 and TGFβ, IL-35 was found to be required for Treg activity both *in vitro* and *in vivo*. IL-35 is formed by the heterodimeric pairing of Ebi3 and IL-12α. The genes for both Ebi3 and IL-12α are preferentially up-regulated in mouse Foxp3$^+$ Treg (Gavin *et al.* 2007), especially Treg that are actively suppressing (Collison *et al.* 2007). Although IL-35 is another addition to the mouse Treg suppression portfolio, its full importance including its target cell type and target cell function remains to be clarified. Furthermore, a recent report by Bardel *et al.*, (2008) showed that human Treg do not express detectable amounts of IL-35, suggesting that it does not play a role in Treg mediated suppression in humans.

1.2.5.2 Suppression by Cytolysis

Typically cytolysis mediated through granzymes is associated with NK cells and cytotoxic $CD8^+$ T cells (reviewed in Lieberman 2003). However, a number of studies have found that granzyme expression is up-regulated in both mouse and human Tregs, suggesting that granzyme mediated cytolysis may be utilised by Tregs (McHugh *et al.* 2002, Grossman *et al.* 2004). Consistent with this, target cell killing by human Treg was shown to be mediated through Granzyme B and perforin (Grossman *et al.* 2004). Treg cytolytic activity has also been demonstrated with mouse Tregs, indeed, mouse Tregs deficient of granzyme B have reduced suppressive activity *in vitro* (Gondek *et al.* 2005). Subsequent studies supporting this finding showed that Treg can kill B cells in a granzyme B dependent and partially perforin dependent manner (Zhao *et al.* 2006). Also, a more recent study by Cao *et al.*, (2007) showed that Treg utilize granzyme B and perforin mediated killing to suppress the ability of NK cells and CTLs to clear tumours. Other cytolytic mechanisms that have been associated with Treg suppressive activity include apoptosis via the TRAIL-DR5 pathway (Ren *et al.* 2007); galectin-1 induced T cell apoptosis (Garín *et al.* 2007); and lysis of antigen-presenting B cells by Fas-FasL interaction (Janssens *et al.* 2003).

1.2.5.3 Metabolic disruption

It has long been proposed that the expression of CD25 (IL-2 receptor alpha chain) by Tregs provides them with a competitive advantage for IL-2 consumption over naïve T cells which only express CD25 after TCR stimulation (Thornton and Shevach 1998). Evidence exists to support this theory which may ultimately result in cytokine deprivation-mediated apoptosis of effector T cells (de la Rosa *et al.* 2004, Barthlott *et al.* 2005, Pandiyan *et al.* 2007). However, there is also compelling evidence (Fontenot *et al.* 2005b), including evidence using human

Treg (Oberle *et al*, 2007) suggesting that this is not a true mechanism of Treg suppression. In particular, Fontenot *et al.*, (2005b) demonstrated that murine Tregs deficient of the IL-2 receptor alpha chain (CD25) were fully able to suppress *in vitro*. Clearly, more work is needed to determine whether CD25 expression and IL-2 deprivation contribute to Treg mediated suppression *in vivo*.

An interesting advancement in the Treg field has been the discovery that Tregs can inhibit target cells via the modulation of intracellular cAMP. An increase in cAMP levels in lymphocytes has been associated with inhibition of proliferation and differentiation for many years (Bodor *et al.* 2001, Kammer 1988). The recent evidence implicates Tregs in two different mechanisms of altering cAMP in target cells. Bopp *et al.*, (2007) found that Tregs, which express high levels of cAMP, can act directly on target cells by delivering cAMP to activated target cells via gap junctions. Whereas three papers published in close succession collectively demonstrated an indirect mechanism whereby Tregs generated extracellular adenosine via the surface expression of the ectonucleotidases CD39 and CD73 (Kobie *et al.* 2006, Deaglio *et al.* 2007, Borsellino *et al.* 2007). In this alternative mechanism, subsequent binding of adenosine to the adenosine A2A receptor on target cells increased intracellular cAMP and lead to the suppression of target cell proliferation and cytokine production.

1.2.5.4 Suppression by targeting dendritic cells

As well as targeting effector cells directly, it has been proposed that Tregs may suppress dendritic cells (DC) which are required for the activation of T cells. Studies using intravital microscopy suggest that Tregs directly interact with DCs (Tang *et al.* 2006) and are also able to reduce the period of interaction between Teff and DCs (Tadokoro *et al.* 2006). The use of

CTLA-4 specific antibodies or CTLA-$4^{-/-}$ deficient Tregs suggests that suppressive interactions between Treg and DCs require CTLA-4 (Oderup *et al.* 2006, Serra *et al.* 2003) which is constitutively expressed by Treg and required for maximal Treg activity *in vivo* (Read, Malmström and Powrie 2000). Some data suggests these CTLA-4 dependant Treg effects on DCs may result in the induction of IDO mediated catabolism of tryptophan (Fallarino *et al.* 2003), a mechanism thought to be involved in suppressing T cell responses and promoting tolerance (Mellor and Munn 2004). Several other papers, including studies with human cells, have reported that Tregs can influence the maturation or function of DCs (Kryczek *et al.* 2006, Lewkowich *et al.* 2005, Houot *et al.* 2006, Misra *et al.* 2004), for example, the downmodulation of costimulatory molecules such as CD80 and CD86 on DCs (Cederbom, Hall and Ivars 2000).

Finally, another molecule that has been implicated in modulating DC function by Tregs is lymphocyte activation gene-3 (LAG-3/CD223). LAG-3 is a CD4 homologue which binds to MHC class II with higher affinity than CD4. LAG-3 is expressed by Tregs and appears to be required for their maximal activity *in vitro* and *in vivo* (Huang *et al.* 2004, Workman and Vignali 2005). A recent study by Liang *et al.*, (Liang *et al.* 2008) showed that Treg inhibit DC maturation via LAG-3 interaction with MHC class II expressed on DCs.

Our current understanding of the mechanisms of Treg suppression suggests that Tregs are capable of inhibiting at multiple levels. Tregs have been shown to inhibit the activation, proliferation, differentiation and effector function of target cells. Furthermore Tregs appear to utilise a plethora of molecular mechanisms for inhibition, for example, cell-cell contact dependant mechanisms, secretion of soluble factors, and competition for cytokines. It is probable that Tregs utilise alternative inhibitory pathways depending on the nature, context,

and extent of the response needing to be suppressed. Crucial to our further understanding of Tregs will be the elucidation of which mechanisms are used when.

1.2.6 Peripheral homeostasis, activation and trafficking of Tregs

1.2.6.1 Activation

Numerous accessory molecules appear to control the peripheral homeostasis, activation and trafficking of Tregs (reviewed in Liston and Rudensky 2007, Wei, Kryczek and Zou 2006). Most of these molecules have also been associated with the regulation of normal T cells. In conventional T cells, CD28 provides a costimulatory signal for IL-2 production, cell survival and proliferation (reviewed in Salomon and Bluestone 2001). Experiments using CD28 deficient mice showed that CD28 signalling is also required for the generation of Tregs in the thymus (discussed above) as well as the peripheral survival of Tregs (Salomon *et al.* 2000). It appears however that CD28 is not completely necessary for Treg function as Tregs from CD28 deficient mice were found to be equally suppressive as CD28 sufficient Tregs *in vitro* (Takahashi *et al.* 2000). As mentioned above, CTLA-4 may also be important for Treg activity. In normal T cells CTLA-4 is up-regulated upon activation and can provide a negative signal which appears to control the induction of anergy (Greenwald *et al.* 2001). However, in Tregs CTLA-4 is constitutively expressed and appears to be important for the maximal suppressive capacity (Read *et al.* 2000, Takahashi *et al.* 2000). Two independent studies showed that blockade of CTLA-4 abrogated Treg function in *in vitro* suppression assays and in the prevention of colitis *in vivo* (Read *et al.* 2000, Takahashi *et al.* 2000). These studies suggested that CTLA-4 engagement is involved in Treg-mediated suppression. Yet, on the contrary, a more recent study showed that CTLA-4 cannot be wholly necessary as CTLA-4 deficient Tregs are still able to suppress *in vitro* (Kataoka *et al.* 2005). GITR

(glucocorticoid-induced tumour necrosis factor receptor family-related gene) is another molecule thought to be involved in Treg activation. Tregs constitutively express high levels of GITR, whereas high levels of GITR are only found on non regulatory T cells, B cells and macrophage following activation (Shimizu *et al.* 2002, McHugh *et al.* 2002). Crosslinking GITR abrogates Treg mediated suppression *in vitro* and anti-GITR treatment can elicit autoimmune disease in mice (Shimizu *et al.* 2002).

1.2.6.2 Proliferation

The cytokines IL-2 and TGFβ have been associated with Treg proliferation and survival. IL-2 is essential for Treg growth and survival and may also be involved in Treg function (Furtado *et al.* 2002). Similarly TGFβ has been found to stimulate human Tregs to proliferate (Yamagiwa *et al.* 2001). Stimulation of Tregs with a high concentration of lipopolysaccaride (LPS) through the toll like receptor (TLR) 4 can induce them to proliferate and prolong their survival (Caramalho *et al.* 2003).

1.2.6.3 Trafficking

It appears that Tregs have unique patterns of expression of adhesion molecules, chemokine receptors and homing receptors which modulate their retention and migration (Wei *et al.* 2006). In particular this may be important for the preferential distribution of Tregs to sites where regulation of immune responses is needed. For example, it has been shown that Tregs infiltrating ovarian carcinomas express the chemokine receptor CCR4 and migrate towards CCL22 which is found within the tumour microenvironment. In addition to chemokine receptor expression, integrin molecules may be important for Treg migration *in vivo*. For example, the integrin αEβ7 (CD103) which may identify a more suppressive subset of Tregs

(Lehmann *et al.* 2002, Banz *et al.* 2003), appears to facilitate the homing/retention of Tregs to inflamed tissue (Suffia *et al.* 2005, Huehn *et al.* 2004).

1.2.7 TCR repertoire of Treg

As mentioned above, it is commonly believed that Treg predominantly recognise self antigens. These assumptions come primarily from the autoimmunity observed when Tregs are abrogated by either day 3 thymectomy (Nishizuka and Sakakura 1969), Treg deletion (Kim *et al.* 2007) or mutations in Foxp3 (Wildin *et al.* 2001). Furthermore, experiments with double transgenic mice for a TCR and its ligand show that a large proportion of TCR transgenic thymocytes become Tregs and that this requires TCR-MHC interactions of relatively high affinity (Jordan *et al.* 2001, Kawahata *et al.* 2002, Apostolou *et al.* 2002). However, studies showing that Tregs are also able to recognise foreign antigens such as those of the protozoa Leishmania (Suffia *et al.* 2006, Belkaid *et al.* 2002), bacteria (Cong *et al.* 2002), transplanted organs/grafts (Joffre *et al.* 2004, Koenen, Fasse and Joosten 2005) and foetal alloantigen (Aluvihare and Betz 2006) suggest Treg have a much broader diversity. The key aspects of the Treg TCR repertoire that have attracted the most scrutiny are: a) do Tregs have a similar level of diversity to conventional T cells; b) do Tregs predominantly or even preferentially recognise self antigens; c) to what extent do the TCR repertoires of Treg and Tconv overlap?

1.2.7.1 Diversity of the Treg TCR repertoire

A number of studies have tried to elucidate whether Treg have as broad a diversity of TCR specificity as conventional T cells (Tconv). Preliminary analyses to compare the levels of diversity between Tconv and Treg simply determined the usage of TCR variable segments Vβ (and/or Vα) on Tconv and Treg cells. A number of groups showed the two cell

populations have similarly diverse usage of the TCR subsets in both mice (Takahashi *et al.* 1998, Romagnoli, Hudrisier and van Meerwijk 2002, Pacholczyk, Kraj and Ignatowicz 2002) and humans (Kasow *et al.* 2004, Fujishima *et al.* 2005). More detailed analysis of the CDR3 lengths, a process called spectratyping, also suggested a similar level of diversity in Tregs to Tconv (Kasow *et al.* 2004, Fujishima *et al.* 2005). Both approaches also suggested there was no particular skewing of either repertoire towards a Vβ subset or clone. These methods do not however determine the identity of individual TCR sequences and thus to compare the true TCR usage of T cell populations, sequence analysis of many individual TCR clones must be performed by clonotyping.

This technique was carried out by Hsieh *et al.* (2004) to compare the TCR repertoires of Treg and Tconv. They sequenced the Vα2 region of TCRα chains associated with a transgenic (fixed) TCRβ chain. Using this approach they could be confident that identification of the same TCRα sequences assured identification of identical TCRs. These studies found that the $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ Tconv repertoires were comparably diverse and displayed few predominant clones. The diversity of the Treg TCR repertoire was also measured using clonotyping by Pacholczyk *et al.* (Pacholczyk *et al.* 2006). This report indicated that the Treg TCR repertoire was actually more diverse than that of naïve T cells.

1.2.7.2 Recognition of self

In an attempt to address the issue of whether Treg predominantly recognise self antigen, Hsieh *et al.*, (Hsieh *et al.* 2004) uncoupled the normal anergic state of Tregs *in vitro* by retroviral transduction of non-regulatory T cells with the Treg TCR sequences. With this approach they found that these Treg TCR sequences, and not Tconv TCR sequences, induced

rapid T cell expansion in lymphopenic hosts. This observation suggests that the Treg population contains a higher number of TCRs that are self-reactive compared to $CD4^+CD25^-$ Tconv. Similar results were obtained when these cells were analysed by limiting dilutions of Treg and Tconv (Romagnoli *et al.* 2002). When stimulated with allogeneic (non-self) or syngeneic (self) APCs, the Treg pool contained a significantly higher frequency of self reactive T cells than Tconv. A more recent study made T cell hybridomas derived from $TCR^{mini}Ep$ mice to investigate the frequency of TCRs directed to self and non-self antigens in naïve T and Treg cells (Pacholczyk *et al.* 2007). The $TCR^{mini}Ep$ mice were obtained by crossing transgenic mice expressing the β chains of TCRs recognising the self peptide Eα52-68 (Ep) (Pacholczyk *et al.* 2006) with mice expressing a single MHC-peptide complex (A^bEp) (Ignatowicz, Kappler and Marrack 1996). In $TCR^{mini}Ep$ mice, the TCRs are therefore selected in the thymus by the expression of a single MHC-peptide complex (A^bEp) resulting in a repertoire that is tolerant to A^bEp and lacks tolerance to natural, endogenous processed peptides. Thus, the A^bEp antigen is perceived as self and natural antigens are perceived as non-self. Notably, this approach showed that high affinity, self reactive TCRs may indeed be more prevalent in the Treg population compared to the Tconv population but are in fact still very low in number in both the Treg and Tconv pools. Indeed, both populations were also found to recognise foreign MHC-peptide complexes as often.

1.2.7.3 Overlap between the Treg and Tconv repertoires

Another key finding that came from Hsieh and colleagues' (2004) study was that the Treg and Tconv TCR repertoires were generally distinct but overlapping by around 10-25%. A much higher level of overlap between the Treg and Tconv repertoires was found in a study using the LTD ("limited") mouse strain which contains a greatly limited TCR repertoire

(Wong *et al.* 2007b). The LTD mouse carries an OT-1 TCRβ transgene and a TCRα minilocus comprising of a single V and two J gene segments, thus TCR variability is vastly reduced in these mice but allows for efficient sequence comparisons (Correia-Neves *et al.* 2001). The results from the comparison of Treg and Tconv cells in LTD mice suggest that rarer TCR sequences may comprise overlapping sequences and that if more individual TCR sequences were analysed in clonotyping studies a greater degree of overlap may be calculated. However, the restriction of the TCR repertoire in LTD mice makes the interpretation of this data difficult. A similar study of human peripheral blood T cells supported the findings in mice (Fazilleau *et al.* 2007). Of 2537 analysed TCR sequences, 615 (24%) were found in both the $CD4^+CD25^{high}$ Treg and $CD4^+CD25^-$ Tconv pools.

1.3. Role for Tregs in tumour development

Given the clear implication for Treg cells in orchestrating self tolerance by preventing autoreactive T cells and the fact that tumours are derived from host cells, it is not surprising that Tregs have been the subject of much scrutiny by cancer immunologists. It is perceivable that T cells reactive to the (self-derived) tumour tissue may be considered as potentially harmful autoreactive T cells and therefore inhibited by the actions of Tregs. Thus, when a tumour emerges, it may be that the balance between autoimmunity and tolerance favours the outgrowth of the tumour by preventing anti-tumour immune responses. Understanding the mechanisms involved in controlling this balance is the goal for these scientists and could aid the design of therapeutics for cancer (and autoimmune diseases).

1.3.1 Tumour induced suppressor T cells

A number of studies performed in the lab of Robert North during the 1980s provided much of the first compelling evidence for the involvement of suppressive T cells in limiting anti-tumour immunity. For example, using the Meth A fibrosarcoma cell line, North's group found that tumour development could induce effector T cell activity capable of tumour rejection if transferred to recipient T cell deficient mice (Berendt and North 1980, North and Bursuker 1984). However, growth of the tumour was also associated with a loss of effector T cell activity and the gradual development of suppressive T cell activity which prevents tumour rejection (Berendt and North 1980, North and Bursuker 1984). Depletion of this suppressive T cell population by cyclophosphamide allowed tumour rejection of established tumours (North 1982). Characterisation of these suppressor T cells found them to be $CD4^+CD8^-$ and showed that specific depletion of $CD4^+$ cells resulted in CD8 T cell mediated tumour rejection (Awwad and North 1988). This group also showed similar findings using the P815 mastocytoma cell line (Dye and North 1981, Mills and North 1983). It is likely that the $CD4^+$ suppressor T cells North described are the same population of cells now more commonly known as $CD4^+$ regulatory T cells (Treg).

1.3.2 Infiltration of Tregs into tumours derived from transplantable tumour cell lines

It has been observed that numerous transplantable tumour cell lines induce the accumulation of tumour infiltrating Tregs. Needham *et al.* (2006) used tumour cells derived from the peritoneal cavity of C57BL/6J mice injected with asbestos fibres. The percentage of Tregs accumulating in these mesotheliomas increased as the tumour developed. Up to 60% of the CD4s infiltrating the tumours were found to be $CD4^+CD25^+$ Tregs. Similarly, Ghiringhelli *et al.* (Ghiringhelli *et al.* 2005) found that the B16F10 melanoma cell line induced a high

percentage and overall number of Tregs in draining lymph nodes and tumours of injected mice. Staining of the T cells with BrdU showed that the Tregs have a high proliferative status at these sites suggesting that this is how Treg accumulate in tumour bearing mice. Other transplantable tumour cell lines that have been associated with an accumulation of Treg include the colon carcinomas PROb (Ghiringhelli *et al.* 2004), and CT26 (Zhou *et al.* 2007), and the carcinogen induced fibrosarcoma Meth A (Ko *et al.* 2005).

1.3.3 $CD4^+CD25^+$ Tregs suppress anti-tumour immunity in mice

Following the identification of CD25 as a marker for $CD4^+$ Tregs numerous studies have been carried out to try and elucidate whether depletion of these cells enhances tumour immunity. Indeed, Onizuka *et al.* (1999) demonstrated that depletion of $CD25^+$ cells using depleting monoclonal antibodies improved the rejection of a range of tumour cell lines. Similar to the early findings of Robert North's group Shimizu *et al.* (1999) found that this rejection was mediated by $CD8^+$ CTLs but also NK like cells. Findings in support of this were later reported by a number of other groups (Sutmuller *et al.* 2001, Chen *et al.* 2005). It has also been found that $CD4^+$ T cells may be involved in tumour rejection and also in establishing long term anti-tumour immunity in mice that rejected tumours as a consequence of $CD25^+$ cell depletion (Jones *et al.* 2002, Casares *et al.* 2003). In addition, Golgher *et al.* (2002) found that long term anti-tumour immunity revealed by Treg depletion was directed towards shared tumour antigens and resulted in rejection of tumours from different origins.

The majority of Treg/tumour studies show that it is possible to induce the rejection of tumours by depleting $CD25^+$ cells prior to tumour inoculation (Onizuka *et al.* 1999, Shimizu *et al.* 1999, Sutmuller *et al.* 2001, Jones *et al.* 2002). However, the rejection of established

tumours by manipulation of Tregs, which is more clinically relevant, has proven less successful. Ghiringhelli *et al.* (2004) showed that depletion of Treg using cyclophosphamide induced the partial regression of established PROb tumours in rats. Nevertheless, complete clearance of the established tumour required a combination of Treg depletion and vaccination. Adoptive T cell transfer, whereby anti-tumour T cells are expanded *ex vivo* and re-infused into the patient, represents an alternative clinical strategy for tumour immunotherapy. Interestingly, Antony *et al.* (2005) showed that the cotransfer of $CD4^+$ Th cells with anti-tumour CD8 T cells combined with vaccination can improve rejection of established tumours in mice. However, this rejection was only possible when the transferred $CD4^+$ T cell population was depleted of $CD4^+CD25^+$ Tregs. These results suggest that Treg depletion may also be necessary for successful adoptive T cell therapy.

1.3.4 Tregs accumulate in MCA induced tumours and prevent tumour rejection

Recently work from our laboratory has suggested that Tregs play an important role in the development of MCA induced tumours (Betts *et al.* 2007). MCA induced fibrosarcomas were found to contain a high percentage of $CD4^+CD25^+Foxp3^+$ Treg. On average, approximately 50% of the $CD4^+$ tumour infiltrating lymphocytes (TILs) were Tregs. This accumulation was also reflected in the tumour draining lymph node, which had significantly more Tregs compared to non tumour draining lymph nodes. Furthermore, these Tregs were found to be in close proximity to $CD8^+$ T cells and $CD4^+Foxp3^-$ conventional T cells within the tumour. Importantly, depletion of Tregs by administration of anti-CD25 depleting antibodies prior to MCA tumour induction resulted in a significant reduction in tumour occurrence. These data provide strong evidence that Tregs prevent effective tumour immunity during MCA tumour development.

1.3.5 Do Treg influence immunosurveillance in human tumours?

As mentioned above, it is apparent that any anti-tumour immune responses that exist in humans have generally become non effective in clearing tumours in patients with established tumours. There is evidence from human cancer patients to suggest that Tregs are involved in suppressing these anti-tumour immune responses (reviewed in Betts *et al.* 2006). The evidence supporting this predominantly comes from observational studies comparing the frequency of Tregs in cancer patients and in healthy controls. The majority of these studies show that the frequency of Treg in the PBMC of cancer patients is significantly elevated compared to healthy controls. Furthermore, some studies suggest that the frequency of Treg in tumour draining lymph nodes is higher than control lymph nodes from non-cancer patients. However, this data is limited because of the difficulty to obtain lymph nodes from healthy controls for comparison. Tregs have also been identified amongst the tumour infiltrating lymphocytes of cancer patients. An indication of the role for these cells in tumour progression is the increased frequency of Tregs observed in some tumours at later stages of disease. For example, Curiel *et al.* (2004) found that stage I ovarian tumours contained less Tregs compared to tumours in stages II to IV. Moreover, they found that there was a significant correlation between tumour Treg cell content and patient survival.

1.4. Summary

The studies discussed above collectively provide evidence that an anti-tumour immunosurveillance process exists in mice and in humans. However, it is also apparent that humans and mice can still develop cancer despite a plethora of anti-tumour mechanisms. Studies also show that a suppressive T cell subset called regulatory T cells (Treg) play a role

in normal homeostasis and in regulating immune responses. Treg cells are a cell type which have a role in many disease models and utilise numerous mechanisms of suppression. The data discussed above also suggest that effective tumour immunity may be inhibited by the action of Treg cells. Moreover, that the accumulation of Treg cells at tumour sites may be a mechanism induced by developing tumours in order to escape deletion by the host immune system.

Phenotypic and functional characterisation of tumour infiltrating T cells.

For the rational design of anti-tumour therapies, is important to understand the network of biological interactions within the tumour microenvironment. A comprehension of the phenotypic and functional characteristics of TILs will allow for the clinical efficacy of current strategies to be improved and the identification of new potential immuno-therapeutic targets. The experiments described in this thesis have utilised a mouse tumour model to investigate Treg cells in tumours. Firstly, a phenotypic and functional characterisation of tumour infiltrating T cells was carried out (Chapter 3). Specifically, four key questions were addressed:

- Do $CD4^+$ and $CD8^+$ cells control tumour development following Treg depletion?
- Do the ratios of Tconv:Treg and CD8:Treg alter in the tumour compared to lymphoid tissue?
- Do tumour infiltrating Treg cells display a phenotype associated with immunosuppression?
- Do tumour infiltrating Tconv and $CD8^+$ T cells express activation markers?

How do Tregs accumulate in tumours?

It has previously been shown that Treg cells accumulate in MCA induced tumours and that these Treg cells can prevent anti-tumour responses. It is therefore important to establish the mechanism(s) of Treg cell accumulation in tumours. Firstly, this may lead to identifying potential therapeutic interventions for the prevention of Treg accumulation. However, an understanding of Treg cell accumulation in tumours may also assist in the rational design of tumour vaccines. The mechanisms leading to accumulation of Treg cells in tumours were investigated (Chapter 4) by addressing the following questions:

- Does conversion of conventional T cells into regulatory T cells account for Treg cell enrichment in tumours?
- Does the proliferation of $CD4^+$ T cells preferentially promote the accumulation of Treg cells in tumours?
- Does the level of cell death within Treg and Tconv cells favour Treg accumulation in tumours?

Chapter 2 – Materials and methods

2.1 Mice

Unless stated otherwise, C57BL/6 mice (Charles River or bred in house), aged 8 to 15 weeks, were injected with carcinogen (see below). Where indicated, to efficiently discriminate between Treg and non Treg cells (Tconv) mice expressing GFP under the control of the Foxp3 promoter (Foxp3_GFP) were used (Fontenot *et al.* 2005c). Where indicated, the spleens and inguinal lymph nodes of naive and aged (30-60 weeks) mice were analysed for comparison. Mice were isolator-bred and housed in filter-top cages for the duration of the experiment.

2.2 Tumour induction

Mice were injected subcutaneously in the left hind leg with 400μg of 3-methylcholanthrene (MCA; Sigma-Aldrich) in 100μl of olive oil under general anaesthetic. Mice were subsequently monitored for tumour development at least once a week for up to 1 year. Tumour bearing mice were culled when their tumours were between 1cm and 2cm in diameter or causing apparent discomfort.

2.3 Depletion of immune cells

Hybridomas secreting CD25- (PC61), CD4- (YTS 191.1.2, YTA 3.1.2), CD8- (YTS 169.4.2.1, YTS 156.7.7), and rat anti-*E. coli beta*-galactosidase- (GL113) specific mAbs have been described previously (Lowenthal *et al.* 1985, Cobbold *et al.* 1984, Qin *et al.* 1987). Depleting antibodies, produced and purified in house as previously described (Jones *et al.*

2002), were injected intraperitoneally in 100 to 500µl of sterile PBS at the amounts and times stated below (Figure 2.1). For Treg depletion, anti-CD25 Abs (clone PC61, 500µg) were given on days -3 and -1 before MCA injection (day 0). Alternatively, rat IgG isotype control (GL113, 500µg) was given as a control. $CD4^+$ cells were depleted by injecting a combination of two Abs (clones YTS-191 and YTA-3, 100µg of each) twice with a two week interval (days 50 and 64). Similarly, $CD8^+$ cells were depleted using a combination of two Abs (clones YTS-156 and YTS-196, 100µg of each) twice with a two week interval (days 50 and 64). Alternatively, as a control, rat IgG (GL113) was injected.

2.4 Dissection of tissues

Spleen and inguinal lymph nodes were removed from tumour bearing and naïve mice. The inguinal lymph node from the left (tumour) side of each tumour bearing mouse was removed for analysis of a tumour draining lymph node. The inguinal lymph node from the right side of the same mouse was taken as a non-draining lymph node. For naïve (control) mice the two inguinal lymph nodes (left and right) were pooled. Tumours were resected from tumour bearing mice avoiding normal muscle and local lymph nodes. In particular, the popliteal lymph node was separated from the tumour mass. However, this lymph node was not used for analysis as the draining popliteal lymph node and in particular the non-draining popliteal lymph node was relatively small.

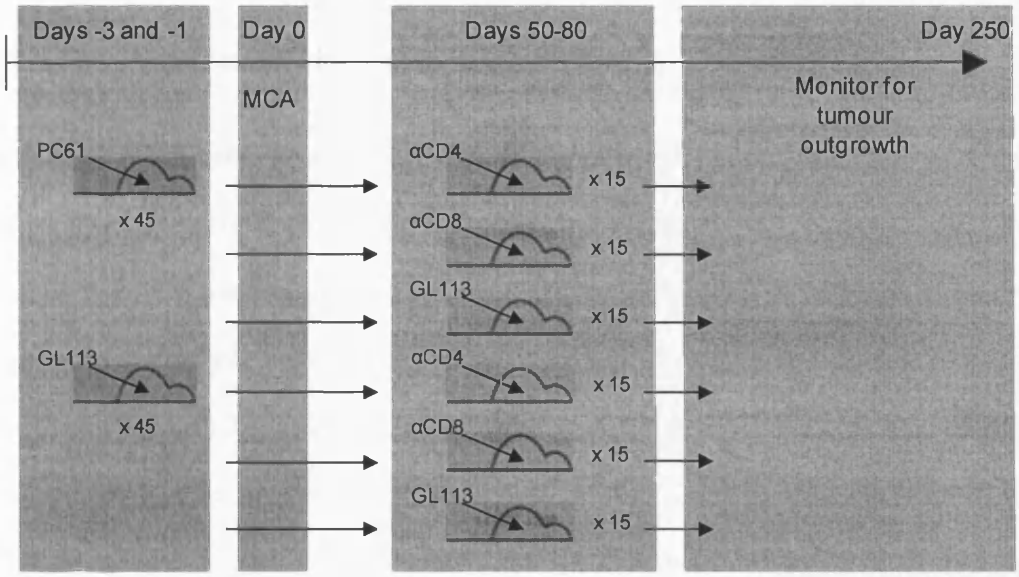


Figure 2.1. Injection program for studying immune factors that control tumour development after Treg cell depletion.
C57BL/6 mice were given Treg cell depleting antibody (PC61, 500µg) or a control antibody (GL113, 500µg) 3 days and 1 day prior to injection with 400µg of MCA. During tumour development (days 50-80) $CD4^+$ or $CD8^+$ cells were depleted with depleting antibodies. Mice were monitored daily for tumour outgrowth.

2.5 Flow cytometry

2.5.1 Preparation of single cell suspensions from tissues

Spleen and lymph nodes were homogenised in a multiwell plate using the back of a syringe plunger. Tumours were diced into small pieces (almost to pulp) then forced through a 70μM cell strainer (BD Biosciences) using the back of a syringe plunger. Homogenised tissue was resuspended in FACS buffer (PBS containing 5mM EDTA and 2% FCS) and passed through a 70μM cell strainer. The resulting single cell suspension was washed once by pelleting the cells by centrifugation at 1,500rpm (~450 x G) for 5 minutes and resuspending in FACS buffer. Before antibody staining, spleen and tumour cell pellets were first subjected to lysis of red blood cells by resuspending in 5ml of RBC lysis buffer (BioLegend) for 90 seconds at room temperature.

2.5.2 Antibody staining

For antibody staining, spleen and lymph node single cell suspensions were plated out into 96 well, round bottom plates at ~0.5 – 1 million cells/well. Enumeration of cells / lymphocytes in the tumour cell suspension is problematic due to the high amounts of cell debris and connective tissue in the homogenised tumour. Therefore, for FACS staining the tumour was simply split into between 1 to 10 wells depending on the size of the tumour cell pellet.

2.5.3 Activation for intracellular cytokine analysis

For analysis of intracellular cytokines by flow cytometry, single cell suspensions (~0.5 to 1.0 million cells per well) were stimulated in a 24 well plate in complete RPMI (RPMI [Invitrogen] supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 50ug/ml

penicillin streptomycin and 10% foetal calf serum) containing 20 nM phorbol myristate acetate (PMA; Sigma-Aldrich) and 1μg/ml ionomycin (Sigma-Aldrich). Cells were incubated at 37°C for a total of 4 hours. After 1 hour 1μl/ml of GolgiStop (containing monensin; BD Bioisciences) was added to each well.

2.5.4 Antibodies and cell staining

Anti-mouse antibodies for flow cytometric analysis are described in the table below. The correct dilutions of the antibodies to give efficient staining were established by titration of the antibodies by different members of our lab group. To eliminate dead cells from flow cytometric analysis a fixable dead cell staining kit (LIVE/DEAD Aqua; Invitrogen) was used prior to antibody staining. For this, cells were washed twice in PBS and 3 to 6μl of diluted (1:10; in PBS) live/dead aqua was added directly to the cell pellet. Cells were left to stain at room temperature for 15 minutes in the dark then washed twice in FACS buffer. For staining of surface markers, 25μl to 50μl of diluted (in FACS buffer) antibody were added to the cells and incubated for 10 to 15 minutes at 4°C in the dark. To remove unbound antibodies cells were washed three times in FACS buffer. For biotinylated antibodies, cells were additionally incubated for 10 to 15 minutes in 25 to 50μl of conjugated streptavidin (SA) diluted to 0.8μg/ml in FACS buffer and washed a further three times in FACS buffer to remove unbound SA. To stain for intracellular antigens cells were firstly fixed and permeabilised by incubating for between 40 minutes and 16 hours in Fixation/Permeabilization buffer (eBioscience). Subsequent washes and incubations were performed using Permeabilization Buffer (eBioscience). Cells were washed once then Fc receptors were blocked for 10 minutes at 4°C in the dark using anti-CD16/32 antibodies diluted to 5μg/ml. Antibodies to intracellular antigens were added to the cells and left for 30 minutes at 4°C in the dark. Cells

Antigen	Conjugate	Clone	Isotype	Company	Final concentration
Surface					
CD3	PeCy5	145-2C11	Hamster IgG	eBioscience	2 µg/ml
CD4	FITC	RM4.5	Rat IgG2a	eBioscience	5 µg/ml
CD4	Alexafluor647	RM4.5	Rat IgG2a	Caltag	2 µg/ml
CD4	Pacific Blue	RM4.5	RatIgG2a	BD	2 µg/ml
CD8	PerCpCy5.5	53-6.7	Rat IgG2a	eBioscience	2 µg/ml
CD25	Alexafluor488	PC-61.5	Rat IgG1	eBioscience	10 µg/ml
CD25	Pe	PC-61.5	Rat IgG1	eBioscience	4 µg/ml
CD39	Pe	24DMS1	Rat IgG2b	eBioscience	2 µg/ml
CD44	FITC	IM7	Rat IgG2b	BD	5 µg/ml
CD44	APC	IM7	Rat IgG2b	BD	2 µg/ml
CD62L	FITC	MEL-14	Rat IgG2a	Caltag	10 µg/ml
CD73	Biotin	TY/11.8	Rat IgG1	eBioscience	5 µg/ml
CD103	FITC	2E7	Hamster IgG	eBioscience	5 µg/ml
CTLA-4	Pe	UC10-4F10-11	Hamster IgG	BD	2 µg/ml
ICOS	Pe	15F9	Hamster IgG	eBioscience	2 µg/ml
ICOS	Biotin	15F9	Hamster IgG	eBioscience	5 µg/ml
GITR	Pe	DTA-1	Rat IgG2b	eBioscience	2 µg/ml
Intracellular					
Bcl-2	Pe	3F11	Hamster IgG	BD	0.3 µg/ml
Foxp3	Pe	FJK-16s	Rat IgG2a	eBioscience	1 µg/ml
Foxp3	PeCy7	FJK-16s	Rat IgG2a	eBioscience	1 µg/ml
IFNγ	FITC	XMG1.2	Rat IgG1	eBioscience	5 µg/ml
IFNγ	APC	XMG1.2	Rat IgG1	BD	2 µg/ml
IL-2	Pe	JES6-5H4	Rat IgG2b	eBioscience	2 µg/ml
IL-4	Pe	11B11	Rat IgG1	eBioscience	2 µg/ml
IL-10	Pe	JES5-16E3	Rat IgG2b	eBioscience	2 µg/ml
IL-10	APC	JES5-16E3	Rat IgG2b	eBioscience	2 µg/ml
IL-17	FITC	TC11-18H10.1	Rat IgG1	Abcam	5 µg/ml
Ki67	FITC	B56	Mouse IgG1	BD	2 µg/ml
TNFα	FITC	MP6-XT22	Rat IgG1	eBioscience	5 µg/ml
Isotype controls					
Isotype	**Conjugate**			**Company**	
Hamster IgG	FITC	-	-	BD	-
Hamster IgG	Pe	-	-	eBioscience	-
Hamster IgG	Biotin	-	-	eBioscience	-
Mouse IgG1	FITC	-	-	BD	-
Rat IgG1	Pe	-	-	eBioscience	-
Rat IgG2a	FITC	-	-	eBioscience	-
Rat IgG2b	Biotin	-	-	eBioscience	-
Rat IgG2b	Pe	-	-	eBioscience	-

were washed three times and resuspended in FACS fix (FACS buffer containing ~0.1% formaldehyde). Where indicated 0.25μg (5μl) of 7-AAD (7-aminoactinomycin D; BD) was added to unfixed cells 15 minutes before flow cytometric analysis.

2.5.5 Flow cytometry analysis

For flow cytometric analysis, samples were acquired using either a FACSCalibur or FACS Canto II flow cytometer (BD Biosciences) and were analysed using Summit v4.3 software (Dako) or FACSDiva software (BD Biosciences).

2.5.6 Gating strategy for identification of $CD8^+$, Tconv and Treg cell populations

To characterise the T lymphocyte populations in tumour bearing mice extensive flow cytometric analysis was performed on samples from the spleens, inguinal lymph nodes and tumours of tumour bearing mice. For analysis of each sample the same gating strategy was adopted. Firstly, the lymphocyte population was gated based on typical lymphocyte forward scatter and side scatter patterns (Figure 2.2 step A). Next, dead cells were gated out as these can bind antibodies non-specifically. A fixable dye (Live/Dead Aqua, Invitrogen) was used for this purpose. Cells with compromised membranes readily stain with the dye and therefore have higher fluorescence intensity than cells with intact cell membranes (Figure 2.2 step B). The $CD4^+$ and $CD8^+$ T cell populations (previously shown to be $CD3^+$, Appendix Figure A1) were gated from the live lymphocyte population based on fluorescent intensity levels from CD4 and CD8 specific antibody staining (Figure 2.2 step C). Based on fluorescent intensity from Foxp3 specific antibody staining, the $CD4^+$ population was subsequently divided into $CD4^+Foxp3^-$ conventional T cells (Tconv) and $CD4^+Foxp3^+$ regulatory T cells (Treg; Figure

2.2 step D). The relative proportions of $CD8^+$, Tconv and Treg cells were determined as shown in the example gating strategy and population hierarchy (Figures 2.2 and 2.3).

2.6 T cell receptor (TCR) clonotyping

Figure 2.4 summarises the clonotyping process which was performed on spleen, non-draining lymph node, draining lymph node and tumour samples from MCA-tumour bearing mice.

2.6.1 Cell sorting

Single cell suspensions were sorted using a MoFlo cell sorter (Dako cytomation) operated by a core facility (Cental Biotechnology Services; CBS) technician. For mouse 1, lymphocytes from spleen and tumour were sorted into two populations based on CD4 and CD25 expression. $CD4^+CD25^-$ cells were sorted as conventional T cells (Tconv). Whereas $CD4^+CD25^+$ cells were sorted as regulatory T cells (Treg.) For subsequent experiments (mouse 2, 3 and 4) cells from spleen, inguinal lymph nodes, and tumour were sorted into Tconv and Treg based on GFP (Foxp3) expression. Tconv were in the $CD4^+GFP^-$ gate. Treg were $CD4^+GFP^+$. Cells were sorted into RNAse free tubes.

2.6.2 RNA extraction

Sorted cells were pelleted by centrifugation at 300 x G for 5 minutes. RNA was extracted from pelleted cells using Qiagen RNA easy kit according to the manufacturer's protocol. RNA was eluted from the column using 14µl of RNase free water. The amount of RNA was measured with a spectrophotometer (NanoDrop, Thermo Scientific) using 1 to 2µl of the eluted RNA.

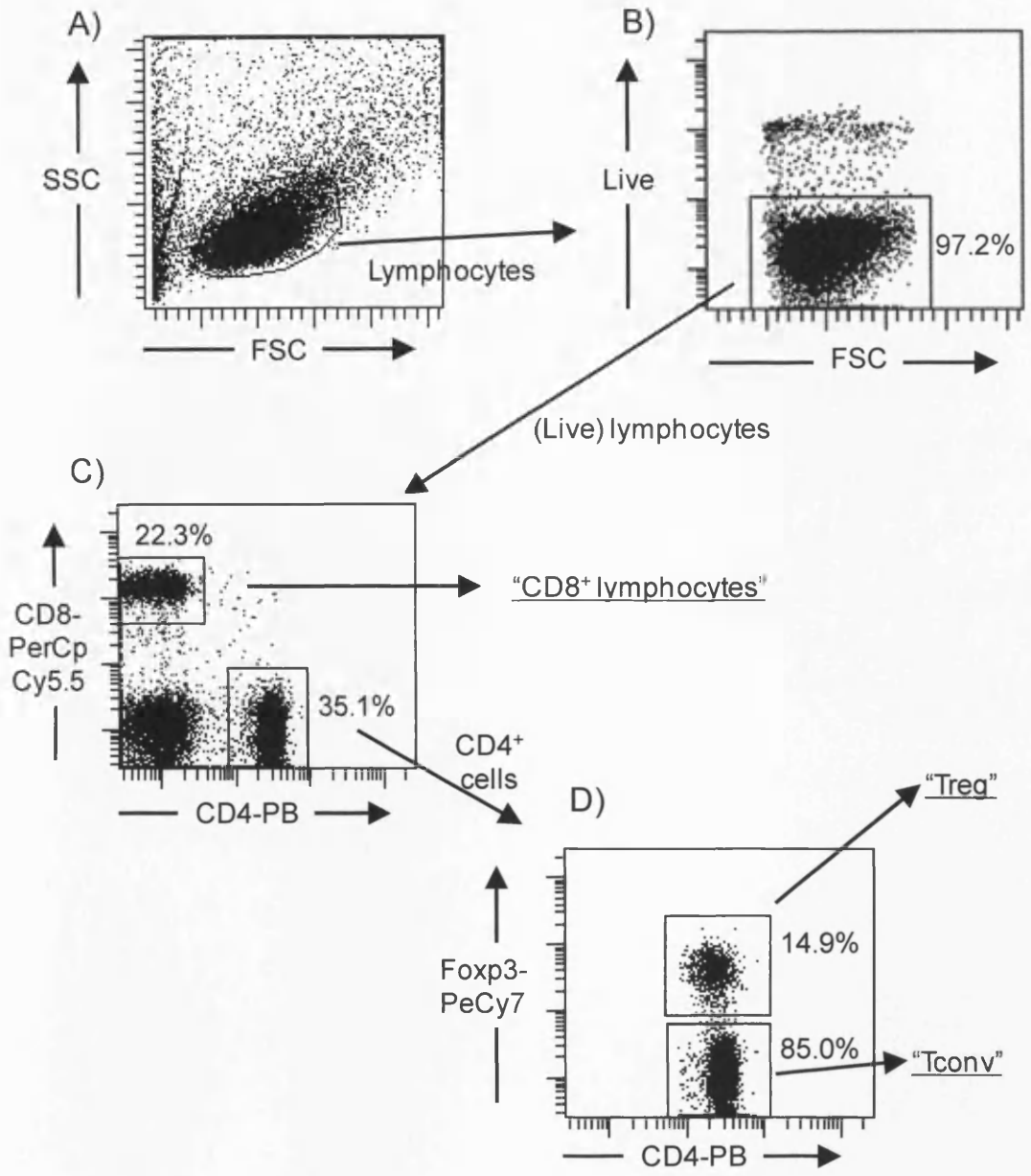


Figure 2.2. Gating strategy for identifying Tconv, Treg and CD8$^+$ cell populations
Single cell suspensions were stained with Live/Dead Aqua viability stain and subsequently with anti-CD4 PB, anti-CD8 PerCpCy5.5 and anti-Foxp3-PeCy7 mAbs. Lymphocytes were defined according to their FSC and SSC (step A). Live lymphocytes were identified as those with low fluorescence levels of Live/Dead (step B). Cells were gated into CD8$^+$ lymphocytes and CD4$^+$ lymphocytes (step C). CD4$^+$ lymphocytes were further divided into CD4$^+$Foxp3$^-$ conventional T cells (Tconv) and CD4$^+$Foxp3$^+$ regulatory T cells (Treg) based on their level of Foxp3 staining (step D).

Population	% of lymphocyte population
Lymphocytes	100.0
→ (Live) lymphocytes	97.2
→ $CD8^+$ cells	22.3
→ $CD4^+$ cells	35.1
→ Treg	5.2 (14.9% of 35.1)
→ Tconv	29.9 (85% of 35.1)

Figure 2.3. Population hierarchy and strategy for determining the frequencies of Tconv, Treg and $CD8^+$ cell populations
The relative proportions of the Tconv, Treg and $CD8^+$ T cell populations within the lymphocyte population was calculated based on the gating strategy described above and the population hierarchy shown. In this example, the $CD8^+$ lymphocyte population makes up 22.3% of the live lymphocyte gate. However, the $CD4^+$ lymphocyte population, which in this example makes up 35.1% of the lymphocytes, is comprised of 14.9% Treg and 85% Tconv. Therefore, the Treg population make up 5.2% of the total lymphocytes. Whereas, the Tconv population makes up 29.9% of the lymphocyte population.

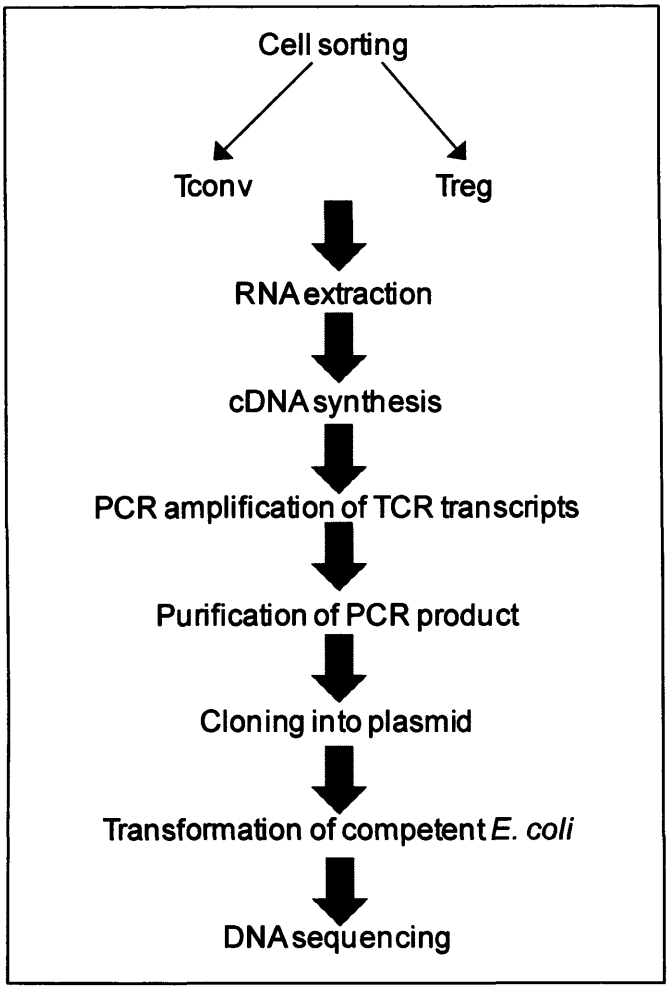


Figure 2.4. Procedure for TCR clonotyping process.
Tconv and Treg cells were sorted by flow cytometry from single cells suspensions of spleen, non-draining lymph node, draining lymph node, and tumour. Immediately after sorting RNA was extracted using a column based kit. cDNA was synthesised from 30-200ng of RNA and used as a template for amplification of TCR transcripts. The resulting PCR product was purified by gel extraction and cloned into a plasmid vector. Cloned vectors were used to tranform chemically competent bacteria and plated onto selective LB plates. At least 96 colonies were picked from each plate and subjected to DNA sequencing of the cloned TCR insert.

2.6.3 cDNA synthesis

cDNA was reverse transcribed from 30 to 200ng of total RNA using superscript III (Invitrogen) with 250ng of random oligos. Briefly, RNA and random primers were denatured by incubating at 65°C for 5mins in the presence of 10mM dNTP mix. Next, RT buffer, 0.1M DTT, RNaseOUT, and superscript III were added and incubated at 25°C for 5mins, then 50°C for 1 hour and finally 70°C for 15mins.

2.6.4 Amplification and purification of TCR transcript

TCR sequences were amplified by PCR using either a TRBV13-2 specific primer (5'-GGTGACATTGAGCTGTAAT-3') paired with a common (TRBC) primer (5'-CACTGATGTTCTGTGTGACAG-3') or using a TRBV13-2 specific primer (5'-GGTGACATTGAGCTGTAATCAGAC-3') paired with a TRBJ2-5 specific primer (5'-TAACACGAGGAGCCGAGTG-3'). PCR products were separated by agarose gel electrophoresis, excised using a clean, sharp knife and subsequently purified from the agarose gel using a gel extraction kit (illustra GFX™ PCR DNA and gel band purification kit, GE Healthcare) according to the manufacturer's instructions. The DNA was eluted with 10µl of DNase free water.

2.6.5 Cloning and transformation of E. Coli

Purified PCR products were cloned into pCR®2.1-TOPO® plasmid vector by TOPO cloning (Invitrogen) according to the manufacturers protocol. The resulting construct was transformed into chemically competent *E. Coli* (TOP10; Invitrogen) by heat shocking the cells at 42°C for 30 seconds. Transformed cells were incubated for only 15 minutes at 37°C

with shaking before spreading onto pre-warmed selective LB plates (100μg/ml Ampicillin with IPTG and X-gal for blue/white screening.) Plates were left overnight (14 to 18 hours) at 37°C for colonies to form.

2.6.6 Sequencing

High throughput sequencing was performed by a sequencing services company (Beckman Coulter Genomics). Briefly, individual white or light blue colonies were picked and subsequently expanded in selective media. DNA was prepared from these cultures and sequenced with a sequencing primer using an ABI 3730*xl* DNA Analyzer. The *TRB* gene usage and CDR3 amino acid composition was established using IMGT/V-QUEST software and used to identify individual TCRs.

2.6.7 Statistical analysis

The level of similarity between the different TCR repertoires was measured using the Morisita-Horn (MH) similarity index. This unitless index ranging from 0 to 1 takes into account the number of shared sequences between two repertoires as well as the contribution of those shared sequences to each repertoire. The EstimateS software package was used to calculate the MH values (Colwell 2005).

Chapter 3 – Phenotypic and functional characterisation of tumour infiltrating T cells

3.1 Introduction

Previous studies suggest that T lymphocytes prevent tumour outgrowth through immune surveillance. However, when cancer arises the host immune response has clearly failed to reject the tumour. The accumulation of regulatory T cells may be one process that is established in the tumour microenvironment to allow for immune evasion of the tumour. Previous studies from our laboratory have shown that Treg cells are abundant within methylcholanthrene induced tumours and that partial depletion of these cells can reduce tumour incidence in mice (Betts *et al.* 2007). It is therefore reasonable to hypothesize that regulatory T cells contribute to the immunosuppressive tumour microenvironment by preventing the anti-tumour action of $CD4^+$Tconv and $CD8^+$ T cells. To investigate this hypothesis a detailed phenotypic and functional characterisation of $CD4^+Foxp3^-$ conventional (Tconv), $CD4^+Foxp3^+$ regulatory (Treg) and $CD8^+$ T cell populations in mice bearing chemically induced (MCA) tumours was performed. Specifically, the following questions were addressed:

- Do $CD4^+$ and $CD8^+$ cells control tumour development following Treg depletion?
- Do the ratios of Tconv:Treg and CD8:Treg alter in the tumour compared to lymphoid tissue?
- Do tumour infiltrating Treg cells display a phenotype associated with immunosupression?
- Do tumour infiltrating Tconv and $CD8^+$ T cells express activation markers?

The answer to the first question should indicate whether T cells control tumours whilst answers to the three subsequent questions should provide insight into whether T cells are suppressed by Tregs within the tumour. This information is necessary for understanding the relationship between tumours and the immune system and how this may be exploited for therapeutic intervention.

3.2 Results

3.2.1 Do $CD4^+$ and $CD8^+$ cells control tumour development following Treg depletion?

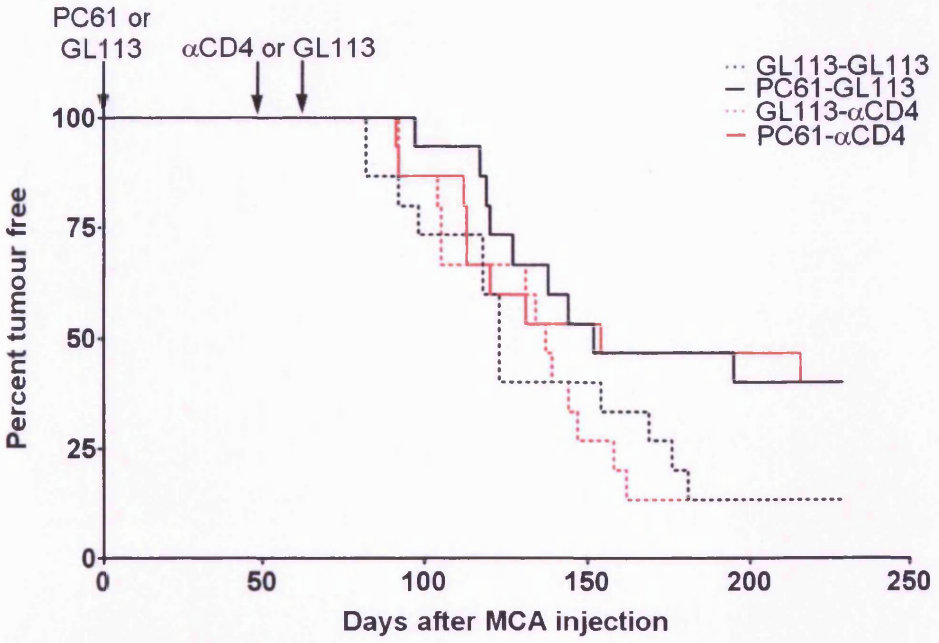
Experiments from our laboratory have shown that tumour induction is reduced if Treg cells are partially depleted prior to injection of carcinogen (Betts *et al.* 2007). To deplete Tregs in these experiments a CD25-specific antibody (PC61) was administered three days and one day before MCA injection. The tumour incidence was significantly reduced from ~70% in control mice to ~40% in mice injected with PC61 (**P=0.001). Whilst approximately 60% of PC61-treated animals appear tumour-free, it is possible that tumours are present in the animals but that the outgrowth of these tumours are controlled by a T cell response induced as a result of Treg depletion. To test this, two groups of mice were set up which received either PC61 Abs or control (non-depleting), GL113, Abs at days -3 and -1 prior to injection with MCA. As shown in Figure 2.1 (page 55), approximately 7 weeks later, a time-point when tumours are not yet visible in either group, the PC61 and GL113 treated mice were injected with depleting antibodies against either CD4 or CD8. If either of these T cell subsets were controlling tumour outgrowth at this stage, tumours would be expected to grow out more rapidly in T cell-depleted animals compared to those receiving the control (non-depleting) Abs (GL113).

3.2.1.1 Anti-CD4 antibody treatment does not affect tumour control

Mice injected with PC61 (days -3 and -1) were later (days 50 and 64) treated with anti-CD4 antibodies or control antibodies (Figure 2.1, page 55). Anti-CD4 antibody treatment did not alter tumour incidence in comparison to control Ab treatment (Figure 3.1). Thus, following PC61 treatment the percentage of tumour free mice after 230 days remained at 40% whether $CD4^+$ cells were subsequently depleted or not. This suggests that $CD4^+$ T cells do not control tumour growth following Treg depletion. Similarly, no effect of CD4-specific Abs was observed in control, Treg-replete animals.

3.2.1.2 Anti-CD8 antibody treatment negates control of tumour development following Treg cell depletion

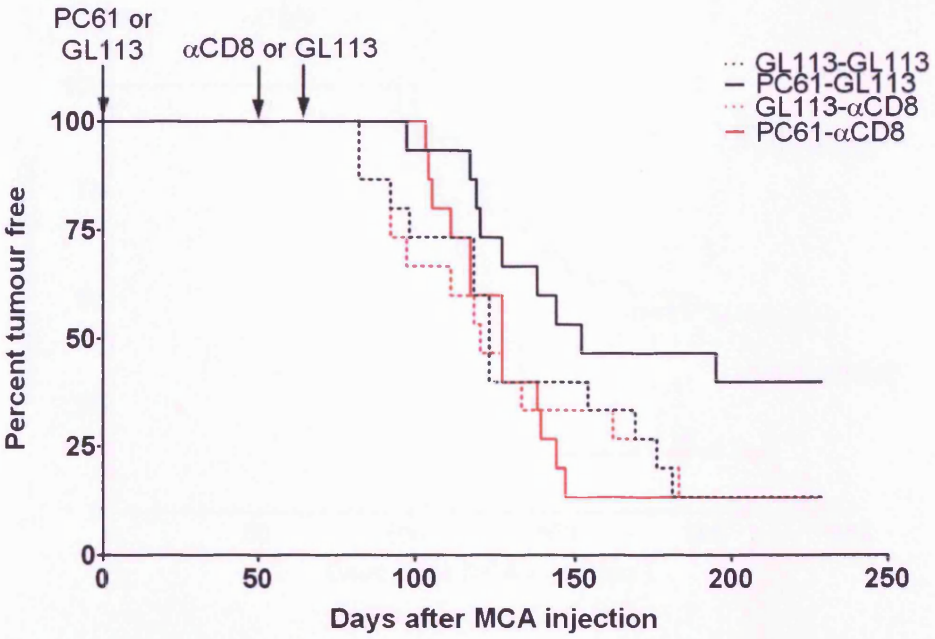
A second group of mice was given anti-CD8 (or control) antibodies following PC61 treatment (Figure 2.1, page 55). The treatment of mice with anti-CD8 Abs had a clear effect on the overall incidence in mice depleted of Treg cells prior to MCA injection (Figure 3.2). Whilst the percentage of mice that remained tumour free following PC61 treatment only was 40%, the percentage of mice that remained tumour free after being treated with PC61 then later with anti-CD8 antibodies was reduced to only 13%, similar to that observed in the control group receiving GL113. Using the data from this experiment alone (n=15), the difference between the PC61 and anti-CD8 antibody treated mice and those given PC61 then control antibodies was approaching statistical significance (P=0.0582). When the results were combined with previous data from our laboratory on the survival of mice following PC61 treatment and compared using a the same statistical test (log rank), depletion of $CD8^+$ cells following PC61 Ab treatment significantly altered survival compared to PC61 Ab treatment alone (**P=0.0058; Figure 3.3). Overall, these results suggest that $CD8^+$ T cells are important



Experimental group		Experimental group	P value	Significant?
GL113-GL113	Vs	PC61-GL113	0.0972	No
GL113-GL113	Vs	GL113-αCD4	0.9265	No
GL113-GL113	Vs	PC61-αCD4	0.1711	No
PC61-GL113	Vs	GL113-αCD4	0.1067	No
PC61-GL113	Vs	PC61-αCD4	0.8205	No
PC61-αCD4	Vs	GL113-αCD4	0.1783	No

Figure 3.1. MCA-induced tumour induction following treatment with PC61 and anti-CD4 .

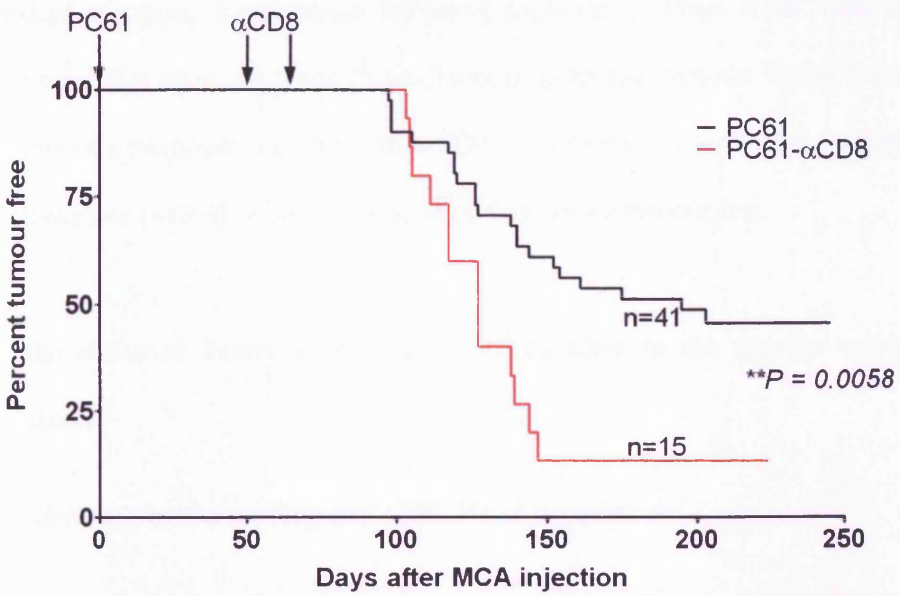
Groups of mice (n=15) were injected with either PC61 or GL113 prior to MCA , then subsequently with either anti-CD4 Abs or GL113 at days 50 and 64. The tumour incidence between experimental groups was analysed using log-rank (Mantel-Cox) tests.



Experimental group		Experimental group	P value	Significant?
GL113-GL113	Vs	PC61-GL113	0.0972	No
GL113-GL113	Vs	GL113-αCD8	0.8643	No
GL113-GL113	Vs	PC61-αCD8	0.8219	No
PC61-GL113	Vs	GL113-αCD8	0.0564	No
PC61-GL113	Vs	PC61-αCD8	0.0582	No
PC61-αCD8	Vs	GL113-αCD8	0.9187	No

Figure 3.2. MCA-induced tumour induction following treatment with PC61 and anti-CD8 .

Groups of mice (n=15) were injected with either PC61 or GL113 prior to MCA , then subsequently with either anti-CD8 Abs or GL113 at days 50 and 64. The tumour incidence between experimental groups was analysed using log-rank (Mantel-Cox) tests.



Experimental group		Experimental group	P value	Significant?
PC61	Vs	PC61-αCD8	0.0058	Yes

Figure 3.3. MCA-induced tumour induction following treatment with PC61 and anti-CD8 .

Groups of mice were injected with PC61 prior to MCA , then subsequently with either anti-CD8 Abs (n=15) or GL113/no treatment (n=41) at days 50 and 64. The tumour incidence between experimental groups was analysed using log-rank (Mantel-Cox) tests.

for the control of tumour development following depletion of Tregs. $CD8^+$ cells were also depleted in mice that were not given PC61. Tumour incidence was not further increased by anti-CD8 antibody treatment suggesting that $CD8^+$ T cells are not controlling tumour growth in the small number (~13%) of Treg-replete mice that remain tumour free.

3.2.2 Do the ratios of Tconv:Treg and CD8:Treg alter in the tumour compared to lymphoid tissue?

3.2.2.1 Analysis of the Tconv, Treg and $CD8^+$ T cell populations

Mouse models have demonstrated that selective removal of Treg cells from naive animals results in catastrophic autoimmune and inflammatory disease (Kim *et al.* 2007). Thus, Treg cells exert potent suppressive effects even under normal homeostatic conditions. As described above, Treg cells accumulate in tumours to levels that are significantly higher than in lymphoid tissue implying that, within the tumour microenvironment, the impact of Tregs on suppression of T cell responses may be even more pronounced at this site. To investigate this further, the proportions of different T cell subsets were analysed in the spleens, draining lymph nodes (LN), non-draining LNs and tumours of tumour bearing mice and also the spleens and LNs of age-matched mice.

A significant (approximately 8 fold; ***$P<0.0001$) expansion of cells was observed within the draining lymph node compared to the non-draining lymph node (Figure 3.4). However, the proportions of the different T cell subsets did not remain consistent throughout the different compartments of tumour bearing mice. The mean percentage of lymphocytes that were Tconv cells in the non-draining lymph nodes of tumour bearing mice was approximately 27% (Figure 3.5 A). Interestingly, the mean percentage of lymphocytes that

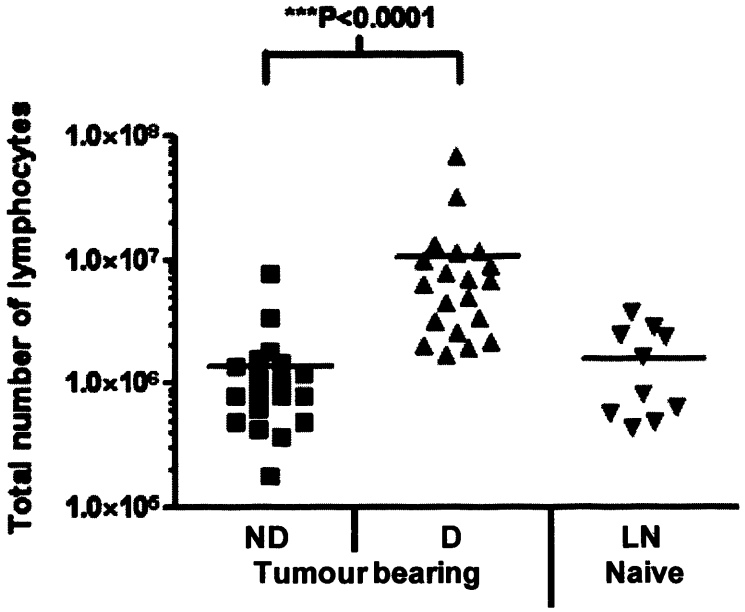


Figure 3.4. Total number of lymphocytes in lymph nodes of tumour bearing and naïve mice.

Single cell suspensions were stained with trypan blue and the number of lymphocytes were counted using a haemocytometer. The total number of lymphocytes from draining (D, n=20) and non-draining (ND, n=20) inguinal lymph nodes of tumour bearing mice and the total number of lymphocytes from inguinal lymph nodes (LN, n=3) of naive age matched mice are shown. The total number of cells from the inguinal lymph nodes of naive mice were normalised by dividing the value by two as both lymph nodes were combined for counting. A paired, non-parametric (Wilcoxon matched) test was used to analyse the difference between ND and D lymph nodes of tumour bearing mice.

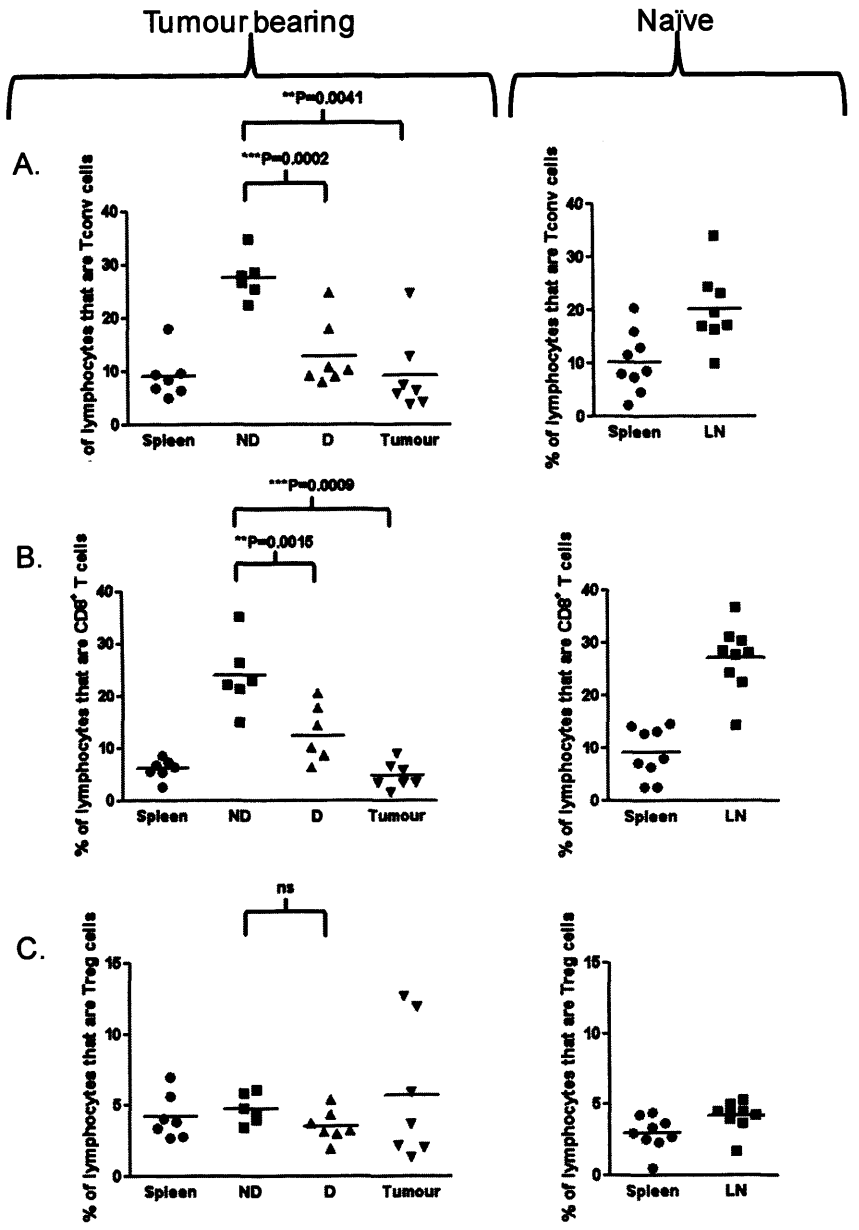


Figure 3.5. Proportions of Tconv, Treg and CD8$^+$ T cell populations in tumour bearing and naïve mice.

The proportions of the total lymphocyte population that were made up of Tconv cells (A), CD8$^+$ T cells (B) and Treg cells (C) were determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=7). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=9). Samples within tumour bearing mice were compared using paired T tests.

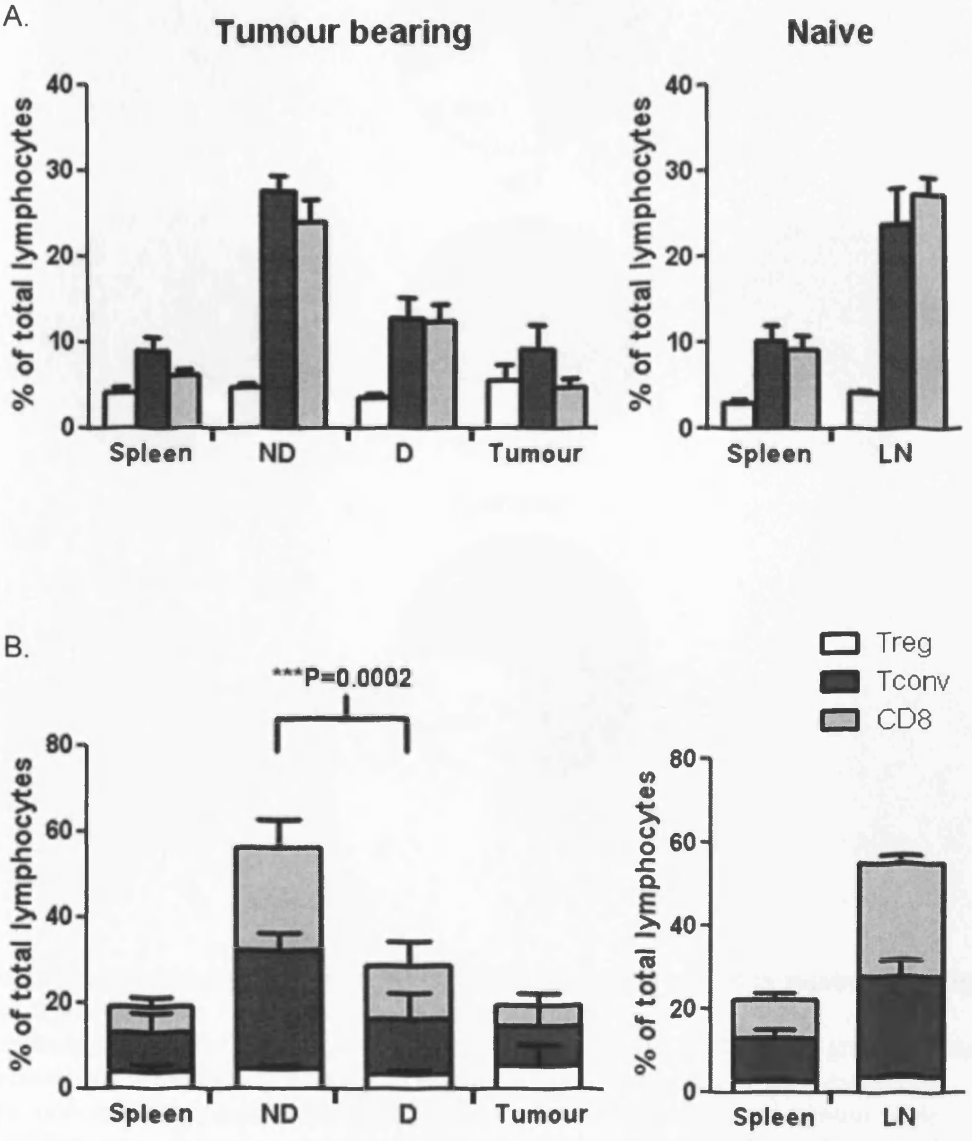
were Tconv was significantly lower within the draining lymph nodes (approximately 13%) compared to the non-draining lymph node. This value for the draining lymph node was similar to the average percentage of lymphocytes that were Tconv in the tumours (approximately 9%) of tumour bearing mice. Thus it appears that within tumour associated tissue the proportion of lymphocytes that are Tconv cells is low.

The pattern with $CD8^+$ T cells was very similar to the pattern of Tconv cells. The mean percentage of lymphocytes that were $CD8^+$ T cells was significantly higher in the non-draining lymph nodes (23.9%) compared to the draining lymph nodes (12.5%; Figure 3.5 B). The tumours contained the lowest percentage of $CD8^+$ T lymphocytes (mean 4.8%). Thus, it appears that within the tumour and tumour associated lymph node a relatively low proportion of the lymphocyte populations are made up of $CD8^+$ T cells.

The changes in proportions of the Tconv and $CD8^+$ T cells were not repeated with the Treg cells. The mean percentage of lymphocytes that were Treg cells (Figure 3.5 C) remained relatively consistent throughout the spleens, non-draining lymph nodes, draining lymph nodes and tumours of tumour bearing mice (means = 3.6% - 5.7%).

It was noticed that in the tumour the T cell populations examined made up a relatively small proportion of the whole lymphocyte population (approximately 20%; Figure 3.6). Furthermore, a significant reduction was also seen when comparing the draining lymph node to the non-draining lymph node (Figure 3.6 B). Thus an accumulation of lymphocytes other than those analysed appears to occur in the draining lymph node and tumour. A preliminary analysis of MCA tumour infiltrating lymphocytes indicates that many $B220(CD45R)^+$ cells and $NK1.1^+$ cells are found within the lymphocyte gate (Appendix Figure A2). Cells

expressing both CD45R and NK1.1 were also found within the tumour lymphocytes gate. Such cells have been previously reported and are proposed to represent a unique subset of natural killer cells (Blasius *et al.* 2007) or dendritic cells (Taieb *et al.* 2006) which may even have anti-tumour activity (Terme *et al.* 2009).



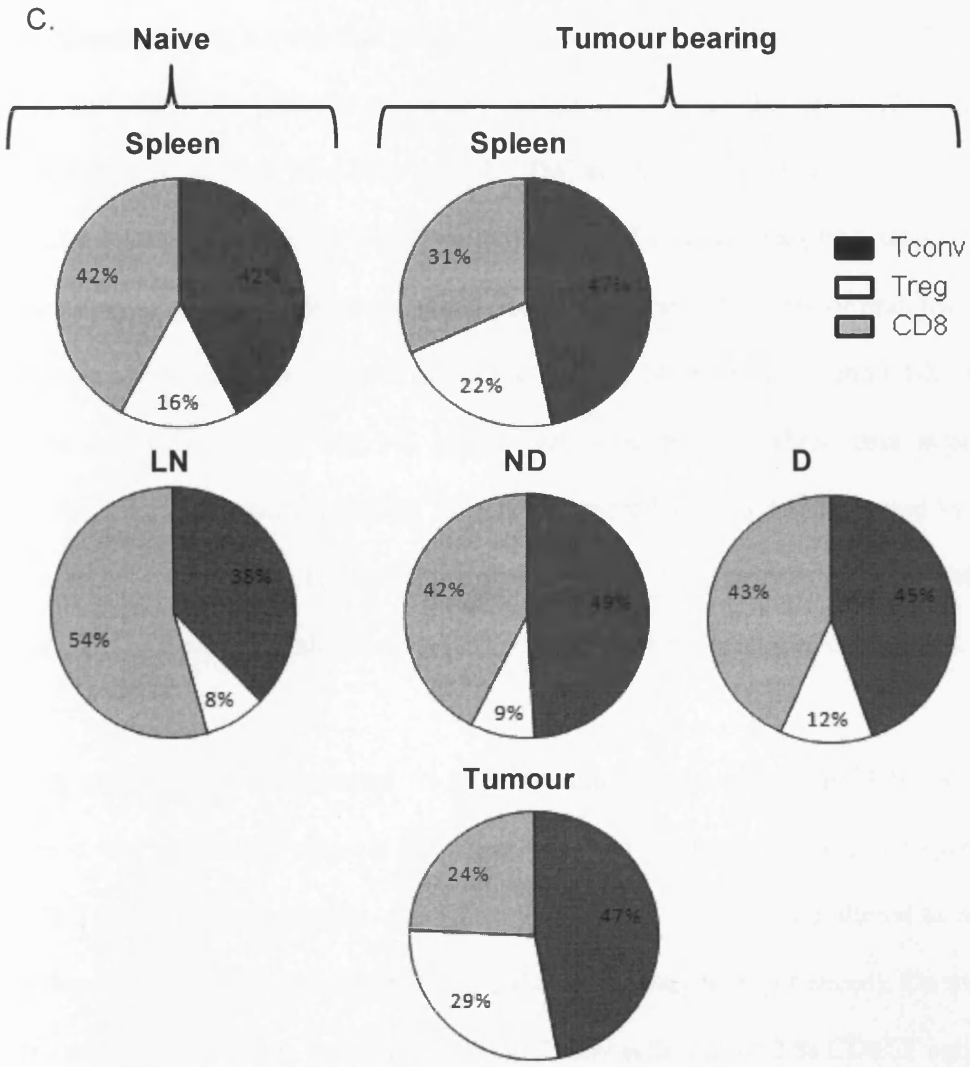


Figure 3.6. Proportions of Tconv, Treg and CD8$^+$ cell populations in tumour bearing and naïve mice.
The proportion of Tconv (dark grey bars), Treg (open bars) and CD8$^+$ (light grey bars) cell populations within the total lymphocyte population was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=7). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=7). Data are presented comparing cell types in individual mean (+/-SEM) bars (A) and stacked mean (+/- SEM) bars (B) and pie charts normalised to percent of T cell population (C). Samples within tumour bearing mice were compared using paired T tests.

3.2.2.2 Relative proportions of Tconv, CD8 and Treg cells

The percentages of lymphocytes that were Tconv, CD8 and Treg were evaluated by flow cytometric analyses of lymphocytes prepared from spleen, tumours and lymphoid organs and used to calculate the ratio of Treg cells to both $CD4^+$ and $CD8^+$ T cells. In the non draining lymph nodes, the ratios of Tregs to Tconv cells and $CD8^+$ T cells were approximately 1:6 and 1:5, respectively (Figure 3.7). However, a difference was observed in tumour draining lymph nodes and even more so in the tumour where the ratio of Tregs to Tconv and $CD8^+$ T cells changed to approximately 1:2 and 1:1, respectively (Figure 3.7). These data imply that tumour infiltrating Tconv cells and $CD8^+$ T cells are more likely to be suppressed by Tregs than those in lymphoid tissue. This alteration in the balance of Treg cells to Tconv cells and particularly $CD8^+$ T cells may aid the tumour in evading immune mediated destruction.

It is likely that any T cell expected to have an anti-tumour effect would be activated. Therefore it was considered relevant to assess the ratio of Tregs to antigen experienced ($CD44^{high}$) T cells. The percentages of the Tconv and $CD8^+$ T cells were altered to account only for the proportion of these cells that were $CD44^{high}$ (antigen experienced). On average, within the non-draining lymph node only 8.7% of Tconv cells and 11.2 % $CD8^+$ T cells were $CD44^{high}$ (Figures 3.8-3.10, discussed below). However, in the tumour, on average, 55.86% of Tconv cells and 41.79% of $CD8^+$ T cells were $CD44^{high}$ (Figure 3.9). Within the non-draining lymph node the ratios of $CD44^{high}$Tconv:Treg and $CD44^{high}$CD8:Treg were both ~1:2, indicating that Treg cells have a numerical advantage over both $CD44^{high}$ Tconv cells and $CD44^{high}$ $CD8^+$ T cells (Figure 3.11). However, within the tumour, the ratio of $CD44^{high}$Tconv:Treg was approximately 1:1 indicating that Treg cells and $CD44^{high}$Tconv cells are present in equal proportions (Figure 3.11A). The trend was not the same for $CD8^+$ T

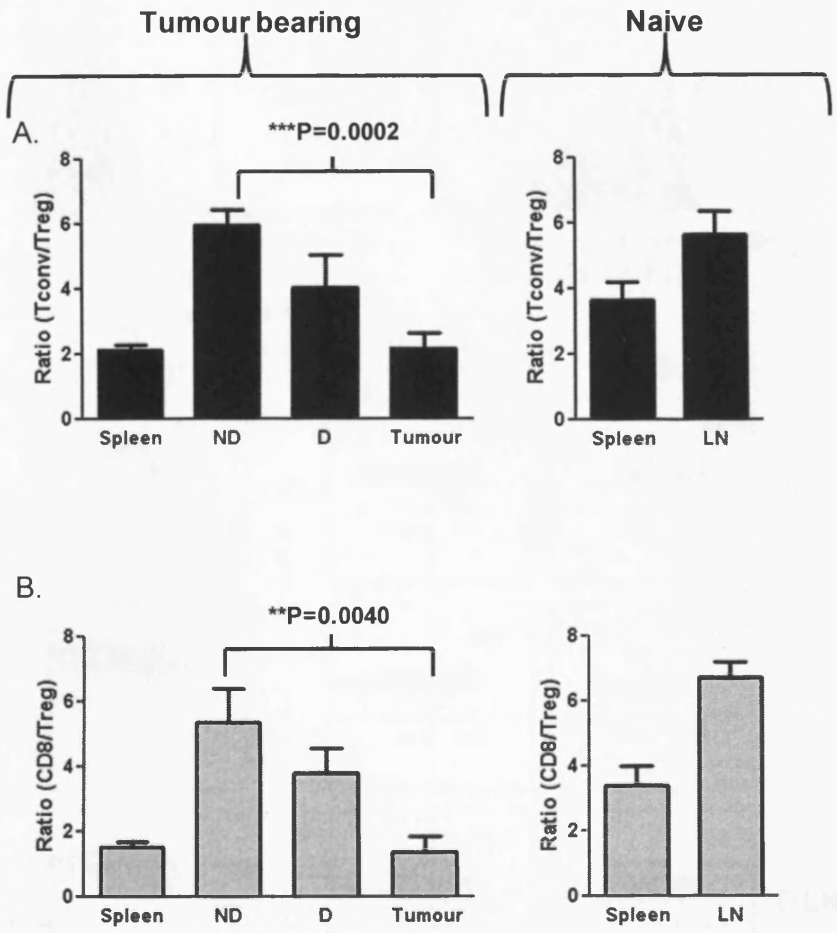


Figure 3.7. Ratios of Tconv, Treg and CD8$^+$ T cell populations in tumour bearing and naïve mice.

The ratio of Tconv/Treg (A, black bars), and CD8$^+$/Treg (B, grey bars) cell populations were determined by dividing the calculated proportion of the first population by the second population. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumours were analysed from tumour bearing mice (n=7). Spleens and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=7). Sample pairs within tumour bearing mice were analysed using paired T tests.

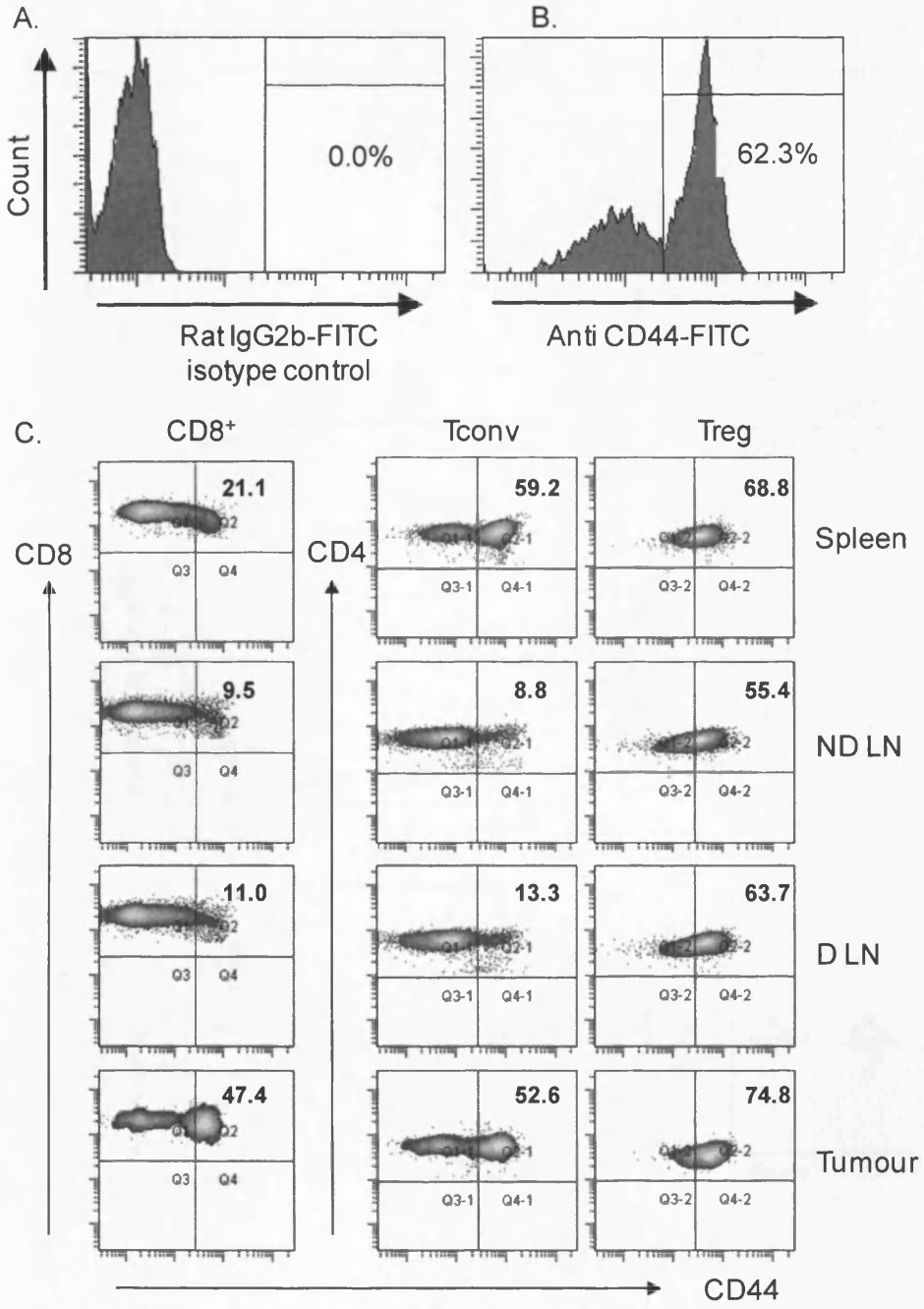


Figure 3.8. Antibody staining and analysis by flow cytometry of CD44.
Single cell suspensions of spleen, non-draining lymph node (ND LN), draining lymph node (D LN) and tumour were analysed for CD44 expression by flow cytometry. Cells were stained with anti CD44-FITC (B) or rat IgG2b-FITC isotype control (A) mAbs. Representative dot plots of $CD8^+$ T cells, $CD4^+Foxp3^-$ Tconv cells and $CD4^+Foxp3^+$ Treg cells from each tissue are shown (C).

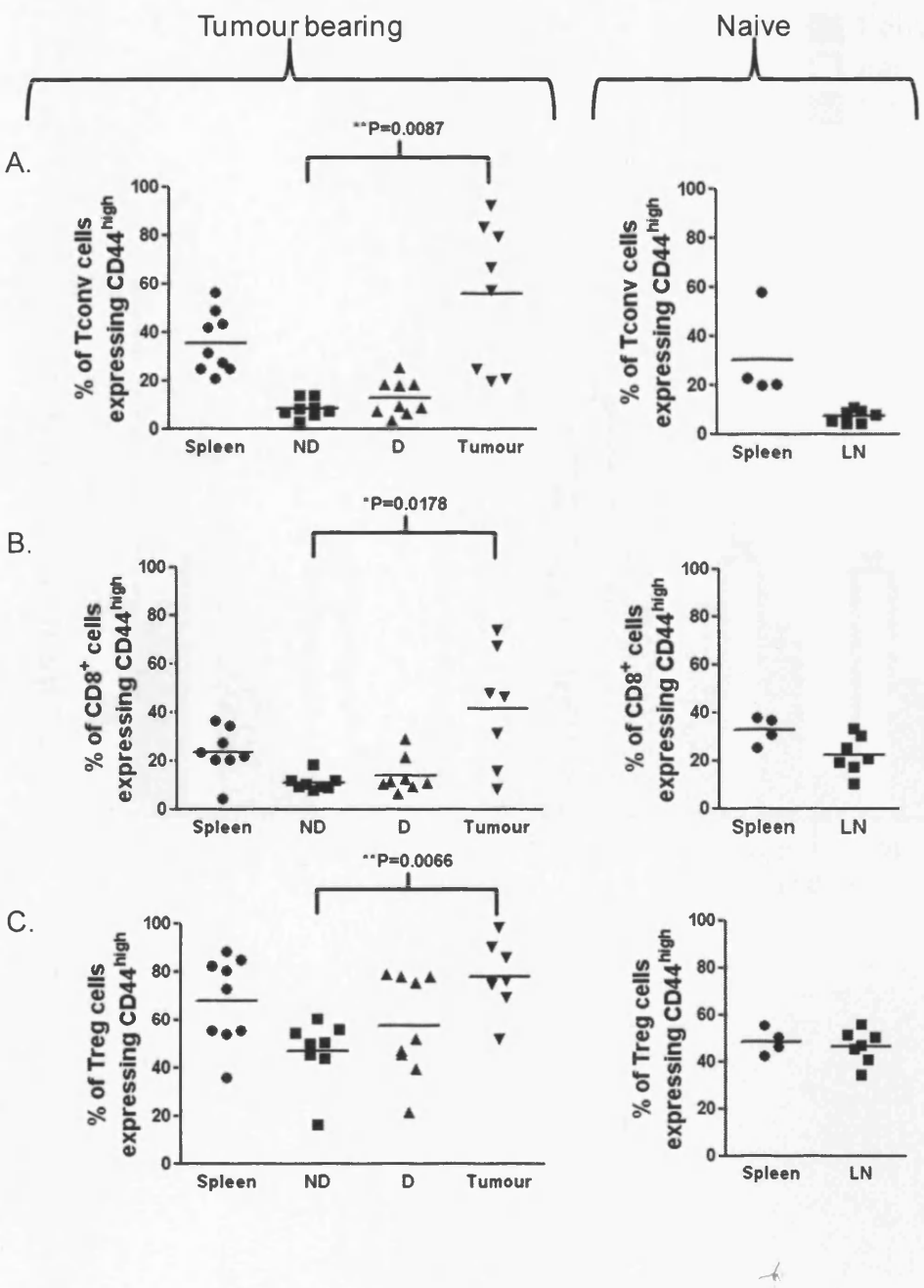


Figure 3.9. Percent of Tconv, Treg and CD8$^+$ cells expressing CD44^{high} in tumour bearing and naïve mice.

The percentage of Tconv (A), CD8$^+$ (B) and Treg (C) cells expressing CD44^{high} was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (Tconv and Treg cells n=9, CD8$^+$ cells n=8). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

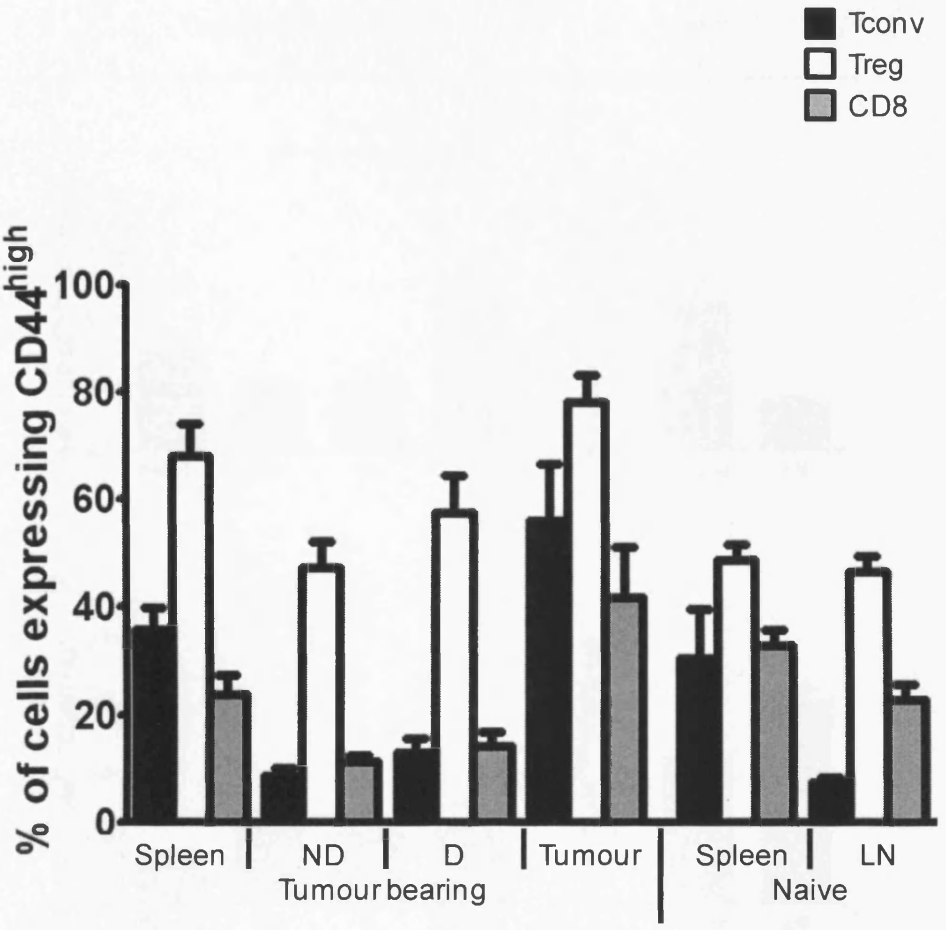


Figure 3.10. Percent of Tconv, Treg and CD8$^+$ cells expressing CD44^{high} in tumour bearing and naïve mice.
The percentage (mean +/-SEM) of Tconv (black bars), Treg (white bars) and CD8$^+$ (grey bars) cells expressing CD44^{high} was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (Tconv and Treg cells n=9, CD8$^+$ cells n=8). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

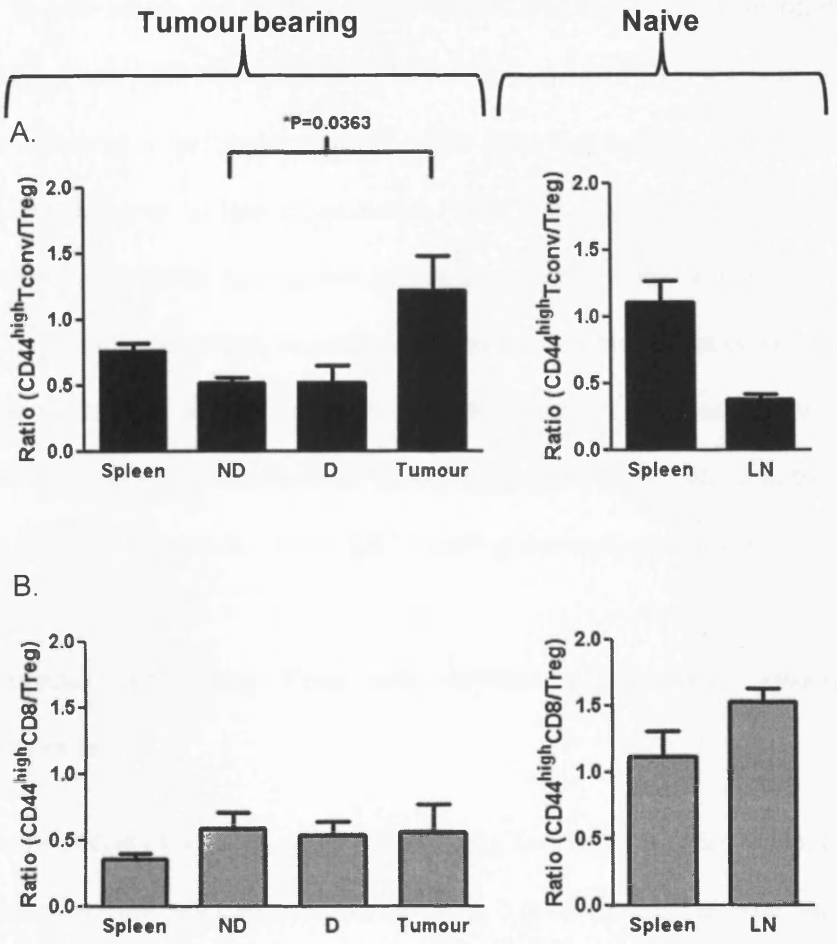


Figure 3.11. Ratios of antigen experienced ($CD44^{high}$) Tconv and $CD8^+$ T cell to Treg cell populations in tumour bearing and naïve mice.
The ratio of $CD44^{high}$Tconv/Treg (A, black bars), and $CD44^{high}CD8^+$/Treg (B, grey bars) cell populations were determined by dividing the calculated proportion of the first population by the second population. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=7). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). Sample pairs within tumour bearing mice were analysed using paired T tests.

cells in the tumour where the ratio of $CD44^{high}CD8$:Treg remained at approximately 1:2 (Figure 3.11B). Thus, $CD44^{high}$ $CD8^{+}$ T cells are outnumbered by Treg cells to a similar extent in the tumour as in the lymph node. Together these data indicate that Treg cells have a numerical advantage over antigen experienced Tconv and CD8 in the lymph node, which may contribute to their ability to suppress autoreactive T cells in the periphery. This balance is altered in the tumour for antigen experienced Tconv cells which are at equal levels to Treg cells, whereas the ratio of antigen experienced $CD8^{+}$ T cells to Treg cells is similar to that observed in the non-draining lymph node. This suggests that Tregs are able to maintain at least the same level of suppression over $CD8^{+}$ T cells in the tumour as in the lymph node.

3.2.3 Do tumour infiltrating Treg cells express a phenotype associated with immunosuppression?

Recently, many studies have focussed on phenotyping Treg cells in order to identify markers that are associated with high suppressive activity. These markers include the activation markers CD25, CD44, CTLA-4, ICOS and GITR as well as the integrin, CD103 and the ectonucleotidases, CD39 and CD73. Treg cells from MCA tumour bearing mice were assessed for the expression of CD25, CTLA-4, CD103, CD39 and CD73 and high expression levels of ICOS, CD44 and GITR as these are associated with the suppressive phenotype of Tregs (Sojka, Huang and Fowell 2008, Sakaguchi 2004, Shevach *et al.* 2006, Miyara and Sakaguchi 2007). An analysis of each of these markers revealed several interesting features of tumour infiltrating Treg cells. The percentage of cells expressing CD25, $GITR^{high}$, CD39, and CD73 each remained high in the tumour (Figures 3.12 – 3.23). The percent of Treg cells expressing $CD44^{high}$, $ICOS^{high}$, and CD103 was approximately 50%, 40% and 30% respectively in the spleen and lymph nodes (Figure 3.9 [above] and Figures 3.24 – 3.29).

Significant increases in the percentage of Treg cells expressing $CD44^{high}$, the percentage of Treg cells expressing $ICOS^{high}$, and the percentage of Treg cells expressing CD103 were seen in the tumour. In contrast to this pattern, the percent of Treg cells expressing CTLA-4 was significantly lower in the tumour (Figures 3.30 – 3.32). An analysis of cytokine expression by intracellular staining revealed Treg cells in tumour bearing mice expressed very little detectable IL-4, IL-17 or IFNγ (Figure 3.33 and Figure 3.34). In the spleen and lymph nodes approximately 10% of Treg cells expressed detectable IL-2, approximately 5% of Treg cells expressed detectable IL-10, and approximately 5-10% of Treg cells expressed detectable TNFα. Interestingly, in the tumour significantly fewer Treg cells expressed detectable levels of each of these cytokines. In summary, these data indicate that most of the markers associated with the suppressive phenotype of Treg cells are maintained or bolstered on tumour infiltrating Treg cells (Figure 3.35). This suggests tumour infiltrating Treg cell are likely to be suppressive within the tumour and that most of the purported mechanisms for Treg cell suppression remain possible in these cells.

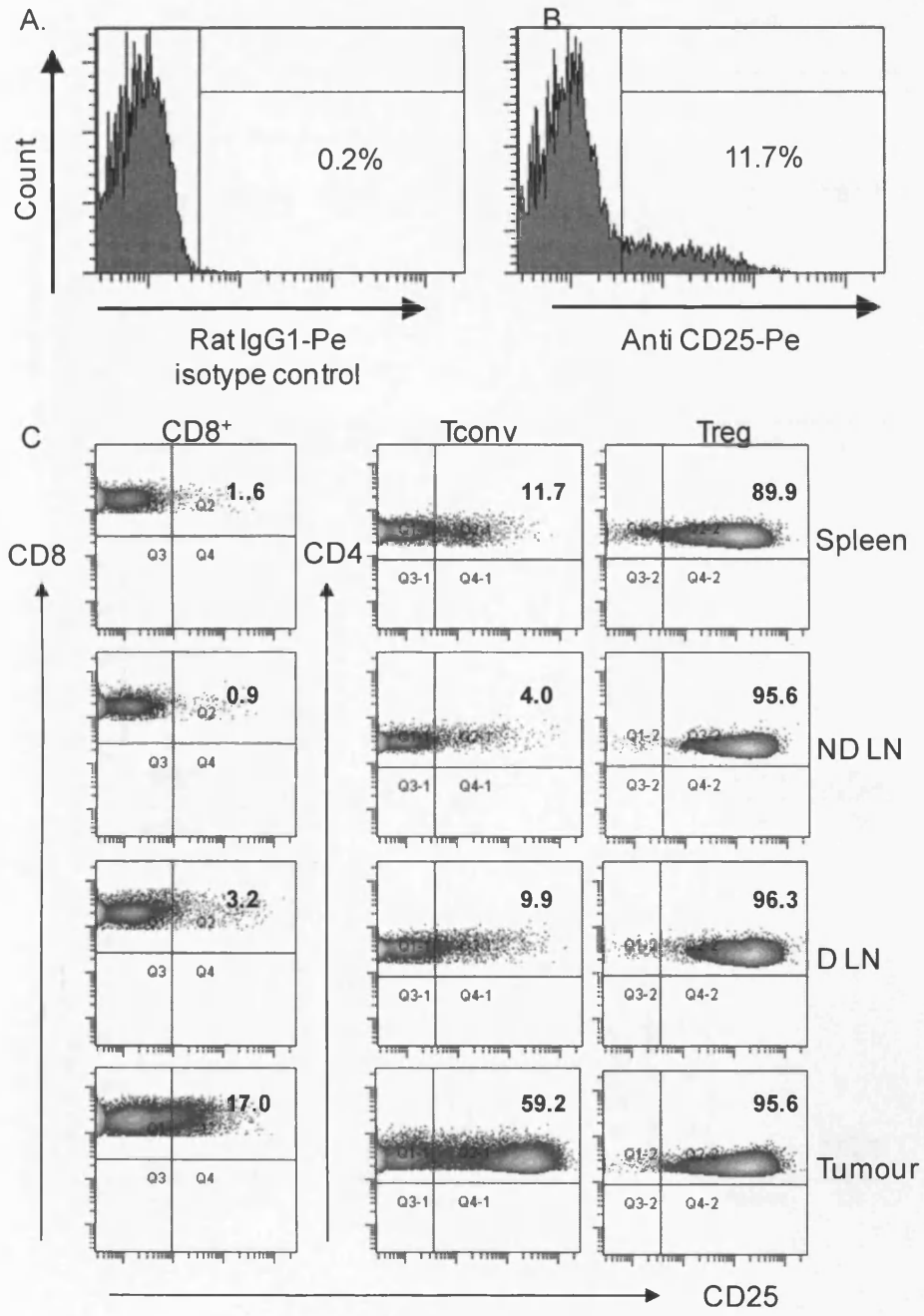


Figure 3.12. Antibody staining and analysis by flow cytometry of CD25.
Single cell suspensions of spleen, non-draining lymph node (ND LN), draining lymph node (D LN) and tumour were analysed for CD25 expression by flow cytometry. Cells were stained with anti CD25-PE (B) or rat IgG1-PE isotype control (A) mAbs. Representative dot plots of $CD8^+$ T cells, $CD4^+Foxp3^-$ Tconv cells and $CD4^+Foxp3^+$ Treg cells from each tissue are shown (C).

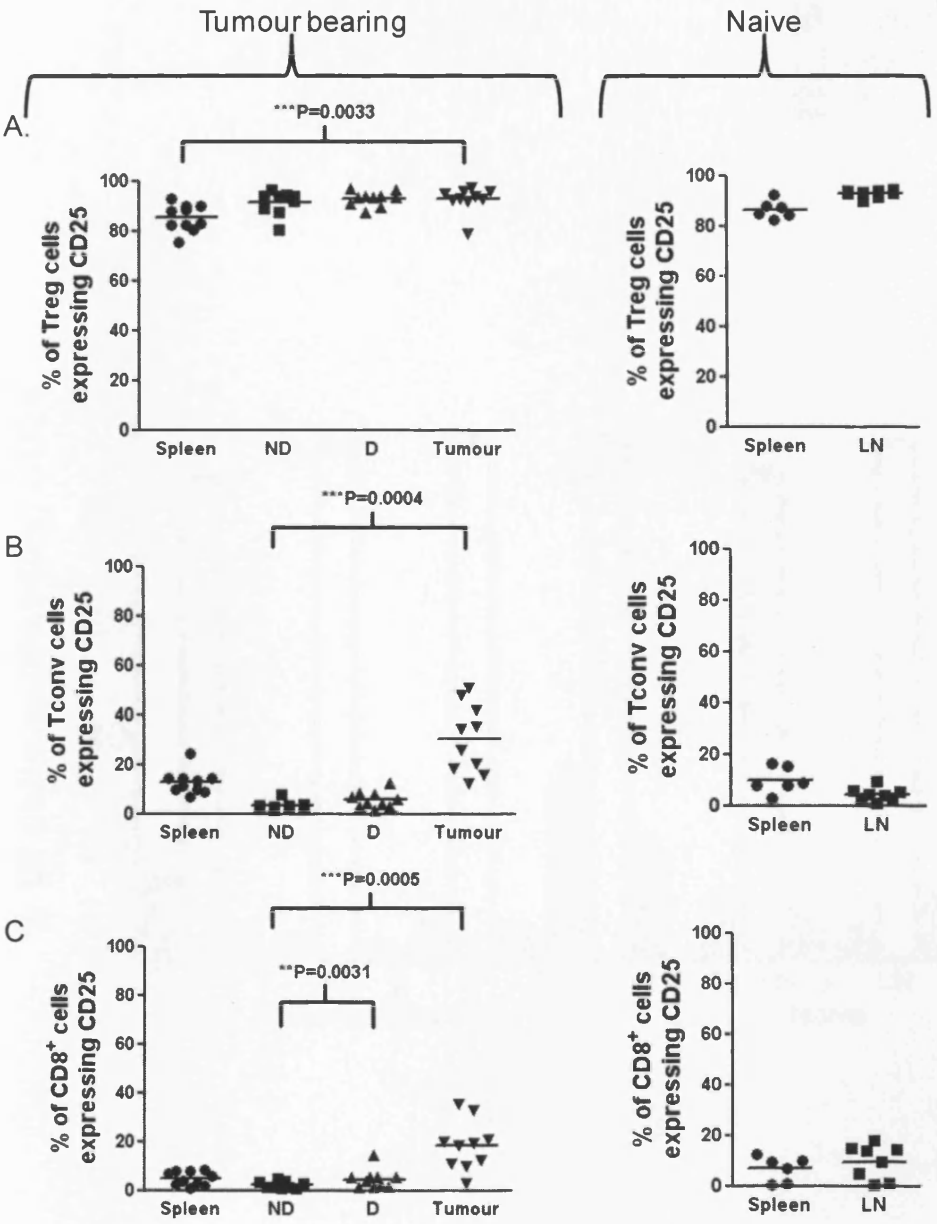


Figure 3.13. Percent of Tconv, Treg and CD8$^+$ cells expressing CD25 in tumour bearing and naïve mice.

The percentage of Treg (A), Tconv (B) and CD8$^+$ (C) cells expressing CD25 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=10). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=6-8). The differences between the means were compared using paired T tests.

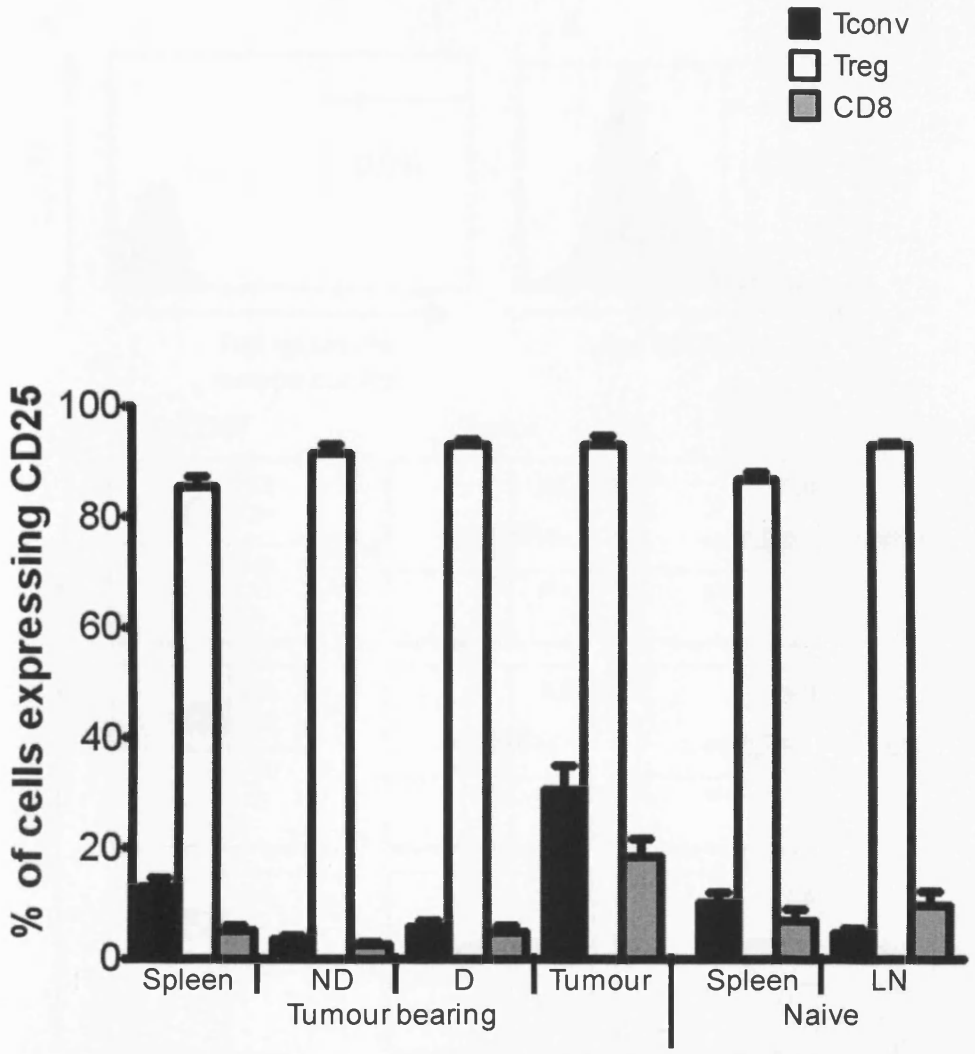


Figure 3.14. Percent of Tconv, Treg and CD8$^+$ cells expressing CD25 in tumour bearing and naïve mice.

The percentage (mean +/-SEM) of Tconv (black bars), Treg (white bars) and CD8$^+$ (grey bars) cells expressing CD25 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=10). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=6-8). The differences between the means were compared using paired T tests.

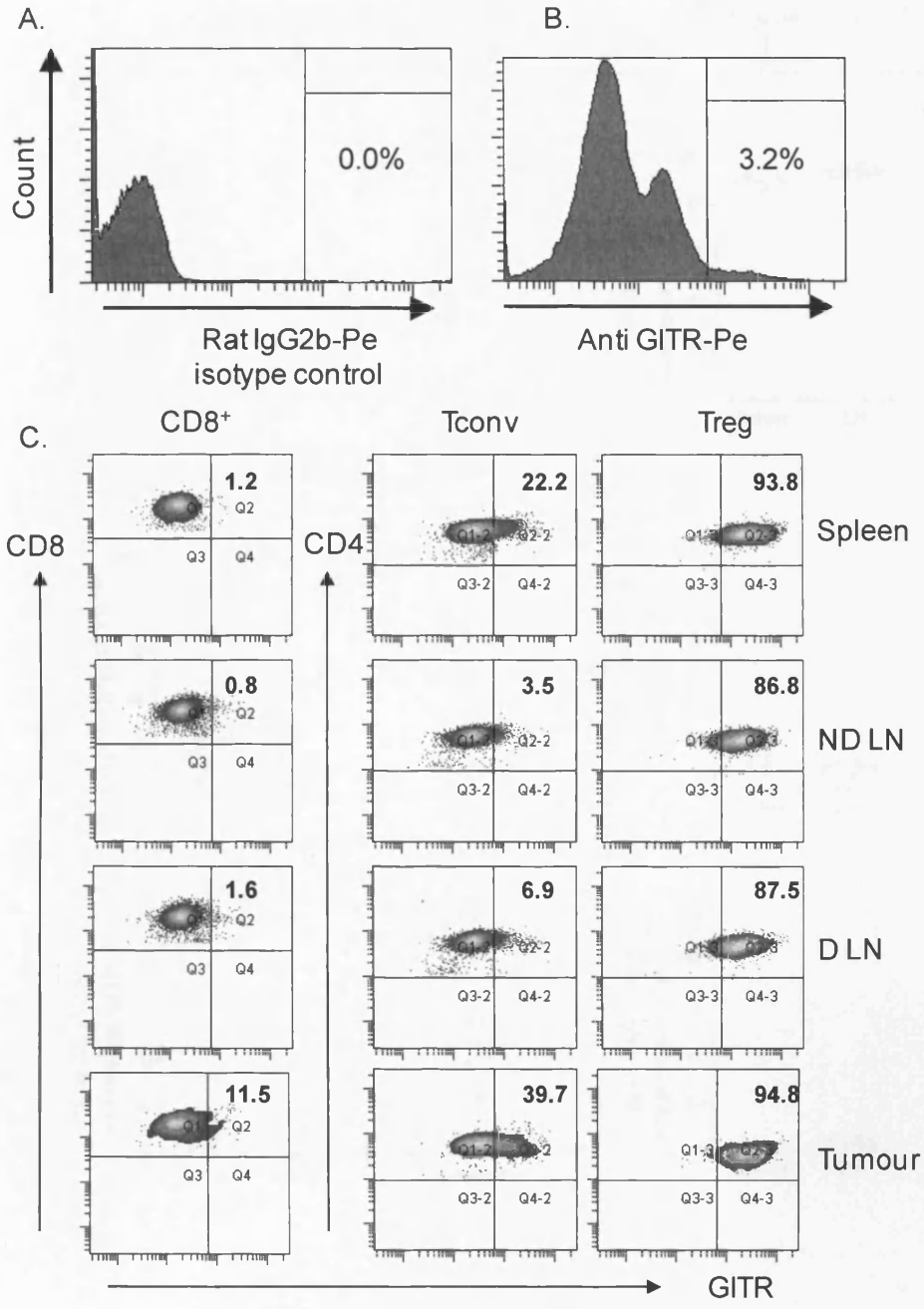


Figure 3.15. Antibody staining and analysis by flow cytometry of GITR.
Single cell suspensions of spleen, non-draining lymph node (ND LN), draining lymph node (D LN) and tumour were analysed for GITR expression by flow cytometry. Cells were stained with anti GITR-PE (B) or rat IgG2b-PE isotype control (A) mAbs. Representative dot plots of $CD8^+$ T cells, $CD4^+Foxp3^-$ Tconv cells and $CD4^+Foxp3^+$ Treg cells from each tissue are shown (C).

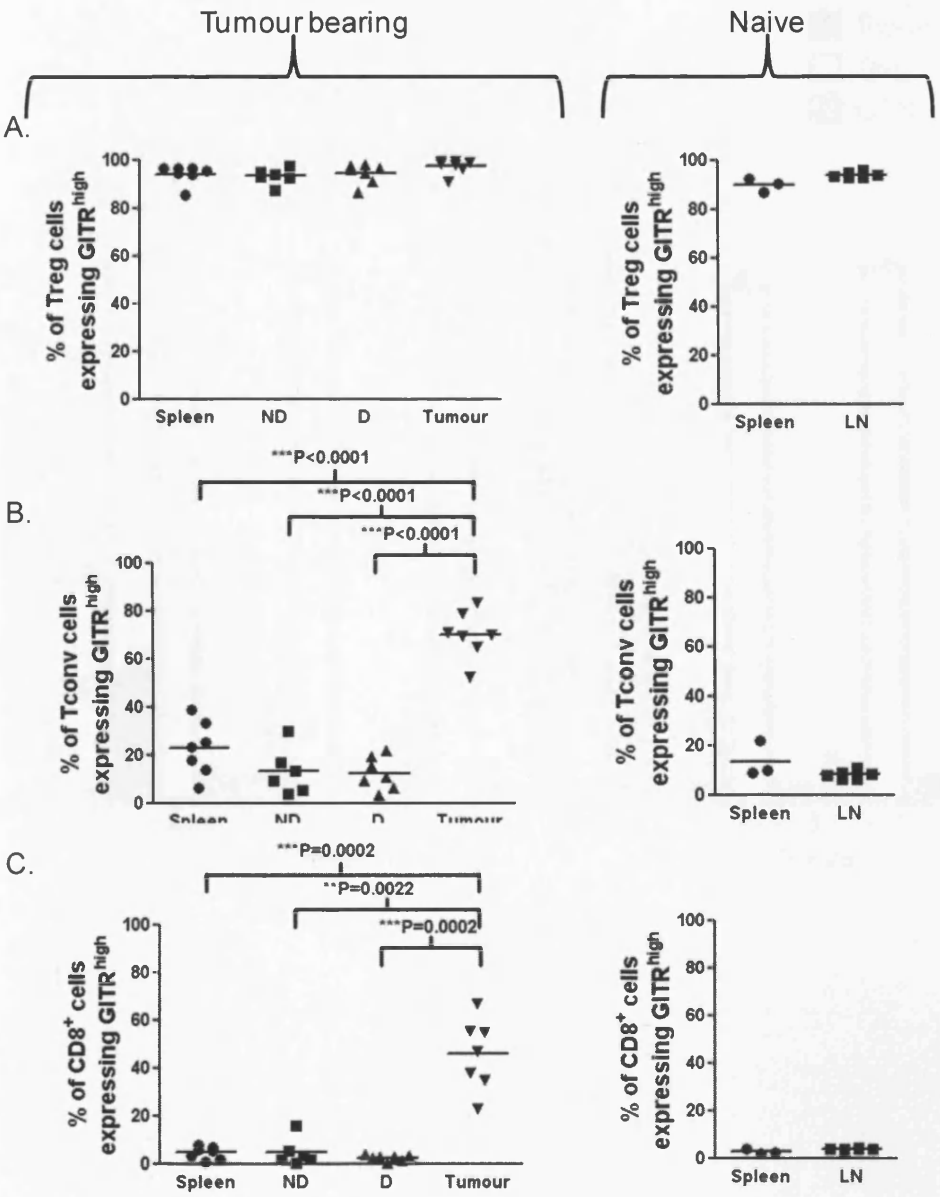


Figure 3.16. Percent of Tconv, Treg and CD8$^+$ cells expressing GITR^{high} in tumour bearing and naïve mice.

The percentage of Treg (A), Tconv (B) and CD8$^+$ (C) cells expressing GITR^{high} was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=7). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

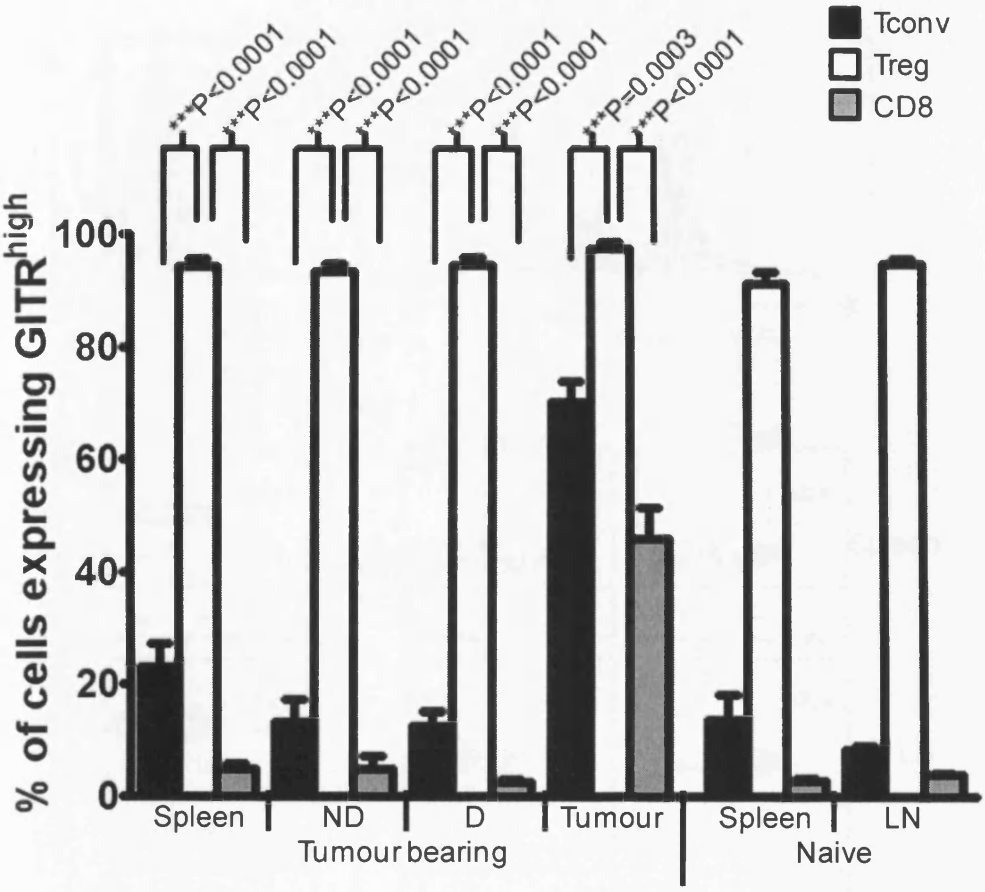


Figure 3.17. Percent of Tconv, Treg and CD8$^+$ cells expressing GITR^{high} in tumour bearing and naïve mice.

The percentage (mean +/-SEM) of Tconv (black bars), Treg (white bars) and CD8$^+$ (grey bars) cells expressing GITR^{high} was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=7). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

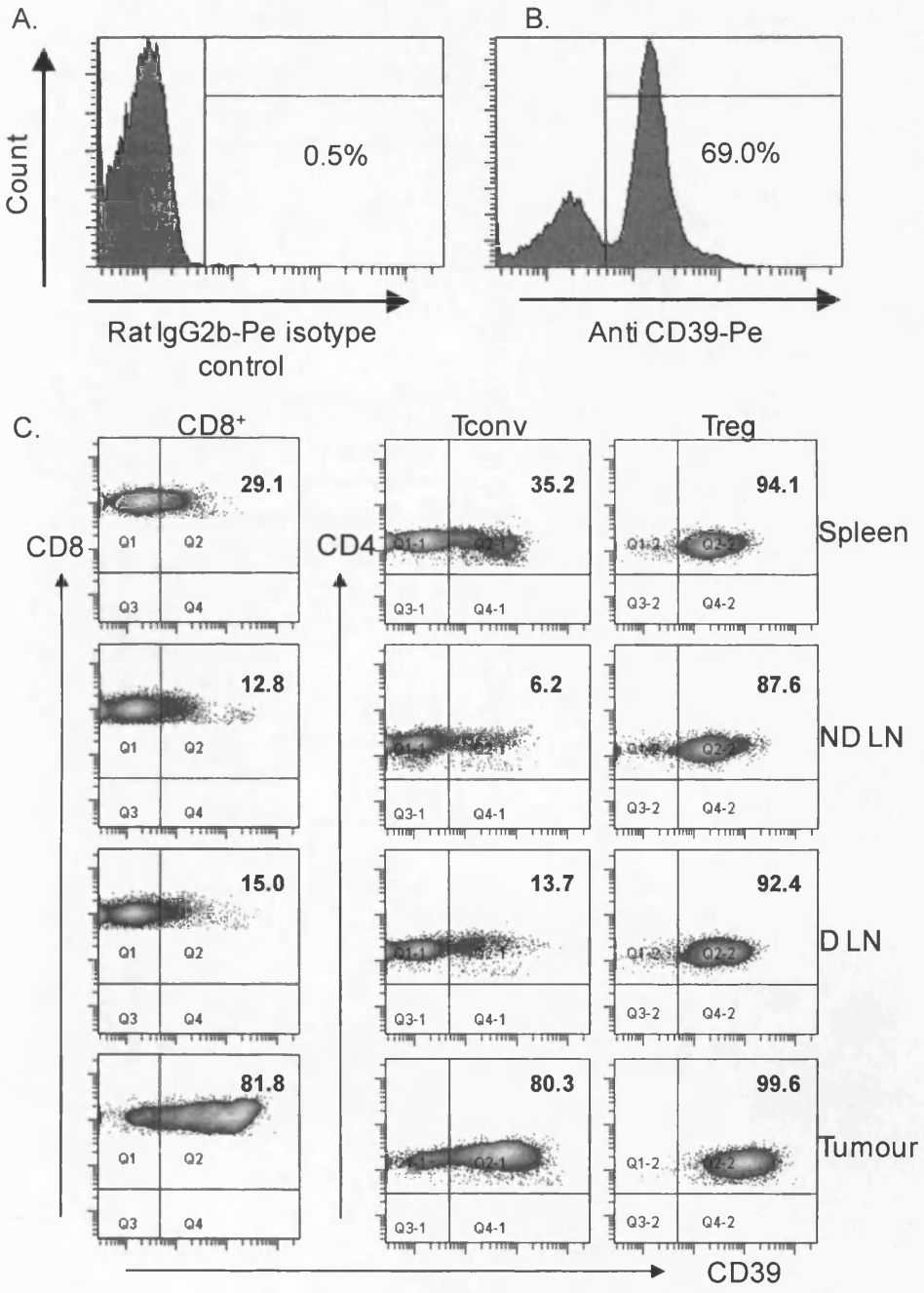


Figure 3.18. Antibody staining and analysis by flow cytometry of CD39.
Single cell suspensions of spleen, non-draining lymph nodeC (ND LN), draining lymph node (D LN) and tumour were analysed for CD39 expression by flow cytometry. Cells were stained with anti CD39-PE (B) or rat IgG2b-PE isotype control (A) mAbs. Representative dot plots of $CD8^+$ T cells, $CD4^+Foxp3^-$ Tconv cells and $CD4^+Foxp3^+$ Treg cells from each tissue are shown (C).

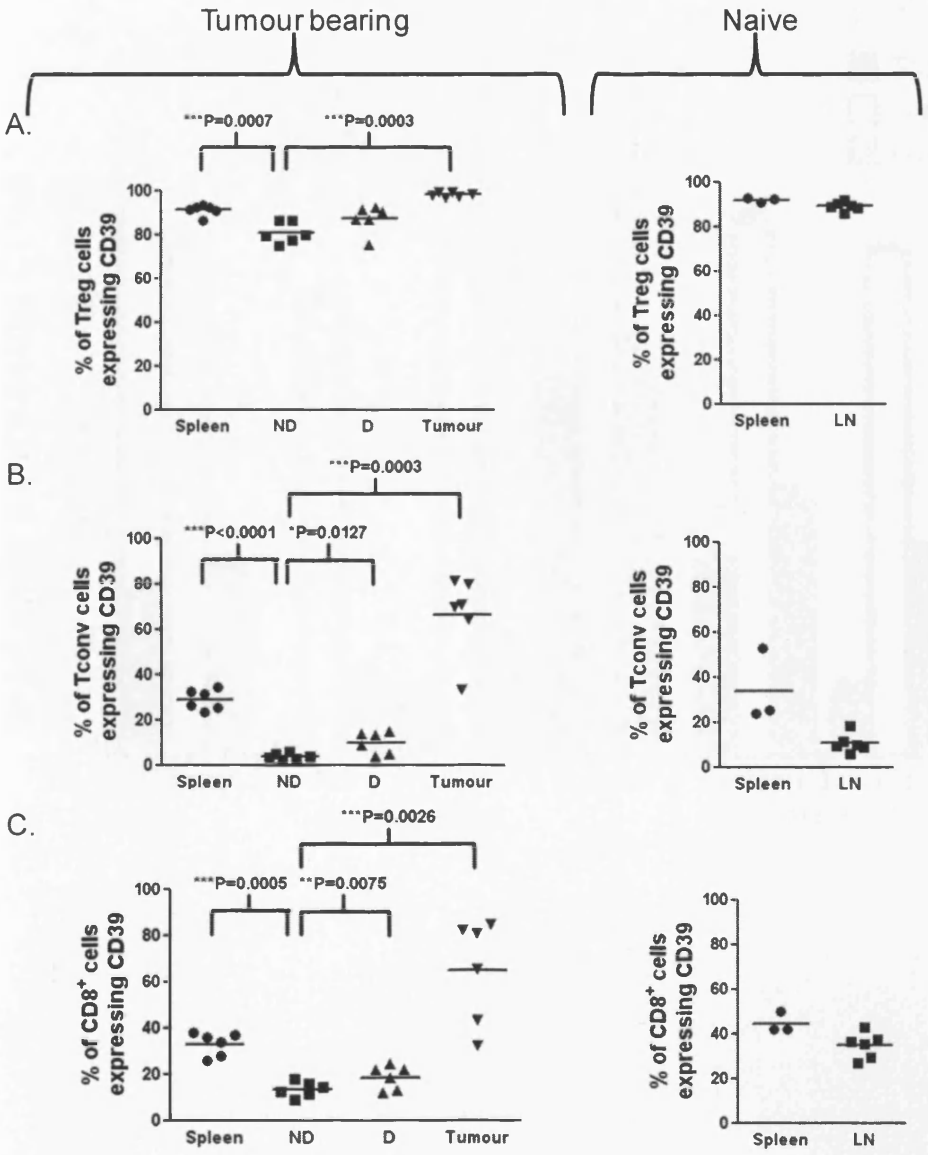


Figure 3.19. Percent of Tconv, Treg and CD8$^+$ cells expressing CD39 in tumour bearing and naïve mice.

The percentage of Treg (A), Tconv (B) and CD8$^+$ (C) cells expressing CD39 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=6). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

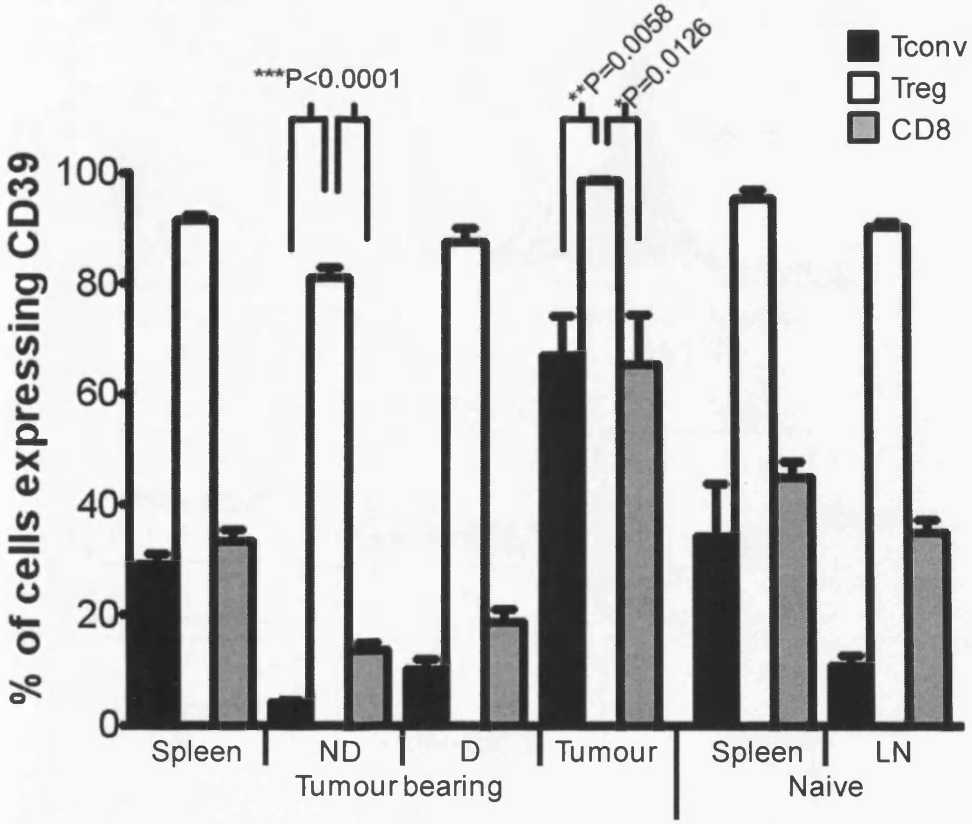


Figure 3.20. Percent of Tconv, Treg and CD8$^+$ cells expressing CD39 in tumour bearing and naïve mice.

The percentage (mean +/-SEM) of Tconv (black bars), Treg (white bars) and CD8$^+$ (grey bars) cells expressing CD39 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=6). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

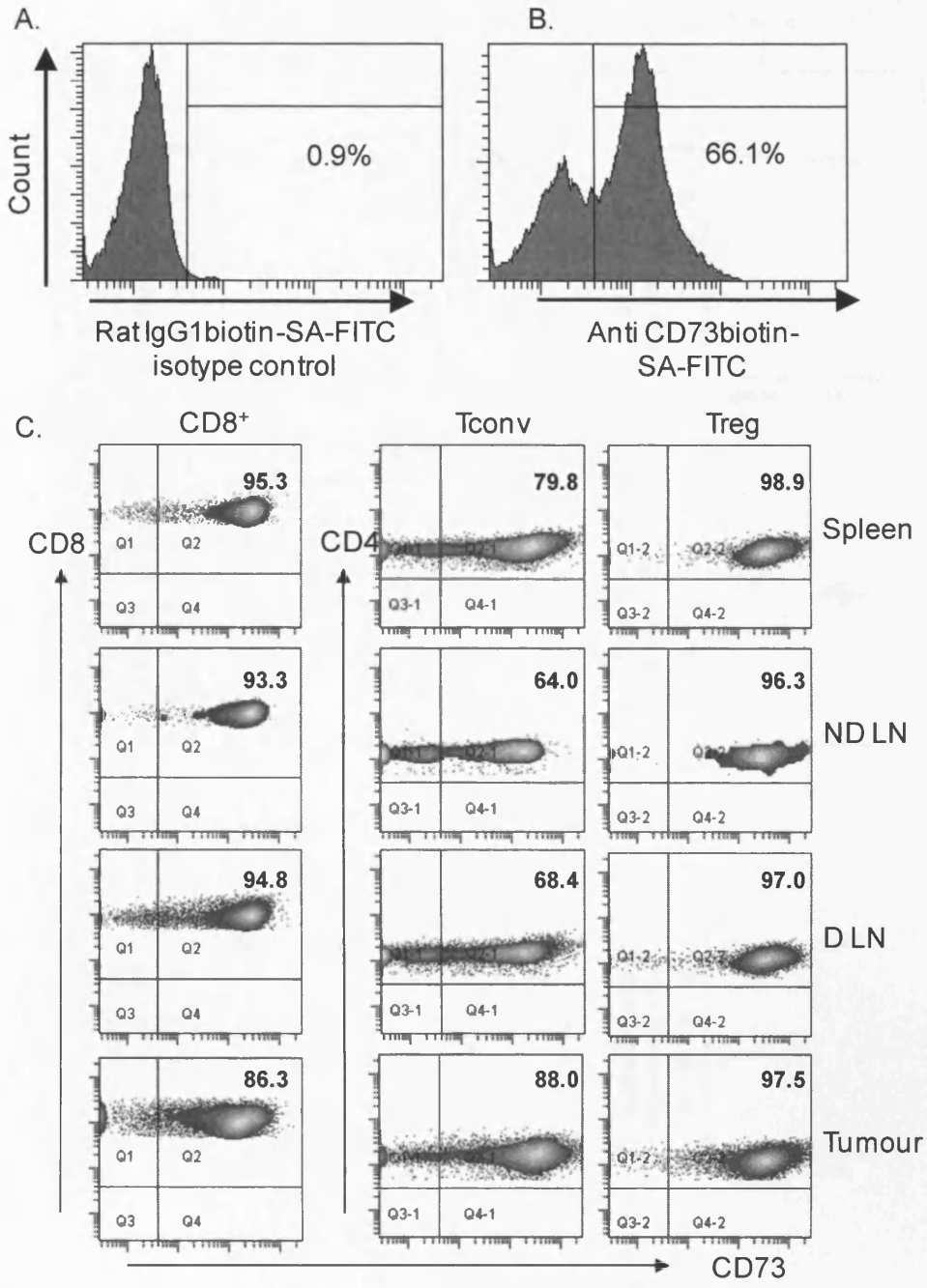


Figure 3.21. Antibody staining and analysis by flow cytometry of CD73.
Single cell suspensions of spleen, non-draining lymph node (ND LN), draining lymph node (D LN) and tumour were analysed for CD73 expression by flow cytometry. Cells were stained with anti CD73-biotin (B) or rat IgG1-biotin isotype control (A) mAbs. Representative dot plots of $CD8^+$ T cells, $CD4^+Foxp3^-$ Tconv cells and $CD4^+Foxp3^+$ Treg cells from each tissue are shown (C).

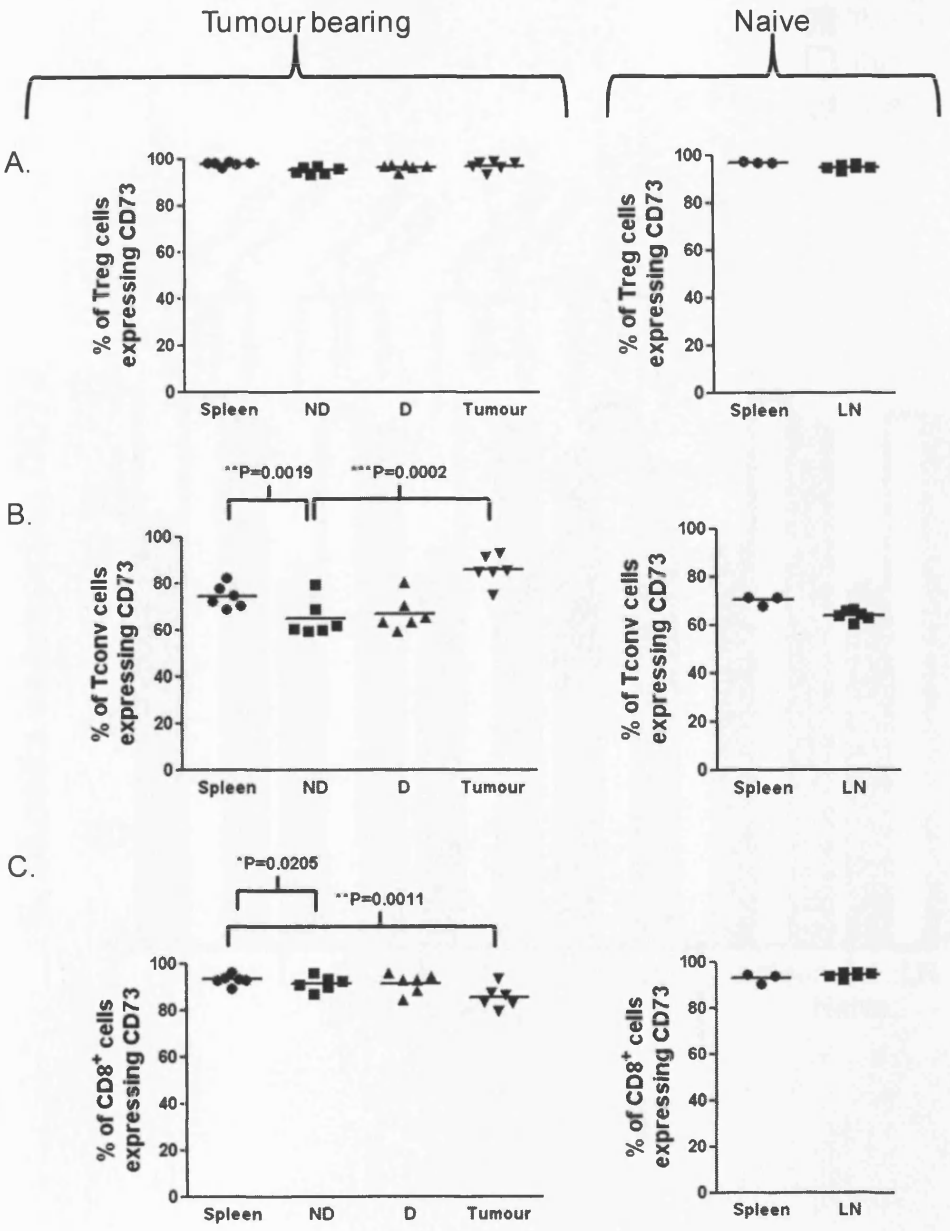


Figure 3.22. Percent of Tconv, Treg and CD8$^+$ cells expressing CD73 in tumour bearing and naïve mice.

The percentage of Treg (A), Tconv (B) and CD8$^+$ (C) cells expressing CD73 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=6). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

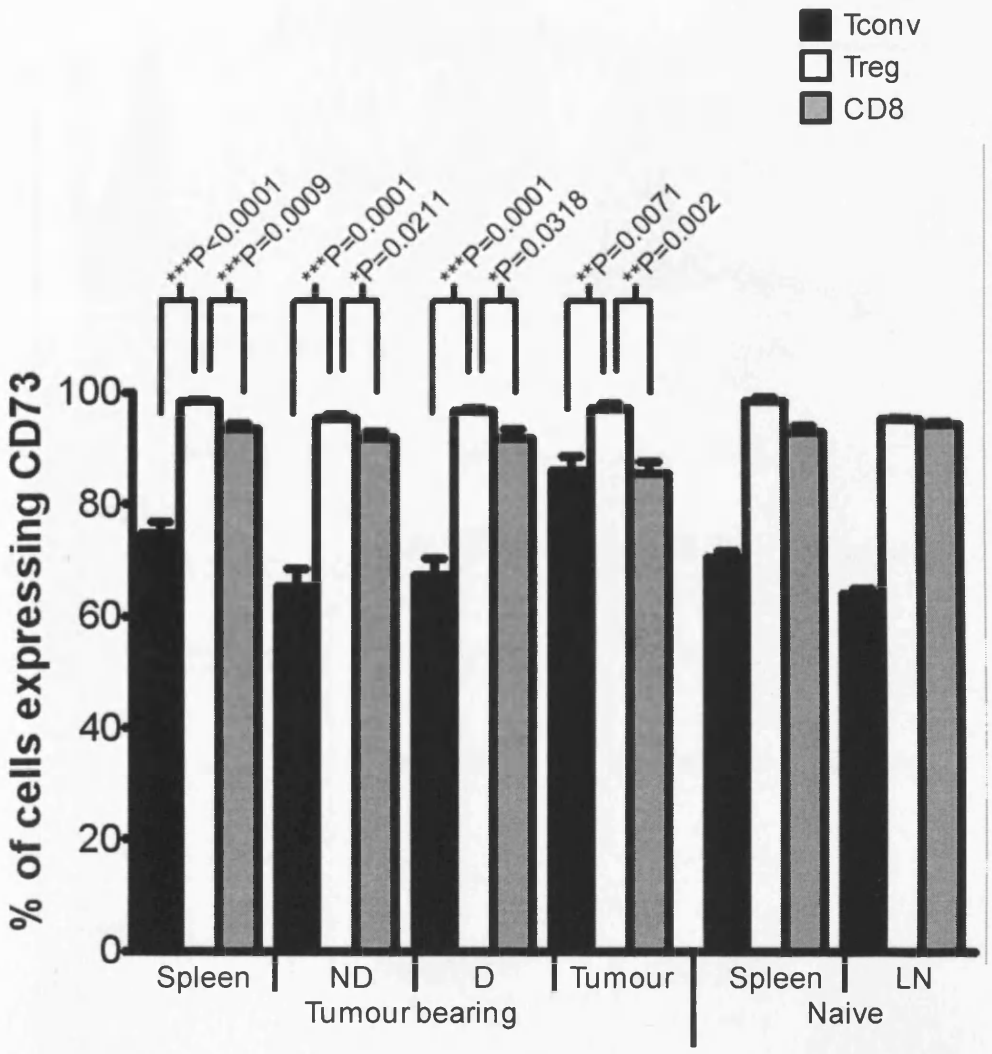


Figure 3.23. Percent of Tconv, Treg and CD8$^+$ cells expressing CD73 in tumour bearing and naïve mice.

The percentage (mean +/-SEM) of Tconv (black bars), Treg (white bars) and CD8$^+$ (grey bars) cells expressing CD73 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (n=6). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

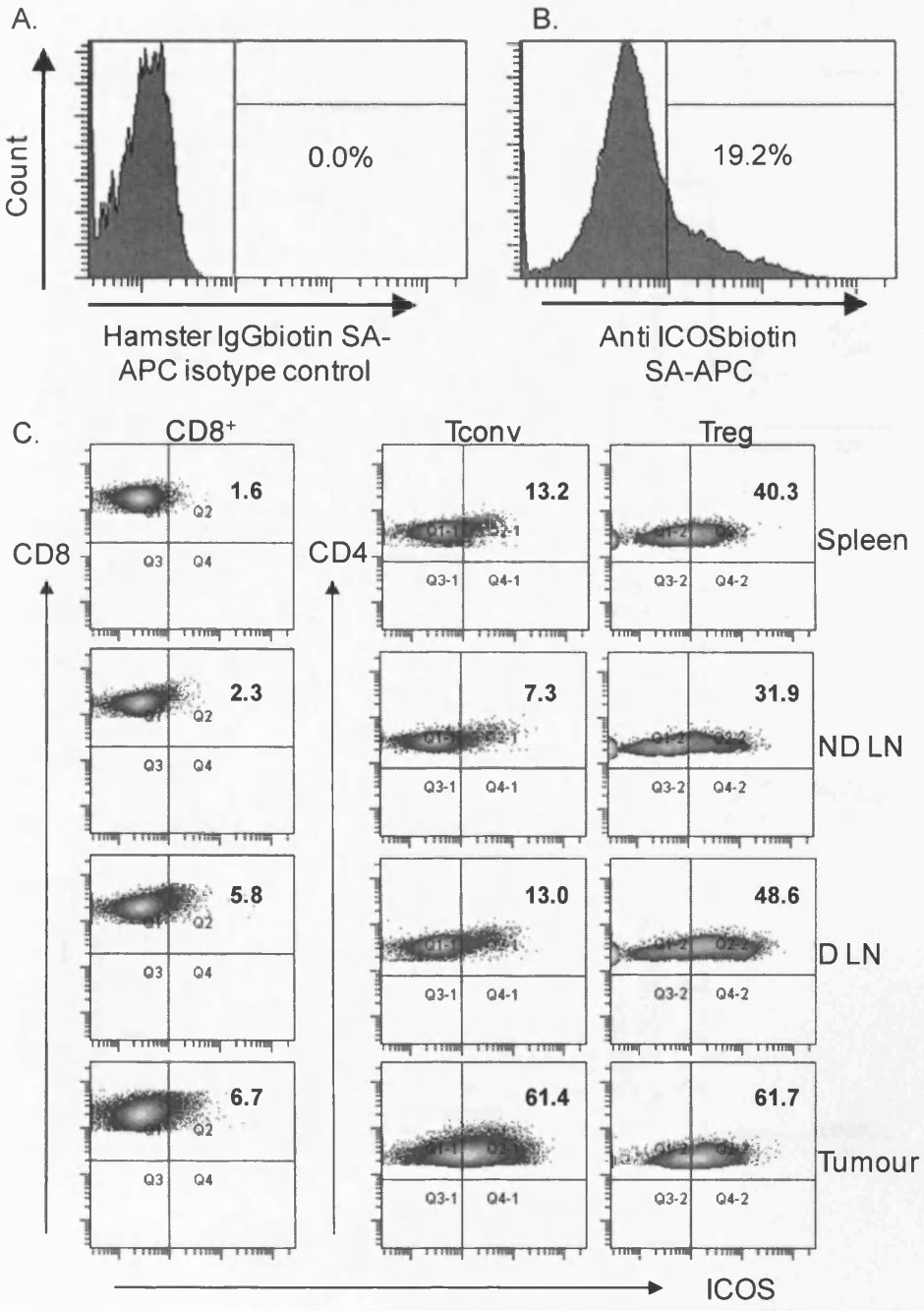


Figure 3.24. Antibody staining and analysis by flow cytometry of ICOS.
Single cell suspensions of spleen, non-draining lymph node (ND LN), draining lymph node (D LN) and tumour were analysed for ICOS expression by flow cytometry. Cells were stained with anti ICOS-biotin (B) or HamsterIgG-biotin isotype control (A) mAbs. Representative dot plots of $CD8^+$ T cells, $CD4^+Foxp3^-$ Tconv cells and $CD4^+Foxp3^+$ Treg cells from each tissue are shown (C).

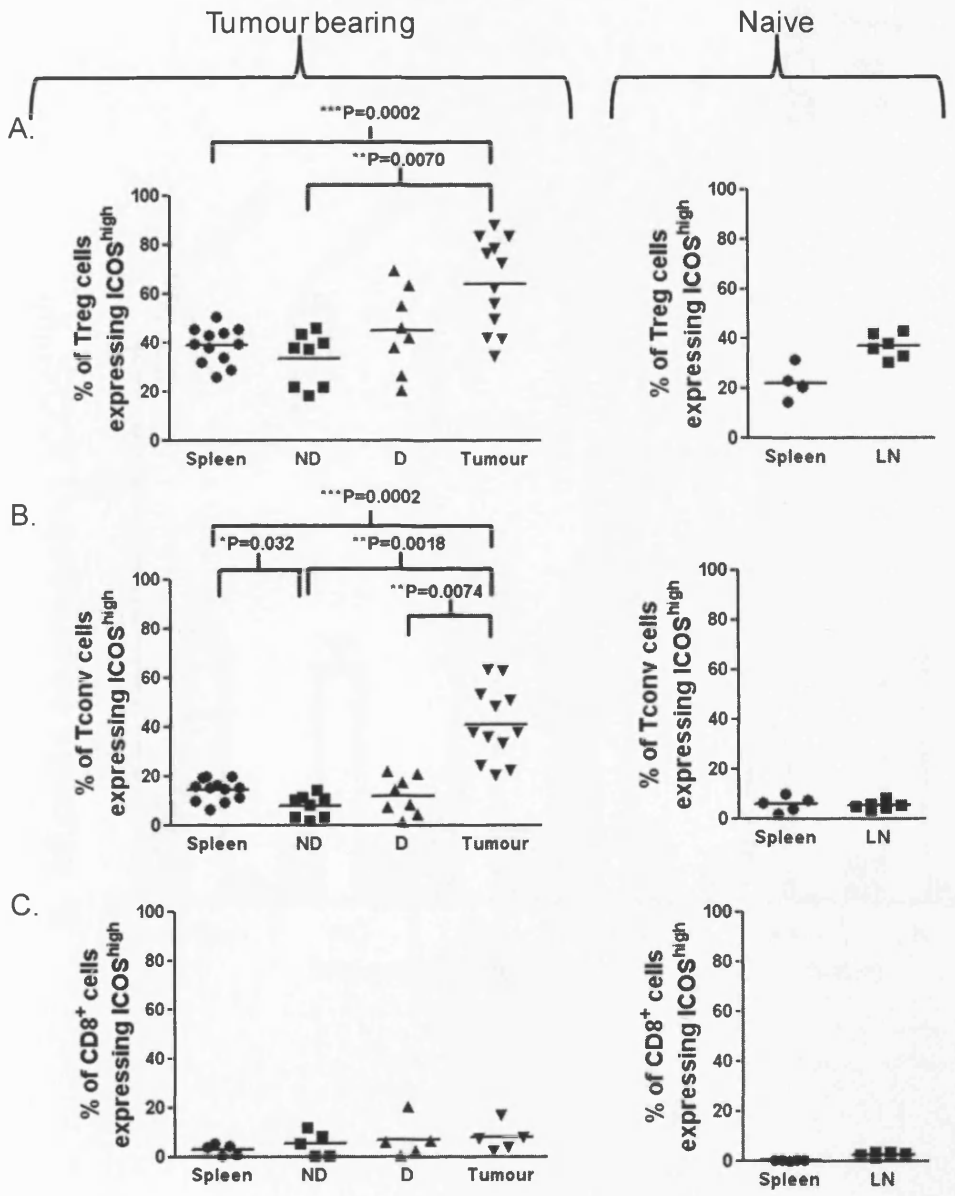


Figure 3.25. Percent of Tconv, Treg and CD8$^+$ cells expressing ICOS^{high} in tumour bearing and naïve mice.

The percentage of Treg (A), Tconv (B) and CD8$^+$ (C) cells expressing ICOS^{high} was determined by flow cytometry. Spleens (n=5-12), non-draining lymph nodes (ND, n=5-8), draining lymph nodes (D, n=5-8) and tumour (n=5-12) were analysed from tumour bearing mice . Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=5-6). The differences between the means were compared using paired T tests.

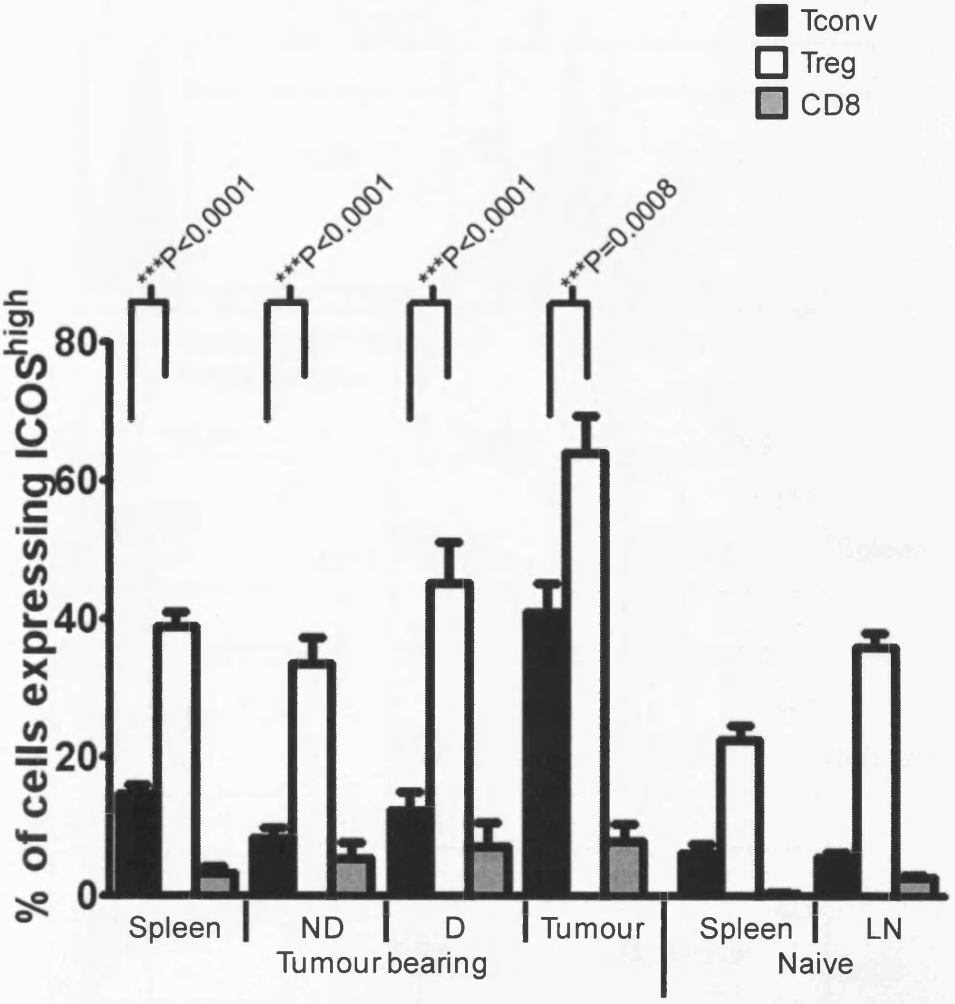


Figure 3.26. Percent of Tconv, Treg and CD8$^+$ cells expressing ICOS^{high} in tumour bearing and naïve mice.

The percentage (mean +/-SEM) of Tconv (black bars), Treg (white bars) and CD8$^+$ (grey bars) cells expressing ICOS^{high} was determined by flow cytometry. Spleens (n=5-12), non-draining lymph nodes (ND n=5-8), draining lymph nodes (D n=5-8) and tumour (n=5-12) were analysed from tumour bearing mice . Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=5-6). The differences between the means were compared using paired T tests.

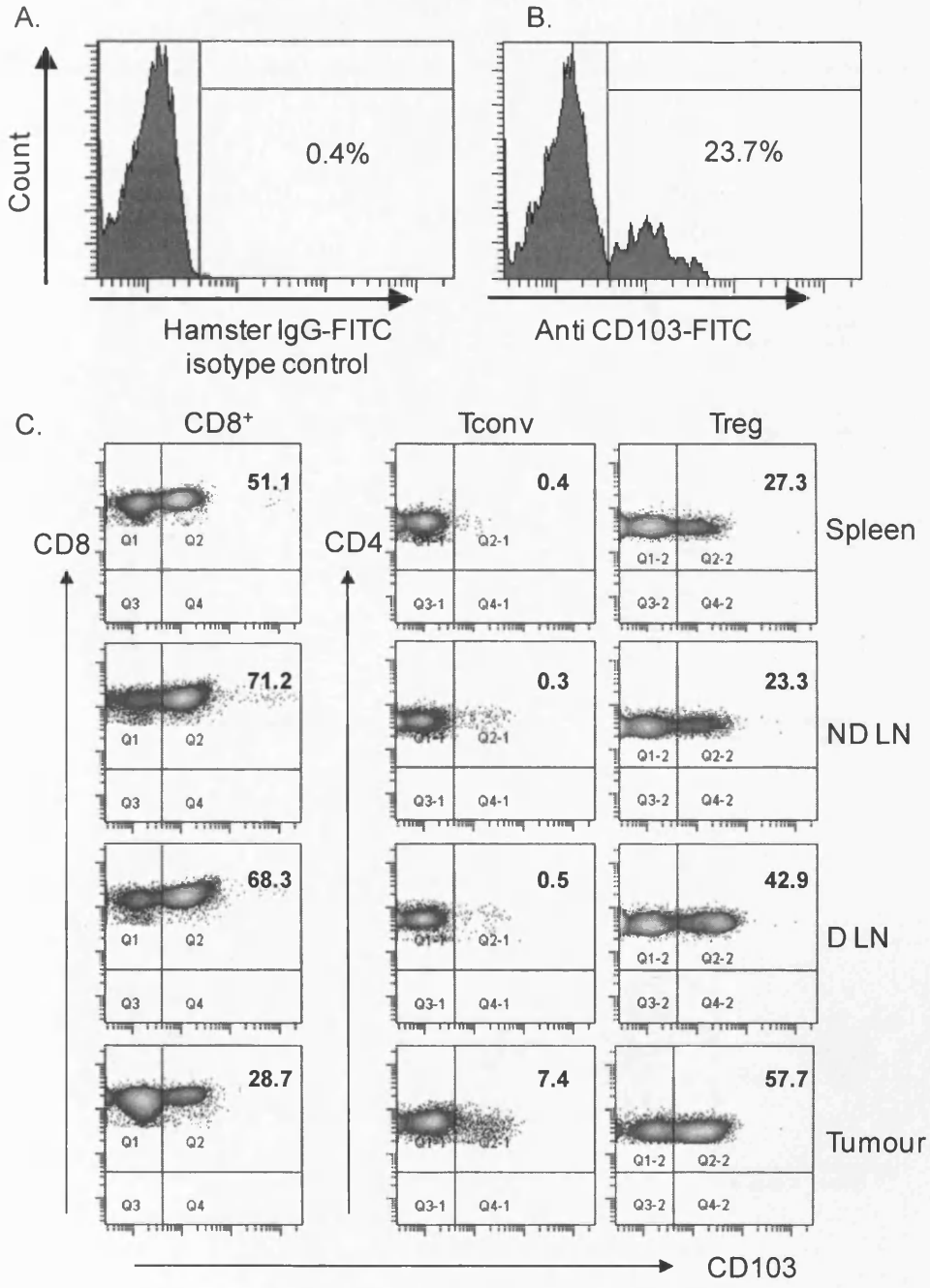


Figure 3.27. Antibody staining and analysis by flow cytometry of CD103.
Single cell suspensions of spleen, non-draining lymph node (ND LN), draining lymph node (D LN) and tumour were analysed for CD103 expression by flow cytometry. Cells were stained with anti CD103-FITC (B) or Hamster IgG-FITC isotype control (A) mAbs. Representative dot plots of $CD8^+$ T cells, $CD4^+Foxp3^-$ Tconv cells and $CD4^+Foxp3^+$ Treg cells from each tissue are shown (C).

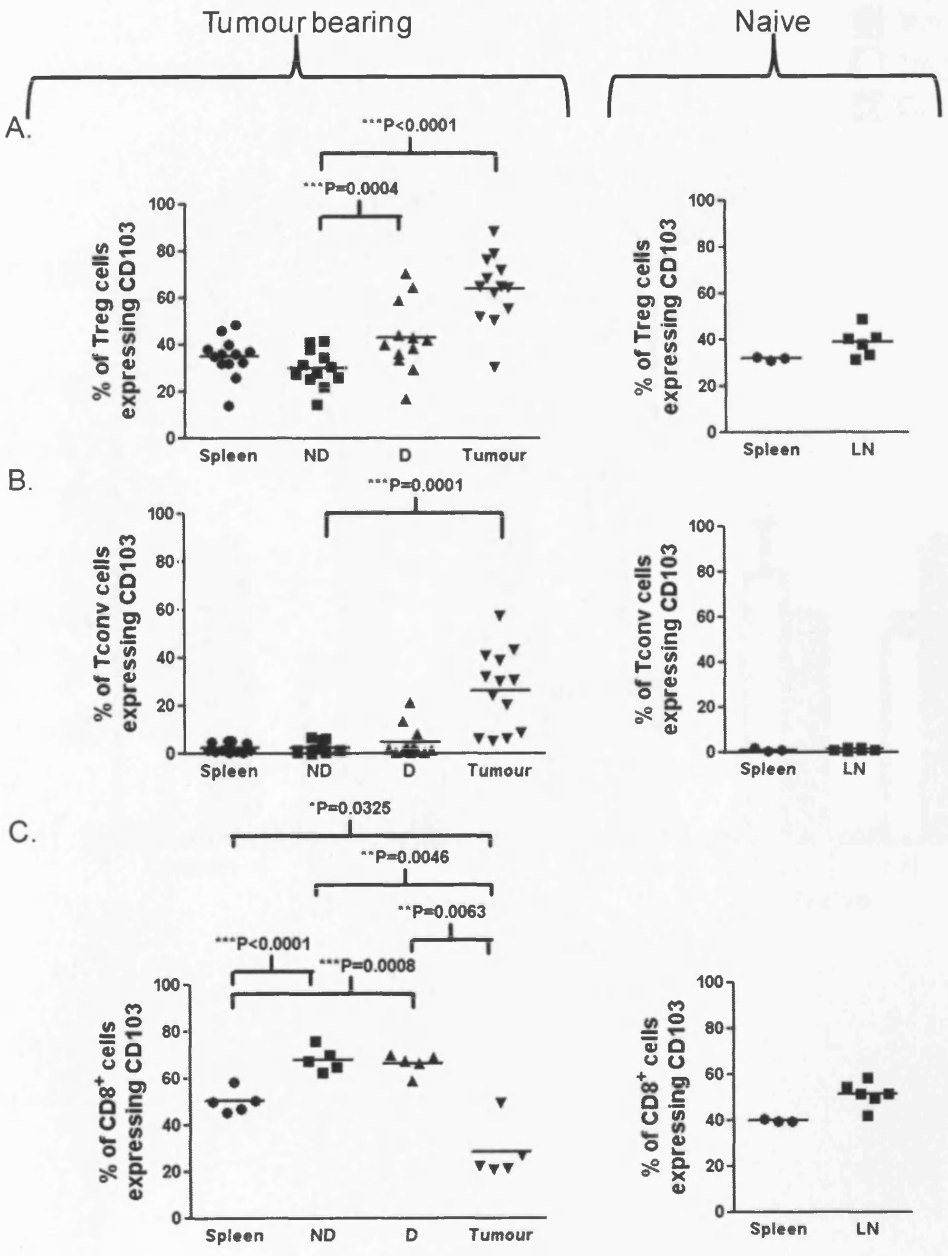


Figure 3.28. Percent of Tconv, Treg and CD8$^+$ cells expressing CD103 in tumour bearing and naïve mice.

The percentage of Treg (A), Tconv (B) and CD8$^+$ (C) cells expressing CD103 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (Tconv and Treg n=13, CD8$^+$ cells n=5). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

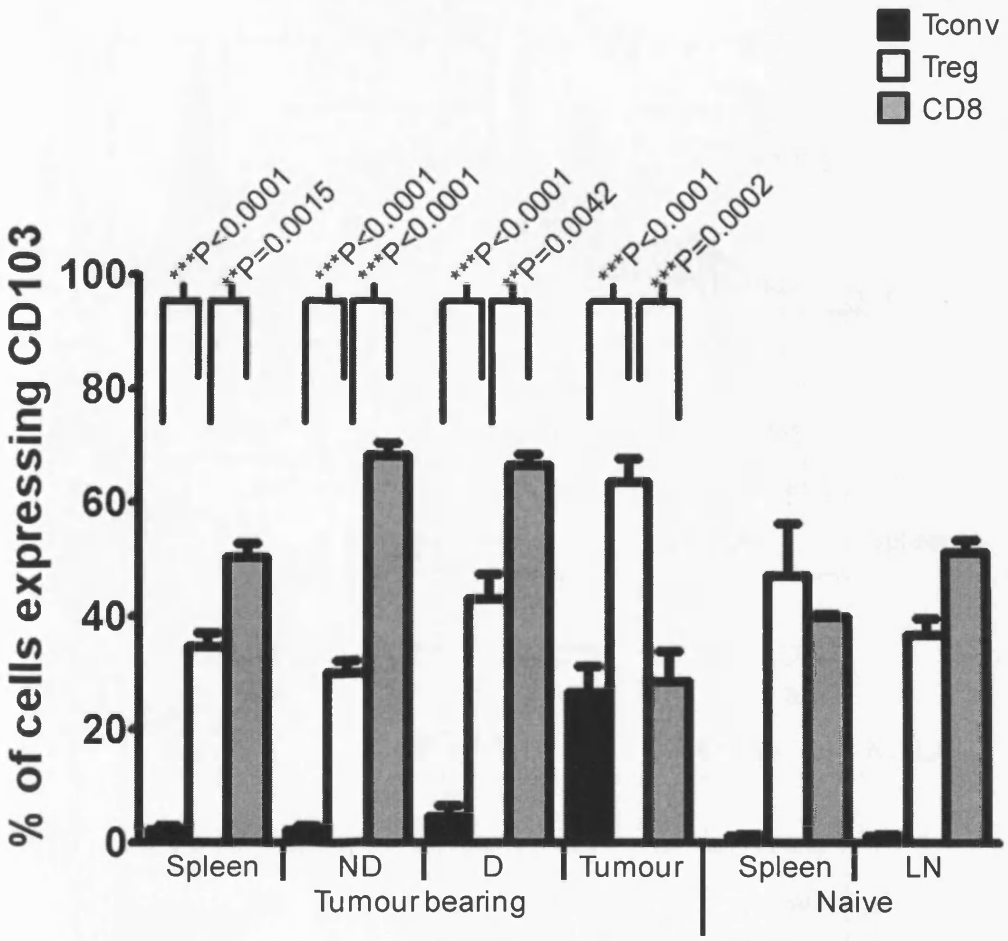


Figure 3.29. Percent of Tconv, Treg and CD8$^+$ cells expressing CD103 in tumour bearing and naïve mice.

The percentage (mean +/-SEM) of Tconv (black bars), Treg (white bars) and CD8$^+$ (grey bars) cells expressing CD103 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (Tconv and Treg n=13, CD8$^+$ cells n=5). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=3-6). The differences between the means were compared using paired T tests.

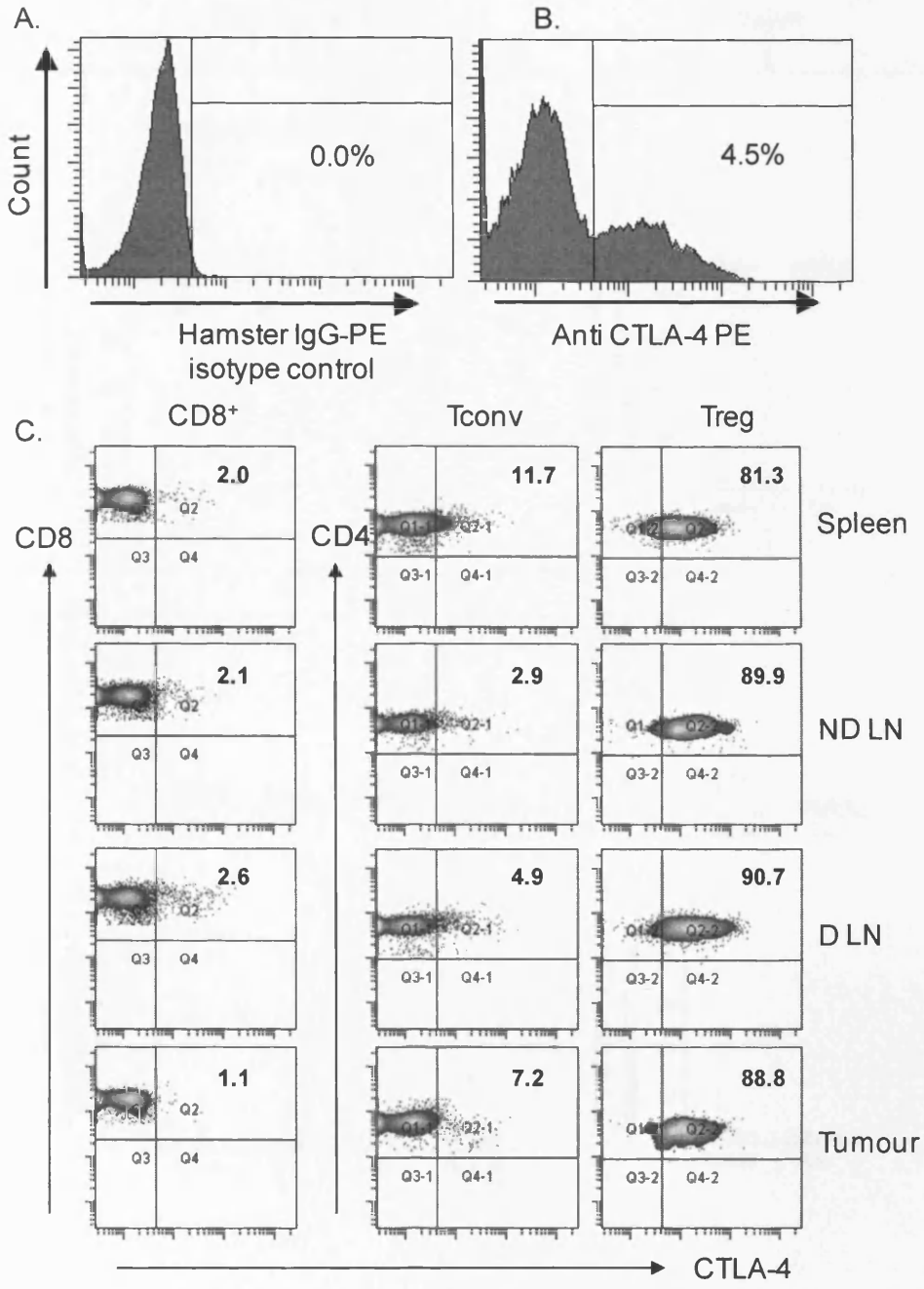


Figure 3.30. Antibody staining and analysis by flow cytometry of CTLA-4.
Single cell suspensions of spleen, non-draining lymph node (ND LN), draining lymph node (D LN) and tumour were analysed for CTLA-4 expression by flow cytometry. Cells were stained with anti CTLA-4-PE (B) or Hamster IgG-PE isotype control (A) mAbs. Representative dot plots of $CD8^+$ T cells, $CD4^+Foxp3^-$ Tconv cells and $CD4^+Foxp3^+$ Treg cells from each tissue are shown (C).

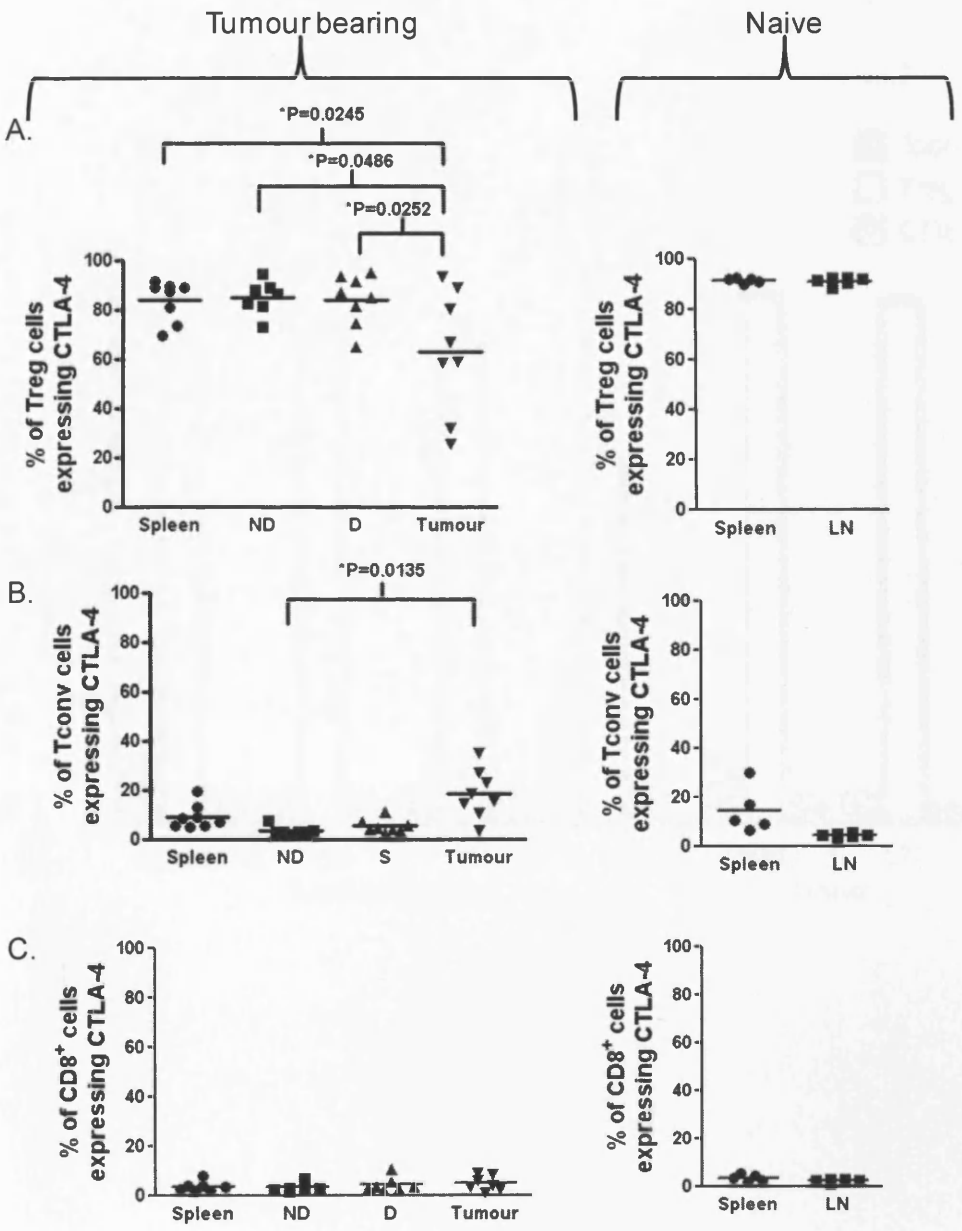


Figure 3.31. Percent of Tconv, Treg and CD8$^+$ cells expressing CTLA-4 in tumour bearing and naïve mice.

The percentage of Treg (A), Tconv (B) and CD8$^+$ (C) cells expressing CTLA-4 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (Tconv and Treg n=8, CD8$^+$ cells n=7). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=5-6). The differences between the means were compared using paired T tests.

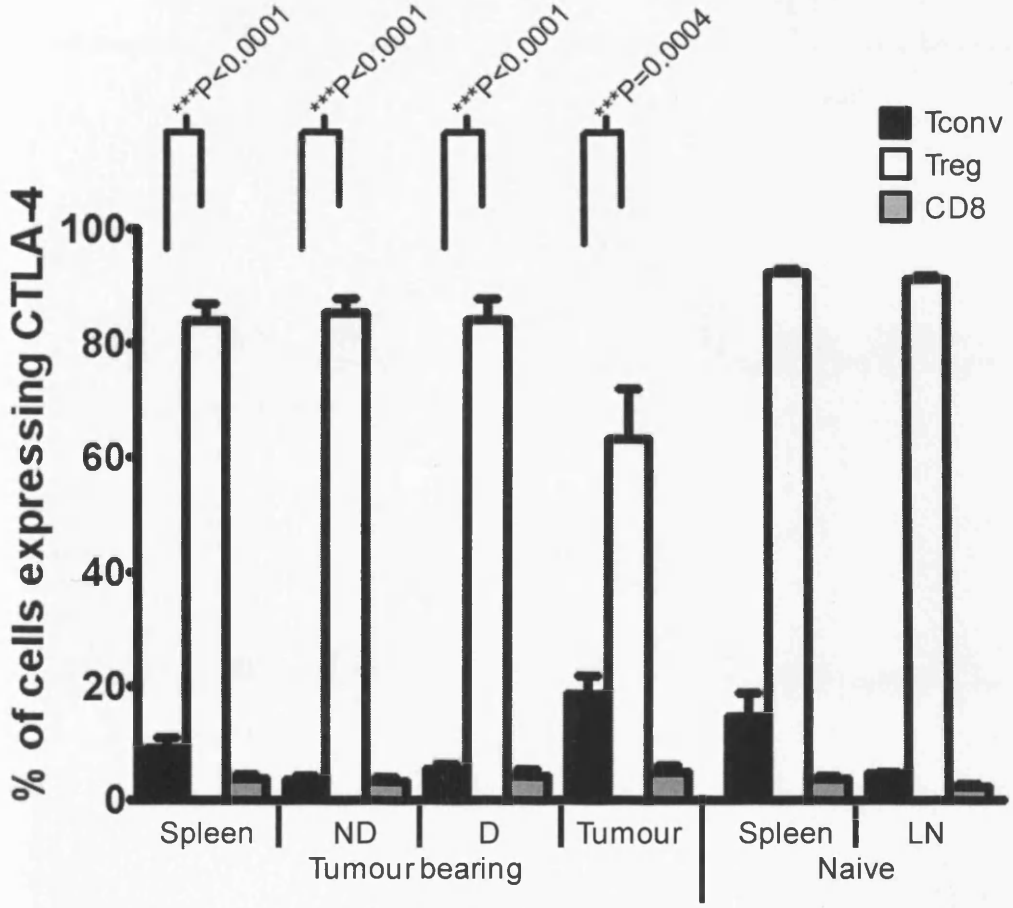


Figure 3.32. Percent of Tconv, Treg and CD8$^+$ cells expressing CTLA-4 in tumour bearing and naïve mice.
The percentage (mean +/-SEM) of Tconv (black bars), Treg (white bars) and CD8$^+$ (grey bars) cells expressing CTLA-4 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumour were analysed from tumour bearing mice (Tconv and Treg n=8, CD8$^+$ cells n=7). Spleen and pooled inguinal lymph nodes (LN) were analysed from naïve mice (n=5-6). The differences between the means were compared using paired T tests.

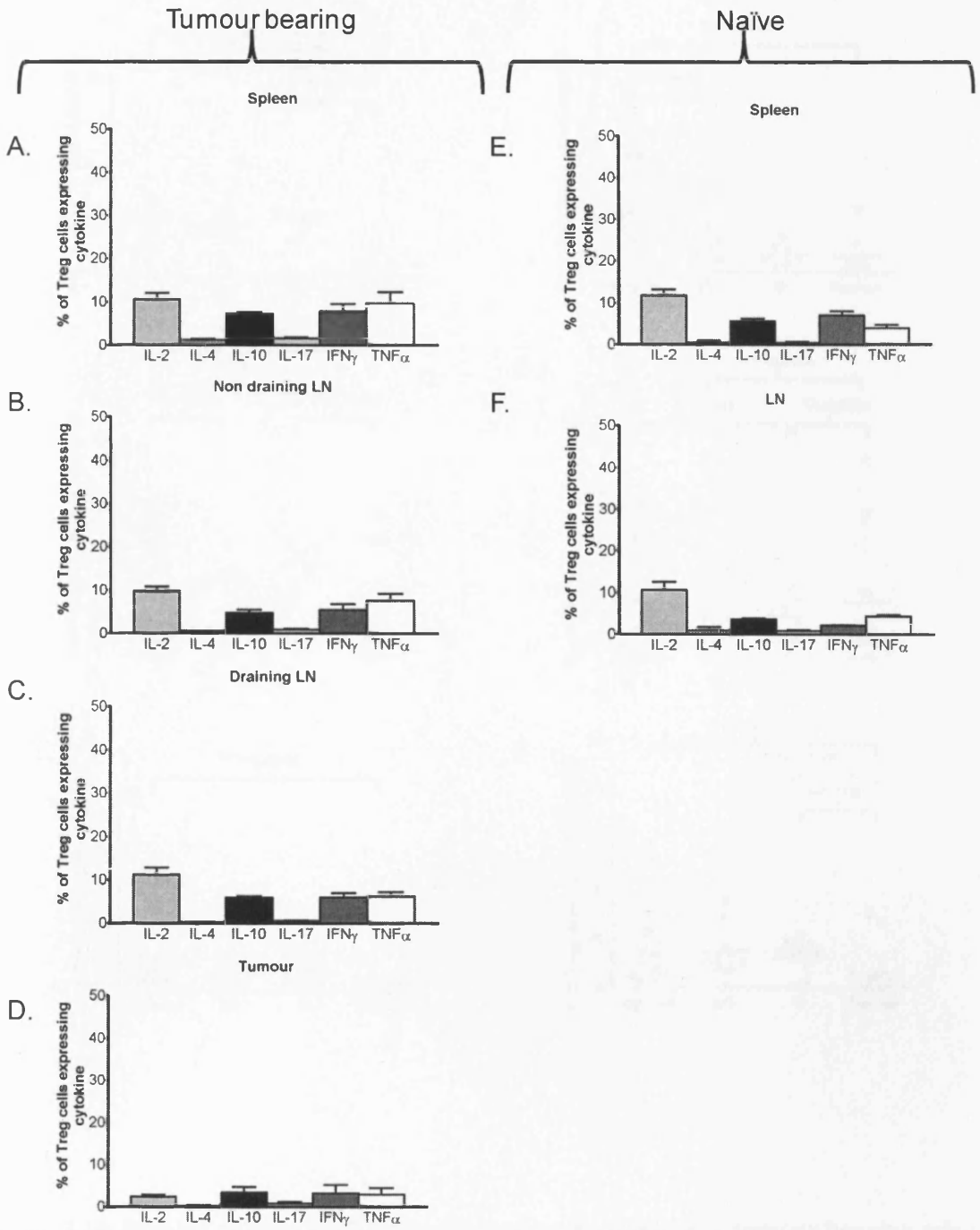


Figure 3.33. Percent of Treg cells expressing various cytokines in tumour bearing and naïve mice.

The percentage of PMA and iomomycin stimulated Treg cells expressing IL-2, IL-4, IL-10, IL-17, IFNγ, and TNFα were determined by flow cytometry. Samples from spleens, non-draining lymph nodes, draining lymph nodes and tumours of tumour bearing mice (n=7-12) and naïve mice (n=3) were analysed, The overall cytokine profile of Treg from spleens (A), ND lymph nodes (B), D lymph nodes (C), and tumours (D) of tumour bearing mice and spleens (E), and lymph nodes (F) of naïve mice are shown.

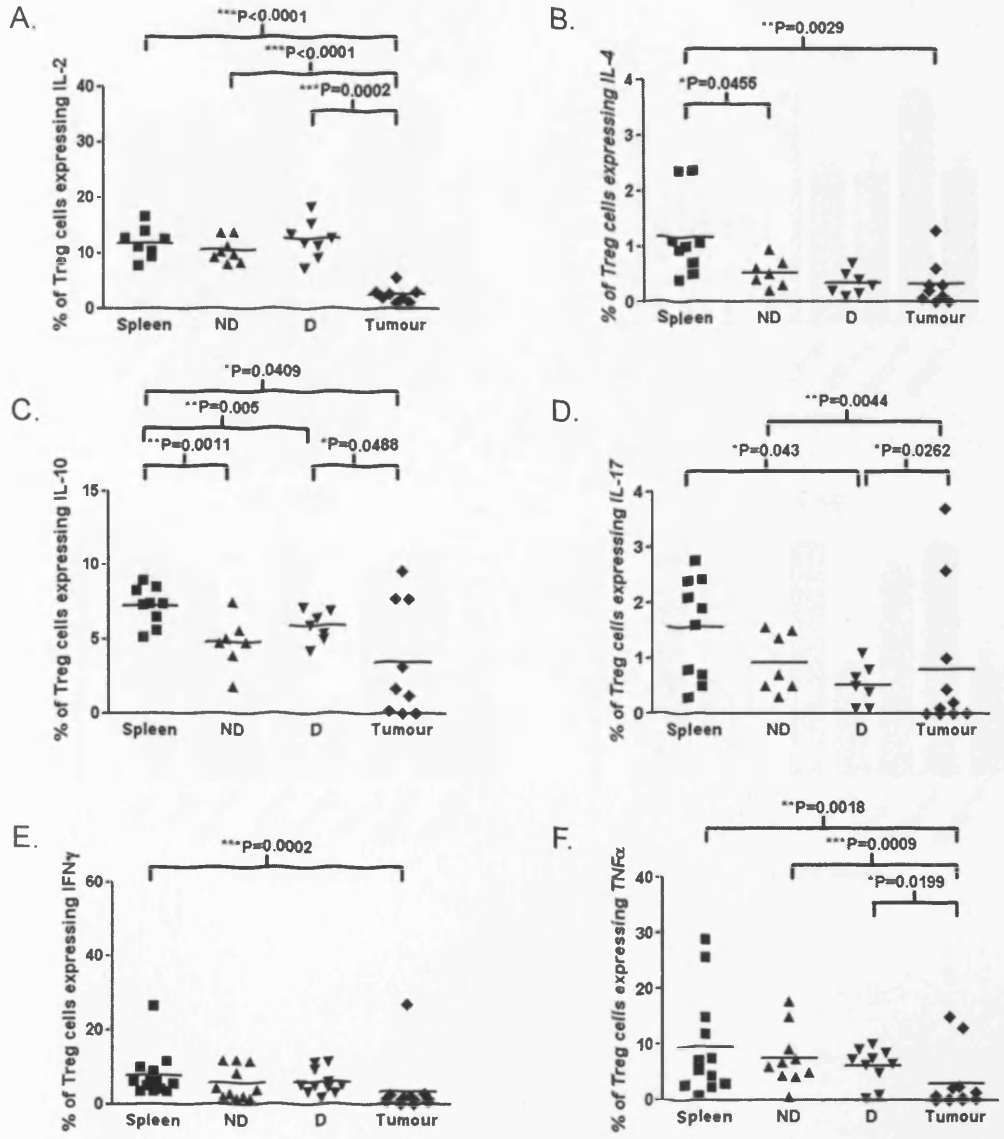


Figure 3.34. Percent of Treg cells expressing various cytokines in tumour bearing mice. The percentage of PMA and iomomycin stimulated Treg cells expressing IL-2, IL-4, IL-10, IL-17, IFNγ, and TNFα were determined by flow cytometry. Samples from spleens, non-draining lymph nodes, draining lymph nodes and tumours of tumour bearing mice (n=7-12) were analysed, The expression of IL-2 (A), IL-4 (B), IL-10 (C), IL-17 (D), IFNγ (E), and TNFα (F) within Treg cells from various locations of tumour bearing mice are compared. The mean percentages were compared using unpaired T tests.

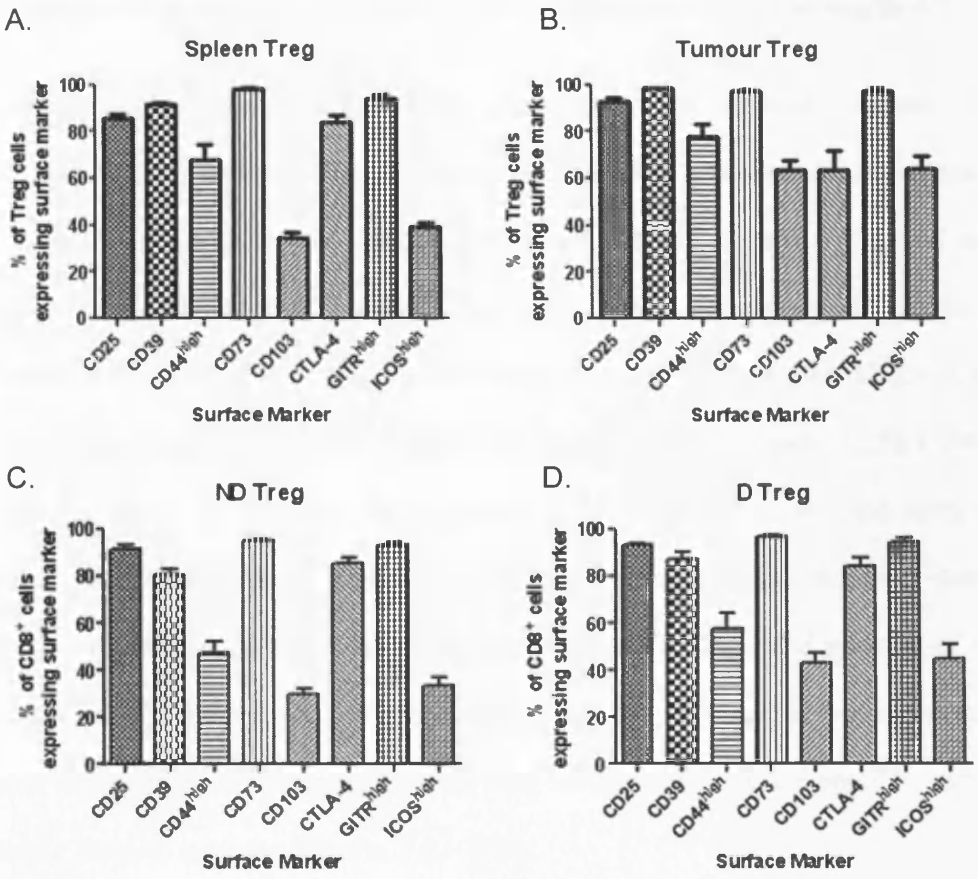


Figure 3.35. Overall phenotype of Treg cells in tumour bearing mice.
The percentage (mean +/-SEM) of Treg cells expressing the markers CD25, CD39, CD44, CD73, CD103, CTLA-4, GITR and ICOS were determined by flow cytometry. The overall phenotype of these cells from the spleens (A), tumours (B), non-draining (C) and draining (D) lymph nodes of tumour bearing mice (n=5-12) are shown.

3.2.4 Do tumour infiltrating Tconv and $CD8^+$ T cells express activation markers?

Many of the markers described above for phenotyping Treg cells are associated with activation of T cells. Tconv and $CD8^+$ T cells were stained with these markers in parallel to Tregs. It was therefore possible to gain some insight into the activation status of tumour infiltrating Tconv cells and $CD8^+$ T cells. Tconv and $CD8^+$ T cells from tumour bearing mice were stained with the following panel of activation markers: CD25, CD44, CTLA-4, GITR and ICOS (Figure 3.13, Figure 3.9, Figure 3.31, Figure 3.16 and Figure 3.25). CD44 and GITR are thought to be constitutively expressed at low levels in T cells and upregulated following TCR activation hence high expression levels of these molecules were examined (Puré and Cuff 2001, Nocentini *et al.* 1997, Shimizu *et al.* 2002). The expression of all the activation markers assessed was elevated significantly on Tconv cells within the tumour compared to control nodes implying that the cells are activated and are possibly part of an anti-tumour immune response (Figures 3.9 – 3.31).

The increase in expression of all activation markers was not observed however, with $CD8^+$ cells. Whilst the percentage of $CD8^+$ T cells expressing CD25, $CD44^{high}$ and $GITR^{high}$ were significantly greater in the tumour compared to non-draining lymph nodes (Figure 3.13, Figure 3.9,Figure 3.16), CTLA-4 and ICOS expression did not change (Figure 3.31 and Figure 3.25). Overall, as for the analysis of Tconv cells, these data imply that significantly more $CD8^+$ T cells within the tumour (compared to lymph node) are activated and possibly part of an anti-tumour immune response (Figure 3.36 and Figure 3.37).

Cytokine production by the same T cell subsets was assessed by intracellular cytokine staining. As shown in Figures 3.38 – 3.41, very little IL-10, IL-4 or IL-17 was detected in

both Tconv cells and $CD8^+$ T cells from the lymph node nodes and tumour. Similar levels of TNFα were detected in Tconv and $CD8^+$ T cells throughout the tumour bearing mice. Interestingly, more $IFN\gamma^+$Tconv cells and $IFN\gamma^+CD8^+$ T cells were detected in the tumour and tumour draining lymph node compared to the non-draining lymph node (Figure 3.39E and 3.41E), suggesting tumour associated Tconv and $CD8^+$ T cells are activated. However, contrary to this, significantly less detectable IL-2 was found in Tconv and $CD8^+$ T cells from the tumour compared to the lymph nodes (Figure 3.39A and 3.41A).

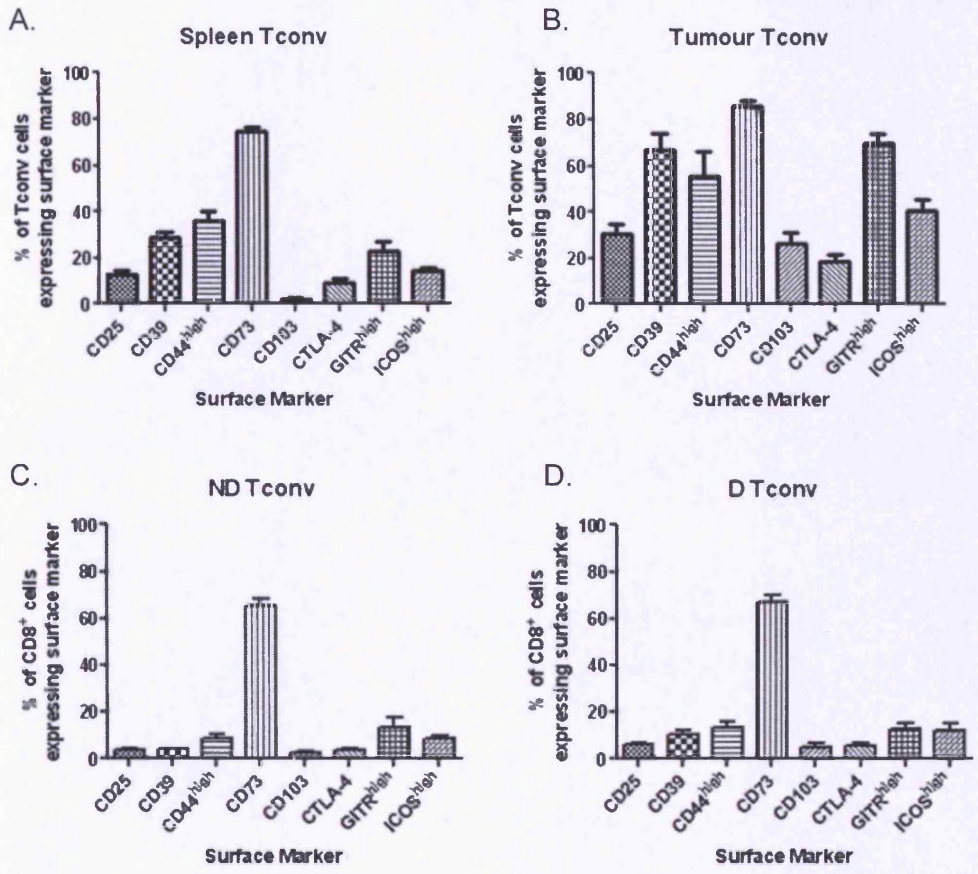


Figure 3.36. Overall phenotype of Tconv cells in tumour bearing mice.
The percentage (mean +/-SEM) of Tconv cells expressing the markers CD25, CD39, CD44, CD73, CD103, CTLA-4, GITR and ICOS was determined by flow cytometry. The overall phenotype of these cells from the spleens (A), tumours (B), non-draining (C) and draining (D) lymph nodes of tumour bearing mice (n=5-12) are shown.

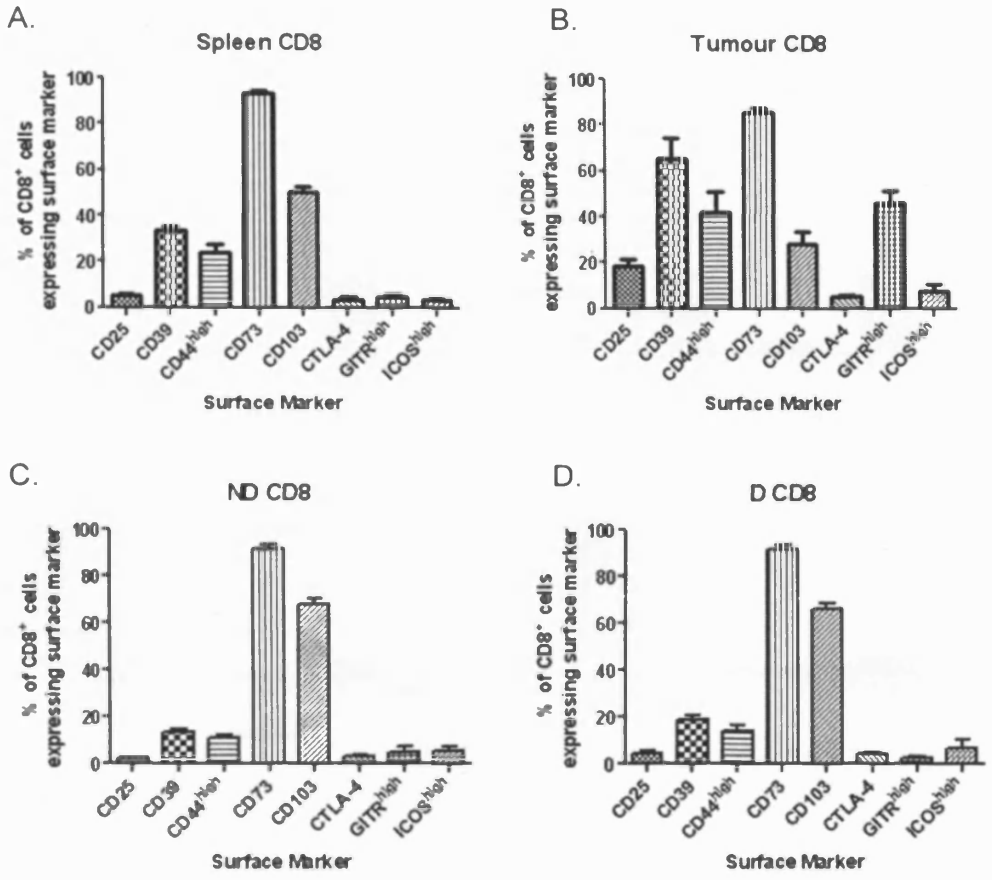


Figure 3.37. Overall phenotype of CD8$^+$ T cells in tumour bearing mice.
The percentage (mean +/-SEM) of CD8$^+$ T cells expressing the markers CD25, CD39, CD44, CD73, CD103, CTLA-4, GITR and ICOS was determined by flow cytometry. The overall phenotype of these cells from the spleens (A), tumours (B), non-draining (C) and draining (D) lymph nodes of tumour bearing mice (n=5-12) are shown.

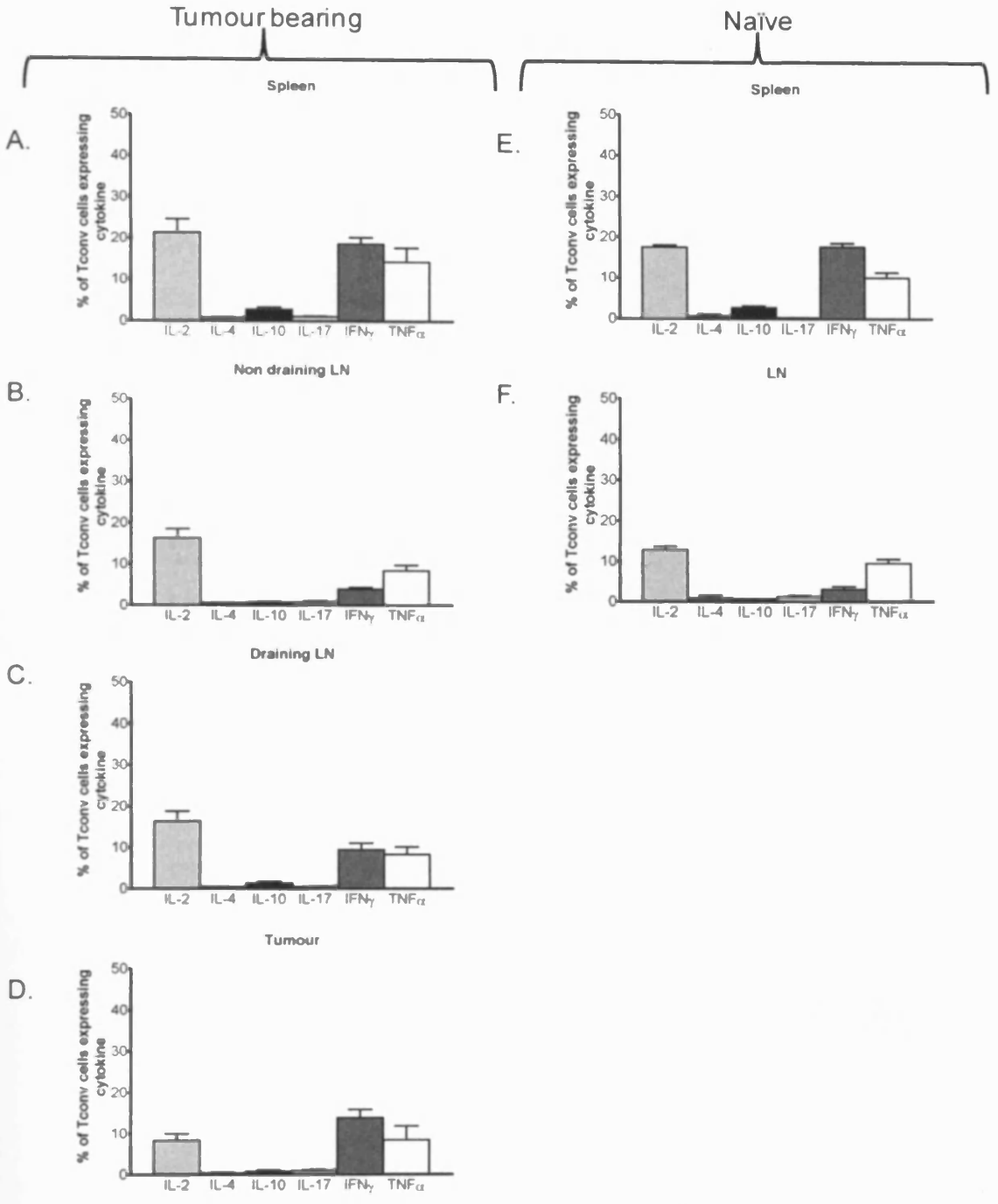


Figure 3.38. Percent of Tconv cells expressing various cytokines in tumour bearing and naïve mice.

The percentage of PMA and iomomycin stimulated Tconv cells expressing IL-2, IL-4, IL-10, IL-17, IFNγ, and TNFα were determined by flow cytometry. Samples from spleens, non-draining lymph nodes, draining lymph nodes and tumours of tumour bearing mice (n=8-13) and naïve mice (n=3) were analysed, The overall cytokine profile of Tconv from spleen (A), ND lymph node (B), D lymph node (C), and tumour (D) of tumour bearing mice and spleens (E), and lymph nodes (F) of naïve mice are shown.

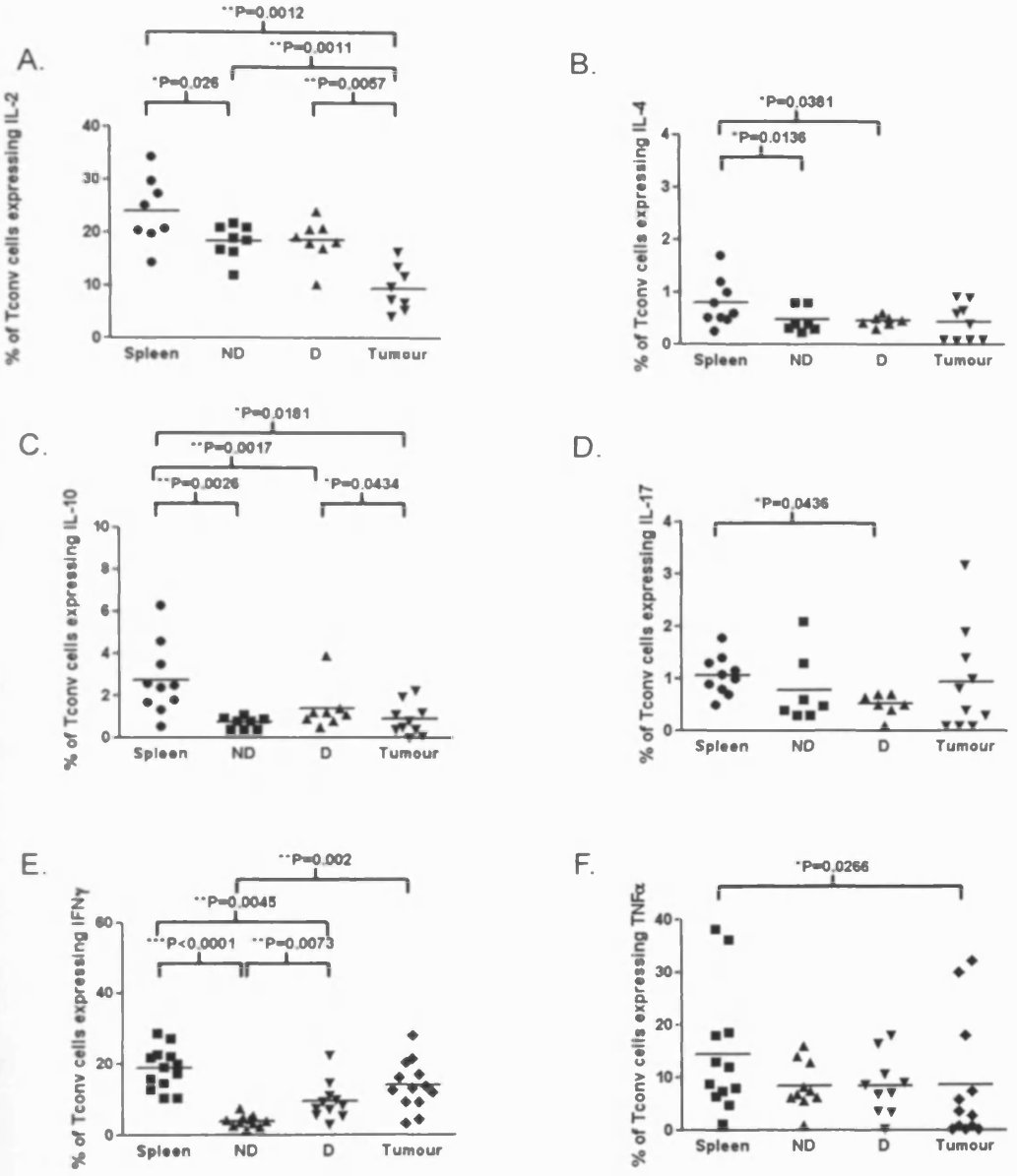


Figure 3.39. Percent of Tconv expressing various cytokines in tumour bearing mice. The percentage of PMA and iomomycin stimulated Tconv cells expressing IL-2, IL-4, IL-10, IL-17, IFNγ, and TNFα were determined by flow cytometry. Samples from spleens, non-draining lymph nodes, draining lymph nodes and tumours of tumour bearing mice (n=8-13) were analysed, The expression of IL-2 (A), IL-4 (B), IL-10 (C), IL-17 (D), IFNγ (E), and TNFα (F) within Tconv cells from various locations of tumour bearing mice are compared. The mean percentages were compared using unpaired T tests.

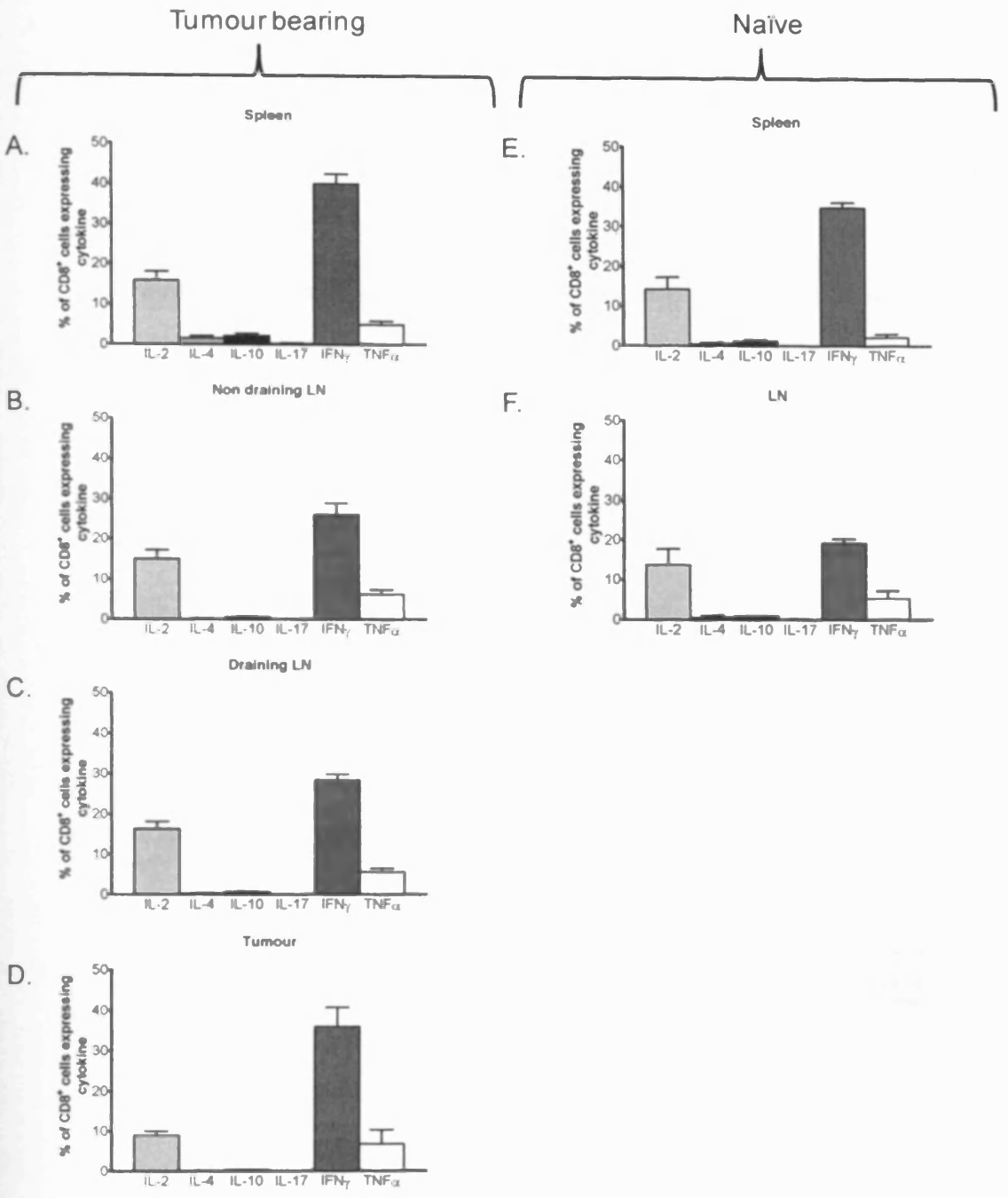


Figure 3.40. Percent of CD8$^+$ T cells expressing various cytokines in tumour bearing and naïve mice.

The percentage of PMA and iomomycin stimulated CD8$^+$ cells expressing IL-2, IL-4, IL-10, IL-17, IFNγ, and TNFα were determined by flow cytometry. Samples from spleens, non-draining lymph nodes, draining lymph nodes and tumours of tumour bearing mice (n=5-8) and naïve mice (n=3) were analysed, The overall cytokine profile of CD8$^+$ cells from spleens (A), ND lymph nodes (B), D lymph nodes (C), and tumours (D) of tumour bearing mice and spleens (E), and lymph nodes (F) of naïve mice are shown.

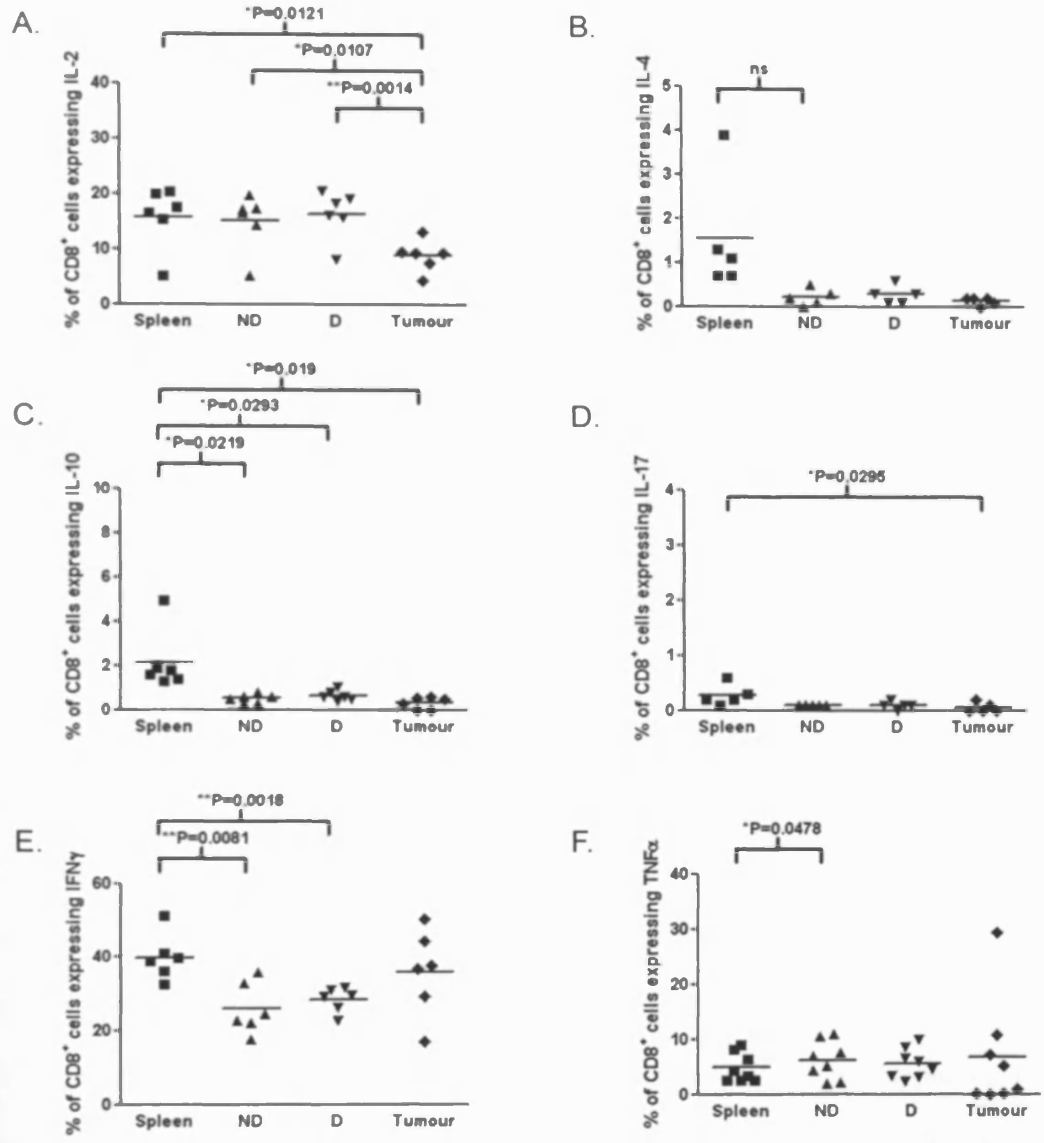


Figure 3.41. Percent of CD8$^+$ T cells expressing various cytokines in tumour bearing mice.

The percentage of PMA and iomomycin stimulated CD8$^+$ T cells expressing IL-2, IL-4, IL-10, IL-17, IFNγ, and TNFα were determined by flow cytometry. Samples from spleens, non-draining lymph nodes, draining lymph nodes and tumours of tumour bearing mice (n=5-8) were analysed. The expression of IL-2 (A), IL-4 (B), IL-10 (C), IL-17 (D), IFNγ (E), and TNFα (F) within CD8$^+$ T cells from various locations of tumour bearing mice are compared. The mean percentages were compared using unpaired T tests.

3.3 Discussion

In this Chapter a phenotypic and functional characterisation of tumour infiltrating T cells was performed. The following four questions were addressed:

- Do $CD4^+$ and $CD8^+$ cells control tumour development following Treg depletion?
- Do the ratios of Tconv:Treg and CD8:Treg alter in the tumour compared to lymphoid tissue?
- Do tumour infiltrating Treg cells display a phenotype associated with immunosupression?
- Do tumour infiltrating Tconv and $CD8^+$ T cells express activation markers?

To address whether $CD4^+$ and $CD8^+$ T cells control tumour development following Treg depletion, CD4 and CD8 specific Abs were given to mice that were earlier injected with CD25 specific Abs and MCA. These experiments revealed that $CD8^+$ cells but not $CD4^+$ cells were vital for the control of tumour development following Treg depletion. The same question has been addressed in other mouse tumour models. Early experiments by Robert North and co-workers in the 1980s demonstrated that removal of a $CD4^+$ suppressor T cell population resulted in spontaneous regression of the lymphoma cell line L5178Y and that this rejection was mediated primarily by $CD8^+$ T cells (Awwad and North 1988). Following the identification of CD25 as a marker for Treg cells Onizuka *et al.* (1999) reported that depletion of $CD25^+$ cells resulted in $CD8^+$ (and in some cases $CD4^+$) T cell dependant control of a variety of tumour cell lines in mice. Subsequently, Shimizu *et al.* (1999) showed that tumour specific $CD8^+$ T cell responses (as well as NK-like responses) were generated in mice inoculated with tumour cells after depletion of $CD25^+$ cells. A number of groups confirmed these findings and showed that $CD8^+$ and $CD4^+$ T cell mediated immunity developed in mice

that rejected tumour cells after depletion of $CD25^+$ cells (Sutmuller *et al.* 2001, Jones *et al.* 2002, Casares *et al.* 2003, Chen *et al.* 2005). Together these findings demonstrate that both $CD8^+$ and $CD4^+$ T cell responses are important in the control of tumours following Treg depletion. The dependence on $CD8^+$ T cells for the control of tumour growth in the experiments described in this chapter are therefore consistent with many previous reports (Awwad and North 1988, Shimizu *et al.* 1999, Sutmuller *et al.* 2001, Casares *et al.* 2003, Chen *et al.* 2005). However, although it is clear that in tumours a direct anti-tumour effect of $CD4^+$ T cells is also important, the experiments in this Chapter indicate this is not the case for MCA tumours. The reasons for the differences between various mouse tumour models are not clear.

$CD8^+$ and $CD4^+$ cells were also depleted in Treg-replete (control Ab treated) mice. Tumour incidence was not further increased by anti-CD8 nor anti-CD4 Ab treatment suggesting that $CD8^+$ and $CD4^+$ T cells are not controlling tumour growth in the ~13% of mice that remain tumour free without Treg depletion. Furthermore the depletion of $CD8^+$ cells following Treg depletion did not reduce tumour incidence beyond ~13%, suggesting that $CD8^+$ cells are not involved in controlling tumour growth in these mice. These findings suggest that in the fraction of mice that remain tumour free, the lack of tumour is not due to an anti-tumour T cell response. At first this may appear to be in contrast to recent findings from Robert Schreiber's laboratory where mice apparently tumour free after 200 days were given a mixture of Abs against CD4, CD8 and IFNγ (Koebel *et al.* 2007). Following anti-CD4/CD8/IFNγ Ab treatment but not control Ab treatment tumour outgrowth occurred. This indicated the immune system can restrain cancer growth for extended periods of time. In apparently tumour free mice occult tumours were kept in a state of equilibrium with the anti-tumour action of the immune system. However, the outgrowth of tumours did not occur in all

mice treated with the mixture of Abs. Only 9 out of 15 (60%) mice developed palpable tumours, thus 40% of mice remained tumour free after anti-CD4/CD8/IFNγ Ab treatment. Therefore, in similarity to the experiments in this Chapter, the results from Koebel *et al.* indicated that in a large proportion of mice that remained tumour free the immune system was not involved in tumour control. Two possible scenarios could account for the lack of tumours in these mice. Firstly, the MCA carcinogen simply did not cause a tumour to arise at any point. Alternatively, an unobserved tumour arose and was completely eliminated prior to the experimental depletion of the immune system.

Under normal physiological circumstances Treg cells prevent autoimmune and inflammatory disease (Sakaguchi *et al.* 1995, Kim *et al.* 2007). This is thought to involve the suppression of $CD4^+$Tconv and $CD8^+$ T cells (Thornton and Shevach 1998, Kim *et al.* 2007, Mempel *et al.* 2006). The suppressive effect of Treg cells on Tconv and $CD8^+$ T cells has been shown to be titratable *in vitro* (Thornton and Shevach 1998, Fontenot *et al.* 2005b), therefore an accumulation of Tregs within tumour tissue compared to lymphoid tissue may indicate that the impact of Tregs on suppression of T cell responses is more pronounced at this site. Using flow cytometry the proportions of Tconv:Treg and CD8:Treg were compared in tumour and lymphoid tissue. Significant differences in the ratios of Tconv:Treg and CD8:Treg were found in the tumour compared to the lymph node implying that $CD8^+$ T cells and Tconv are more likely to be suppressed by Tregs in the tumour than in the lymph node. The difference between the CD8:Treg ratio was even more prominent when only the antigen experienced fraction of $CD8^+$ T cells were considered. However, the trend was not the same for Tconv cells. Together this supports the hypothesis that within the tumour Treg cells become dominant favouring immunosuppression. In order to fully establish whether tumour infiltrating Treg cells are suppressive during tumour development more studies are clearly

necessary. A functional analysis of these cells, for example an *in vitro* suppression assay may help to further support this hypothesis. Preliminary experiments utilising transgenic mice (Foxp3_DTR) expressing the diphtheria toxin receptor on $Foxp3^+$ cells have been carried out to specifically deplete $Foxp3^+$ cells in MCA tumour bearing mice. These experiments have suggested that Treg depletion can slow tumour growth (my own unpublished data).

As mentioned above the Tconv:Treg ratio and particularly the CD8:Treg ratio were altered in the tumour tissue compared to lymphoid tissue. However, an additional observation was made which showed an interesting link between the Treg and CD8 cell populations. An increase in the proportion of T cells that were Treg cells was matched by a decrease in the proportion of T cells that were $CD8^+$ T cells. When these two values were plotted against each other a clear correlation between the $CD8^+$ T cells and Treg cells was observed (***$P<0.0001$; Figure 3.42A). In contrast, no correlation between Tconv and Treg cells was observed (Figure 3.42B). It was noticed that generally tumour tissue (blue circles) contained a relatively high proportion of Treg cells and low proportion of $CD8^+$ T cells which is in contrast to non-draining lymph node (green circles) which generally contained a high proportion of $CD8^+$ T cells and low proportion of Treg cells. This finding suggests that a reciprocal relationship between Treg cells and $CD8^+$ T cells exists within tumour bearing mice. This may be due to Treg cells preventing the expansion of $CD8^+$ T cells. The findings of the depletion experiments above also implicated a relationship between $CD8^+$ T cells and Tregs in tumour bearing mice suggesting that Treg cells normally inhibit $CD8^+$ T cells which can prevent tumour development.

In agreement with the finding above, Sato *et al.* (2005) found that the ratio of $CD8^+$ T cells:Treg correlated with survival in epithelial ovarian carcinoma patients. High

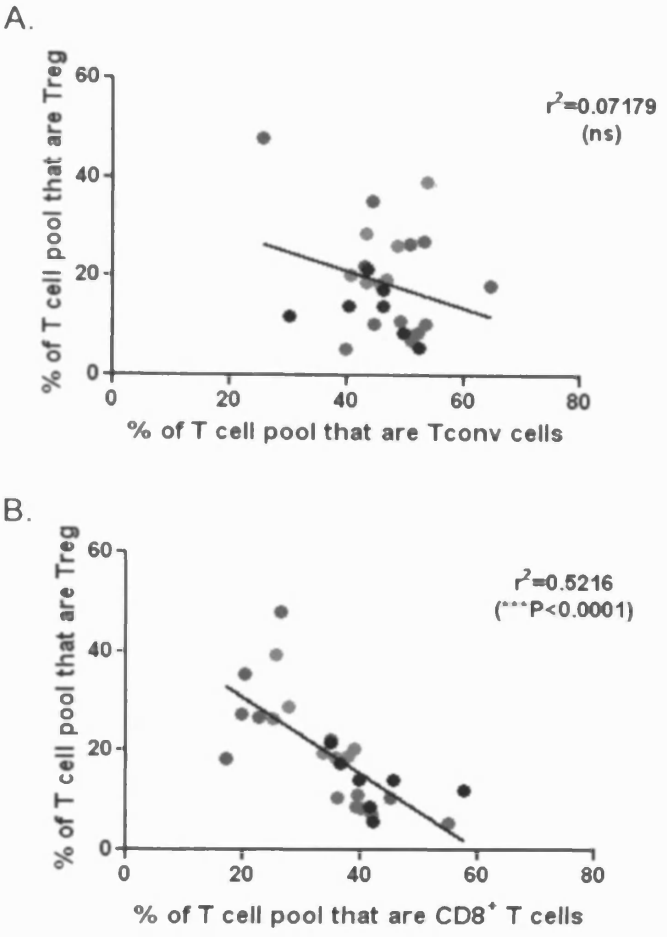


Figure 3.42. Correlation between percentage of Treg cells and other T cells.
The proportion of the T cell pool made up by each subset (Treg, Tconv and and $CD8^+$ T cells) were determined by flow cytometry. The percentage of the T cell pool made up by Treg (y axis) was plotted against the percentage of the T cell pool made up by either Tconv cells (A) or CD8+ T cells (B; y axis). Samples from spleens (red), tumours (blue), non-draining lymph nodes (green) and draining lymph nodes (black) of tumour bearing mice were plotted. The r^2 value from the Pearson correlation coefficient was determined.

intratumoural $CD8^+$ T cell:Treg ratio correlated with favourable prognosis. Two recentreports also assessed the association between T cells, Tregs and patient outcome in patients with colorectal tumours. Sinicrope *et al.* (2009) revealed that a low intraepithelial $CD3^+$:$Foxp3^+$ cell ratio was associated with shorter patient survival time. Furthermore, Salama *et al.* (2009) investigated $CD8^+$ T cells and $Foxp3^+$ T cells in normal and tumour tissue from colorectal cancer patients and found that although the ratio of $CD8^+$ T cell:Treg showed no significance in relation to patient outcome, a high density of $CD8^+$ T cells was associated with good patient outcome and a high density of $Foxp3^+$ cells in normal tissue was associated with significantly worse outcome. However, they also found that a high density of $Foxp3^+$ cells in tumour tissue was actually associated with good patient outcome. Thus although these studies suggest $Foxp3^+$ Tregs cells can be associated with poor prognosis, in particular when in conjunction with a low $CD8^+$ T cell numbers the precise location and function of the $Foxp3^+$ cell infiltration may also be significant in predicting patient outcome. Although in this thesis the $CD8^+$ T cell:Treg ratio was not compared to outcome or survival time, the correlation between $CD8^+$ T cells and Treg cells throughout tumour bearing mice suggests that the proportions of these two cell types may alter to establish an immunosuppressive environment within tumour bearing mice. In particular, in tumour tissue relatively high Treg numbers are associated with low CD8 numbers and therefore potentially an immunosuppressive environment.

From the analysis of tumour infiltrating lymphocyte populations it was noticed that a large proportion of the lymphocyte population was not made up of $CD4^+$ T cells or $CD8^+$ T cells. Thus other cells expand and dominate the lymphocyte pool in tumours and also tumour-draining lymph nodes. An initial analysis of tumour bearing mice by flow cytometry with a panel of Abs against other lymphocyte markers suggested that these cells are made up

primarily by B cells and also NK cells (Appendix Figure A2). However, their role in MCA tumour development remains to be fully understood.

The expression of numerous markers has been associated with the suppressive capacity of Treg cells. To assess whether these markers were upregulated within tumour infiltrating T cells a detailed phenotypic analysis of Treg cells from tumour bearing mice was performed. This revealed that tumour infiltrating Treg cells display a phenotype associated with immunosuppression (Being $CD25^{+}$, $CD44^{high}$, $CD39^{+}$, $CD73^{+}$, $CD103^{+}$, CTLA-4^{+}, $ICOS^{high}$, $GITR^{high}$; Figure 3.35). For most of the markers analysed the percentage of Treg cells expressing that marker either remained high in all tissues tested or was significantly higher in the tumour compared to the lymph node. Studies where Treg cells are depleted *in vivo* have revealed that Treg cells are continuously suppressing autoimmune and inflammatory responses in the periphery (Kim *et al.* 2007). The findings in this Chapter suggest that within the tumour, Treg cells are equal, if not better equipped to suppress immune responses than Treg cells in lymphoid tissue. Further investigations are now necessary to identify which of these markers are important for the function of Treg cells in tumours.

Although it is not known which mechanism Treg cells use for suppressing immune responses within tumours, the results in this Chapter indicate that many of the purported mechanisms remain possible. CD25 has been linked with Treg activity through its ability to bind to IL-2 and act as an 'IL-2 sink' (Thornton and Shevach 1998, de la Rosa *et al.* 2004, Barthlott *et al.* 2005, Pandiyan *et al.* 2007). It has been suggested that constitutive expression of CD25 (IL-2R) endows Treg cells a competitive advantage over other T cells which have relatively fewer cells expressing CD25 (Barthlott *et al.* 2005, de la Rosa *et al.* 2004). Thus Treg cells consume IL-2 thereby inhibiting other T cells (de la Rosa *et al.* 2004, Barthlott *et al.* 2005,

Pandiyan *et al.* 2007). In this Chapter it was demonstrated that within the tumour significantly more Treg cells express CD25 compared to Tconv and $CD8^+$ T cells. Furthermore, an assessment of the expression of IL-2 by tumour infiltrating T cells indicated that compared to lymph node within the tumour low levels of IL-2 are produced by these cells (Figure 3.39 and Figure 3.41). This suggests that relatively small amounts of IL-2 may be available within the tumour. Furthermore, Treg cells within the tumour may 'mop up' the limited IL-2 that is available within the tumour preventing Tconv and $CD8^+$ T cells to expand. To investigate this possibility further experiments are underway within our laboratory to more accurately measure IL-2 levels within the tumour using RT-PCR and ELISA (enzyme linked immunoassay). Together these findings also indicate that administration of IL-2 could act as a possible treatment strategy by providing enough IL-2 for all T cells within the tumour and therefore aiding the expansion of Tconv and $CD8^+$ T cells within the tumour. Interestingly, this strategy has already shown modest efficacy in the treatment of melanoma, renal carcinoma and other types of cancer (Smith *et al.* 2008, Rosenberg *et al.* 1998), but this treatment is also associated with significant toxicity.

Extracellular adenosine is a potent immunosuppressor that accumulates during tumour growth and can inhibit the activity of anti-tumour T cells (Ohta *et al.* 2006, Pellegatti *et al.* 2008). Expression of the ectonucleotidases CD39 and CD73 on Treg cells is thought to play a role in Treg activity through catabolism of adenosine derivatives into adenosine (Kobie *et al.* 2006, Deaglio *et al.* 2007, Borsellino *et al.* 2007). Furthermore, these studies showed that specific inhibitors of adenosine production can reverse the suppressive effects of Treg cells *in vitro*. In this Chapter it was found that the percentage of Treg cells expressing CD39 and the percentage of Treg cells expressing CD73 were very high (mean >95%) in the tumour. To further examine whether adenosine catabolism by these enzymes contributes to suppression

of the immune responses to tumour, inhibitors of these ectonucleotidases described by other groups (Kobie *et al.* 2006, Deaglio *et al.* 2007, Borsellino *et al.* 2007) could be tested in this model, initially perhaps in an *in vitro* suppression assay using Treg cells derived from the tumour and ultimately *in vivo* in tumour bearing mice. Interestingly, within the tumour a large percentage (mean >60%) of Tconv and $CD8^+$ T cells also expressed CD39 and CD73. Therefore if a suppressive role for CD39 and CD73 was discovered within tumours, their expression on tumour infiltrating Tconv and $CD8^+$ T cells may suggest that suppression via this mechanism is not restricted to the action $Foxp3^+$Treg cells.

It has been suggested that a distinct subset of (effector/memory) Treg cells exists that is discriminated by the expression of CD103. $CD103^+$ Tregs are reported to have a distinct cytokine profile, be highly proliferative and have a unique ability to home to sites of inflammation therefore providing a first line of defence against harmful inflammatory reactions (Lehmann *et al.* 2002, Siewert *et al.* 2008, Huehn *et al.* 2004, Suffia *et al.* 2005). The expression of CD103 in T cells is upregulated by TGFβ (Robinson *et al.* 2001), a cytokine that can be highly expressed at sites of inflammation (reviewed in Letterio and Roberts 1998) and also in MCA tumours (unpublished data from Dr B. Ondondo and Dr E. Jones). Recent findings indicated that an accumulation of $CD103^+$Treg cells in the tumour and spleen was responsible for the loss of concomitant immunity in mice bearing the colon cancer cell line CT-26 (Lin *et al.* 2009). In this Chapter it was found that the percent of Treg cells expressing CD103 was significantly greater within the tumours than in the lymph nodes suggesting that $CD103^+$Tregs may play a role in preventing anti-tumour immunity. In contrast to the findings of Lin *et al.* the percentage of Treg cells expressing CD103 was not significantly altered within the spleen of tumour bearing mice suggesting that these cells do not lead to a systemic inhibition of anti-tumour immunity in mice with MCA tumours. The

primary ligand for CD103 (αEβ7 integrin), E-Cadherin has been found to be expressed in numerous tumours, although its loss is actually associated with invasive phenotypes (Schipper *et al.* 1991, Umbas *et al.* 1992, Oka *et al.* 1993, Mayer *et al.* 1993, Bringuier *et al.* 1993, Gamallo *et al.* 1993). However, through binding to ligands, such as E-Cadherin, CD103 expression may give cells an ability to be retained within tumour tissue, particularly at early stages of tumour development. Given that within MCA tumours the percentage of Tconv and $CD8^+$ T cells which express CD103 is approximately 30% (Figure 3.28 B and 3.28 C), whereas in contrast approximately 70% of Treg cells express CD103 (Figure 3.28 A), Treg cells may have an added ability to be retained within the tumours compared to Tconv and $CD8^+$ T cells. To further support this hypothesis E-Cadherin expression should be examined within MCA tumours.

It has previously been shown that Treg cells can express ICOS and that ICOS may be linked to the suppressive capacity and proliferation of Treg cells (Ito *et al.* 2008). $ICOS^{high}$ Tregs appear to be more suppressive than $ICOS^{low}$ Tregs suggesting a possible role for ICOS in the immunosuppressive function of these cells (Ito *et al.* 2008, Strauss *et al.* 2008). Indeed, high ICOS levels were shown to be associated with higher Foxp3 expression and ICOS has been implicated as being important for the stimulation of IL-10 production by T cells (Ito *et al.* 2008, Akbari *et al.* 2002, Tuettenberg *et al.* 2009, Strauss *et al.* 2008). A significantly higher percentage of Treg cells were found to be $ICOS^{high}$ in the tumour compared to the lymph node. However, no significant difference in IL-10 production was detected in tumour infiltrating Tregs compared to Treg cells from the lymph node suggesting tumour infiltrating Treg may not suppress via IL-10 and that in this environment an increase in ICOS expression is not linked to an increase in IL-10 production.

Another molecule which was found to be expressed on significantly more Treg cells in the tumour than in the lymph node was CD44. CD44 is an adhesion molecule that is expressed on most cells (reviewed in Ponta, Sherman and Herrlich 2003). It is commonly used as an activation marker for T cells as its expression is upregulated following TCR activation and remains highly expressed on memory T cells (Puré and Cuff 2001). It is thought that the majority of Treg cells express CD44 at high levels (Fisson *et al.* 2003). Within the non-draining lymph node of MCA tumour bearing mice approximately 50% of Treg cells expressed $CD44^{high}$ whereas in the tumour ~80% of Treg cells expressed $CD44^{high}$. Although it is commonly used as a marker of T cells which have been activated, the role of CD44 has remained elusive. Recent evidence from $CD44^{-/-}$ mice showed CD44 played an essential role in the survival and memory development of Th1 cells (Baaten *et al.* 2010). CD44 positively correlated with Foxp3 expression and suppressive capacity of Treg cells (Liu *et al.* 2009a). There is also evidence to show that an activated (hylanuronic acid binding) form of CD44 correlates with more suppressive regulatory T cells (Firan *et al.* 2006). Furthermore, it has recently been shown that CD44 costimulation (using antibodies to CD44 or by binding to its ligand hyaluronic acid) can promote Foxp3 expression and in certain circumstances the production of IL-2, IL-10 and TGFβ (Bollyky *et al.* 2009). Hyaluronic acid (HA) is a glucosaminoglycan distributed widely throughout connective tissue and epithelial tissue which is thought be required for extracellular matrix formation and facilitating cell behaviour, such as cell proliferation, differentiation and migration (reviewed in Laurent and Fraser 1992). There is also evidence to suggest a role for HA in tumour progression through interactions with cell surface receptors, such as CD44 (Ahrens *et al.* 2001a, Ahrens *et al.* 2001b). Interestingly, it has been demonstrated that MCA tumours contain several types of gylcosaminoglycans, of which hyaluronic acid is the major component (Wolańska *et al.* 1996, Asokan *et al.* 1989). Furthermore, the amount of glycosaminoglycans increased during

tumour growth. Clearly the role of HA and its potential role in promoting Treg survival or activity requires further experimentation to be verified. Another ligand for CD44, osteopontin has been shown to contribute to various cell functions including chemotaxis, cell survival and proliferation (reviewed in Mazzali *et al.* 2002). Analysis of MCA tumours has shown that this molecule is abundantly expressed in MCA tumours (Dr B. Ondondo unpublished). Osteopontin may therefore provide important survival signals to cells expressing CD44 such as Treg cells.

The percentage of Treg cells expressing high levels of GITR was consistently high throughout tumour bearing mice. This finding is in agreement with previous reports which show constitutively high expression of GITR in Tregs (McHugh *et al.* 2002, Shimizu *et al.* 2002). Removal of $GITR^+$ T cells or administration of GITR-specific Abs produces organ-specific autoimmune disease in mice similar to that observed in mice depleted of Tregs suggesting that GITR plays a role in Treg cell mediated self tolerance (Itoh *et al.* 1999). More recent studies have shown that administration of Abs against GITR enables immune mediated rejection of the melanoma cell line B16 in mice (Ramirez-Montagut *et al.* 2006) and can result in concomitant immunity to B16 tumours (Turk *et al.* 2004). Furthermore, reports indicate that GITR is required for Treg activity *in vitro* (Itoh *et al.* 1999, McHugh *et al.* 2002, Tone *et al.* 2003, Ji *et al.* 2004). However, the role of GITR interaction in Treg cell mediated suppression has not been fully determined. More recent studies suggest GITR ligation can induce preferential expansion of Treg cells *in vitro* and *in vivo* (Ramirez-Montagut *et al.* 2006, Nishioka *et al.* 2008, Liao *et al.* 2010, van Olffen *et al.* 2009), thus GITR may be important for the accumulation of Treg cells in tumours. To support this hypothesis the presence of the ligand for GITR (GITR-L), which can be found on APCs (Kim *et al.* 2003, Suvas *et al.* 2005) must be investigated in MCA tumours.

The expression of CTLA-4 was also analysed in MCA tumour bearing mice. CTLA-4 has been reported to be constitutively expressed on Treg cells (Read *et al.* 2000, Takahashi *et al.* 2000). Furthermore, these studies identified that CTLA-4 is an important component of the immunosuppressive capacity of Treg cells. In contrast to many of the markers assessed, the percentage of Treg cells expressing CTLA-4 was significantly less within the tumour than within the lymphoid tissue (Figure 3.31). Therefore, although CTLA-4 expression remains on many of the tumour infiltrating Treg cells this finding suggest that CTLA-4 may be dispensable for Treg mediated suppression within the tumour. This hypothesis is supported by some *in vitro* studies which show that CTLA-4 is not required for all normal Treg activities (Tang *et al.* 2004, Kataoka *et al.* 2005). However, Treg-specific deletion of CTLA-4 causes spontaneous development of systemic lymphoproliferation and fatal disease in mice highlighting its importance in Treg cell development (Wing *et al.* 2008, Sojka, Hughson and Fowell 2009). Furthermore, Treg cells from CTLA-4 deficient mice are unable to control pancreatic tissue destruction by autoantigen-specific T cells (Schmidt *et al.* 2009, Ise *et al.* 2010). Collectively these studies show that CTLA-4 is essential for Treg development and acquisition of a full suppressive capacity. However, the requirement of CTLA-4 for the action of Treg mediated suppression needs further examination.

Most of the markers assessed for investigating the Treg cell phenotype are associated with activation of conventional T cells. As Abs to both CD4 and CD8 were used in the Ab panels described above it was possible to also assess the expression of these activation markers on Tconv and $CD8^+$ T cells from tumour bearing mice. This analysis revealed that many markers associated with activation were expressed on tumour infiltrating Tconv and $CD8^+$ T cells suggesting that an increased proportion of Tconv and $CD8^+$ T cells are activated in the

tumour compared to the lymph node. An increase in the percentage of cells expressing IFNγ was also observed in the tumour compared to lymph nodes suggesting that Tconv and $CD8^+$ T cells are also functionally more active in the tumour than in the lymph nodes. For a more complete analysis of the activation status of tumour infiltrating T cells a number of other markers could have been measured, for example CD69, CD62L, CD45RA, CD45RO and OX40.

It is also interesting to note that despite an 8 fold increase in the number of cells within the draining lymph node compared to the non-draining lymph node (Figure 3.4) most of the Tconv cells and $CD8^+$ T cells within the draining lymph node were not $CD44^{high}$ (Figure 3.9). The reasons for this apparent paradox are not clear. It may be that simply the vasculature of the draining lymph node is increased therefore allowing an influx of cells non-specifically (Steeber *et al.* 1987, Herman *et al.* 1972, Anderson *et al*, 1975). However, it has also been shown that during chronic infection and in tumours exhausted ($PD\text{-}1^+$) T cells can show markedly lower levels of CD44 expression (Sakuishi *et al.* 2010, Wherry *et al.* 2003).

Very few Tconv and $CD8^+$ T cells expressed IL-4, IL-10 or IL-17. These results indicate that the T cell response in the tumour is primarily a Th1 associated response. This is in agreement with numerous reports from other laboratories that show Th1 associated cytokine production in response to tumours (Zitvogel *et al.* 1996, Matsui *et al.* 1999, Nishimura *et al.* 1999b, Egeter *et al.* 2000).

Although in the tumour numerous activation markers are upregulated on both Tconv and $CD8^+$ T cells and many of these cells express an antigen experienced phenotype, it is clear that these cells were not able to effectively control tumour development. This may indicate

that many of the Tconv and $CD8^+$ T cells within the tumour are exhausted, anergic or suppressed by tumour infiltrating Treg cells. Indeed, although an increase in the percentage of T cells expressing an activated phenotype was observed in the tumour compared to the lymph node, it still remained that the majority of tumour infiltrating Tconv and $CD8^+$ T cells did not express an activated phenotype. This was particularly noticeable for $CD8^+$ T cells. The expression of CTLA-4 and ICOS are two clear examples of this. These two costimulatory molecules are reported to be upregulated following activation of T cells (Freeman *et al.* 1992, McAdam *et al.* 2000, Hutloff *et al.* 1999, Brunet *et al.* 1987), however >90% of tumour infiltrating $CD8^+$ T cells did not upregulate expression of these markers. Similarly, although an increase in the expression of the cytokine IFNγ was observed in tumour infiltrating T cells versus T cells from lymph node, it remains that the majority of tumour infiltrating Tconv and $CD8^+$ T cells do not express any detectable cytokines. Furthermore, a significant reduction in the expression of IL-2 was found in tumour infiltrating Tconv and $CD8^+$ T cells compared to lymph node resident Tconv and $CD8^+$ T cells. It has been shown that following T cell activation IL-2 is produced, however co-stimulation is required for this process (Thompson *et al.* 1989, reviewed in Linsley and Ledbetter 1993). CD28 ligation, for example, is thought to stabilise IL-2 mRNA and also activate transcription factors that increase the transcription of IL-2 mRNA (Ragheb, Deen and Schwartz 1999, Sanchez-Lockhart *et al.* 2004). However, antigen recognition in the absence of co-stimulation inactivates T cells inducing anergy (Harding *et al.* 1992, Tan *et al.* 1993, Chen and Nabavi 1994). The reduction in IL-2 production by tumour infiltrating Tconv and CD8 may be due to inefficient T cell stimulation, particularly co-stimulation, leading to anergy of these cells and an ineffective immune response. Indeed, a hallmark of anergic T cells is their inability to produce IL-2 (Macián *et al.* 2002, Jenkins *et al.* 1987). However, contrary to this, it has been shown that memory responses do not necessarily require

costimulation for activation (reviewed in Duttagupta et al, 2009). Clearly more work is needed to test the reasons for reduced IL-2 production by T cells in tumours.

A significant co-inhibitory molecule which was not investigated in this thesis is Programmed Death-1 (PD-1). PD-1 is expressed on the surface of T cells following activation and is thought to negatively regulate immune responses (reviewed in Riley 2009). Specifically, engagement of PD-1 with its ligands PD-L1 or PD-L2 can inhibit T cell proliferation, cytokine production and cytolytic function. Experiments using mice lacking PD-1 or blocking antibodies against PD-1 ligands suggest that the induction and maintenance of tolerance requires PD-1 (Nishimura *et al.* 1999a, Nishimura *et al.* 2001, Okazaki *et al.* 2003, Ansari *et al.* 2003, Keir *et al.* 2006). However, the PD-1:PD-L pathway may also enable the persistence of chronic infections and tumour survival (reviewed in Keir *et al.* 2008). Tumour cells may escape anti-tumour immune responses by expressing PD-1 ligands. Indeed, PD-1 ligands are found on many human cancer tissues (reviewed in Okazaki and Honjo 2007) and PD-1 expression is found on tumour infiltrating lymphocytes (Matsuzaki *et al.* 2010, Sfanos *et al.* 2009, Ahmadzadeh *et al.* 2009, Fourcade *et al.* 2009). The expression of PD-1 and its ligands in MCA tumours has not yet been investigated, but may be important in modulating the immune response generated in these mice.

Another interesting pattern was observed in the expression of CD103 on $CD8^+$ T cells from MCA tumour bearing mice. The percentage of $CD8^+$ T cells that expressed CD103 was found to be significantly less within tumour compared to lymph node. Although the implications of this are not clear, CD103 expression on $CD8^+$ T cells has recently been shown to correlate with CTL function and retention in tumours (Franciszkiewicz *et al.* 2009). Studies have also shown that CD103 endows peripheral $CD8^+$ cells with a unique capacity to access and

ultimately destroy graft renal epithelial cells (Feng *et al.* 2002, El-Asady *et al.* 2005). Furthermore, a recent finding by Webb *et al.* (2010) suggested that CD103 marked activated and tumour reactive CD8 T cells in high-grade serous ovarian cancer. However, an infiltration of these cells was observed in these cancer patients, and in the MCA tumour analysed herein indicating that the presence of $CD103^+$ $CD8^+$ T cells is unable to prevent tumour outgrowth. Therefore more work is clearly necessary to determine whether CD103 expression on $CD8^+$ T cells infiltrating tumours is required for its destruction.

In summary, in this Chapter a phenotypic and functional analysis of T cells from MCA tumour bearing mice was performed. This analysis revealed several interesting findings. Firstly, it was demonstrated that $CD8^+$ T cells control tumour development following Treg depletion. Also, it was found that the ratios of Tconv and particularly $CD8^+$ T cells to Treg cells altered significantly within the tumour supporting the hypothesis that an environment favouring immunosuppression is established in tumours. Tumour infiltrating Treg cells displayed a highly suppressive phenotype and although the expression of numerous activation markers was elevated in tumour infiltrating Tconv and $CD8^+$ T cells compared to these cells from other locations, for each marker only a small proportion of tumour infiltrating Tconv and particularly $CD8^+$ T cells appeared to show expression. Collectively, the data presented in this Chapter support the hypothesis that Treg cells accumulate in tumours and gain control of $CD8^+$ T cells, impairing their function and preventing anti-tumour immunity.

Chapter 4 – Investigation into the mechanisms leading to accumulation of regulatory T cells in tumours

4.1 Introduction

One mechanism through which the immune response to tumours might be subverted is by the activity of regulatory T cells (Tregs) within the tumour tissue and tumour draining lymph nodes. The normal functions of Tregs are to maintain immune homeostasis, prevent autoimmunity and limit immunopathology (reviewed in Sakaguchi 2004). However, many groups have reported, in studies of both mouse models and patients with cancer, that tumour development is often associated with a significant enrichment of Tregs in peripheral blood, local lymph nodes and tumour tissue (reviewed in Betts *et al.* 2006). Using the chemical carcinogen 3-methylcholanthrene (MCA), our laboratory previously examined the impact of Tregs on tumour immunosurveillance (Betts *et al.* 2007). Tregs are significantly enriched in MCA-induced tumours (fibrosarcomas) compared to lymphoid tissue and even a partial and transient depletion of these cells results in a marked reduction in tumour incidence. This observation, along with other studies, supports the hypothesis that tumours can utilize Tregs for their own advantage by promoting Treg activity (Shimizu *et al.* 1999, Jones *et al.* 2002, Turk *et al.* 2004, Yu *et al.* 2005). As well as their potential for limiting the effectiveness of tumour immunosurveillance, it is likely that Tregs also represent a significant obstacle to successful immunotherapy (reviewed in Zou 2006). It is important, therefore, to understand the factors that lead to the enrichment of Tregs during tumour progression to enable the development of inhibitory strategies.

The majority of Tregs which express the transcription factor Foxp3 (termed naturally occurring Tregs) are generated in the thymus as a distinct cell lineage (Fazilleau *et al.* 2007, Hsieh *et al.* 2006, Pacholczyk *et al.* 2006). $Foxp3^+$ Tregs may also be generated in the periphery through the conversion of conventional $Foxp3^-$ T cells (Tconv) into $Foxp3^+$ Tregs; these have been termed adaptive (or induced) Tregs (reviewed in Bluestone and Abbas 2003). Experiments using mice with restricted TCR repertoires and limited studies in humans have suggested that the TCR repertoires of Tregs and Tconv cells are largely distinct, overlapping by approximately 10 – 20% (Hsieh *et al.* 2006, Pacholczyk *et al.* 2006, Scheinberg *et al.* 2007, Wong *et al.* 2007b). It has been suggested that Tconv cells with TCRs overlapping with those of the Treg repertoire represent cells that recognize self-antigens (Hsieh *et al.* 2006). Adaptive Tregs that have arisen by conversion of Tconv cells in the periphery may also contribute to the observed overlap in the TCR repertoires. The role and significance of adaptive Tregs *in vivo*, however, is unclear as the majority of the peripheral Treg repertoire is also represented within the thymic Treg repertoire (Hsieh *et al.* 2006). Furthermore, TCR repertoire studies of diabetogenic NOD mice indicated a lack of conversion in autoimmune disease (Wong, Mathis and Benoist 2007a). However, studies showing that tumours can facilitate conversion of Tconv cells into Treg cells *in vitro* (Liu *et al.* 2007) and, more recently, *in vivo*, imply that conversion can potentially contribute to the accumulation of tumour-infiltrating Tregs (Valzasina *et al.* 2006, Zhou and Levitsky 2007). In many of these studies, TGFβ has been identified as a key cytokine for the induction of Foxp3. Work from our laboratory has shown that MCA tumours contain large amounts of TGFβ (Dr E. Jones and Dr B. Ondondo, unpublished, Appendix Figure A3). This finding indicates that the tumour microenvironment established in MCA tumours may promote the conversion of Tconv cells into Treg cells. The influence of the tumour environment on Treg proliferation and survival might also make an important contribution to the enrichment of Tregs within

TIL populations. It is known that Tregs exhibit a higher turnover than Tconv cells under homeostatic conditions and that tumours can license dendritic cells (DCs) to promote the proliferation of Tregs in lymph nodes via TGFβ (Ghiringhelli *et al.* 2005).

Whilst informative, the tumour models described here, often using tumour cell lines and tracking the fate of adoptively transferred T cells, omit interactions between the immune system and the tumour during the early stages of tumour development; the reciprocal influences of the immune system and the tumour during these stages are likely to have a significant impact on the nature of their relationship during the period of tumour outgrowth. In this Chapter, the MCA tumour induction model, which takes these early interactions into account, was used to analyze the TCR repertoires of conventional and regulatory T cells in order to assess the contribution of conversion as well as proliferation and survival to Treg enrichment in tumours.

4.2 Results

4.2.1 Does conversion of conventional T cells into regulatory T cells account for Treg cell enrichment in tumours?

To investigate whether conversion of conventional $CD4^+$ T cells (Tconv) into Treg cells contributes to Treg accumulation in tumours the TCR repertoires of the two populations were compared. As described in Chapter 1 the TCR repertoires of Tconv and Treg cells have been previously investigated. Experiments using mice with restricted TCR repertoires and limited studies in humans have suggested that the TCR repertoires of Tregs and Tconv cells are largely distinct, overlapping by approximately 10 – 20% (Hsieh *et al.* 2006, Pacholczyk *et al.* 2006, Fazilleau *et al.* 2007, Scheinberg *et al.* 2007). For the studies described in this Chapter,

it was surmised that if Treg enrichment in tumours is due to the conversion of Tconv cells into Treg cells, then the degree of overlap between their TCR repertoires would be significantly greater in the tumour compared to other lymphoid tissues where Treg enrichment is not observed (Figure 4.1). Thus, the extent of TCR repertoire overlap in Treg and Tconv populations isolated from tumour, spleen, non-draining inguinal lymph node and draining inguinal lymph node was compared. For this purpose, the $CD4^+$ T cells from tumour-bearing mice were purified by flow cytometry.

4.2.2 Similarity between the TRBV13 TCR repertoires of $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells

Given that many thousands of unique clonotypes can comprise a single T cell receptor β chain variable domain (TRBV) subset it was decided that a single TRBV subset would be analysed in detail. Based on previous work from our laboratory performed by Dr Gareth Betts it was decided that the TRBV13 (Vβ8) subset would be used as this Vβ gene was well represented (approximately 15-20%) within the total Vβ TCR repertoires of both Tconv and Treg cells from tumour and spleen of tumour bearing mice (Appendix Figure A4 and Figure A5). The TRBV13 chain consists of three further subsets 13-1, 13-2 and 13-3. Initially all three were analysed.

For this initial experiment, cells were sorted based on expression of CD25. CD25 has been extensively used as a marker for Tregs because the majority of $Foxp3^+$ Treg cells express CD25. The advantage of CD25 over Foxp3 as a marker for Tregs is that CD25 is expressed on the cell surface allowing for sorting of the cells without permeabilisation. $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells (simply referred to as $CD25^-$ and $CD25^+$ T cells hereafter) were

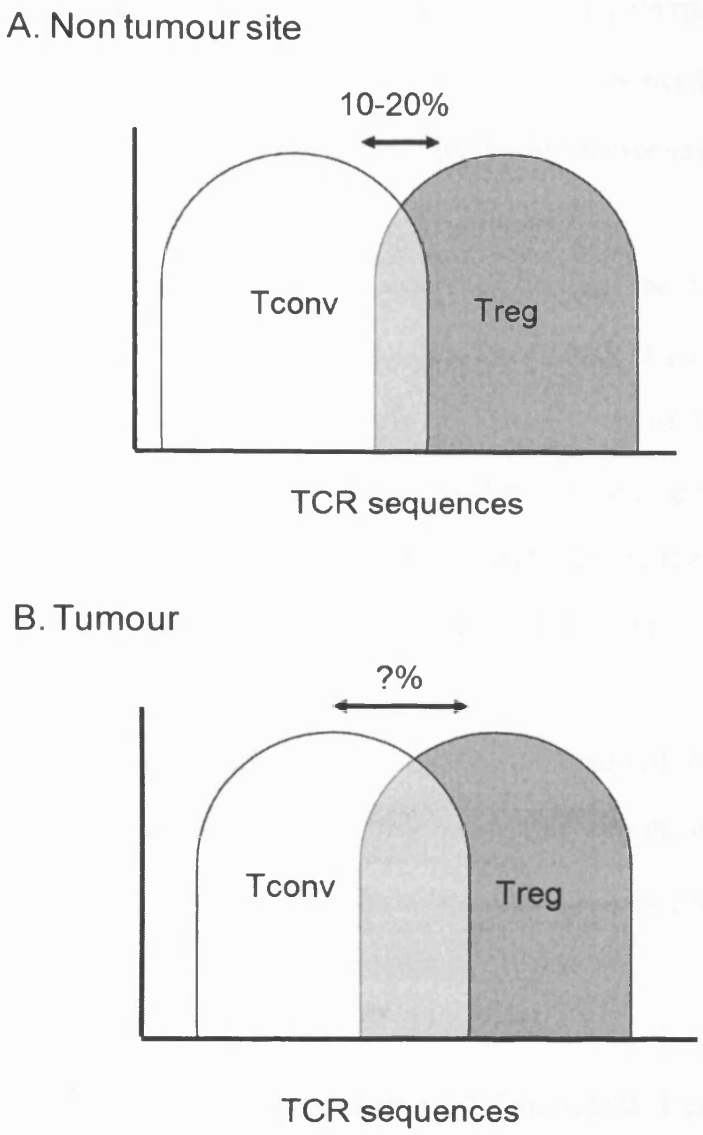


Figure 4.1. Diagrammatic representation of the hypothesis for studying conversion of Tconv into Treg by TCR repertoire analysis
Previous studies have found that the TCR repertoires of Tconv and Treg are generally distinct. Approximately 10-20% of the repertoires were found to be overlapping. Therefore in non-tumour sites such as the spleen and non-draining lymph node a low level of overlap was expected (A) whereas in the tumour (and possibly tumour draining lymph node) if Treg cells were accumulating through the conversion of Tconv cells it was surmised that a significant increase in the degree of overlap between the Treg repertoire and Tconv repertoire would be observed (B).

sorted from the spleen and tumour of an MCA tumour bearing C57BL/6 (WT) mouse. Clonotyping for the three TRBV13 subsets was performed as described in Materials and Methods. In total 873 TCR sequences were analysed for this initial experiment (Table 4.1).

Firstly, the number of overlapping or shared sequences between the TCR repertoires of $CD25^+$ and $CD25^-$ T cells were analysed for each of the three TRBV13 subsets for the spleen and tumour samples. For the TRBV13-1 subset in the spleen, 1 out of 30 (3.3%) different $CD25^+$ TCR clonotypes overlapped with the $CD25^-$ TCR repertoire (Figure 4.2A). This one TCR clonotype represented 2.2% (2 out of 90) of the spleen $CD25^+$ TCR repertoire analysed. Thus the $CD25^+$ and $CD25^-$ T cell TCR repertoires were generally distinct within the spleen.

In contrast, in the tumour, more overlapping sequences were observed. Out of 11 different TRBV13-1 $CD25^+$ T cell TCR sequences, 3 (27%) overlapped with the tumour TRBV13-1 $CD25^-$ T cell TCR repertoire (Figure 4.2B). These represented a large proportion (58%; 28 out of 48) of the tumour $CD25^+$ T cell TCR repertoire.

The number of overlapping sequences between the $CD25^+$ and $CD25^-$ T cell repertoires was also greater within the tumour than in the spleen for the TRBV13-2 and TRBV13-3 TCR subsets (Table 4.2 and Figure 4.3 & Figure 4.4). For the TRBV13-2 subset, in the spleen, 3 out of 50 (6%) different $CD25^+$ TCR clonotypes overlapped with the $CD25^-$ TCR repertoire (Figure 4.3A). These 3 clonotypes made up 13.5% (12 out of 89) of the total spleen $CD25^+$ TRVB13-2 repertoire. In contrast, in the tumour 3 out of 4 (75%) different $CD25^+$ TRBV13-2 clonotypes were found to be overlapping with the $CD25^-$ TCR repertoire (Figure 4.3B). This made up 48% (46 out of 95) of the tumour $CD25^+$ TRBV13-2 repertoire. Finally, the TRBV13-3 repertoires also showed similar patterns. In the spleen, 3 out of 38 (8%) different

Experiment / Mouse number	TCR subset	Cell type	Spleen	ND	D	Tumour	Total
1	TRBV 13-1	$CD25^-$	82	*	*	44	126
		$CD25^+$	90	*	*	48	138
	TRBV 13-2	$CD25^-$	88	*	*	84	172
		$CD25^+$	89	*	*	95	184
	TRBV 13-3	$CD25^-$	90	*	*	41	131
		$CD25^+$	79	*	*	43	122
		Total:	*518*	*	*	*355*	*873*
2	TRBV 13-2	Tconv	80	83	82	84	329
		Treg	80	88	86	79	333
		Total:	*160*	*171*	*168*	*163*	*662*
3	TRBV 13-2	Tconv	84	81	78	93	336
		Treg	79	92	82	90	343
		Total:	*163*	*173*	*160*	*183*	*679*
4	TRBV 13-2 / TRBJ 2-5	Tconv	51	64	53	40	208
		Treg	55	62	53	63	233
		Total:	106	126	106	103	441
		Grand total:	***947***	***470***	***434***	***804***	***2655***

Table 4.1. Number of successful traces / sequences analysed.
The number of successful traces that were obtained for the spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumours from each experiment are given (* = not done).

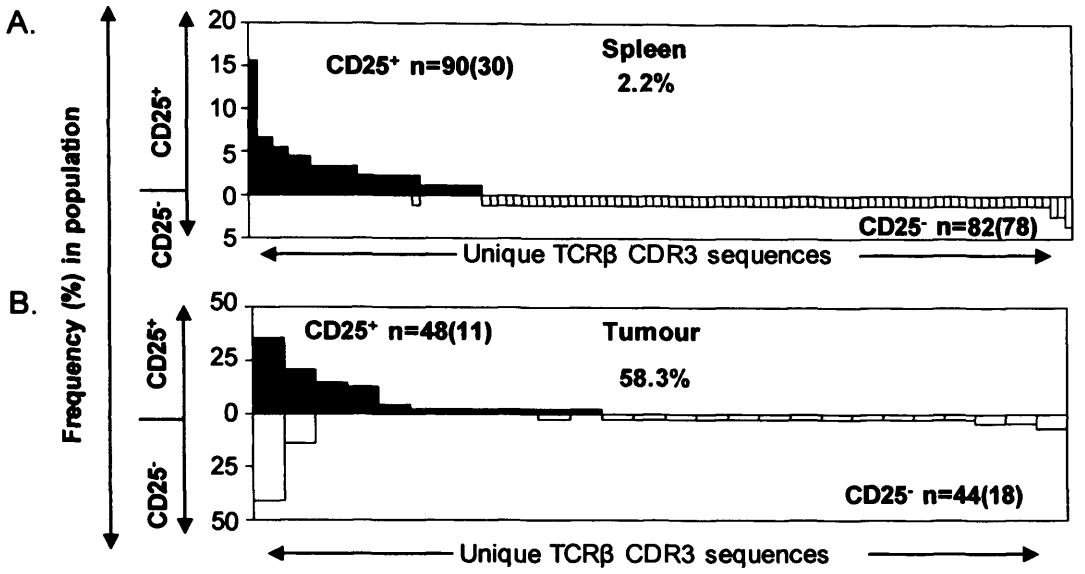


Figure 4.2. TRBV13-1 TCR repertoires of $CD4^+CD25^-$ and $CD4^+CD25^+$ cells
T cells were FACS sorted based on their expression of CD4 and CD25 and cells were analysed by TCR clonotyping. CDR3 amino acid sequences of the TRBV13-1 subset were determined and used to identify individual TCRs for each T cell subset from the spleen (A), tumour (B) of mice given MCA. Each graph displays the different TCR sequences observed (x axis) within the $CD25^+$ (filled & above) and $CD25^-$ (open & below) repertoires and the frequency (y axis) of each sequence within each subset. The total number of TCR sequences analyzed is displayed as n=, with the number of unique TCR sequences observed in parentheses. The proportion of the $CD25^+$ repertoire that has shared sequences with the $CD25^-$ repertoire is also shown.

TRBV subset	Tissue	Number of $CD25^+$ TCR sequences that overlap with $CD25^-$ TCR repertoire	Frequency of overlapping sequences within $CD25^+$ TCR repertoire?	Morisita-Horn index
13-1	*Spleen*	1 out of 30 (3.3%)	2 out of 90 (2.2%)	0.008
	Tumour	3 out of 11 (27.3%)	28 out of 48 (58.3%)	0.837
13-2	*Spleen*	3 out of 50 (6.0%)	12 out of 89 (13.5%)	0.015
	Tumour	3 out of 4 (75.0%)	46 out of 95 (48.4%)	0.269
13-3	*Spleen*	3 out of 38 (7.9%)	3 out of 79 (3.8%)	0.034
	Tumour	2 out of 12 (16.7%)	13 out of 43 (30.2%)	0.083
Total / Average	*Spleen*	7 out of 118 (5.9%)	17 out of 258 (6.6%)	0.019
	Tumour	8 out of 27 (29.6%)	87 out of 186 (46.8%)	0.396

Table 4.2. Similarity between $CD25^+$ and $CD25^-$ TCR repertoires in mouse 1
The number of $CD4^+CD25^+$ TCR sequences that overlap with the $CD4^+CD25^-$ TCR repertoire was determined for each TRBV13 subset for the spleen and tumour. Data are also displayed to show the proportion of the $CD25^+$ TCR repertoire that overlapped with the $CD25^-$ TCR repertoire. The Morisita-Horn similarity index between the $CD25^-$ and $CD25^+$ TCR repertoires was also determined.

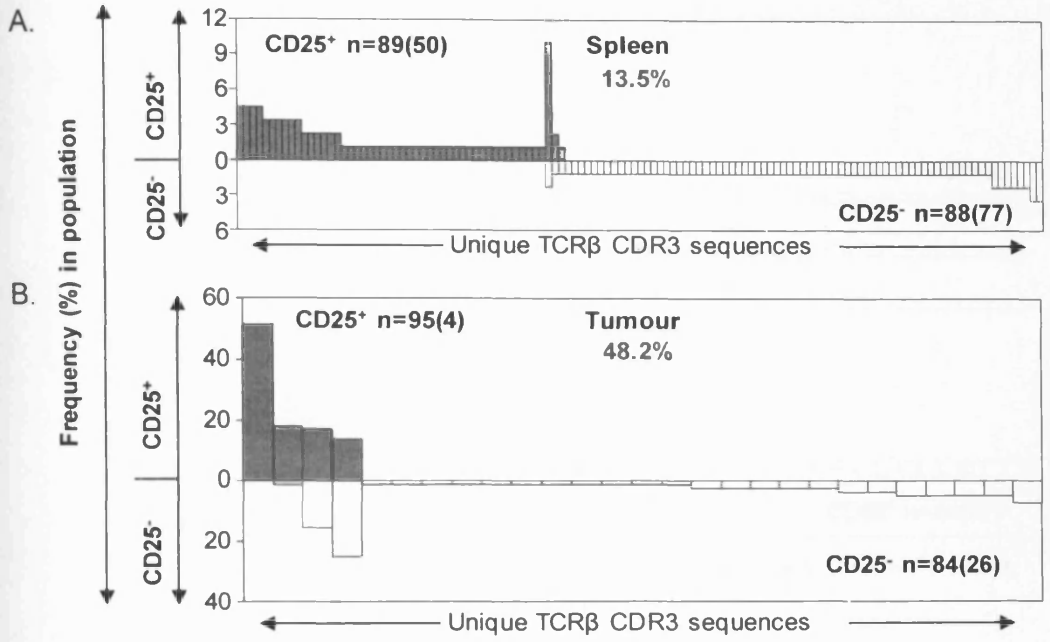


Figure 4.3. TRBV13-2 TCR repertoires of $CD4^+CD25^-$ and $CD4^+CD25^+$ cells
T cells were FACS sorted based on their expression of CD4 and CD25 and analysed by TCR clonotyping. CDR3 amino acid sequences of the TRBV13-2 subset were determined and used to identify individual TCRs for each T cell subset from the spleen (A), tumour (B) of mice given MCA. Graphs are displayed as described for Figure legend 4.2.

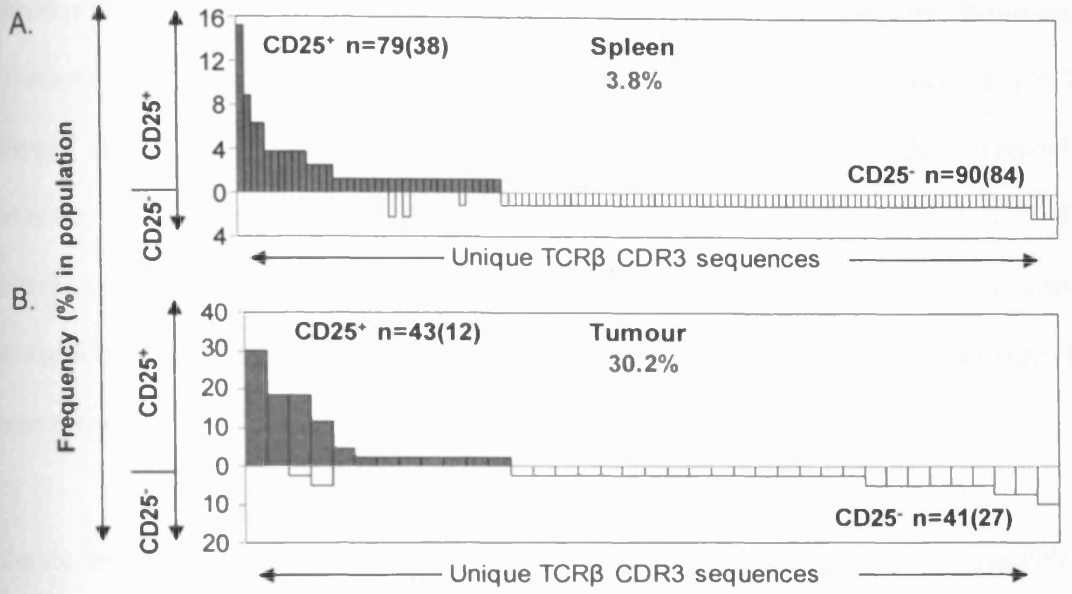


Figure 4.4. TRBV13-3 TCR repertoires of $CD4^+CD25^-$ and $CD4^+CD25^+$ cells
T cells were FACS sorted based on their expression of CD4 and CD25 and analysed by TCR clonotyping. CDR3 amino acid sequences of the TRBV13-3 subset were determined and used to identify individual TCRs for each T cell subset from the spleen (A), tumour (B) of mice given MCA. Graphs are displayed as described for Figure legend 4.2.

$CD25^+$ sequences were found to overlap with the $CD25^-$ repertoire (Figure 4.4A). These 3 sequences represented 3.8% (3 out of 79) of the $CD25^+$ TRBV13-3 repertoire. However, in the tumour, more overlap was observed. Out of 12 different clonotypes, 2 (16.7%) overlapped (Figure 4.4B). This equated to 30% (13 out of 43) of the $CD25^+$ TRBV13-3 repertoire in the tumour. On average, in the spleen approximately 6% of the $CD25^+$ TCR repertoire overlapped with the $CD25^-$ T cell repertoire. In the tumour, on average approximately 46% of the $CD25^+$ TCR repertoire overlapped with the $CD25^-$ repertoire. This difference was statistically significant (*P=0.0473; Figure 4.5A).

To further this analysis the Morisita-Horn (MH) similarity index was used to quantify the degree of similarity between each subset. This index is more complex than simply measuring the proportion of the $CD25^+$ TCR repertoire that overlaps with the $CD25^-$ TCR repertoire. The MH index ranges from 0 to 1, representing complete dissimilarity to identical. It takes into account the following: a) the number of overlapping TCR sequences; b) how well represented those overlapping sequences are; and c) the number and representation of those sequences within both repertoires. The MH similarity index between $CD25^-$ and $CD25^+$ cells was calculated for each tissue (Table 4.2). Across the three TRBV13 subsets, the TCR repertoires of $CD25^+$ and $CD25^-$ T cells had an average MH similarity value of 0.396 in the tumour and only 0.019 in the spleen (Table 4.2 and Figure 4.5B). Using the values from this index the difference was approaching statistical significance, suggesting that the $CD25^+$ and $CD25^-$ cells were more similar in the tumour than in the spleen.

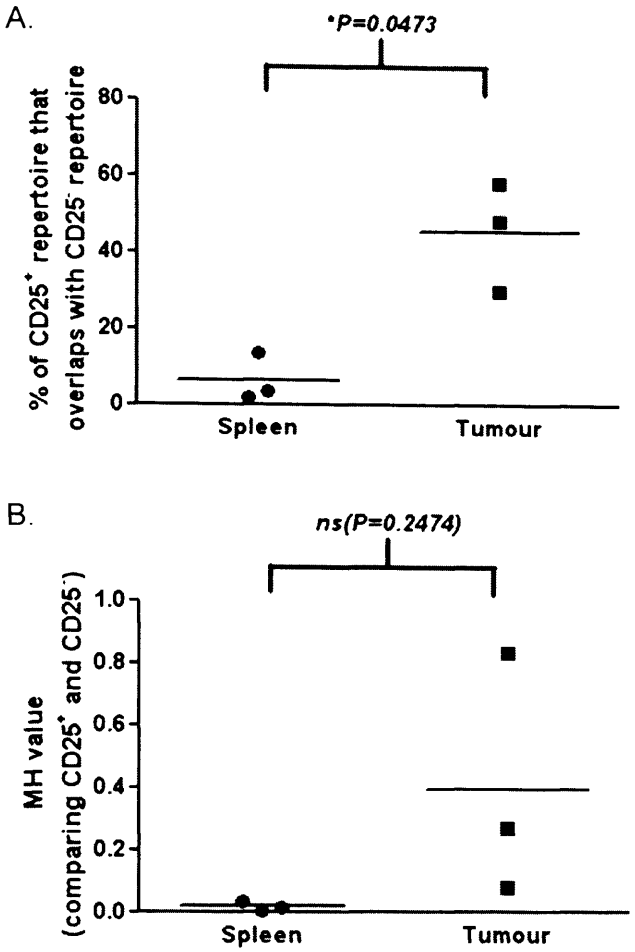


Figure 4.5. Comparison of $CD25^-$ and $CD25^+$ cell similarity levels in spleen and tumour
The similarity between $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells was analysed for spleen (●) and tumour (■) for the three different TRBV subsets (13-1, 13-2 and 13-3). The percentage of $CD25^+$ repertoire that overlaps with the $CD25^-$ repertoire (A) or the mean Morisita-Horn (MH) similarity value (B) is displayed. The differences between the means were compared using paired T tests.

In summary, initial data from T cells sorted based on CD25 showed that a significantly higher percentage of the $CD4^+CD25^+$ repertoire overlapped with the $CD4^+CD25^-$ repertoire in the tumour compared to the spleen supporting the hypothesis that conversion of Tconv cells into Tregs contributes to the accumulation of Treg cells in the tumour. This trend was matched when a more sophisticated method (MH index) of analysis was applied to the data.

4.2.3 Analysis of the TRBV13-2 TCR repertoires of Tconv and Treg cells sorted based on Foxp3

As mentioned in Chapter 1, the separation of Tregs from Tconv cells using CD25 as a marker is not ideal. Not all $Foxp3^+$ Treg cells express CD25. Therefore, it is likely that the $CD25^-$ "Tconv" pool of mouse 1 was contaminated with $CD25^-Foxp3^+$Tregs. Results from Chapter 3 demonstrate this point (Figure 3.13, page 87). On average, between 7% and 14% of the $Foxp3^+$Treg population does not express CD25. Conversely, activated $Foxp3^-CD4^+$ non-regulatory T cells express CD25. As shown in Chapter 3, on average between 3.5% (non-draining lymph node) and 31% (tumour) of the $Foxp3^-$Tconv populations expressed CD25 (Figure 3.13). Therefore, it is also likely that very many $CD25^+Foxp3^-$Tconv cells contaminated the $CD25^+$ "Treg" pool. Due to the contamination of both cell populations it is clear that these data must be interpreted cautiously. This problem is compounded by the fact that more $CD4^+Foxp3^-CD25^+$ activated T cells exist in the tumour (mean 31%) compared to the spleen (mean 13%) or lymph nodes (3.5-6%). Thus, in the tumour more contamination of the $CD25^+$ "Treg" pool would occur, and hence an increase in the level of overlap in the tumour may reflect sampling of more $CD25^+Foxp3^-$ cells. It was imperative to reduce this contamination during the FACS process to avoid any skewing of the measurement of similarity / overlap. This was especially important since the levels of contamination may be

greater in the tumour than in the spleen. For this purpose, it was decided that subsequent analyses would use mice expressing GFP under the control of the Foxp3 promoter (Foxp3_GFP mice) such that lymphocytes could be sorted based on GFP (Foxp3) expression.

The analysis was also further refined to include T cells isolated from the lymph nodes of the tumour bearing mouse. Inguinal lymph nodes from the tumour draining and non-tumour draining sides of the MCA mouse were taken. This would allow for a direct comparison of the two lymph nodes and also provide an additional non-tumour associated site for comparison to the tumour.

In the initial experiment described above three TRBV13 subsets were analysed. As the patterns of TCR clonotypes observed were similar in terms of diversity and overlap across the three different subsets, only one TRBV subset was analysed in further experiments. TRBV13-2 was chosen as it gave the highest number of successful sequences for analysis (Table 4.1).

Mouse 2 (1st GFP mouse)

The clonotyping experiment was repeated with a second mouse with a number of changes. An MCA tumour bearing Foxp3_GFP mouse was used to sort Treg and Tconv cells based on Foxp3 expression. $CD4^{+}Foxp3^{+}$ Tregs and $CD4^{+}Foxp3^{-}$ Tconv cells were sorted from spleen, non-draining lymph node, draining lymph nodes and tumour samples. The TRBV13-2 TCR repertoires of each population were determined by clonotyping. In total 662 TCR sequences were analysed (Table 4.1).

As in the previous analyses very little overlap was observed between the Tconv and Treg TCR repertoires of splenic T cells (Table 4.3 and Figure 4.6A). Only 1 out of 72 Treg TCR clones overlapped with the Tconv repertoire. This represented only 1.3% of the spleen Treg repertoire. Very little overlap was observed between the Treg and Tconv TCR repertoires from the non draining LN (Figure 4.6B). Only one Treg sequence (representing 1.1%; 1 out of 88) overlapped with the Tconv repertoire in the non-draining lymph node. Hence, the TCR repertoires of Tconv and Treg cells were mostly distinct.

Interestingly the Treg and Tconv TCR repertoires in the draining lymph node and tumour were also generally distinct (Table 4.3). This is in contrast to the result obtained previously when the cells were sorted based on CD25. In the draining lymph node only one sequence (representing 1.16%, 1 out of 86) of the Treg TCR repertoire was found to overlap with the Tconv TCR repertoire (Figure 4.6C). A slight increase in the number of Treg sequences overlapping with Tconv sequences was observed in the tumour compared to other locations. Out of 38 different Treg TCR clones 3 were overlapping (Figure 4.6D). This represented ~8% of the Treg clones identified and 14% (11 out of 79 sequences) of the Treg TCR repertoire. Although an increase in the degree of overlap between the Treg and Tconv repertoires was observed in the tumour in comparison to the other sites, it was apparent that in the tumour, the vast majority (85%) of the Treg TCR repertoire was distinct from the Tconv TCR repertoire.

To analyse the similarity between the Tconv and Treg TCR repertoires in more depth, the Morisita-Horn similarity index was calculated for each tissue (Table 4.3). A MH value of 0.01 was calculated for the similarity between the Tconv and Treg cell subsets from the spleen, and both lymph nodes. A higher MH similarity index of 0.034 was calculated for the

Tissue	Number of Treg TCR sequences that overlap with Tconv TCR repertoire	Frequency of overlapping sequences within Treg TCR repertoire?	Morisita-Horn index
Spleen	1 out of 72 (1.39%)	1 out of 80 (1.25%)	0.010
ND	1 out of 81 (1.23%)	1 out of 88 (1.14%)	0.010
D	1 out of 80 (1.25%)	1 out of 86 (1.16%)	0.010
Tumour	3 out of 38 (7.89%)	11 out of 79 (13.92%)	0.034

Table 4.3. Similarity between Treg and Tconv TCR repertoires in mouse 2
The number of Treg TCR sequences that overlap with the Tconv TCR repertoire was determined for the spleen, non-draining lymph node (ND), draining lymph node (D), and tumour. Data are also displayed to show the proportion of the Treg TCR repertoire that overlapped with the Tconv TCR repertoire. The Morisita-Horn similarity index between the Tconv and Treg TCR repertoires was also determined.

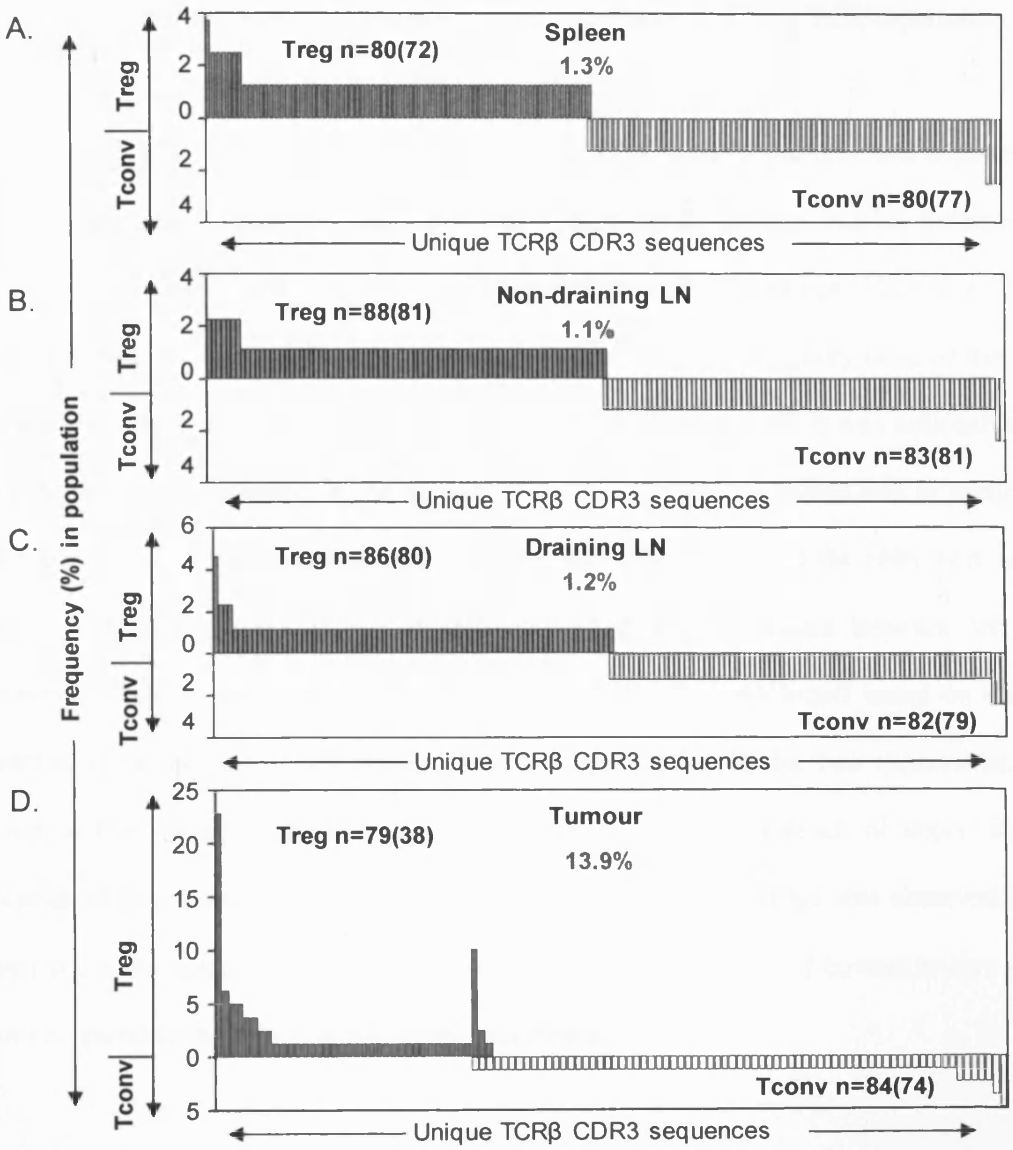


Figure 4.6. TRBV13-2 TCR repertoires of $CD4^+Foxp3^-$ Tconv and $CD4^+Foxp3^+$Treg in tumour mice. Tconv ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) cells were FACS sorted based on Foxp3 expression and analyzed by TCR clonotyping. CDR3 amino acid sequences of the TRBV13-2 subset were determined and used to identify individual TCRs for each T cell subset from the spleen (A), tumour (D), non-draining (B) and draining (C) lymph nodes of mice given MCA. Graphs are displayed as described for Figure legend 4.2.

Treg and Tconv TCR repertoires in the tumour. However, this value remained very low indicating that very little similarity existed between the Tconv and Treg TCR repertoires.

Therefore, the results from this experiment contrasted with those of the previous experiment. The initial experiment suggested that there was a significantly greater overlap between the Treg and Tconv TCR repertoires in the tumour compared to the spleen. However, in the experiment where Tconv and Treg cells were sorted based on Foxp3, very little of the Treg repertoire overlapped with the Tconv repertoire in all tissues examined. It was noticeable that the degree of overlap observed in the analysis of T cells from both spleen and in particular, tumour, was lower in the later analysis than previously observed when the cells were sorted based on CD25. The most likely explanation for these differences between the two experiments is that in the initial experiments, (in which cells were sorted based on CD25,) contamination of the two T cell pools resulted in an overlap in the two repertoires. The reduction in the overlap in the later experiment was therefore as a result of improving the purification of Tconv and Treg cells. Furthermore, a much larger change was observed in the tumour than in the spleen. This was probably due to the higher level of contamination in the tumour compared to the spleen in the initial experiment.

Mouse 3 (2nd GFP mouse)

The experiment was repeated with another set of samples from a different Foxp3_GFP MCA tumour bearing mouse. This was a straight repeat of the experiment performed with the previous mouse (mouse 2). $CD4^{+}Foxp3^{+}$ Treg and $CD4^{+}Foxp3^{-}$ Tconv cells were sorted from spleen, non-draining lymph node, draining lymph node and tumour. The TRBV13-2 TCR repertoires of each population were determined by clonotyping. In total 679 TCR sequences were analysed (Table 4.1).

In the spleen, out of 68 different TRBV13-2 Treg clonotypes, only 1 overlapped with the Tconv repertoire; this accounted for only 1.3% of the Treg TCR repertoire (Figure 4.7A). Similarly, none of the 73 different Treg clonotypes overlapped in the non-draining lymph node (Figure 4.7B). Thus, in normal lymphoid tissue, the TCR repertoires of Tconv and Treg cells were generally distinct. MH values of 0.01 and 0.0 were measured for the spleen and non-draining lymph node, respectively (Table 4.4). The lack of overlap between the Tconv and Treg TCR repertoires was also reflected in the tumour draining lymph node (Figure 4.7C), where less than 2.5% (2 out of 82) of the Treg TCR repertoire overlapped with the Tconv TCR repertoire. More overlap was observed between the Treg and Tconv TCR repertoires in the tumour (Figure 4.7D). Of 77 different Treg clonotypes, 2 overlapped with Tconv TCRs. However, this only accounted for around 3.3% of the Treg TCR repertoire. The MH values for the draining lymph node and tumour remained less than 0.05 (Table 4.4). Hence, although there was slightly more overlap in the TCR repertoires between Tregs and Tconv cells in the tumour, the vast majority (>96%) of the Treg repertoire was distinct from the Tconv repertoire in all locations.

The Morisita-Horn similarity index was used to measure the similarity between the TCR repertoires from all locations and cell populations (Figure 4.8). Using, the average similarity from the two analyses of cells sorted based on Foxp3, the levels of similarity between the TRBV13-2 TCR repertoires of Tconv and Treg cells were consistently low (MH < 0.04) throughout all four tissues measured. A small increase in the MH values was observed in the draining lymph node and tumour but generally the Treg and Tconv TCR repertoires were distinct in these tissues as well as the spleen and non-draining lymph node.

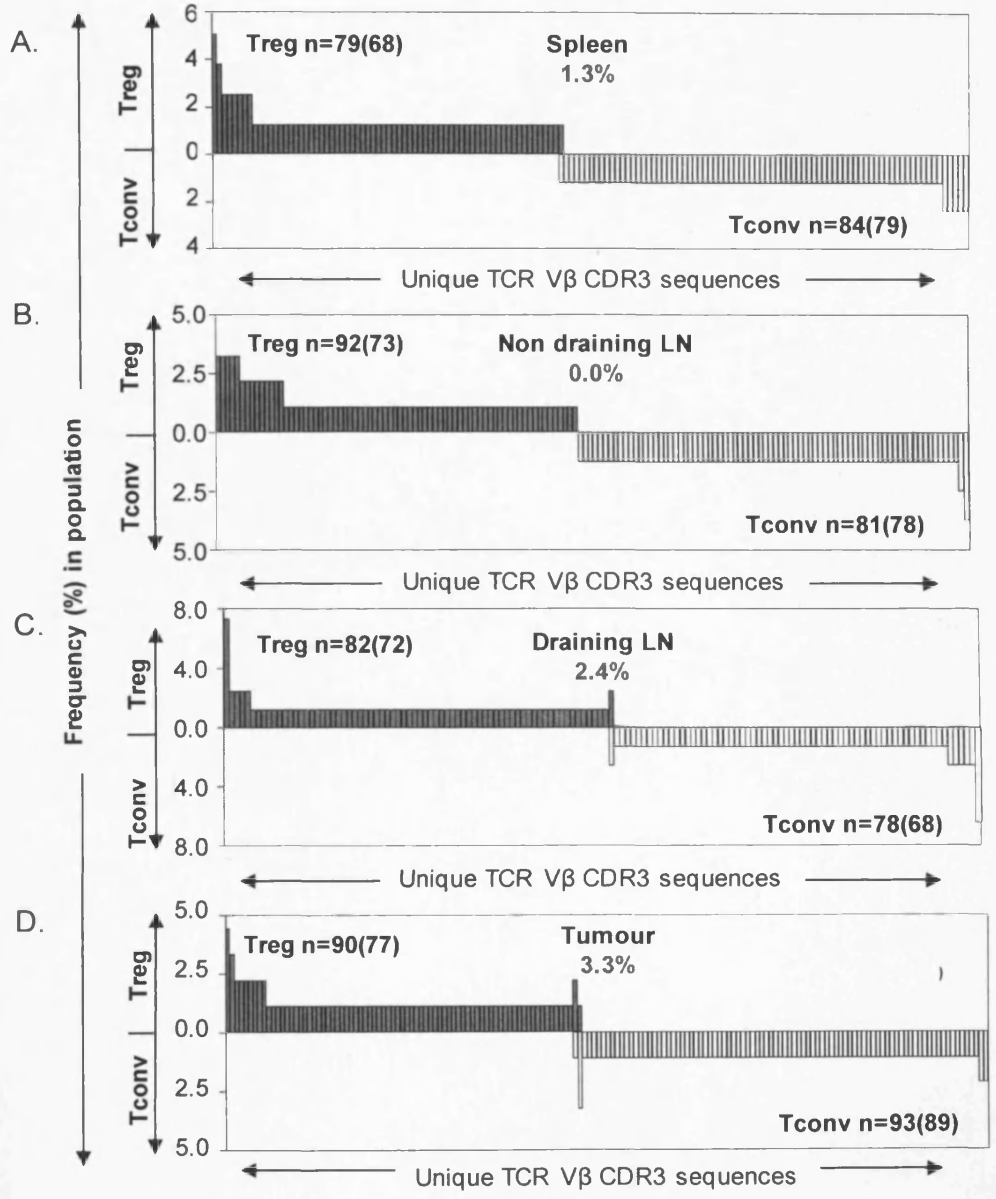


Figure 4.7. TRBV13-2 TCR repertoires of Tconv and Treg in tumour mice. Tconv ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) FACS sorted cells were analyzed by TCR clonotyping. CDR3 amino acid sequences of the TRBV13-2 subset were determined and used to identify individual TCRs for each T cell subset from the spleen (A), tumour (D), non-draining (B) and draining (C) lymph nodes of mice given MCA. Graphs are displayed as described for Figure legend 4.2.

Tissue	Number of Treg TCR sequences that overlap with Tconv TCR repertoire	Frequency of overlapping sequences within Treg TCR repertoire?	Morisita-Horn index
Spleen	1 out of 68 (1.5%)	1 out of 79 (1.3%)	0.010
ND	0 out of 73 (0%)	0 out of 92 (0%)	0.000
D	1 out of 72 (1.4%)	2 out of 82 (2.4%)	0.035
Tumour	2 out of 77 (2.6%)	4 out of 90 (4.4%)	0.044

Table 4.4. Similarity between Treg and Tconv TCR repertoires in mouse 3
The number of Treg TCR sequences that overlap with the Tconv TCR repertoire was determined for the spleen, non-draining lymph node (ND), draining lymph node (D), and tumour. Data are also displayed to show the proportion of the Treg TCR repertoire that overlapped with the Tconv TCR repertoire. The Morisita-Horn similarity index between the Tconv and Treg TCR repertoires was also determined.

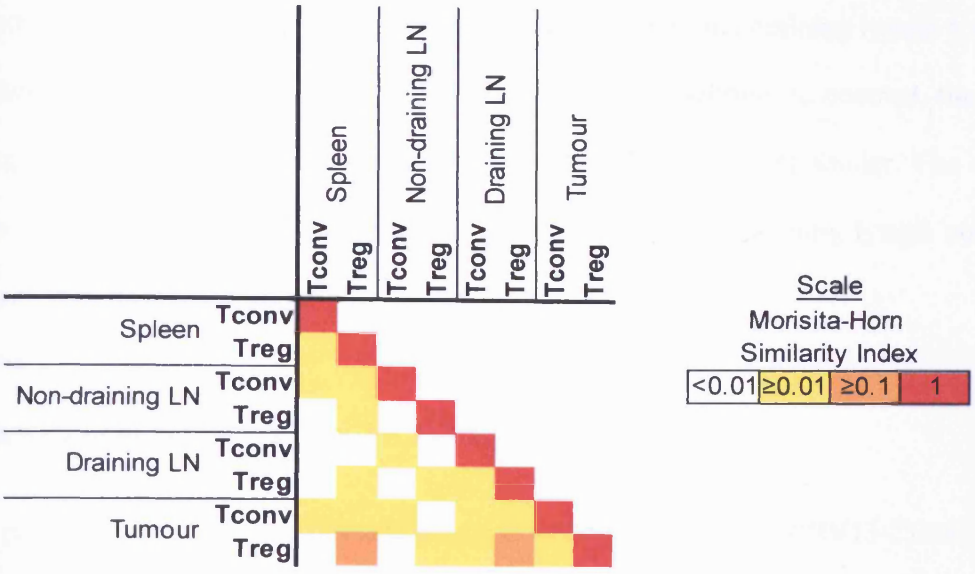


Figure 4.8. Morisita-Horn similarity values for comparison of all TRBV13-2 TCR repertoires.

Similarity between each TCR repertoire was measured using the Morisita-Horn index. This index measures similarity based on the number of shared sequences between two populations as well as how well represented those overlapping sequences are within the two populations. A value between 0 (no similarity) and 1 (identical) was calculated by comparing the TCR repertoire from each population and coloured according to the scale given. Values used are mean similarity taken from two mice and two independent experiments.

The greatest degree of similarity was observed between the Treg repertoires from draining lymph node and tumour (Figure 4.8). The Treg repertoire from the draining lymph node was on average ~20% (MH = 0.19) similar to the tumour Treg repertoire. In contrast, the Tconv and Treg repertoires from the tumour were only 3.8% (MH = 0.038) similar. The overlap between the Treg TCR repertoires from the tumour and tumour draining lymph node was clear for both mice (Figure 4.9).

4.2.4 Large amount of diversity in TRBV13-2 repertoires

For the previous two TCR repertoire analyses a single TRBV subset (TRBV13-2) was examined. A single TRBV subset was chosen in order to reduce the potential clonotypic diversity in the TCR repertoires analysed. However, the identification of very many different clonotypes suggested that the diversity in the TCR repertoires was nevertheless still very high.

Using data generated from the accumulation of TCR sequences it is possible to estimate the number of different clonotypes (diversity, or more accurately termed richness) that would be found within each group. The Michaelis-Menten equation (originally designed for measuring enzyme kinetics) can be used to calculate the diversity (richness) of a TCR repertoire in the same way in which species diversity for ecological system can be estimated from a quadrant or sample (Magurran 2004). As the number of TCR sequences analysed (x) increases so too does the accumulated number of new/unique TCR clones (y; Figure 4.10). However, when an analysed sequence is a repeat of a previously identified clonotype the number of new clonotypes (y) stays the same. A curve of cumulative number of sequences (x) versus new TCR clones identified (y) therefore follows a non-linear regression (unless all sequences

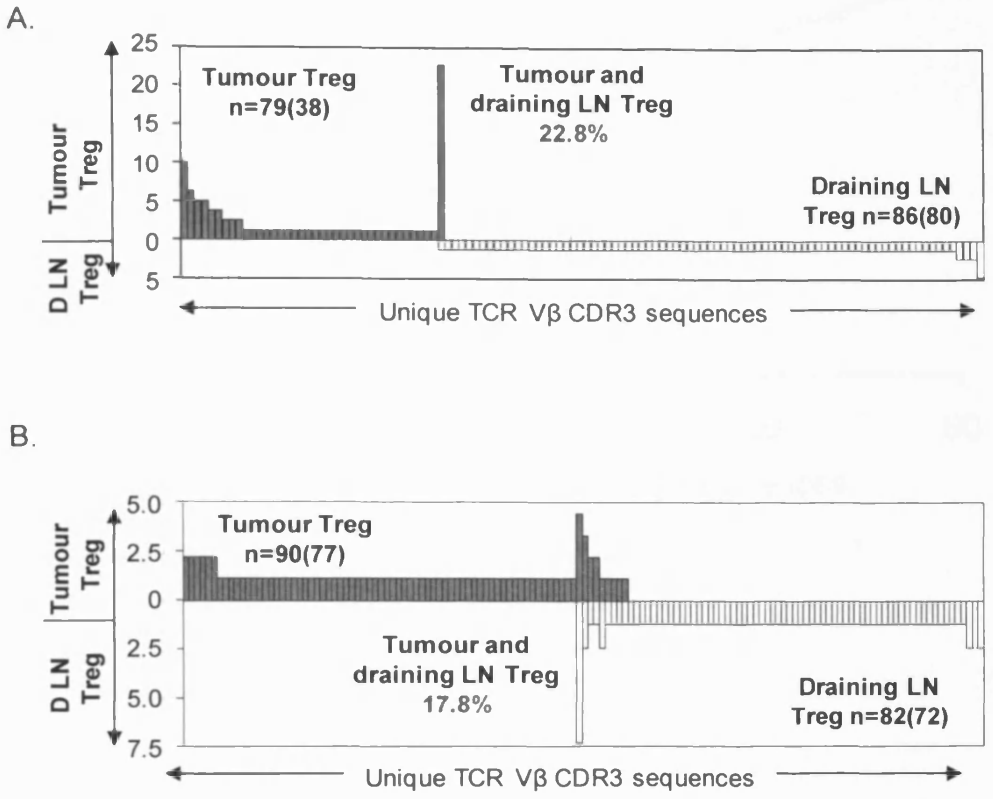


Figure 4.9. TRBV13-2 TCR repertoires of Treg TCR repertoires from draining lymph node and tumour.

The TRBV13-2 TCR sequences were determined by clonotyping and used to identify individual TCRs. The TCR repertoires of Treg cells from tumour and Treg cells from draining lymph node were compared for mouse 2 (A)and mouse 3 (B). Different TCR sequences observed (x axis) within the tumour Treg (filled & above) and draining lymph node Treg (open & below) repertoires and the frequency (y axis) of each sequence within that subset are displayed. The total number of TCR sequences analysed is displayed as n=, with the number of unique TCR sequences observed in parentheses. The proportion of the tumour Treg repertoire that has shared sequences with the draining lymph node Treg repertoire is given.

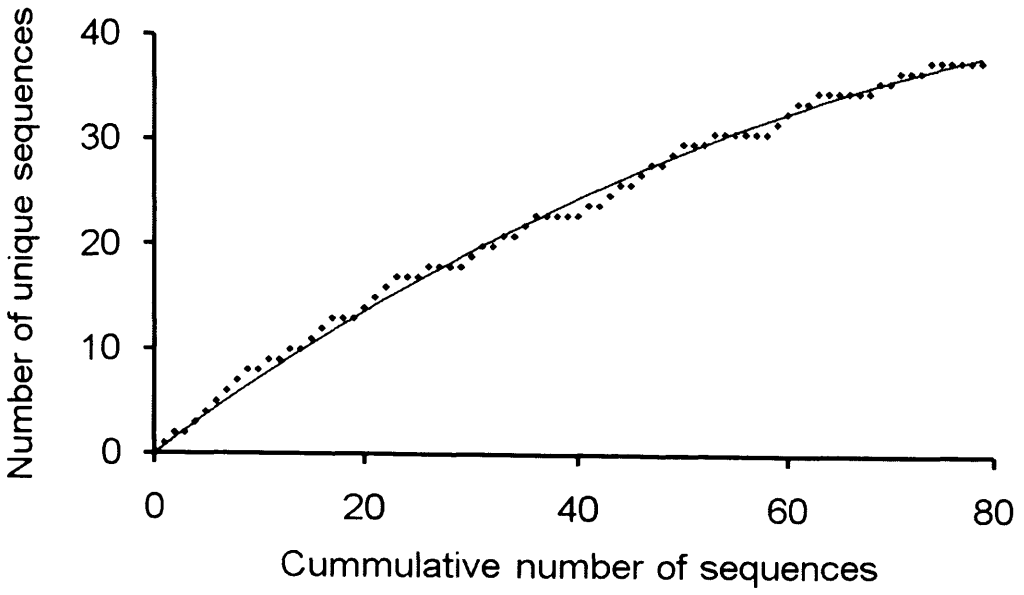


Figure 4.10 Non-linear regression of accumulation of TCR sequences
The accumulation of sequence data from mouse 2 tumour Treg was plotted. It demonstrates that as the cumulative number of sequences increases so too does the number of unique sequences. However, each time a repeat sequence is discovered the number of unique sequences does not rise. Therefore, (unless all sequences are unique) the graph follows a non-linear regression.

analysed are unique). From this non-linear regression the Michaelis-Menten equation can be used to estimate the total number of different sequences (S_{max}) that would be observed if an infinite number of sequences were analysed (Figure 4.11).

Using the data generated through the accumulation of TCR sequences and the Michaelis-Menten equation, the size of the TCR repertoires were first approximated for mouse 2 (Table 4.5 and Figure 4.12). The lowest level of diversity was estimated for the tumour Treg sample where 97 clonotypes were predicted to exist in the TRBV13-2 repertoire. The highest diversity was estimated in the draining lymph node Tconv cell sample where 3628 clonotypes were predicted. Using this information it is possible to predict how much of the repertoire was analysed in the experiment. In the tumour Treg sample, 38 different clonotypes were identified. Therefore 39% (38 out of 97) of the estimated clonotpyes were identified (Figure 4.13). However, in the draining lymph node 79 clonotypes were identified, this equated to just 2% (79 out of 3628) of the estimated number of clonotypes. On average, it was estimated that 10% of the possible TRBV13-2 clonotypes were identified in the TCR repertoire analysis of mouse 2 (Figure 4.13).

These analyses suggested that a high amount of diversity existed and that many of the TCR sequences may have been missed in the initial TRBV13-2 analysis. Using the Michaelis-Menten equation it was estimated that to identify 90% of the predicted 97 clonotypes in the tumour a total of 1076 sequences would need to be analysed (Table 4.5). This problem is even greater in the draining lymph node. To identify 90% of the clonotypes it was estimated that more than 32,000 sequences would need to be analysed.

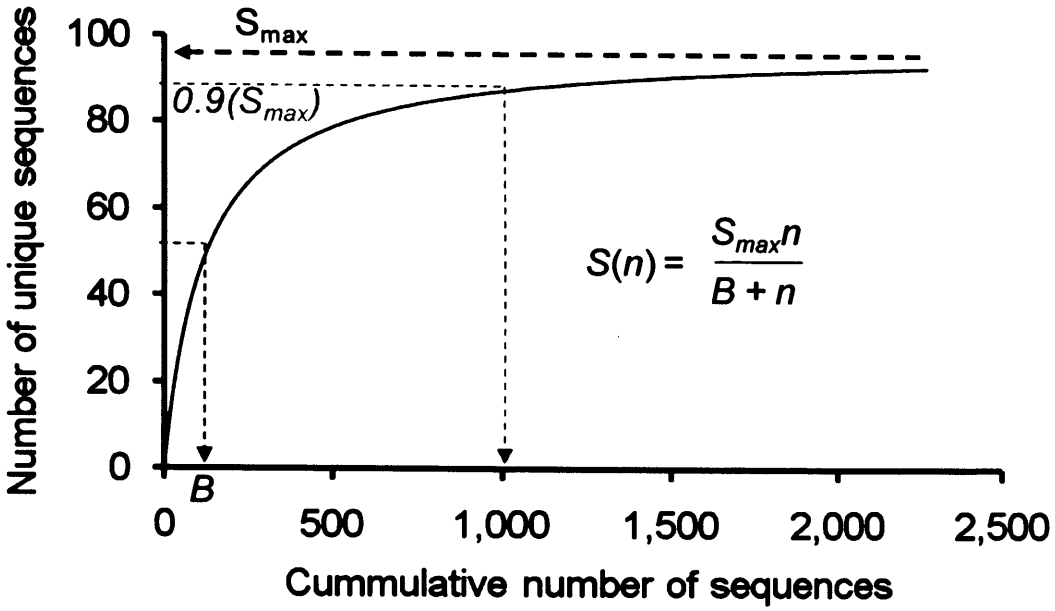


Figure 4.11 Extrapolated Michaelis-Menten curve
Using the data plotted in Figure 4.10, GraphPad Prism was used to determine the parameters of the Michaelis-Menten equation. Where S(n) = the number of new TCR sequences observed after *n* sequences; S_{max} = the total number of sequences in the repertoire; and *B* is the amount of sequences required to detect 50% of the S_{max}. The parameters were used to extrapolate the data and generate the graph shown above. These parameters were also used to estimate the total size of the TCR repertoire (S_{max}) and the number of sequences needed to identify 90% of the different clonotypes (0.9 x S_{max}).

Tissue	Cell type	Number of different TCR clonotypes identified	Estimated number of TCR clonotypes (S_{max})	% of clonotypes identified	Number of sequences required to identify 90% of clonotypes
Spleen	Tconv	77	1977	4%	17,739
	Treg	72	531	14%	4,596
ND	Tconv	81	1666	5%	14,697
	Treg	81	1575	5%	14,616
D	Tconv	79	3628	2%	32,814
	Treg	80	962	8%	8,530
Tumour	Tconv	74	1770	4%	17,397
	Treg	38	97	39%	1,076

Table 4.5 Size and diversity estimates for Treg and Tconv TRBV13-2 TCR repertoires in mouse 2
The number of different clonotypes that were identified from TCR repertoire analysis of Tconv and Treg cells isolated from the spleen, non draining lymph node (ND), draining lymph node (D) and tumour of tumour bearing mouse 2. From these data the number of clonotypes was estimated as well as the number of sequences that would be required to identify 90% of these clonotypes. It was also estimated what percentage of the clonotypes were identified in this analysis.

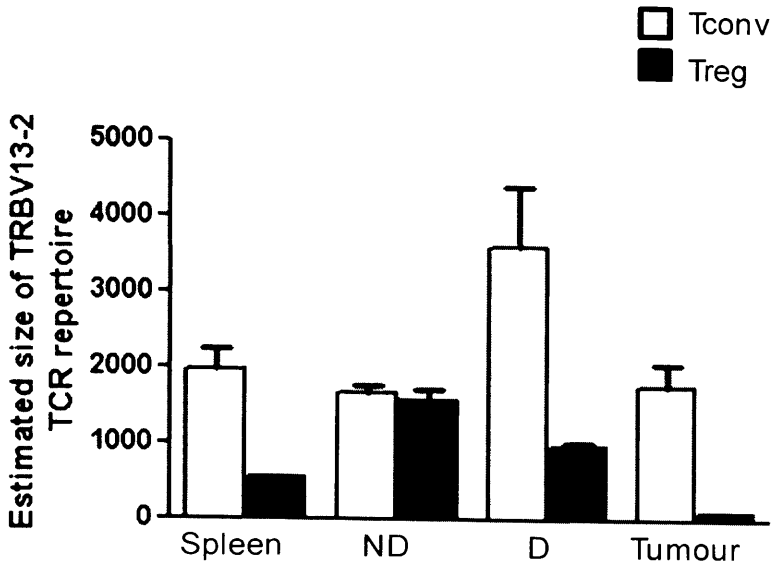


Figure 4.12 Estimated size of the TRBV13-2 TCR repertoires of Tconv and Treg from mouse 2

The accumulated data from TRBV13-2 TCR repertoire analysis of Tconv (open bars) and Treg (filled bars) cells from spleen, non-draining lymph node (ND), draining lymph node (D) and tumour was used to plot non-linear regression curves and estimate the size of the TCR repertoire using the Michaelis-Menten equation. The estimated the number of clonotypes that would be discovered after analysing an infinite number of sequences (S_{max}) +95% confidence intervals are displayed.

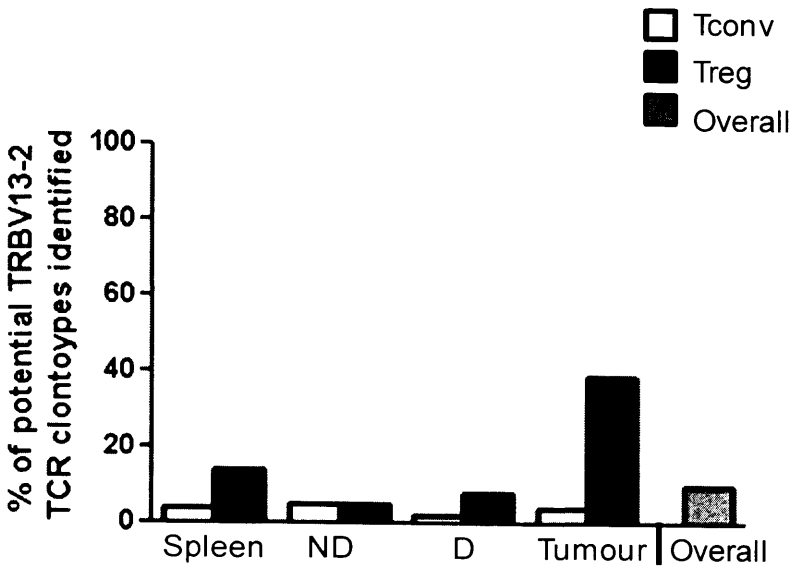


Figure 4.13 Estimated percent of the TRBV13-2 TCR clonotypes identified in mouse 2
From the estimated number of clonotypes predicted and the number of clonotypes actually discovered it was estimated what percentage of the possible TRBV13-2 TCR clonotypes were identified within the Tconv (open bars) and Treg (filled black bars) cell repertoires from spleen, non-draining lymph node, draining lymph node and tumour. These values were averaged to estimate the overall (filled grey bars) percent of the TRBV13-2 TCR clonotypes that was identified.

The sizes of the TCR repertoires were also estimated for the samples from mouse 3 (Table 4.6 and Figure 4.14). The highest repertoire size was estimated in the tumour Tconv cell sample where 3371 clontoypes were predicted. The lowest was for the non-draining Treg repertoire where 297 clonotypes were predicted. It was estimated that throughout all tissues on average only 11% of the potential clonotypes were identified (Figure 4.15). In the largest estimated sample, the tumour Tconv, more than 31,000 sequences would need to be analysed to identify 90% of the total repertoire (Table 4.6). In the smallest, the non-draining Treg repertoire, 2387 sequences would need to be analysed to identify 90% of the total repertoire.

The diversity estimates from the TRBV13-2 repertoires of mouse 2 and mouse 3 indicated that the diversity of the TCR repertoires was often very large and that many of the clonotypes may have been missed in the analysis. They also suggested that to identify a large majority (90%) of the clonotypes extremely large sample sizes (>30,000) are required.

4.2.5 Analysis of the TRBV13-2/TRBJ2-5 repertoires of Tconv and Treg cells

Rather than performing more sequencing in the TRBV13-2 repertoire a different approach was taken. The examination of the TRBV13-2 repertoire was further focused and investigated in more detail by performing the sequencing on a single TRBJ subset within the TRBV13-2 subfamily. As for the Vβ usage analysis, no significant skewing towards any particular Jβ subset was observed within the Treg or Tconv repertoires from the spleen, tumour or lymph node (Figure 4.16). Therefore, as above, the relatively common TRBJ2-5 gene was selected as a representative subset for the focused analysis.

Tissue	Cell type	Number of different TCR clonotypes identified	Estimated number of TCR clonotypes (S_{max})	% of clonotypes identified	Number of sequences required to identify 90% of clonotypes
Spleen	Tconv	79	1153	7%	10,251
	Treg	68	499	14%	4,635
ND	Tconv	78	1299	6%	11,556
	Treg	73	297	25%	2,387
D	Tconv	68	925	7%	9,018
	Treg	72	418	17%	3,688
Tumour	Tconv	89	3371	3%	31,077
	Treg	77	707	11%	6,509

Table 4.6 Size and diversity estimates for Treg and Tconv TRBV13-2 TCR repertoires in mouse 3
The number of different clonotypes that were identified from TCR repertoire analysis of Tconv and Treg cells isolated from the spleen, non draining lymph node (ND), draining lymph node (D) and tumour of tumour bearing mouse 3. From these data the number of clonotypes was estimated as well as the number of sequences that would be required to identify 90% of these clonotypes. It was also estimated what percentage of the clonotypes were identified in this analysis.

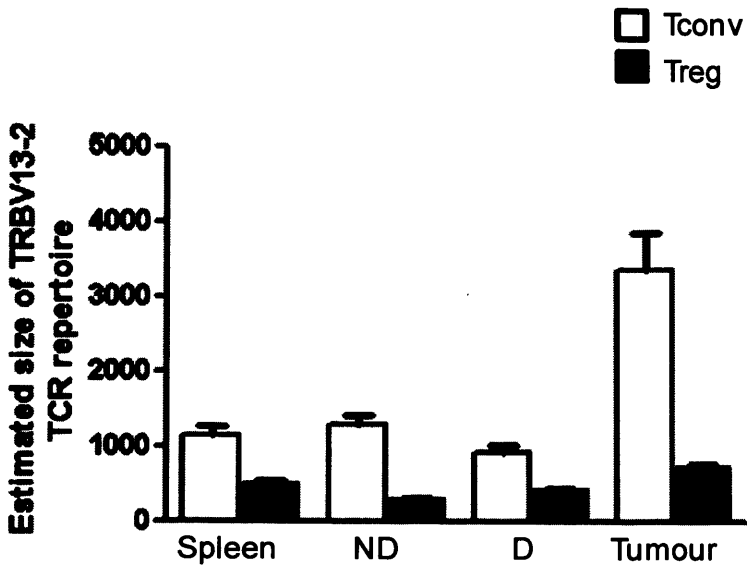


Figure 4.14 Estimated size of the TRBV13-2 TCR repertoires of Tconv and Treg from mouse 3

The accumulated data from TRBV13-2 TCR repertoire analysis of Tconv (open bars) and Treg (filled bars) cells from spleen, non-draining lymph node (ND), draining lymph node (D) and tumour was used to plot non-linear regression curves and estimate the size of the TCR repertoire using the Michaelis-Menten equation. The estimated the number of clonotypes that would be discovered after analysing an infinite number of sequences (S_{max}) +95% confidence intervals are displayed.

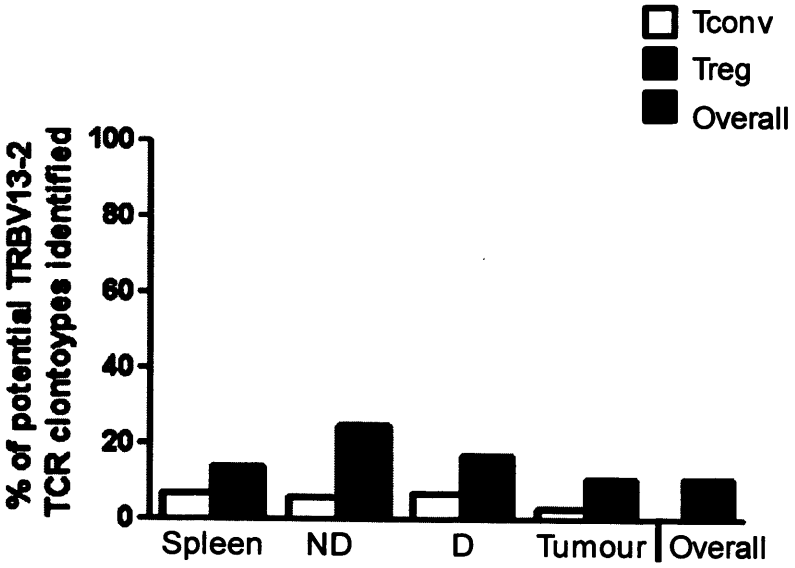


Figure 4.15 Estimated percent of the TRBV13-2 TCR clonotypes identified in mouse 3 From the estimated number of clonotypes predicted and the number of clonotypes actually discovered it was estimated what percentage of the possible TRBV13-2 TCR clonotypes were identified within the Tconv (open bars) and Treg (filled black bars) cell repertoires from spleen, non-draining lymph node, draining lymph node and tumour. These values were averaged to estimate the overall (filled grey bars) percent of the TRBV13-2 TCR clonotypes that was identified.

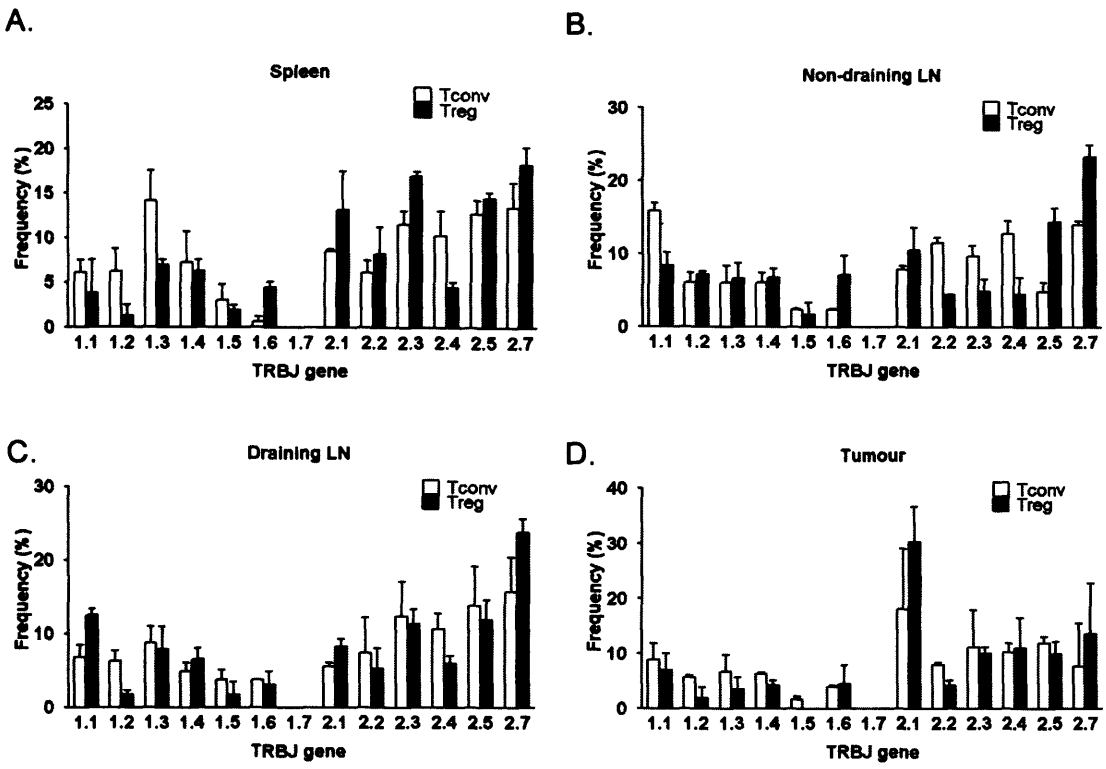


Figure 4.16. TRBJ gene usage within Tconv and Treg from tumour bearing mice. Tconv ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) FACS sorted cells were analyzed by TCR clonotyping. The TRBJ gene usage for Tconv (open bars) and Treg (filled bars) cells were determined for each population from spleen (A), tumour (D), non-draining (B) and draining (C) lymph nodes. Data shown are the mean frequencies (+/- SEM) obtained using two mice from two independent experiments.

In agreement with the findings from the TRBV13-2 TCR repertoire analysis, the TRBV13-2/TRBJ2-5 TCR repertoires of Tconv and Treg cells were generally non-overlapping within all tissues including the tumour (Table 4.7 and Figure 4.17). No shared TCR sequences were found within the tumour or the non-draining lymph node. Furthermore, only 1 overlapping sequence was found within the spleen and draining lymph node repertoires.

Conversely, when the TRBV13-2/TRBJ2-5 TCR repertoires of Tregs from the draining lymph node and Tregs from the tumour were compared, a high degree of overlap was clearly observed (Figure 4.18). Out of 17 different clonotypes, 6 (35.3%) were found to be overlapping. These 6 overlapping clonotypes made up 33.3% (21 out of 63 sequences) of the tumour Treg repertoire. The similarity between the Treg subsets was highlighted when analyzed using the Morisita-Horn similarity index (Figure 4.19). The greatest level of similarity (MH = 0.16) was observed between the TCR repertoires of the tumour-infiltrating Tregs and draining lymph node Tregs. In contrast, the similarity between the Treg and Tconv repertoires was less in both the tumour and the draining lymph node (MH = 0.0 and 0.017, respectively). These data are in agreement with previous analysis of the whole TRBV13-2 TCR repertoire.

4.2.6 Size estimates of TRBV13-2/TRBJ2-5 TCR repertoires

The TCR sequence data was used to estimate the size of the TRBV13-2/TRBJ5-5 TCR repertoires. Using the Michaelis-Menten equation it was estimated that the largest TRBV13-2/TRBJ2-5 TCR repertoire was the spleen Tconv where 211 different clonotypes were predicted (Table 4.8 and Figure 4.20). The tumour Treg repertoire was predicted to be the smallest repertoire at 24 different clonotypes. From the spleen Tconv repertoire 42 different

Tissue	Number of Treg TCR sequences that overlap with Tconv TCR repertoire	Frequency of overlapping sequences within Treg TCR repertoire?	Morisita-Horn index
Spleen	1 out of 33 (3.0%)	7 out of 55 (12.7%)	0.127
ND	0 out of 41 (0.0%)	0 out of 62 (0.0%)	0.000
D	1 out of 33 (3.0%)	2 out of 53 (3.8%)	0.017
Tumour	0 out of 17 (0.0%)	0 out of 63 (0.0%)	0.000

Table 4.7. Similarity between Treg and Tconv TCR repertoires in mouse 4
The number of Treg TCR sequences that overlap with the Tconv TCR repertoire was determined for the spleen, non-draining lymph node (ND), draining lymph node (D), and tumour. Data are also displayed to show the proportion of the Treg TCR repertoire that overlapped with the Tconv TCR repertoire. The Morisita-Horn similarity index between the Tconv and Treg TCR repertoires was also determined.

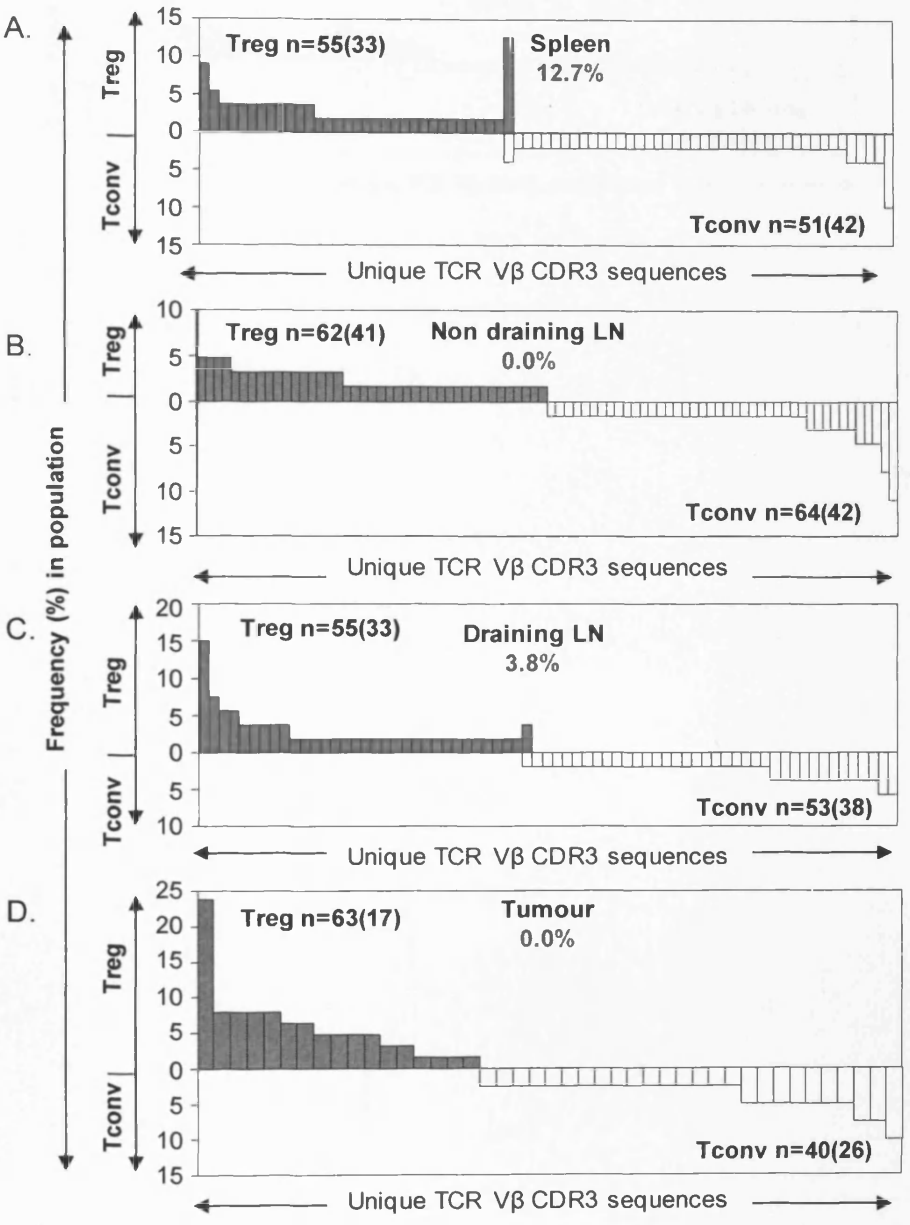


Figure 4.17. TRBJ2-5,TRBV13-2 TCR repertoires of Tconv and Treg in tumour mice. Tconv ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) FACS sorted cells were analyzed by TCR clonotyping. CDR3 amino acid sequences of the TRBV13-2, TRBJ2-5 subset were determined and used to identify individual TCRs for each T cell subset from the spleen (A), tumour (D), non-draining (B) and draining (C) lymph nodes of mice given MCA. Graphs are displayed as described for Figure legend 4.2.

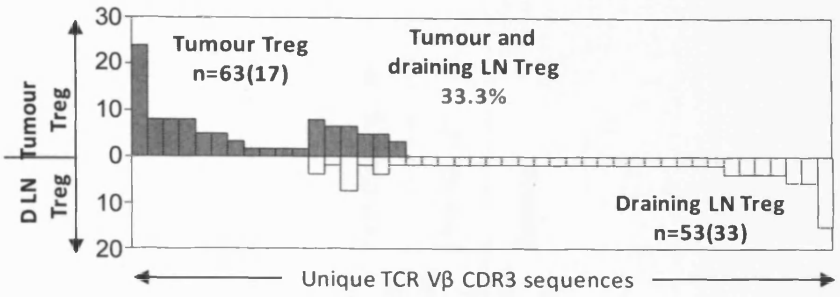


Figure 4.18. TRBJ2-5, TRBV13-2 TCR repertoires of Treg TCR repertoires from draining lymph node and tumour.
The TRBJ2-5, TRBV13-2 TCR sequences were determined by clonotyping and used to identify individual TCRs. The TCR repertoires of Treg from tumour and Treg from draining lymph node were compared. Graphs are displayed as described for Figure legend 4.9.

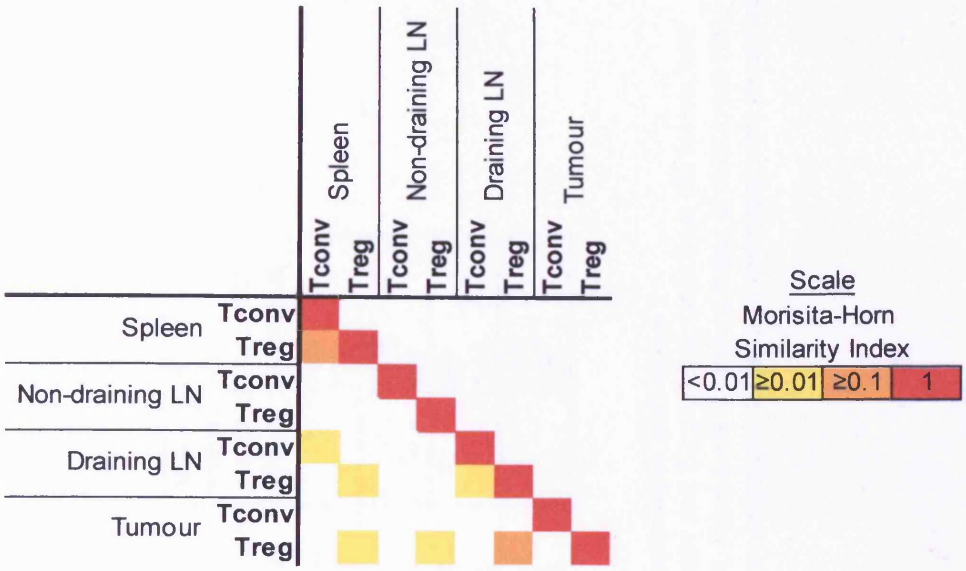


Figure 4.19. Morisita-Horn similarity values for comparison of all TRBJ2-5, TRBV13-2 TCR repertoires.
Similarity between each TCR repertoire was measured using the Morisita-Horn index. This index measures similarity based on the number of shared sequences between two populations as well as how well represented those overlapping sequences are within the two populations. A value between 0 (no similarity) and 1 (identical) was calculated by comparing the TCR repertoire from each population and coloured according to the scale given.

Tissue	Cell type	Number of different TCR clonotypes identified	Estimated number of TCR clonotypes (S_{max})	% of clonotypes identified	Number of sequences required to identify 90% of clonotypes
Spleen	Tconv	42	211	20%	1765
	Treg	33	83	40%	728
ND	Tconv	42	118	36%	1064
	Treg	41	103	40%	892
D	Tconv	38	120	32%	997
	Treg	33	121	27%	1311
Tumour	Tconv	26	97	27%	1011
	Treg	17	24	71%	239

Table 4.8 Size and diversity estimates for Treg and Tconv TRBV13-2/TRBJ2-5 TCR repertoires in mouse 4
The number of different clonotypes that were identified from TCR repertoire analysis of Tconv and Treg cells isolated from the spleen, non draining lymph node (ND), draining lymph node (D) and tumour of tumour bearing mouse 4. From these data the number of clonotypes was estimated as well as the number of sequences that would be required to identify 90% of these clonotypes. It was also estimated what percentage of the clonotypes were identified in this analysis.

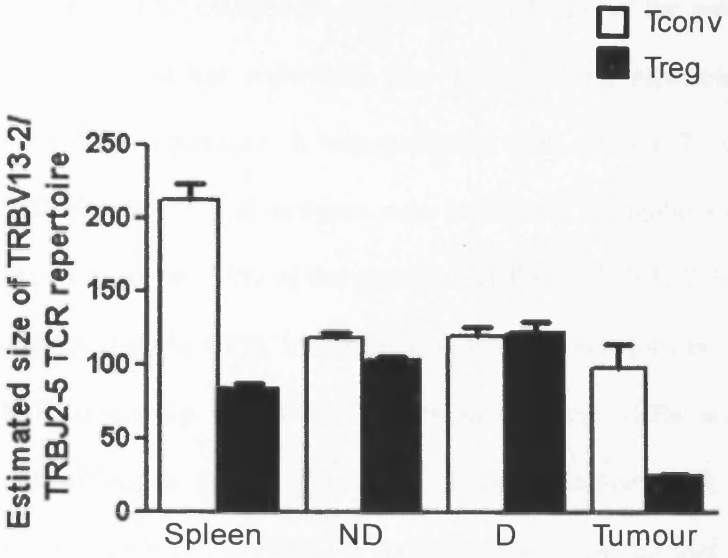


Figure 4.20 Estimated size of the TRBV13-2/TRBJ2-5 TCR repertoires of Tconv and Treg from mouse 4

The accumulated data from TRBV13-2/TRBJ5-5 TCR repertoire analysis of Tconv (open bars) and Treg (filled bars) cells from spleen, non-draining lymph node (ND), draining lymph node (D) and tumour was used to plot non-linear regression curves and estimate the size of the TCR repertoire using the Michaelis-Menten equation. The estimated the number of clonotypes that would be discovered after analysing an infinite number of sequences (S_{max}) +95% confidence intervals are displayed.

clonotypes were identified. This equated to ~20% (42 out of 211) of the predicted repertoire (Figure 4.21). From the smallest repertoire (the tumour Treg repertoire) 17 different clonotypes were identified. Therefore, it was predicted that ~70% (17 out of 24) of the potential TRBV13-2/TRBJ2-5 TCR clonotypes were identified. Throughout the whole mouse it was predicted that on average ~36% of the potential TRBV13-2/TRBJ2-5 clonotypes were identified in the analysis (Figure 4.21). In comparison with the previous two analyses of just the TRBV13-2 TCR repertoire (where it was estimated that ~10% and ~11% of the clonotypes were identified) a larger proportion of the potential TCR clonotypes was identified (~36%) in the TRBV13-2/TRBJ2-5 analysis. This indicates that the analysis was refined so that fewer of the potential TCR clonotypes were missed and hence the chance of missing any overlapping clones was also reduced.

4.2.7 Summary of TCR repertoire data

Analysis of the TRBV13-2 and TRBV13-2/TRBJ2-5 TCR repertoires of Tconv and Treg cells in tumour bearing mice showed that the Tconv and Treg repertoires were by and large distinct from each other. In collaboration with Professor David Price, Dr Kristin Ladell and Dr Katherine Wynn of Cardiff University, additional analyses of the TCR repertoires of Tconv and Treg cells from MCA tumour bearing mice were made in parallel to the experiments described above. Using an unbiased template-switch anchored RT-PCR (Price *et al.* 2005) TCR clonotyping of the total TCR repertoire was performed on $CD4^{+}Foxp3^{-}$Tconv and $CD4^{+}Foxp3^{+}$Treg cells sorted by flow cytometry. In agreement with the focused TCR repertoire analyses, the total TCR repertoires of Tconv and Treg cells were found to be non-overlapping. No TCR clonotypes from the Treg repertoire were found within the corresponding Tconv repertoire from the same location (Figure 4.22).

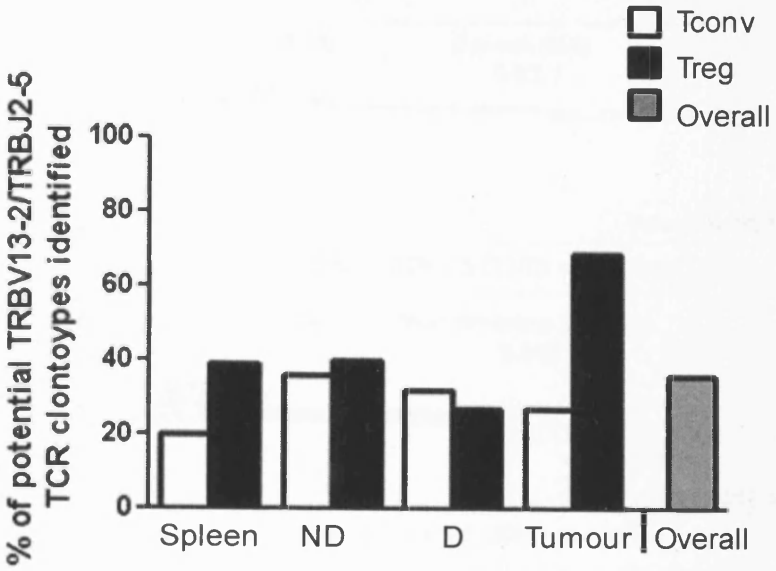


Figure 4.21 Estimated percent of the TRBV13-2/TRBJ2-5 TCR clonotypes identified in mouse 4

From the estimated number of clonotypes predicted and the number of clonotypes actually discovered it was estimated what percentage of the possible TRBV13-2/TRBJ2-5 TCR clonotypes were identified within the Tconv (open bars) and Treg (filled black bars) cell repertoires from spleen, non-draining lymph node, draining lymph node and tumour. These values were averaged to estimate the overall (filled grey bars) percent of the TRBV13-2/TRBJ2-5 TCR clonotypes that was identified.

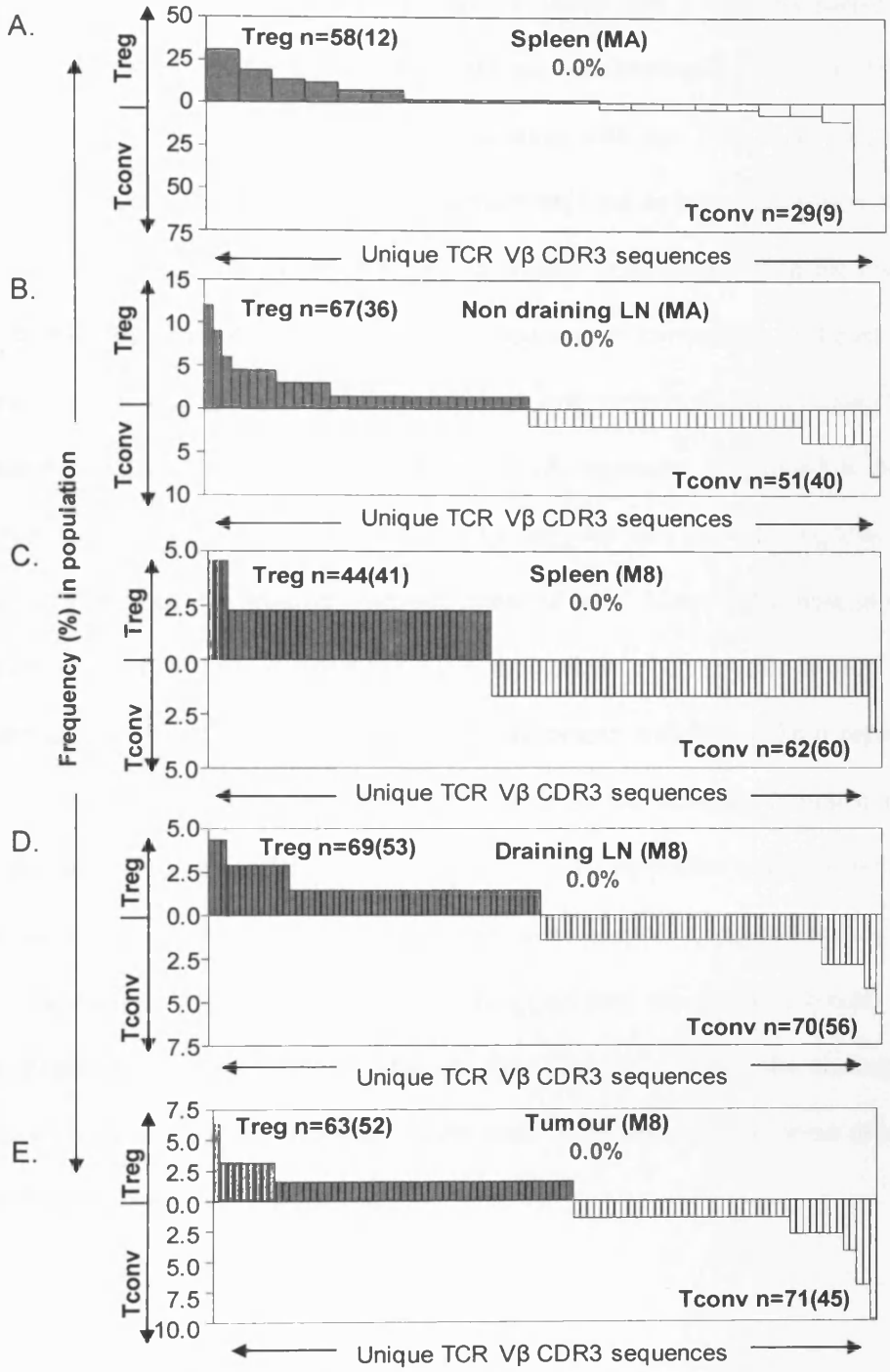


Figure 4.22. TCR repertoires of Tconv and Treg in tumour mice.
Tconv ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) FACS sorted cells from mice bearing MCA tumours were analyzed using an unbiased TCR clonotyping method. CDR3 amino acid sequences were determined and used to identify individual TCRs for each T cell subset from the spleen (A), and non-draining lymph node (B) of mouse A (MA) and the from the spleen (C) draining lymph node (D) and tumour (E) of mouse 8 (M8). Graphs are displayed as described for Figure legend 4.2.

The findings from the TCR repertoire analyses of Tconv and Treg cells sorted based on Foxp3 expression were compiled. Taken together, on average 4.58% of the tumour infiltrating Treg TCR repertoire was found to overlap with the TCR repertoire of tumour infiltrating Tconv cells (Figure 4.23). This was more than the average proportion of the Treg TCR repertoire (0.285%) that overlapped with the Tconv TCR repertoire in the non-draining lymph node. Although this may indicate that an increase in conversion of Tconv cells into Tregs occurs within the tumour, this increase was not statistically significant. Moreover, greater than 95% of the tumour infiltrating Treg TCR repertoire was found to not overlap with the Tconv TCR repertoire (Figure 4.24). This suggests that the vast majority of tumour infiltrating Treg cells do not arise through the conversion of Tconv cells. Instead the largest degree of overlap was found between the TCR repertoires of Treg cells from the draining lymph node and tumour. On average, ~20% of the tumour infiltrating Treg repertoire was found to overlap with the TCR repertoire of Treg from the draining lymph node (Figure 4.23). In the most focused (TRBV13-2/TRBJ2-5) TCR repertoire analysis where it was estimated that ~36% of the potential clonotypes were identified approximately one third (33.3%) of the tumour Treg TCR repertoire overlapped with the draining lymph node Treg repertoire (Figure 4.18). Together this suggests that Treg cells within the tumour are more likely to have derived from the draining lymph node Treg pool or *vice versa* than from the tumour infiltrating Tconv cell population.

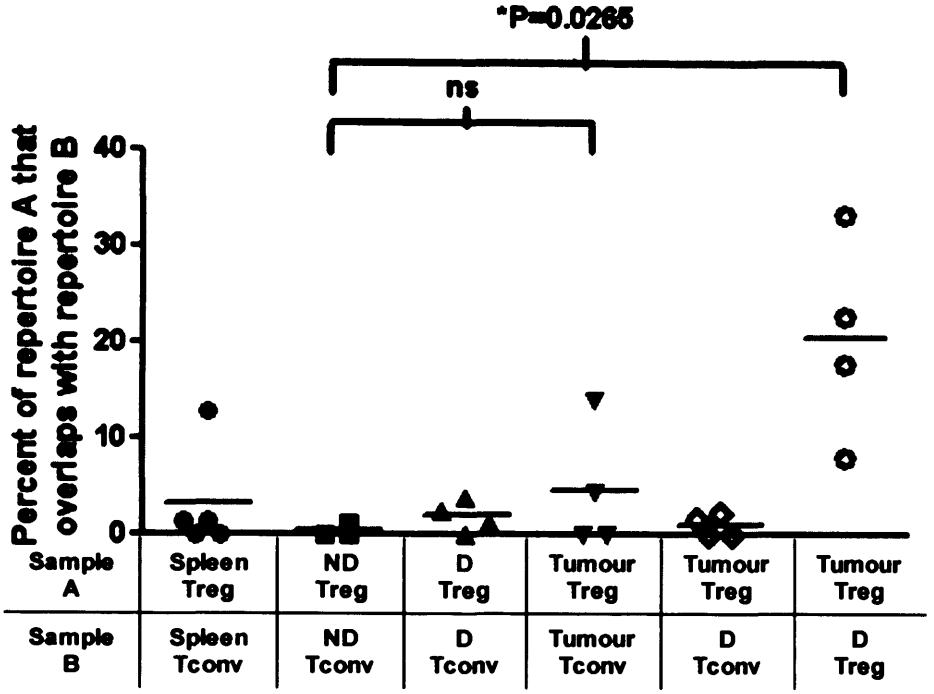


Figure 4.23 Percent overlap between various TCR repertoires
The mean percent of repertoire (sample) A that overlaps with repertoire (sample) B is given (n=4-5). Data from all TCR repertoire analyses on Foxp3 sorted cells from this thesis were compiled. Comparisons are displayed as indicated in the table. The differences between the mean of two groups were compared using unpaired T tests.

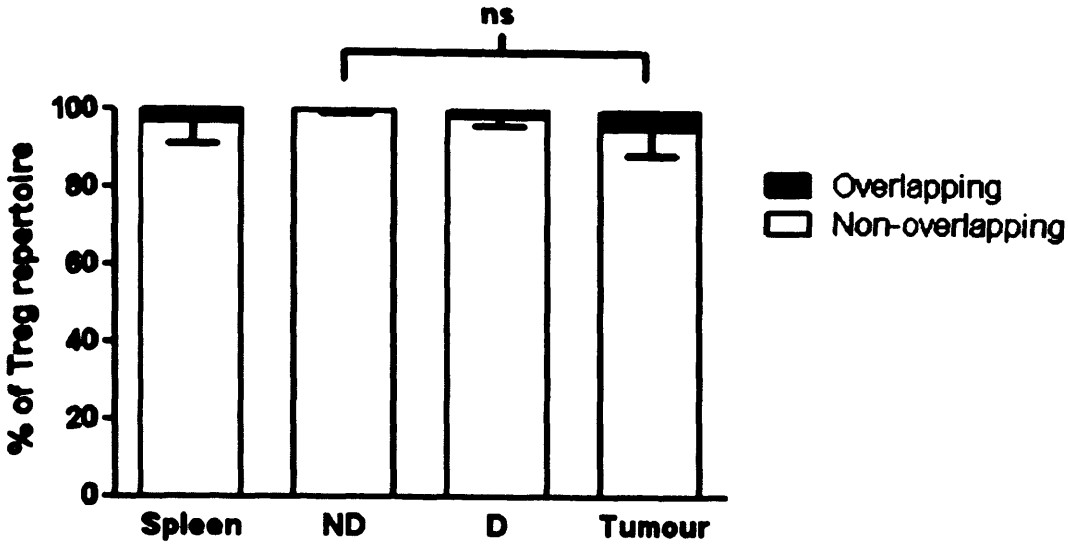


Figure 4.24 Degree of the Treg TCR repertoire that is found to overlap (and not-overlap) with the corresponding Tconv TCR repertoire
The mean (+/-SEM) percentage of the Treg TCR repertoire that was found to be overlapping (filled) and not-overlapping (open) with the corresponding Tconv TCR repertoire was determined. Data from all analyses on Foxp3 sorted cells from this thesis for spleen, non-draining lymph node (ND), draining lymph node (D) and tumour were compiled (n=4-5). Groups were compared using unpaired T tests.

4.2.8 Does the proliferation of CD4+ T cells preferentially promote the accumulation of Treg cells in tumours?

Originally Tregs were described as being non-proliferative. These conclusions were based on the finding that Tregs stimulated through the TCR did not proliferate *in vitro*. More recently it was demonstrated that the addition of exogenous IL-2, or stimulation with purified DCs allowed for the expansion of Tregs *in vitro* (Thornton and Shevach 1998, Takahashi *et al.* 1998, Yamazaki *et al.* 2003). Furthermore, Gavin *et al.* (2002) found that upon adoptive transfer into lymphopenic hosts both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells proliferate extensively. Numerous studies have now confirmed that proliferation of Treg cells is possible *in vivo* and is dependent on both antigen and MHC class-II (Klein, Khazaie and von Boehmer 2003, Walker *et al.* 2003a, Cozzo, Larkin and Caton 2003, Gavin *et al.* 2002). However, the proliferative behaviour of Treg cells under normal and disease states remained to be fully determined. Mottet *et al.* (2003) investigated the proliferation of Tregs in mice with experimental colitis and found that Tregs were proliferating in the spleen, and lymph nodes of mice with colitis. Interestingly this proliferation was reduced when the inflammatory response had subsided, suggesting that Treg cell proliferation is induced by the inflammatory response they control. Treg cells were also shown to be more proliferative than Tconv cells in the draining lymph node and tumour of mice with transplanted tumours (Bui *et al.* 2006). In addition, the results of one *in vivo* human study indicated that Tregs in peripheral blood (identified by high levels of CD25 expression) are highly proliferative (Vukmanovic-Stejic *et al.* 2006). In this study, the authors used deuterium-labelled glucose to label proliferating cells with deuterium. They found that the rate of proliferation was significantly higher in Treg cells than in $CD4^+$ memory or naive T cells. This paper also showed that the Treg cells appear more prone to apoptosis and have shorter telomere lengths than other $CD4^+$ T cells

suggesting their survival is also impaired. Thus, collectively these data suggest that Treg cells have a high rate of turnover in humans *in vivo* under normal circumstances. Given that the proliferation and survival characteristics of Treg cells appeared to vary from Tconv cells under normal physiological conditions in humans and in disease states in mice, it seemed plausible that these Treg cells may also have altered characteristics within the tumour microenvironment which favour their accumulation.

To investigate the proliferative status of Tconv cells and Tregs within tumour-bearing mice, Ki67 expression was analysed within each of the $CD4^+$ T cell populations. Ki67 is a protein that is expressed during all active phases of the cell cycle and therefore demarcates cells that are proliferating (Brown and Gatter 2002). The proportion of $CD4^+Foxp3^-$ Tconv and $CD4^+Foxp3^+$ Treg cells expressing Ki67 in tumour-bearing mice was analysed by flow cytometry (Figure 4.25). A higher proportion of Treg cells compared to Tconv cells were found to express Ki67 within all of the sites analyzed in both tumour-bearing and naive mice (Figure 4.26A). The difference between the percentage of Tconv cells expressing Ki67 and the percentage of Treg cells expressing Ki67 was most significant in the tumour (***P=0.0002; Figure 4.26A). For each tissue the numerical difference (differential) between the proportion of Tconv cells and Treg cells that were $Ki67^+$ was determined (Figure 4.26B). In the spleen the differential between the percentage of Tconv cells and Treg cells that were Ki67 was on average ~15%. In the tumour the differential between the percentage of Tconv cells and Treg cells that were Ki67 was significantly (**P = 0.0019) greater where ~31% more Tregs expressed Ki67 over Tconv cells (Figure 4.26B). A similar pattern was also observed when the draining and the non-draining lymph nodes were compared. The differential between the proportion of proliferating Tconv cells and Treg cells was ~16% in the non-draining lymph node (Figure 4.26B). The differential between the proportion of

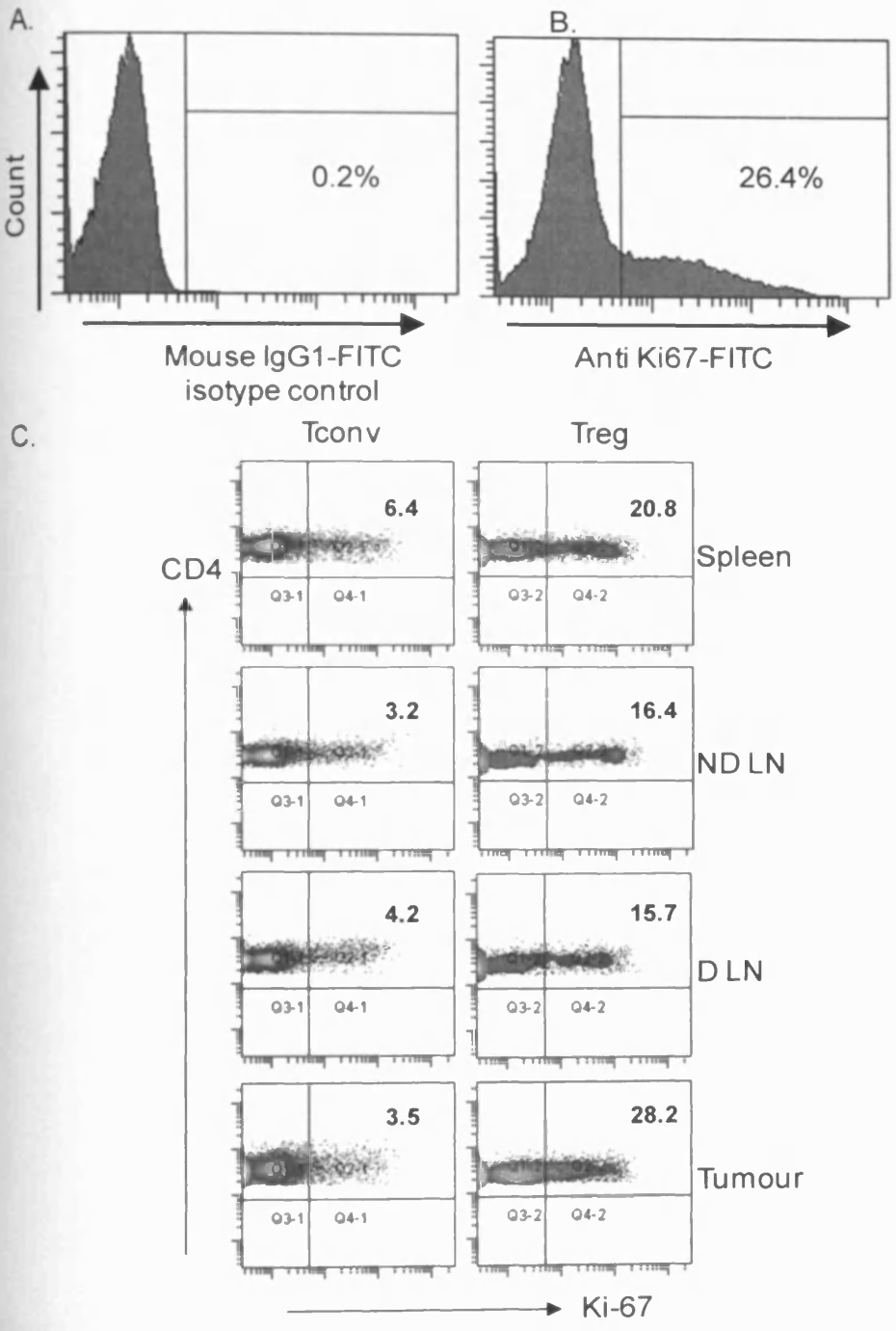


Figure 4.25. Antibody staining and analysis by flow cytometry of Ki67
Single cell suspensions of spleen, non-draining lymph node (ND LN), draining lymph node (D LN) and tumour were analysed for Ki67expression by flow cytometry. Cells were stained with anti Ki67-FITC (B) or Mouse IgG1-FITC isotype control (A) mAbs. Representative dot plots of $CD8^+$ T cells, $CD4^+Foxp3^-$ Tconv cells and $CD4^+Foxp3^+$ Treg cells from each tissue are shown (C).

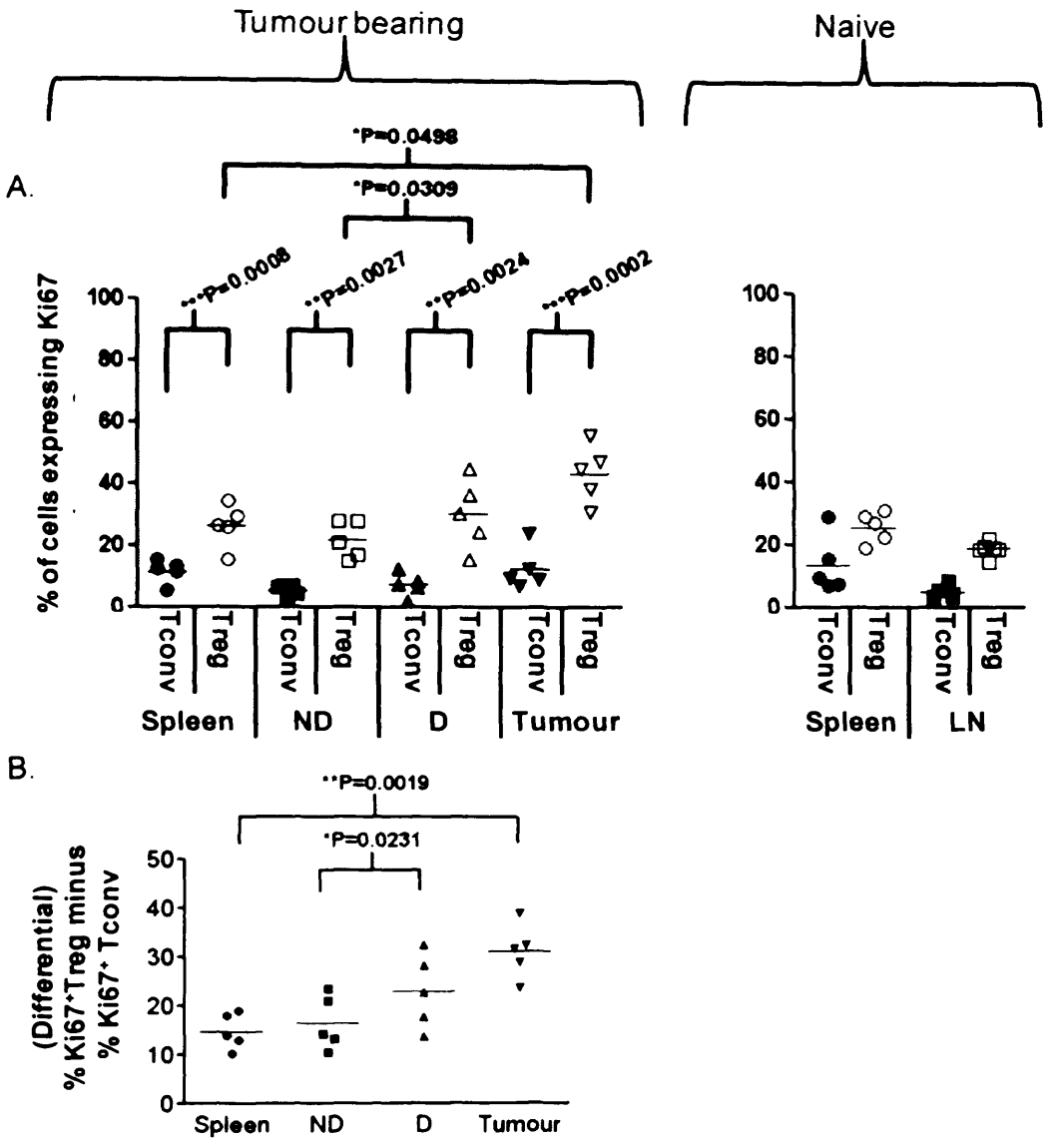


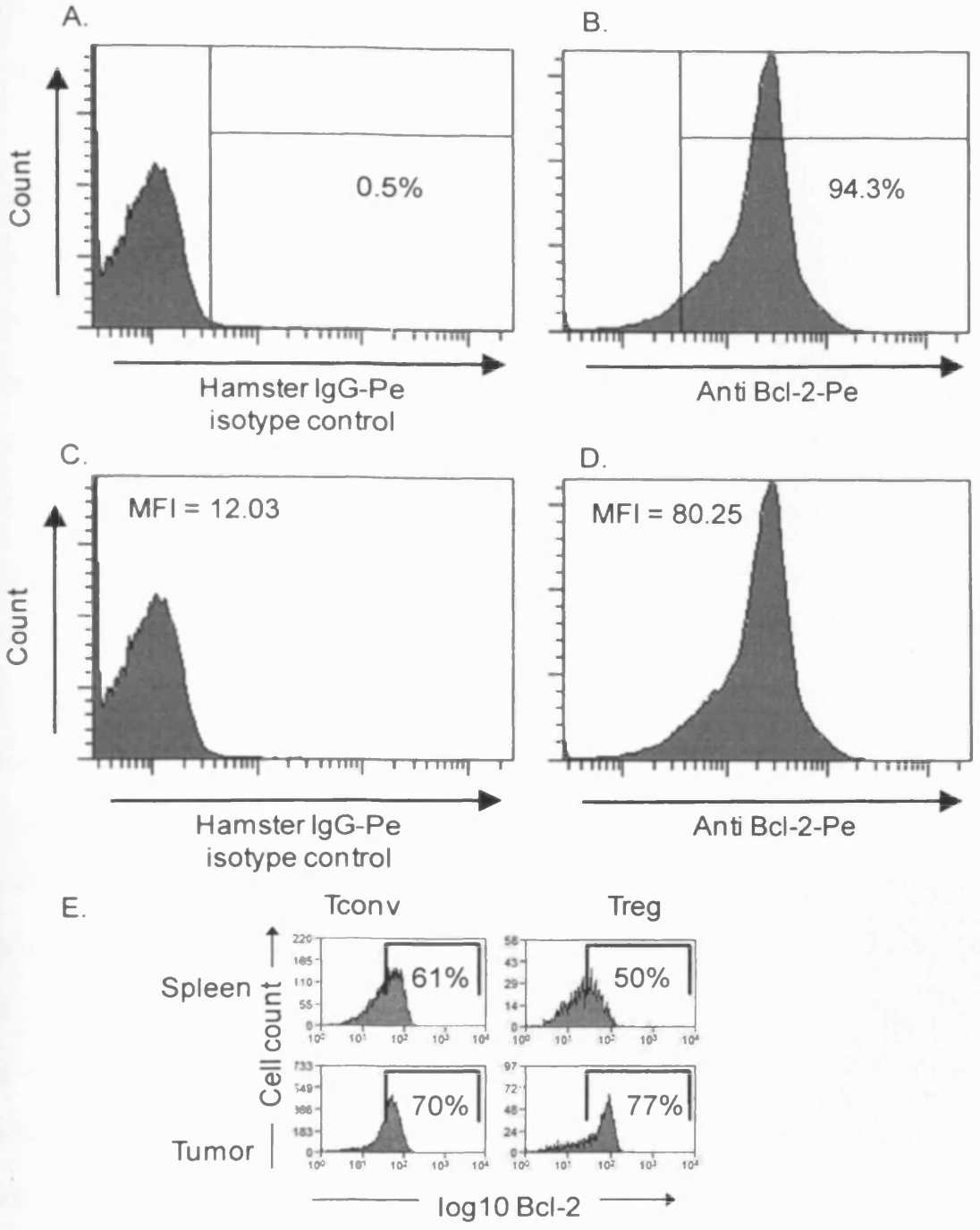
Figure 4.26. Percent of Tconv and Treg cells expressing Ki67 in tumour bearing and naïve mice

The percentage of Tconv (filled) and Treg (open) cells expressing Ki67 was determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumours were analysed from tumour bearing mice (n=5). Spleen and pooled inguinal lymph nodes (ND) were analysed from naïve mice (n=5-6). Data are displayed as (A) percentage of cells expressing Ki67 and (B) proliferation differential (percent of $Ki67^{+}$Treg minus percent of $Ki67^{+}$Tconv). The differences between the means were compared using paired T tests.

proliferating Tconv cells and Treg cells was significantly greater (*P=0.0231) in the draining lymph node (average ~23%). It is apparent that these increases in the differentials were primarily due to significant increases in the percentage of proliferating Tregs within the tumour (~45%, *P 0.0498) and the tumour draining lymph node (~30%, *P=0.0309) compared to normal lymphoid tissue (~20%; Figure 4.26A). Thus, Treg cells are more proliferative than Tconv cells in all tissues. However, Treg cells in the tumour and to a lesser extent in the tumour draining lymph node are significantly more proliferative. Furthermore, this increase in proliferation is Treg cell specific as it is not observed within the tumour-infiltrating Tconv cell population. These data suggest that tumour-specific factors lead to Treg cell enrichment due to increased Treg cell proliferation.

4.2.9 Does the level of cell death within Treg and Tconv cells favour Treg cell accumulation in tumours?

Previous studies have indicated that under normal physiological conditions Treg cells are more proliferative than Tconv cells (Vukmanovic-Stejic *et al.* 2006). However, this increase in proliferation is also coupled to a higher level of cell death compared to Tconv cells. Thus, an increase in Treg cell proliferation, as observed in MCA-induced tumours, might not necessarily lead to their accumulation but may rather reflect higher cell turnover due to an accompanying elevation in cell death. To address this question, firstly, the susceptibility of tumour-infiltrating Tregs to cell death was assessed by measuring expression of the anti-apoptotic factor, Bcl-2, in Tconv and Treg populations isolated from tumours and lymphoid tissue of tumour-bearing mice (Figure 4.27). Part of the analysis of Bcl-2 was carried out by Dr Sarah Lauder, a post-doctoral researcher in our laboratory. Secondly, the frequency of



igure 4.27. Antibody staining and analysis by flow cytometry of Bcl-2
Iouse splenocytes were stained with anti Bcl-2-Pe (B & D) or Hamster IgG-Pe isotype ontrol (A & C) mAbs and analysed by flow cytometry. The percentage of lymphocytes ithin gate (A & B) and the MFI (mean fluorescence intensity; C & D) were determined. epresentative plots for the percentage of Tconv cells and Treg cells expressing Bcl-2 are own for spleen and tumour.

cells which were unable to exclude the cell viability dye 7-aminoactinomycin D (7-AAD) was compared in Tconv and Treg cells from tumour bearing mice.

In agreement with previous findings, it was observed that within normal lymphoid tissue (spleen and non-draining lymph node) Treg cells expressed significantly less Bcl-2 than Tconv cells. This was found for both the percent of cells expressing Bcl-2 (Figure 4.28A) and the level (MFI) of Bcl-2 within the whole cell population (Figure 4.28B). In contrast, no significant difference in the proportion of tumour-infiltrating Treg and Tconv cells expressing Bcl-2 was found. Furthermore, the mean levels (MFI) of Bcl-2 expression in tumour infiltrating Treg and Tconv cells were not significantly different. The draining lymph node resembled normal lymphoid tissue in that the percentage of Treg cells expressing Bcl-2 was significantly less than the percent of Tconv cells but the difference between the level (MFI) of Bcl-2 expression in the Tconv cells and the level of Bcl-2 expression in the Treg cells was not significantly different (Figure 4.28B). Overall, these data imply that Tregs within the tumour environment are no more prone to cell death by apoptosis than Tconv cells. This is particularly interesting as it is in contrast to what is observed in normal lymphoid tissue where Treg cells appear more prone to apoptosis than Tconv cells. However, in mammals there are at least 12 members of the BCL-2 family, including Bcl-2 itself, which can contribute to the induction or inhibition of apoptosis (reviewed in Youle and Strasser 2008). Therefore, although the expression of Bcl-2 is known to contribute substantially to T lymphocyte survival, these data can only indicate cells that are more prone to apoptosis and do not demonstrate the actual level of cell death within the cell population (Veis *et al.* 1993).

To support the findings with Bcl-2 staining and compare the level of cell death in Tconv and Treg cell populations, the percentage of dying Tconv and Treg cells was measured by

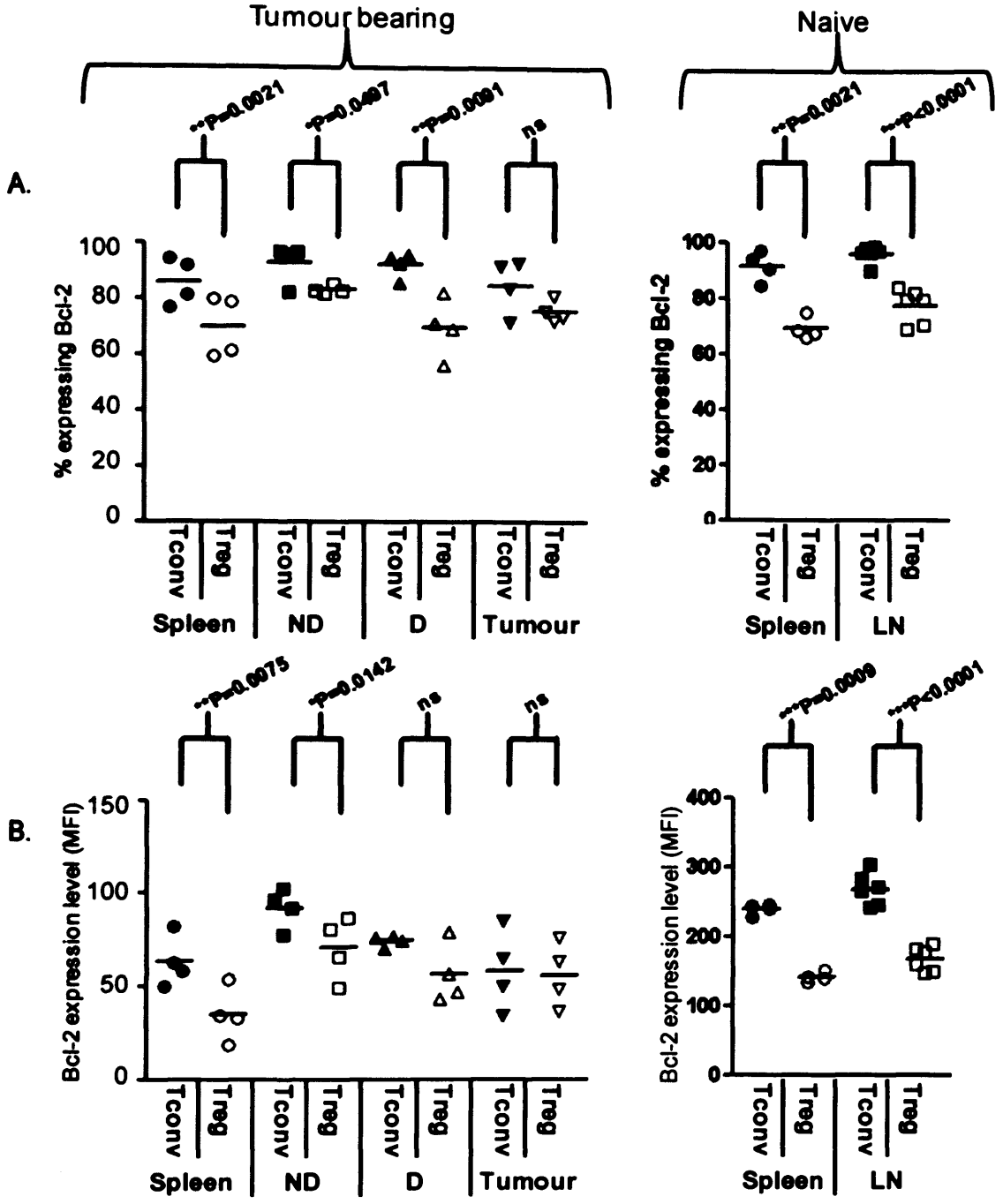


Figure 4.28. Expression of Bcl-2 in Tconv and Treg cells in tumour bearing mice
Tconv (filled) and Treg (open) cells were stained for Bcl-2 expression and analysed by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumours were analysed from tumour bearing mice (n=4). Spleens and lymph nodes (LN) were analysed from naïve mice (n=4-6). Data are displayed as (A) percentage of cells expressing Bcl-2 and (B) MFI (mean fluorescence intensity) of cells. The differences between the means were compared using paired T tests.

staining with the cell viability marker 7-AAD (Figure 4.29). In agreement with the pattern of Bcl-2 expression shown in Figure 4.28, it was found that significantly higher levels of 7-AAD+ Treg cells rather than 7-AAD+ Tconv cells exist in lymph nodes (Figure 4.30). However, this was not the case in tumour where, in contrast, the opposite was observed (Figure 4.30). In the tumour, the percentage of 7-AAD+ Tconv cells was higher than the percentage of 7-AAD+ Tregs. In the spleen, the mean percentages of Tconv cell and Treg cells that were 7-AAD+ were not significantly different. Taken together, these data suggest that tumour infiltrating Treg cells have equal if not enhanced survival over tumour infiltrating Tconv cells. This is particularly interesting given that the difference between these two cell types is reversed in the lymph nodes of the same mice where fewer Tconv cells appear to be dying than Treg cells.

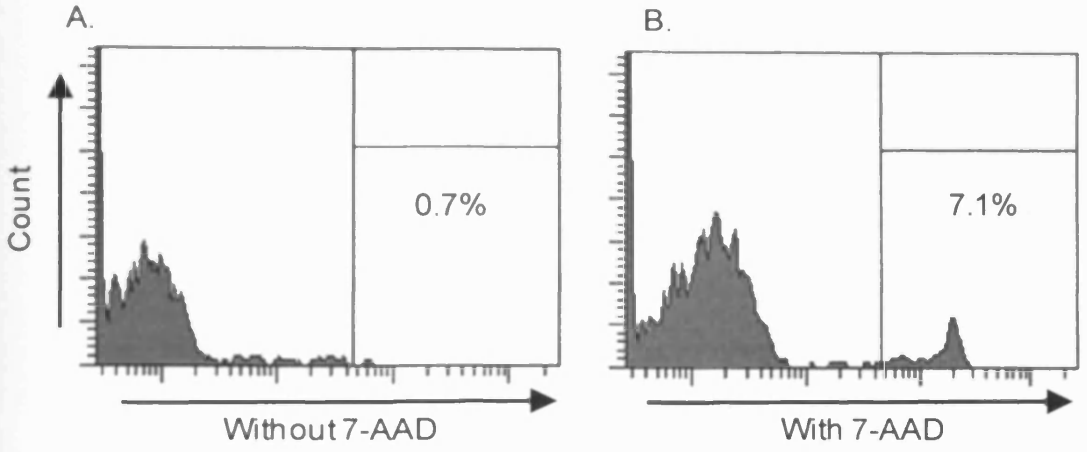


Figure 4.29. Staining and analysis by flow cytometry with 7-AAD
Mouse splenocytes were stained with (B) or without (A) 7-AAD and analysed by flow cytometry.

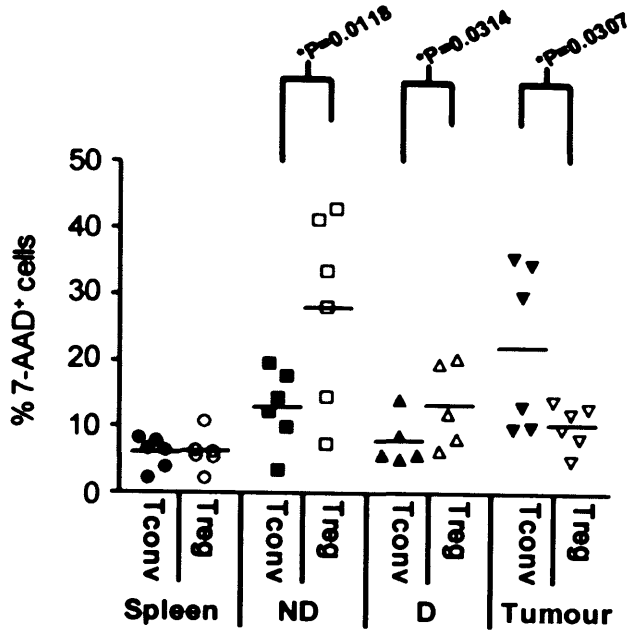


Figure 4.30. Percent of Tconv and Treg cells stained with 7-AAD in tumour bearing mice

The percentage of 7-AAD$^+$ Tconv (filled) and Treg (open) cells were determined by flow cytometry. Spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumours were analysed from tumour bearing mice (n=5-6). The differences between the means were compared using paired T tests.

4.3 Discussion

In this Chapter, I have investigated mechanisms leading to the accumulation of regulatory T cells in tumours. For this purpose, mice bearing chemically (methylcholanthrene) induced tumours were analysed. Two potential mechanisms of accumulation were independently tested. Firstly, the contribution of conversion of Tconv cells into Tregs was assessed by TCR repertoire analysis. Tconv and Treg cell populations in tumour and normal lymphoid tissue were found to have generally non-overlapping TCR repertoires, suggesting that conversion of Tconv cells does not account for the accumulation of Treg cells in the tumour. Secondly, proliferation and survival of Tconv and Treg cells were compared in tumour bearing mice. Tumour infiltrating Treg cells were found to be significantly more proliferative than tumour infiltrating Tconv cells as well as Treg cells from other locations. Furthermore, in the tumour, in contrast to other tissues, fewer Treg cells than Tconv cells appeared to be dying. This may be due to alterations in the levels of anti-apoptotic factors such as Bcl-2.

Initially, the TCR repertoires of Tconv and Treg cells were analysed by sorting the two populations based on the expression of CD25. $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were sorted as Tconv and Treg cells, respectively. The results from these initial studies suggested that the two cell populations had a large degree of overlap in the tumour which was not present in the spleen. This would suggest that conversion of Tconv cells into Treg cells was plausible. However, a full interpretation of this initial data was difficult because the cells were sorted based on CD25. As discussed above, sorting the two populations based on CD25 is imperfect and contamination of both the Tconv and Treg cell populations was likely. Firstly, with this approach it was likely that $CD4^+CD25^+Foxp3^-$ Tconv cells were sorted into the Treg pool and secondly, $CD4^+CD25^-Foxp3^+$ Treg cells were sorted into the Tconv pool. It is also clear from

the data presented in Chapter 3 that the problem of contamination was likely compounded further by the fact that more activated $CD4^+CD25^+Foxp3^-$ Tconv cells are found within the tumour compared to the spleen. Thus, the contamination of the two populations was probably greater in the tumour than in the spleen and may have led to an increase in the level of overlap in the tumour compared to the spleen. Indeed, this appears to be the case, as when Tconv and Treg cells were later more stringently sorted based on Foxp3 using the Foxp3_GFP reporter mice much less overlap was observed between the two cell populations. Thus, it seems most likely that the reduction in the overlap was due to the improved sorting criteria.

In later experiments, where cells were sorted based on Foxp3 the degree of overlap between the two repertoires was consistently low. The highest degree of overlap between the TCR repertoires of Tconv and Treg cells was observed in the tumour of mouse 2 where 13.9% of the Treg TRBV13-2 TCR repertoire was found to overlap with the Tconv TCR repertoire. Other measurements of the similarity between the TCR repertoires of tumour infiltrating Tconv and Treg cells showed less overlap at 3.3% and 0.0%, in mouse 3 and mouse 4, respectively. The overlap between the Treg and Tconv TCR repertoires was also consistently low within other tissues. Thus it appears that the majority of the Treg repertoire does not overlap with the Tconv repertoire in both tumour and normal lymphoid tissue. In the TRBV13-2 analyses (but not the TRBJ2-5 analysis) a small increase was observed for the degree of overlap between the Tconv and Treg TCR repertoires in the tumour compared to the spleen or lymph node which may indicate that an increase in conversion occurs in the tumour. However, when all the TCR data were compiled the difference between the spleen or lymph node and the tumour was not statistically significant. Furthermore, on average, more

than 95% of the tumour infiltrating Treg TCR repertoire remained non-overlapping with the Tconv TCR repertoire.

Based on the hypothesis that a high degree of overlap would be observed if the Treg cells arose through conversion, these data would suggest that the majority of Treg cells in the tumour do not arise through conversion of Tconv cells. However, it may be difficult to assess the level of conversion when looking at one time point. For example, if naive Tconv cells entering the draining lymph node of tumour, encounter their cognate antigen, become activated and upregulate Foxp3 immediately, very few of these potentially convertible cells would exist within the Tconv population at the time of sampling. Thus, when the cells are sorted late in the development of the tumour, many Tconv cells may have converted into Treg cells and would therefore not appear in the Tconv repertoire as they have already upregulated Foxp3. The kinetics of Foxp3 induction have been previously investigated *in vitro* (Selvaraj and Geiger 2007). These studies show that the upregulation of Foxp3 in naive $CD4^+$ T cells is not immediate. It takes between 24 and 48 hours for detectable levels of $Foxp3^+$ cells to appear in these assays and between 3 to 4 days for more than 50% of the cells to convert. Furthermore, most studies suggest that tumour specific factors, for example an immunosuppressive cytokine microenvironment, induce the conversion of Tconv cells into Treg cells. If the conversion of Tconv cells into Treg cells is tumour specific an overlap in the TCR repertoires of Treg cells from tumour and Tconv cells from the draining lymph node would be evident. This would be observed even if once entering the tumour Tconv cells are immediately converted. However, this was not observed. On average less than 1% of the tumour Treg repertoire overlapped with the draining lymph node Tconv cell TCR repertoire (Figure 4.23). Instead, the TCR repertoires of Treg cells from tumour were most similar to the TCR repertoires of Treg cells from the draining lymph node. This was an important

control in these experiments and supports the hypothesis that Treg cells traffic between the two sites and remain unconverted.

Previous investigations based largely on adoptive transfer of Tconv cells, despite showing that conversion of conventional T cells into Tregs is possible, do not demonstrate the extent to which conversion might occur in a natural context and whether it accounts for the large enrichment of Tregs often seen in tumours (Liu *et al.* 2007, Valzasina *et al.* 2006, Zhou and Levitsky 2007). Whilst the data in this thesis do not exclude conversion of Tconv cells as a mechanism contributing to Treg cell enrichment in tumours, they do demonstrate, in the case of a spontaneously developing tumour, that the majority of tumour-infiltrating Tregs probably do not arise through conversion. Accurately measuring the true degree of overlap between two populations is difficult. This is primarily due to the inherent diversity in the TCR repertoire. This is particularly true for samples from the spleen and lymph nodes, however, a large amount of diversity was also detected in the tumour infiltrating T cell populations. This was, to some extent, not predicted, as expansions of T cells in immune responses to viruses (for example CMV, EBV and Influenza) tend to be dominated by a limited number of clones (Day *et al.* 2007, Miles *et al.* 2005, Khan *et al.* 2002, Price *et al.* 2005, Annels *et al.* 2000; and personal communication with Dr Ian Humphreys and Professor David A. Price). However, very few data on the diversity of T cell responses, particularly $CD4^+$ T cell responses to whole tumours have been reported. Given the vast diversity in the TCR repertoires, the limited number (up to 100) of CDR3 sequences analysed may not provide a true measurement of overlap since additional overlapping sequences may be present in the fraction of the TCR repertoire not sequenced. A more accurate measurement of the degree of overlap between two populations would be possible either if the two populations are clearly dominated by a limited number of clonotypes or with exhaustive sequencing. Given that the

TCR repertoire may contain many thousands of potential clonotypes, sequencing the required number of TCR sequences would be extremely expensive. However, with the advent of deep sequencing (Freeman *et al.* 2009) this may feasibly become cost effective.

In an attempt to overcome the problem posed by the vast diversity of the TCR repertoire, a focused analysis of the TCR repertoire was performed whereby a smaller subset of the TCR repertoire was analysed. The TRBJ2-5/TRBV13-2 subset was analysed instead of the whole TRBV13-2 subset. Using this approach, the ~50-100 analysed sequences covered a larger proportion of the TRBJ2-5/TRVJ13-2 repertoire since the potential diversity was reduced compared to the whole TRBV13-2 repertoire. Thus, a more in depth analysis of one particular subset was achieved. The diversity in this repertoire appeared reduced as fewer single sequences and more repetitive sequences were found. Indeed, it was estimated that approximately 36% of the potential TCR clonotypes was identified with this focused analysis, compared to approximately 10% in the previous TRBV13-2 analyses. Ultimately, the results of this focused analysis were very similar to the previous findings of the thesis. The Tconv and Treg TCR repertoires in the tumour were generally non-overlapping, supporting the view that the majority of tumour infiltrating Treg cells do not arise through the conversion of Tconv cells. However, even with this focused analysis, the repertoires were still diverse particularly in the spleen and lymph node. Furthermore, the TCR repertoire analysed using this method represents a limited sampling of the extensive TCR repertoire. Interestingly, the degree of overlap between the TCR repertoires of Tregs from the draining lymph node and Tregs from the tumour was higher with the TRBJ2-5/TRVB13-2 specific analysis in comparison to simply the TRJV13-2 analysis. This supports the notion that the in depth analysis improved the capacity to find any potential overlap between two repertoires.

The degree of overlap between the TCR repertoires of Tconv and Treg cells has been previously investigated (Hsieh *et al.* 2004, Pacholczyk *et al.* 2006). Using mice with a fixed Vβ chain, Hsieh *et al.* (2004) found that the TRAV14 repertoires of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells overlapped by approximately 10-20%. Pacholczyk and colleagues (2006) compared the TCR repertoires of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-Foxp3^-$ cells in transgenic (TCR^{mini}) mice which express a single TCRβ chain and a TCRα chain that allows for rearrangement of only a single Vα (Vα2.9) segment to one of two Jα (Jα26 and Jα2) segments and found that the TCR repertoires of these cells overlapped by 10-20%. Furthermore, they found that the highest degree of overlap was between the repertoires of TCRs found on thymocytes and peripheral T cells belonging to the same group of cells (70-80% for $Foxp3^-$ cells and 65% for $Foxp3^+$ cells) suggesting that these cells are distinct populations both in the thymus and in the periphery (Pacholczyk *et al.* 2006). In this thesis, the degree of overlap between the TCR repertoires of the Treg and Tconv cell populations was lower than that reported by other groups which had used TCR-transgenic mice (Hsieh *et al.* 2004, Pacholczyk *et al.* 2006). This disparity is expected given the curtailed TCR repertoires found within these mice. In these models, a limited number of TCR structural combinations pass thymic selection resulting in a restriction in peripheral diversity and, accordingly, an increased chance of identify recurring overlapping clonotypes. The T cell dynamics during a natural tumour response in a TCR intact animal is considerably more complex considering the full $2x10^6$ (and $25x10^6$ in humans) receptor pool available in mice (Arstila *et al.* 1999, Casrouge *et al.* 2000). In this thesis, non-TCR-transgenic mice were used to investigate whether the degree of repertoire overlap between the two T cell populations was significantly different in cells isolated from the tumour compared to lymphoid tissue.

The analysis of the Tconv and Treg TCR repertoires was further scrutinised by Lathrop *et al.* (Lathrop *et al.* 2008). In this paper, the requirement for TCR engagement for Foxp3 upregulation was considered when analysing the repertoires of the two cell types. The Tconv cell population was analysed separately as $CD44^{high}$ (antigen experienced) and $CD44^{low}$ (naive) cells. In similarity to the reports mentioned above which analysed the whole Tconv pool (Hsieh *et al.* 2004, Pacholczyk *et al.* 2006), Lathrop *et al.* (2008) found that the TCR repertoires of $CD44^{high}$ Tconv and Treg cells were generally non-overlapping. The analysis of the TCR repertoires of Tconv and Treg cells in MCA tumour bearing could be improved by comparing the TCR repertoires of $CD44^{high}$Tconv and Treg cells. However, since the majority (approximately 60%) of tumour infiltrating Tconv cells were found to be $CD44^{high}$ (Figure 3.9 page 81) it seems unlikely that the similarity between TCR repertoires of Tconv and Treg cells from the tumour will change dramatically when further categorised based upon CD44 expression. In contrast, the percentage of Tconv cells that were $CD44^{high}$ within the lymphoid tissues (spleen, draining and non-draining lymph nodes) was found to be only approximately 10-30% (Figure 3.9 page 81) thus a change in the similarity between the lymphoid Tconv TCR repertoire and other TCR repertoires may be seen. In particular the TCR repertoires of Tconv cells from the draining lymph node and tumour may be more similar when only antigen experienced, $CD44^{high}$ Tconv cells are considered.

The findings in this Chapter are also similar to those of a study which analysed the degree of conversion in self antigen-specific T cells in the context of a mouse model of type I diabetes (Wong *et al.* 2007a). The TCRs of Treg and Tconv cells isolated from the pancreas and pancreas-draining lymph nodes of BDC2.5/NOD TCR transgenic mice were distinct, thereby indicating no role for conversion in response to a pancreatic autoantigen. Similarly, a more recent study into the TCR repertoire and specificity of T cells in experimental allergic

encephalomyelitis (EAE) concluded that, despite sharing autoantigen specificity, effector T cells and Treg cells had largely distinct TCR repertoires, similarly suggesting that conversion between these two populations was minor (Liu *et al.* 2009b). Thus, the combined results of these studies imply that tolerance in the context of autoimmunity and tumour immunity is achieved without substantial conversion of conventional T cells into Tregs.

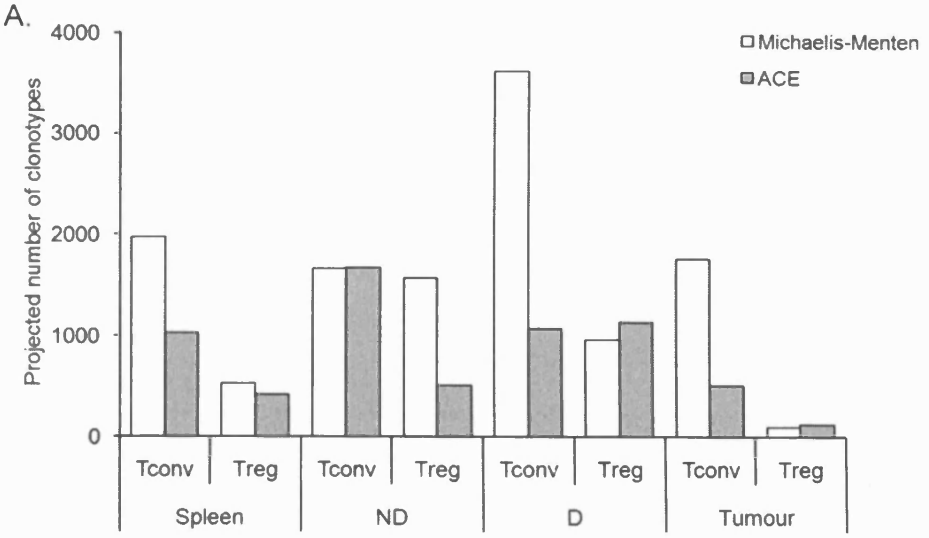
In contrast, a role for conversion may exist in preventing immune responses to some foreign antigens such as those of commensal bacteria in the gut (induced tolerance). In the intestine, the immune system must resist infection from occasional pathogens whilst maintaining tolerance to commensal flora and dietary antigens. The generation of Tregs by conversion from Tconv cells has been suggested as an important mechanism for establishing this tolerance to persistent antigens in the gut (Coombes *et al.* 2007). Interestingly, recent data suggests that the Foxp3 locus contains a dedicated (non-coding) region (CNS1) that controls the induction of Foxp3 in the periphery (Zheng *et al.* 2010). Although mice lacking CNS1 had no defect in thymic production of Treg, cells from CNS1-deficient (but not CNS1-sufficient) mice were unable to induce Foxp3 and therefore generate Treg cells in gut-associated lymphoid tissues.

Using the accumulated data from TCR repertoire analyses it was possible to estimate the sizes of the TCR repertoires of Tconv and Treg cells from tumour bearing mice. The Michaelis-Menten equation was used for estimating the size of the TCR repertoires as it is relatively simple to use and can be calculated using the statistical software package, Prism (Graphpad). Numerous methods have also been used to estimate T cell diversity. Summary statistics such as Simpson's diversity and Shannon's entropy indexes have been used as measures of diversity in TCR repertoires (Ferreira *et al.* 2009, Venturi *et al.* 2007). However,

these indexes provide limited information about the size of the TCR repertoire from which the sample was taken. One approach is to use a method which assumes that all clonotypes are equally represented within a sample. The MLE (maximum likelihood estimation) method is based on this assumption and been used to estimate the TCR repertoire diversity for various data sets (Casrouge *et al.* 2000, Barth *et al.* 1985, Hsieh *et al.* 2004). However, TCR repertoire samples usually show significant skewing therefore the assumption of this test is often not applicable. The abundance-coverage and incidence-coverage estimators take into account some heterogeneity in the clonality of a population and are therefore probably the most robust methods (Pacholczyk *et al.* 2006, Hsieh *et al.* 2004). Nevertheless, it is clear that none of these methods are considered ideal and new methods for estimating TCR diversity have been suggested (Venturi *et al.* 2007, Sepúlveda, Paulino and Carneiro 2010).

To confirm the validity of the method used in this thesis (Michaelis-Menten) the size estimates generated for each mouse were compared to the size estimates generated using the abundance coverage estimator (ACE). Using the software package, EstimateS (Colwell 2005), the ACE method was applied to estimate the diversity of the TCR repertoires of Tconv and Treg cells from the spleen, lymph nodes and tumour of mouse 2, mouse 3 and mouse 4. A significant correlation (R^2=0.7543, ***P=<0.0001) between the size estimates generated using the two methods was found (Figure 4.31), indicating that the Michaelis-Menten and ACE methods are comparable for estimating the size of the TCR repertoires. Although it has been suggested that the Michaelis-Menten method is likely to underestimate the total size of the repertoire (Magurran 2004), this was not consistent with the findings from the comparison of the two methods. Overall, the size estimates made using the Michaelis-Menten method may not be completely accurate, however, they provided a good estimate of the number of TCR clonotypes that would be found within each repertoire and also the number of sequences

A.


Michaelis-Menten
ACE
Projected number of clonotypes
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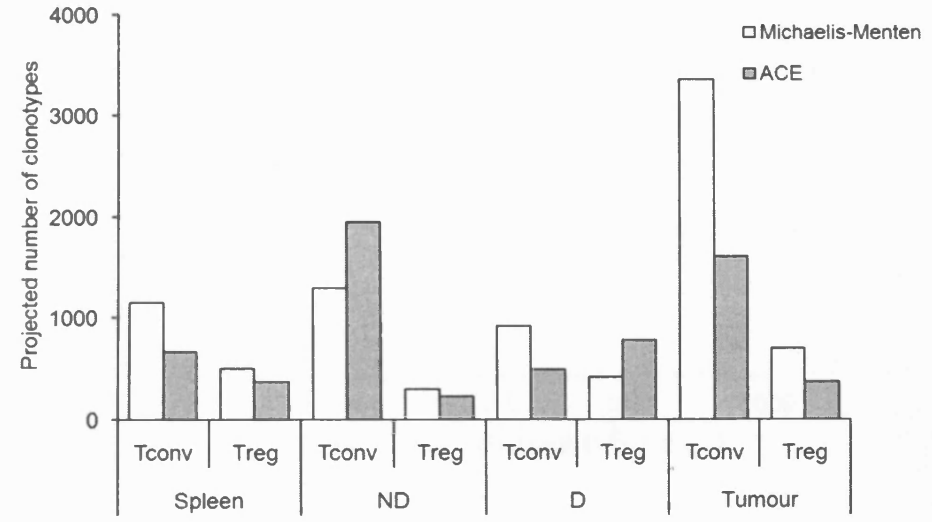
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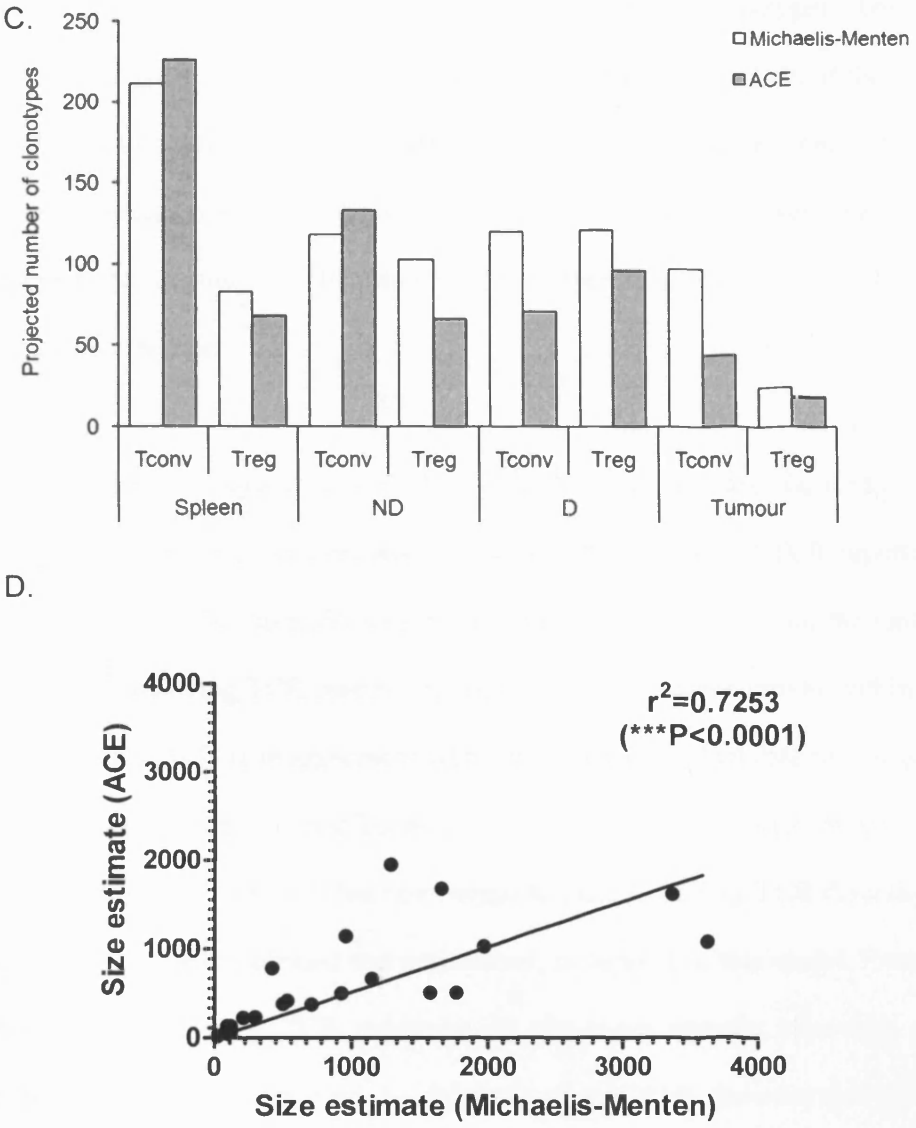

Figure 4.31. Size estimates of TCR repertoires using Michaelis-Menten and ACE methods

The sizes (estimated number of clonotypes) of the TCR repertoires of Tconv and Treg cells from tumour bearing mouse 2 (A), mouse 3 (B) and mouse 4 (C) were estimated using the Michaelis-Menten (open bars) and ACE (filled bars) methods. The ACE method was applied using the statistical software package EstimateS. Samples from spleens, non-draining lymph nodes (ND), draining lymph nodes (D) and tumours were analysed. The correlation between the size estimates generated using the two methods was also plotted and the Pearson's r^2 value determined (D).

that would be required to identify for example 90% of those clonotypes. The diversity estimates indicated that in the TRBV13-2 analyses only approximately 10% of the clonotypes were identified and when the TCR repertoire analysis was focused onto the TRBJ2-5/TRBV13-2 sequences approximately 36% of the potential clonotypes were identified. This finding supports the reasoning for focusing the TCR repertoire analysis using TRBJ2-5 and TRBV13-2 specific primers.

An interesting pattern that appeared from these size estimates was that the size of the Treg cell TCR repertoire was often smaller than the size of the Tconv cell TCR repertoire. The restriction of the Treg TCR repertoire was most consistently noticed within the tumour. The sizes of the Tconv and Treg TCR repertoires were found to be most similar within the non-draining lymph node. This is in agreement with previously published data that suggests that the TCR repertoires of Treg cells and Tconv cells in naive mice are equally diverse (Hsieh *et al.* 2004, Pacholczyk *et al.* 2006). It has been suggested that high Treg TCR diversity ensures that self tolerance can be established and maintained. In support of this model, Ferreira *et al.* (2009) showed that the Treg TCR repertoire of non-obese diabetic mice was markedly restricted. Interestingly, a recent study by Adeegbe *et al.* (2010) demonstrated that when a severe breakdown in immune tolerance is induced (mice lacking IL-2Rβ), control of autoimmunity is achieved by only a fraction of the Treg repertoire. However, individual mice exhibited unique patterns of skewing of the Treg TCR repertoire suggesting that a large diversity is necessary to ensure that self tolerance is readily established towards the random autoimmune cell(s) generated in each individual. The restriction of the Treg TCR diversity observed in MCA tumours may therefore resemble the response required to control autoimmunity.

Due to the small size of the Treg TCR repertoire in the tumour in each of the analyses in this Chapter a relatively large proportion of the potential TCR clonotypes were identified. In mice 2, 3 and 4 the average percentage of the potential Treg and Tconv TCR clonotypes that were actually identified was estimated to be 10%, 11% and 36%, respectively. However, it was estimated that in mice 2, 3 and 4 approximately 39%, 11% and 69% of the potential tumour Treg TCR clonotypes were identified, respectively. Therefore, it was estimated that the proportion of the potential tumour Treg TCR repertoire identified was often above average. This indicates that within the tumour the likelihood of identifying any potentially overlapping sequences was greater than within other locations where fewer of the potential clonotypes were identified. Furthermore, this strengthens the finding that very little of the tumour Treg TCR repertoire is overlapping with the Tconv TCR repertoire and therefore conversion of Tconv cells into Treg cells is unlikely to account for the accumulation of Treg cells within the tumour. Instead, it seems most likely that tumour infiltrating Treg cells derive from naturally occurring Treg cells. This finding is in agreement with Bui *et al.* (2006) who demonstrated that following adoptive transfer of Thy1.2 $CD4^+CD25^+$ T cells into Thy1.1 Treg depleted mice, the $CD25^+CD4^+$ cells within TILs derived mostly from the adoptively transferred Thy1.2 naturally occurring $CD4^+CD25^+$ Tregs.

To examine the mechanisms that underlie Treg cell enrichment in tumours, the proliferation and survival of Tregs and Tconv cells in TILs and lymphoid tissues were compared. The expression of Ki67, a marker of proliferating cells, was analysed by flow cytometry. The analysis of this molecule which is present during all active phases of the cell cycle has been extensively used in the analysis of tumours in clinical laboratories (Scholzen and Gerdes 2000). It has also become widely used by immunologists using flow cytometry to analyse the proliferating fraction of cell populations. The analysis of Ki67 provided a snap shot of the

proliferative status of cells within tumour bearing mice. Tumour infiltrating Treg cells were found to be significantly more proliferative than tumour infiltrating Tconv cells as well as Treg cells from other locations.

A more traditional method for measuring proliferation is the use of Bromodeoxyuridine (BrdU). BrdU is incorporated into the DNA of cells as they divide, and hence can indicate any cell that has divided. By injecting BrdU directly into the mouse, Tconv and Treg cells that have recently divided would become stained. Since Ki67 is upregulated during all active phases of the cell cycle and BrdU is only incorporated following the S phase, Ki67 provides a more comprehensive measurement of the current proliferative state of a cell. However, it has also been suggested that Ki67 can be expressed in cells that are arrested in the cell cycle and therefore not actively proliferating (van Oijen *et al.* 1998). Therefore, to confirm the results of staining with Ki67, BrdU incorporation in tumour bearing mice is currently being examined in our laboratory. The incorporation of BrdU was previously used to compare the proliferation of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from TILs and draining lymph node of mice with transplanted MCA sarcoma cell lines (Bui *et al.* 2006). These studies suggest that $CD25^+$ Treg cells are more proliferative than $CD25^-$ Tconv cells in both the tumour and tumour draining lymph node. Furthermore, tumour infiltrating $CD25^+$ cells were more proliferative than $CD25^+$ cells from the draining lymph node. However, in this study Bui *et al.* (2006) did not separate these cells based on Foxp3 expression making a full interpretation of these data difficult.

Previous studies of Treg cell turnover have shown that Treg cells are more proliferative than Tconv cells but are also more prone to cell death (Vukmanovic-Stejic *et al.* 2006). Given these findings, attempts were made to measure the level of cell death within Tconv and Treg

cells from tumour bearing mice. Firstly, the anti-apoptotic factor Bcl-2 was assessed by flow cytometry. In agreement with previous studies, in normal lymphoid tissue the level (MFI) of Bcl-2 expression and the percent of cells expressing Bcl-2 were found to be significantly lower in Treg cells than Tconv cells. However, within the tumour, both the percent of cells expressing Bcl-2 and the level of Bcl-2 were similar in the Tconv and Treg cell populations suggesting that in the tumour, Treg and Tconv cell turnover are similar. Since there are at least 12 members of the BCL-2 family which can contribute to induction or inhibition of cell death another method of measuring cell death was also investigated to support the findings of the Bcl-2 staining (Youle and Strasser 2008). For this purpose, Tconv and Treg cells were stained directly *ex vivo* with the cell viability dye 7-AAD. In the lymph node, a significantly higher percentage of the Treg cells were 7-AAD$^+$ compared to Tconv cells, suggesting more Treg cells were dying than Tconv cells. However, in the tumour the opposite was observed, more Tconv cells than Treg cells were 7-AAD$^+$ suggesting that Treg cells are less prone to die than Tconv cells in the tumour. This may reflect the nature of antigens, costimulation or cytokines found within the tumour microenvironment which favour Treg cell survival.

Accurately measuring cell death *in vivo* and *ex vivo* is considered difficult. Firstly, it is thought that the intrinsic mechanisms for the removal of dead/dying cells are rapid (Kerr, Wyllie and Currie 1972, Parnaik, Raff and Scholes 2000, Savill *et al.* 2002), therefore dying cells are difficult to observe. Furthermore, once dissected from the mouse, it is thought that the dynamics of cell death and regulation of cell death markers can alter in cells particularly following extensive handling in the laboratory (Carbonari, Cibati and Fiorilli 1995, Darzynkiewicz *et al.* 1997, Listman *et al.* 1998, Mattes 2007). Despite these caveats, staining for cell death with Bcl-2 and 7-AAD was found to be quite consistent and the differences between various groups reached statistical significance. Furthermore, the levels of these

markers were compared between the two different cell types which were stained together. Thus the Tconv and Treg cells that were compared were treated in exactly the same manner. Therefore, although the exact amount of cell death within the tissues may not be correct, and therefore comparisons between the various tissues cannot be accurately made, it is likely that the difference between the two cell types from the same location is authentic. To confirm these findings, work is currently underway within our laboratory to measure the level of Caspase-3 within tumour infiltrating Tconv and Treg cells by immunohistochemistry. Using this method, only minor disruption of the tissue occurs, and the time between dissection from the mouse and fixation is minimal allowing very little time for changes in the expression of markers for cell death, such as Caspase-3.

In summary, the results in this Chapter have shown that the conversion of Tconv cells into Treg cells is unlikely to account for the accumulation of Treg cells within the tumour tissue. However, Treg cells were found to proliferate more than Tconv cells in all sites examined and this difference was most significant in the tumour. Furthermore, in contrast to lymphoid tissue, the tumour microenvironment was seen to offer Treg cells protection against apoptosis as fewer dying Treg than Tconv cells were observed in the tumour. It is therefore reasonable to conclude that the enrichment of Tregs within the $CD4^+$ TIL population is due to the tumour environment preferentially promoting proliferation and survival of Tregs. The causes for this are as yet unknown. This may reflect the nature of the antigens expressed by tumour cells. The antigens recognised by Treg cells remain largely unknown, however, there is some evidence to suggest that compared to Tconv cells, Treg cells preferentially recognize self-antigens (Hsieh *et al.* 2004, Pacholczyk *et al.* 2006). Most antigens expressed by cancer cells and recognised by the immune system are non-mutated self antigens (reviewed in Houghton and Guevara-Patiño 2004), thus Tregs may receive stronger antigen-driven signals than

conventional T cells in the tumour environment. The stimulation of Treg cells may further support Treg proliferation and survival through the secretion of cytokines such as IL-2 and TGFβ.

The highly proliferative status of tumour infiltrating Treg cells and lack of overlap in the Treg and Tconv TCR repertoires suggest that the accumulation of Treg cells in the tumour is through the expansion of naturally occurring T cells. A restriction in the diversity of the tumour Treg TCR repertoire was noted. This restriction is in agreement with the suggestion that these cells are expanding. Nevertheless, it is clear that the diversity of the tumour infiltrating Treg population remains large, suggesting many Treg cell clones are stimulated to expand. There was no evidence that a restriction in the Tconv TCR repertoire occurs in tumour bearing mice. This suggests that the strong stimulation of particular Tconv cell clones does not occur, and may also signify that a robust anti-tumour immune response is not generated in these mice. In agreement with this hypothesis, the proportion of Tconv cells expressing Ki67 in the tumour was similar to the proportion of Tconv cells expressing Ki67 in lymphoid tissue. However, it was demonstrated in Chapter 3 that the immune response to MCA tumours is primarily associated with the effects of $CD8^+$ T cells rather than $CD4^+$ T cells. An analysis of the TCR repertoire of tumour infiltrating $CD8^+$ T cells may therefore be more indicative of the anti-tumour immune response generated in MCA tumours. Early data generated from our laboratory suggest that the TCR repertoire of $CD8^+$ T cells isolated from MCA tumours is also diverse.

Chapter 5 – Final Discussion

For this thesis I have investigated the accumulation, phenotype and function of $CD4^+$ $Foxp3^+$ regulatory T cells in chemically induced (MCA) mouse tumours. The results of these experiments indicate that control of tumour growth following Treg cell depletion is dependent on $CD8^+$ cells. In tumour bearing mice the ratio of Treg cells to conventional $CD4^+$ T (Tconv) cells and particularly $CD8^+$ T cells altered within tumour tissue to favour immune suppression. Furthermore, tumour infiltrating Treg cells displayed a phenotype associated with immunosuppression whereas only a small fraction of tumour infiltrating Tconv and $CD8^+$ T cells appeared activated. Together these findings support the hypothesis that Treg cells accumulate within tumours and gain control of T cells preventing anti-tumour immunity. To investigate how Treg cells accumulate within MCA tumours a detailed analysis of the TCR repertoires of Tconv cells and Treg cells from tumour bearing mice was performed. Little overlap between the repertoires of these cells was found suggesting that the majority of tumour infiltrating Treg cells do not arise through the conversion of Tconv cells. Instead, it was found that the proliferation and survival characteristics of tumour infiltrating Treg cells favoured their accumulation over Tconv cells. These findings support the rational for inhibition of Treg cells in the treatment of cancer (reviewed in Curiel 2008, and Zou 2006). They also indicate that it is necessary to investigate tumour specific factors which cause their preferential proliferation and survival as this may enable manipulation of these cells for therapy.

The role of the immune system in preventing tumour growth in humans has been controversial (reviewed in Dunn *et al.* 2002). It has been demonstrated that tumour reactive T cells can be isolated from the tumours and blood of patients with cancer (Lee *et al.* 1999,

Germeau *et al.* 2005, Coulie *et al.* 1992, Mazzocchi *et al.* 1994). However, the reasons for the inability of tumour reactive immune cells to eliminate tumours in cancer patients remain to be fully determined. The findings from this thesis and many other studies suggest that Treg cells can limit the immune response to tumours (reviewed in Betts *et al.* 2006). It has also been shown that many other mechanisms of immune suppression exist in tumours (reviewed in Rabinovich, Gabrilovich and Sotomayor 2007). The recruitment of myeloid derived suppressor cells (MDSC) to tumours may contribute to hampering an effective anti-tumour immune response (reviewed in Serafini, Borrello and Bronte 2006, and Kusmartsev and Gabrilovich 2006). These cells suppress cellular immune responses primarily through the production arginase-1 and nitric oxide synthase 2A (reviewed in Gabrilovich and Nagaraj 2009). Tumour cells may also secrete soluble factors with direct immunosuppressive effects such as IL-10, TGFbeta, and IDO or express receptors/ligands with immune inhibitory effects (reviewed in Kim *et al.* 2006b, Raffaghello *et al.* 2004, Teicher 2007, Uyttenhove *et al.* 2003, reviewed in Muller and Prendergast 2007). In addition, tumours can evade immune detection through downmodulation of the protein machinery involved in antigen processing and presentation such as MHC and TAP (reviewed in Seliger, Maeurer and Ferrone 2000, Algarra *et al.* 2004, Vitale *et al.* 1998, Kaklamanis *et al.* 1995, Cromme *et al.* 1994). Therefore, evidence suggests that tumours can evade immune detection and elimination through the induction of multiple mechanisms which lead to local immune suppression and the outgrowth of non-immunogenic cancer cells. This poses a significant obstacle to generating effective immunotherapy to cancer. To overcome the immune suppressive environment within tumours and generate a successful immune response to cancerous cells may require the inhibition of several mechanisms. Indeed preliminary experiments using Foxp3_DTR mice have shown that in a number of subjects the complete removal of Treg cells in MCA tumour bearing mice does not induce any obvious signs of tumour control (my own unpublished observation).

One approach to cancer immunotherapy which shows promise is cancer vaccination (reviewed in Emens 2008). Cancer vaccines are designed to awaken the immune system to the presence of cancer by presenting it with antigens associated with tumour cells. To date, a limited number of cancer vaccines have been approved for use (Goldman and DeFrancesco 2009). Numerous other cancer vaccines have failed to show clinical efficacy (Goldman and DeFrancesco 2009). It is hoped that a more comprehensive understanding of tumour immunology will allow for the design of more successful cancer vaccine approaches. The discovery and characterisation of tumour antigens which can generate robust immune responses forms a vital part of this understanding. Many of the antigens used in cancer vaccines are tumour-associated antigens: antigens expressed by tumours but also expressed by other tissues at lower levels. Since these are self antigens, tolerance to these antigens has been previously established by the host. This may have contributed to the failure of many previous trials. Breaking the tolerance to these antigens, but also avoiding detrimental autoimmune disease may be necessary for the success of the cancer vaccine. Depletion or inhibition of Treg cells may aid the reversal of tolerance although the side effect of autoimmunity may also remain. Alternatively, to avoid the induction of autoimmunity, vaccination with tumour-specific antigens (antigens expressed only by tumour cells) may also improve immunotherapy. These antigens normally consist of mutated self proteins, but may be considered as immunologically foreign. Unfortunately, however, expression of the same tumour-specific antigen by the tumours of many patients appears to be rare (Parmiani *et al.* 2007). This makes the design of a vaccine to such antigens less attractive as only a fraction of patients may respond to treatment. Furthermore, it still remains that the body has already been exposed to the antigen so it is likely that the immune system has become tolerant to the antigen targeted. Therefore, breaking tolerance to tumour-specific antigens may also be required for the success of cancer vaccines which target them.

In order to generate the best therapeutic outcomes in cancer patients it appears that combinatorial therapies will be required. Results from James Allison's lab demonstrate that CTLA-4 blockade in combination with a vaccine consisting of irradiated tumour transduced to express the cytokine GM-CSF (Gvax) can eradicate established tumours in mice (van Elsas *et al.* 2001, van Elsas, Hurwitz and Allison 1999, Hurwitz *et al.* 1998). Interestingly, this therapy causes priming of effector T cells through Gvax and removal of inhibitory effects by CTLA-4 blockade which synergise to increase the intratumour frequency and activity of effector T cells resulting in tumour rejection (Quezada *et al.* 2006). In humans, anti-CTLA-4 treatment also resulted in an increase in anti-tumour immunity when given to patients previously vaccinated with Gvax (Hodi *et al.* 2003). Furthermore, anti-CTLA-4 treatment in conjunction with vaccination with tumour-antigen derived peptide also resulted in tumour regression in patients with metastatic melanoma (Phan *et al.* 2003). However, this treatment was also associated with the induction of autoimmunity in a number of patients indicating the importance of CTLA-4 in regulating tolerance to self antigens (Phan *et al.* 2003).

Adoptive cell therapy (ACT) represents another potential approach for immunotherapy of cancer (reviewed in June 2007a, June 2007b). This involves the infusion of anti-tumour lymphocytes, often in large numbers, to cancer patients. The latest studies in humans have shown impressive response rates (Dudley *et al.* 2008). More recent ACT regimes have combined ACT with the administration of irradiaton, lymphodepleting agents, vaccines or growth factors that aid the *in vivo* impact of the transferred cells (Dudley *et al.* 2008, reviewed in Rosenberg and Dudley 2009). The immunosuppressive effect of Treg cells is thought to hamper the effectiveness of adoptively transferred anti-tumour cells (Antony and Restifo 2005). Animal studies suggest that a major effect of lymphodepletion is the removal of suppressive cells such as Treg cells (Klebanoff *et al.* 2005).

Of major importance for the success of immunotherapeutics such as ACT and cancer vaccination is the determination of which antigens are recognised by tumour infiltrating Treg cells and tumour infiltrating Tconv/$CD8^+$ T cells. There is evidence to show that vaccination of tumour antigens may expand populations of Treg cells (Nishikawa *et al.* 2005, Chakraborty *et al.* 2004, Wang *et al.* 2004, Zhou, Drake and Levitsky 2006). Therefore careful selection of antigens and/or epitopes for vaccination is required to avoid simultaneous induction of Treg cells. Epitope mapping of tumour infiltrating T cells including Treg cells will uncover these details. However, as the results of Chapter 4 demonstrate the diversity of the T cell repertoires of tumour infiltrating Treg cells and in particular Tconv cells may be large. Consequently it is likely that numerous epitopes and antigens which stimulate tumour infiltrating T cells exist in tumours making comprehensive epitope mapping demanding. Clearly, the TCR repertoires of T cells infiltrating human tumours must be determined to know if the large diversity observed in MCA tumours also exists in other tumours. The results of Chapter 4 indicate that the TCR repertoires of tumour infiltrating Tconv cells and Treg cells are distinct, suggesting that they recognise different epitopes. This would suggest that careful selection of epitopes for vaccination or *in vitro* stimulation of cells for ACT will allow for expansion of Tconv cells and not concurrent stimulation of Treg cells. In accordance with the findings in this thesis, a recent study of T cell responses in patients with colorectal carcinoma demonstrated that Treg and Tconv cells tend to recognise distinct antigens (Bonertz *et al.* 2009). However, in contrast Vence *et al.* (2007) demonstrated that patients with metastatic melanoma contain Treg cells that recognize tumour antigens known to also stimulate Tconv cells and $CD8^+$ T cells. This finding was further corroborated by recent findings which showed that two tumour antigen derived peptides known to stimulate $CD4^+$ Th cells could also stimulate Tregs (Fourcade *et al.* 2010). Collectively, these studies

demonstrate the complexity of immune responses to tumours and highlight the necessity for further investigation of multiple cancer types.

In this thesis, the TCR repertoire of $CD8^+$ T cells was not determined. These cells were identified as being important for anti-tumour immune responses (following Treg cell depletion). It would therefore be interesting to determine the TCR repertoire of tumour infiltrating $CD8^+$ T cells. Of particular interest would be the diversity of the TCR repertoire of tumour infiltrating $CD8^+$ T cells. A large diversity in the TCR repertoire of tumour infiltrating Tconv cells was observed in this thesis. This suggests that the expansion of particular Tconv clones was not induced in tumours. This is also corroborated by the finding that the majority of tumour infiltrating Tconv and $CD8^+$ T cells display no signs of activation and that the majority of tumour infiltrating Tconv are not proliferating. Several reasons for this may exist. Firstly, the diverse TCR repertoire may reflect low avidity interactions between the T cells and target resulting in poor stimulation (Price *et al.* 2005). Alternatively, the milieu of costimulatory factors and cytokines within the tumour may result in suboptimal stimulation and a lack of clonal expansion (Smith-Garvin, Koretzky and Jordan 2009). Clonal expansion of anti-tumoural Tconv and $CD8^+$ T cells may allow for tumour regression. Therefore it is necessary to determine the factors which prevent this expansion. The suppressive effects of Treg cells may contribute to preventing the proliferation of anti-tumour T cells and thus therapeutic removal of Treg cells may allow for clonal expansion of T cells and subsequently tumour regression.

It is evident that the phenotype of Treg cells overlaps significantly with that of other tumour infiltrating T cells, in particular Tconv cells (presumably following their activation). This presents a major problem for therapeutic targeting of Treg cells. Any approach to deplete

Treg cells through targeting the surface markers described herein are likely to also cause simultaneous depletion of activated anti-tumour Tconv and $CD8^+$ T cells. Therefore the identification of Treg cell specific surface molecules which allow for their exclusive depletion remains paramount for this approach to be successful. Current treatments which target Treg cells through the CD25 receptor, such as ONTAK (Barnett *et al.* 2005), may not show significant efficacy due to this problem. An alternative approach which may circumvent this is to limit the preferential accumulation of Treg cells within tumours, for example, by targeting tumour specific factors which cause their preferential proliferation and survival. The particular antigens, cytokines or costimulatory factors found within the tumour environment which mediate this remain to be determined.

In this thesis, two potential mechanisms for Treg cell accumulation in MCA tumours were investigated, namely conversion of Tconv cells into Treg cells and preferential proliferation/survival of Treg cells. An additional mechanism of accumulation which was not investigated in this thesis was the preferential recruitment and/or retention of Treg cells in tumours. For this purpose the expression patterns of chemokines and chemokine receptors on T cells in MCA tumour bearing mice could be examined. High expression levels of chemokine receptors may allow Treg cells to accumulate and be retained within tumour tissue more readily. Two independent studies, one of B-cell non-Hodgkin lymphoma the other of ovarian carcinoma, have found that that tumours can produce the chemokine CCL22 which attracts $CCR4^+$ Treg cells into tumour tissue (Curiel *et al.* 2004, Yang *et al.* 2006). Furthermore, Curiel *et al.* (2004) utilised NOD/SCID mice to demonstrate that the *in vivo* migration of human Treg cells to transplanted human ovarian tumours was dependant on CCL22. The potential role for CCL22 and CCR4 expression should be investigated in the recruitment of Treg cells into MCA tumours and more importantly, other human tumours.

As mentioned above, mice specifically depleted of Treg cells succumb to severe autoimmune disease within 7-14 days (Kim *et al.* 2007). Therefore, any approach to remove Treg cells is likely to be met by the induction of autoimmunity. To prevent autoimmune disease partial Treg cell depletion may be more efficacious. Alternatively, this may be better achieved through tumour specific ablation of Treg cells. However, the development of tumour specific targeting drugs represents another significant obstacle to successful cancer treatment. Finally, it is thought that tumours develop through a Darwinian-like process - selecting less immunogenic variants over time (reviewed in Dunn *et al.* 2004). Therefore, the late stage tumours targeted in cancer vaccinations and ACT approaches may not be susceptible to immunotherapy as they have selected for variants which are no longer recognisable by the immune system. Thus, the window of opportunity for successful immunotherapy may be early in tumour development which may be significantly earlier than the time of tumour discovery and treatment.

In conclusion, over the last few decades great strides in understanding tumour immunology have allowed for the development of immunotherapies for the treatment of cancer. These therapies have shown real promise, but have been met by significant obstacles limiting their clinical effectiveness. The suppressive effect of regulatory T cells appears to represent one such obstacle. Further studies are necessary to elucidate the interactions between the immune system and cancer and to continue the improvements in immunotherapeutic strategies. I remain cautiously optimistic that the immune system will one day be utilised for the treatment of significant numbers of cancer patients.

Appendix

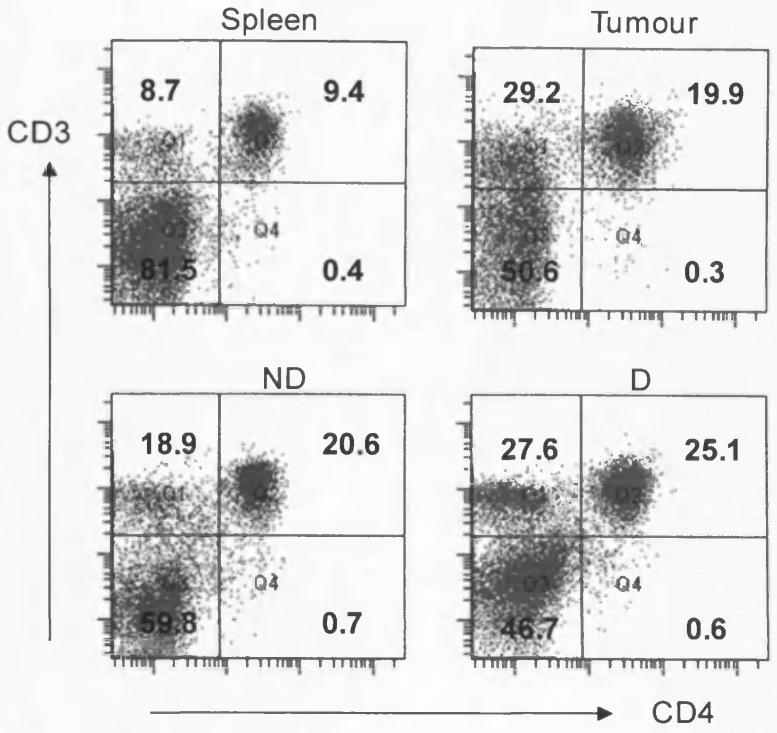

Figure A1. Staining of lymphocytes from tumour bearing mouse with anti-CD3 and anti-CD4 antibodies.
The percercentage of lymphocytes expressing CD3 and CD4 were deteremined by flow cytometry. Single cell suspensions from the spleens, draining lymph node (D), non-draining lymph node (ND) and tumours of a tumour bearing mouse were stained with conjugated antibodies against CD3 and CD4 and analysed using a flow cytometer.

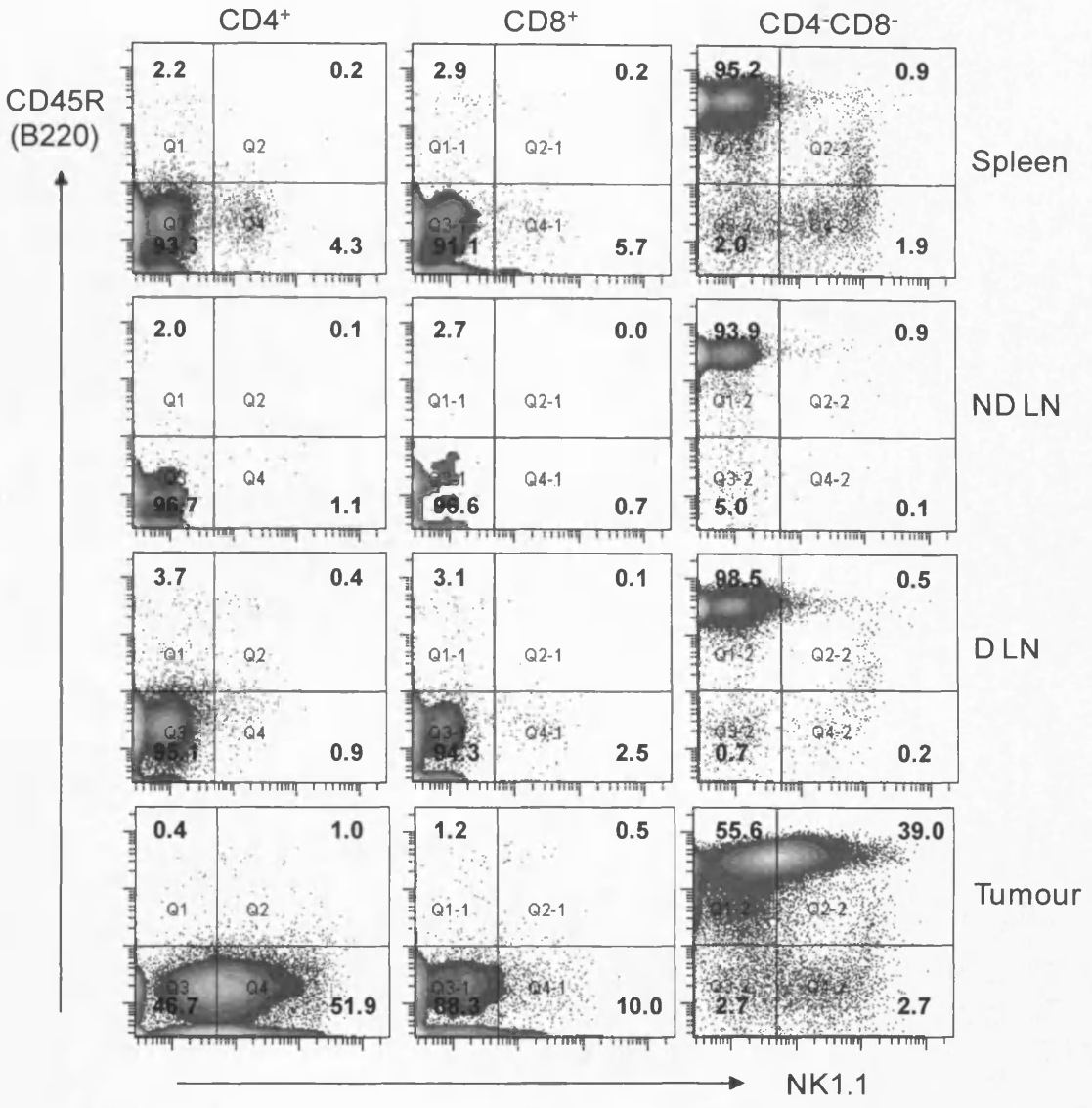

Figure A2. Expression of CD45R (B220) and NK1.1 on lymphocytes in tumour bearing mouse.

Single cell suspensions of spleen, non-draining lymph node (ND LN), draining lymph node (D LN) and tumour were stained with anti-CD4, anti-CD8, anti-CD45R(B220) and anti-NK1.1 antibodies and analysed by flow cytometry. Lymphocytes (gated based on FSC/SSC) were further categorised into $CD4^+$ single positive, $CD8^+$ single positive, and $CD4^-CD8^-$ populations. The percentage of cells expressing CD45R(B220) and NK1.1 within these populations were determined.

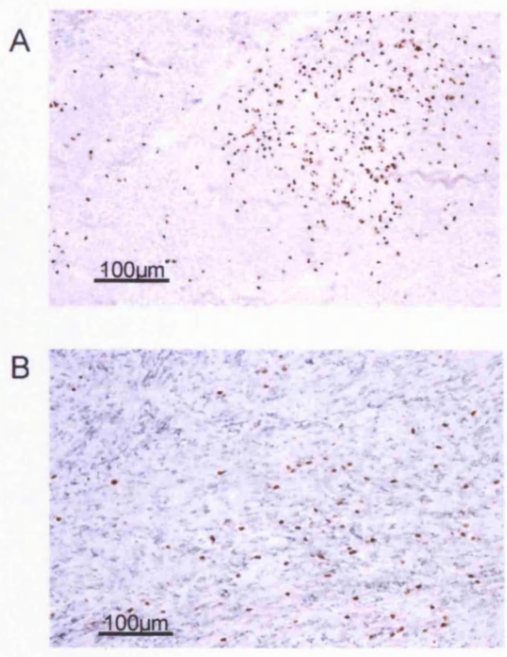

Figure A3. TGFβ containing MCA-induced tumours
Serial sections of spleen (A) and tumour tissue (B) from mice bearing MCA induced tumours were stained with TGFβ (grey) and Foxp3 (brown) -specific antibodies.

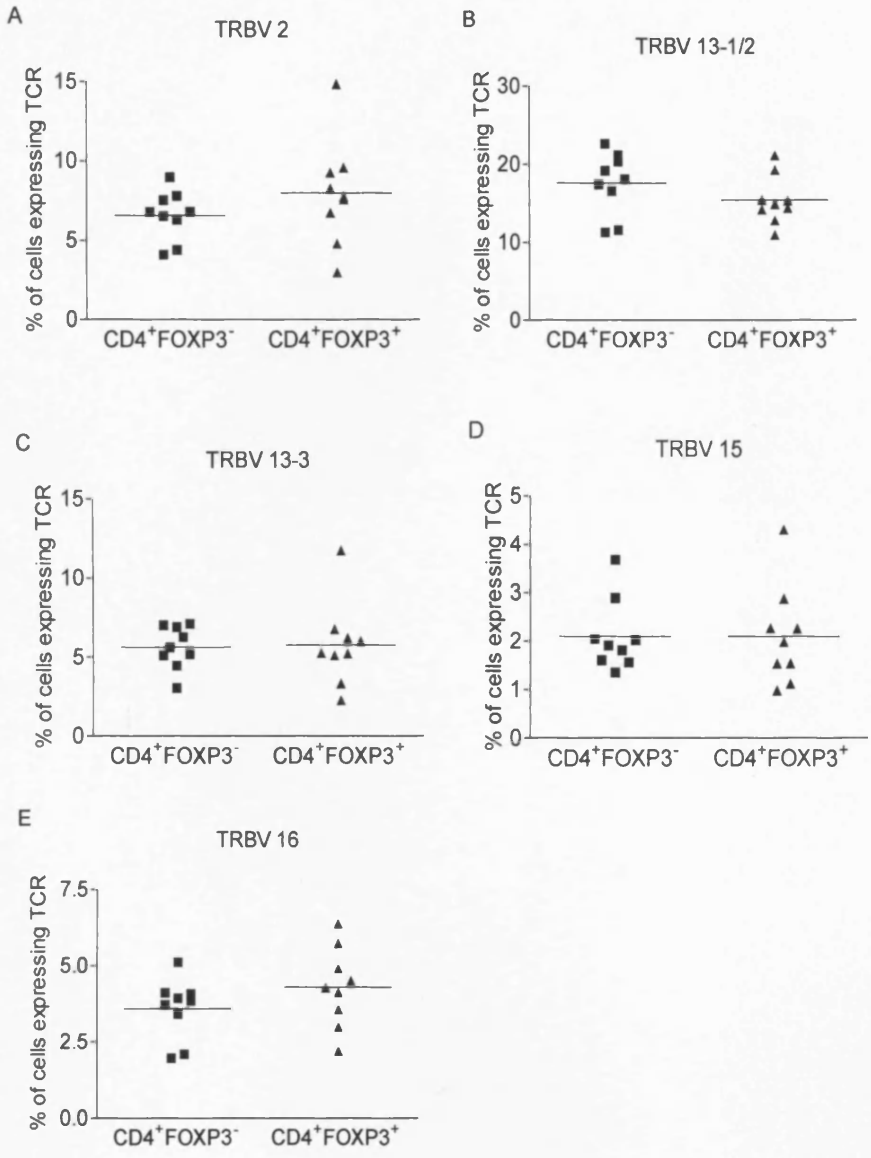

Figure A4. TRBV usage of tumour infiltrating $CD4^+FOXP3^+$ Treg and $CD4^+FOXP3^-$ Tconv cells.

Single cell suspensions of tumour tissue were stained with anti-CD4, -FOXP3 and a series of anti-TCRvβ antibodies specific to TRBV 2, 13-1/2, 13-3, 15 and 16. The percentage of $CD4^+Foxp3^-$ Tconv and $CD4^+Foxp3^+$ Treg cells that expressed TRBV 2 (A), 13-1/2 (B), 13-3 (C), 15 (D) and 16 (E) is shown.

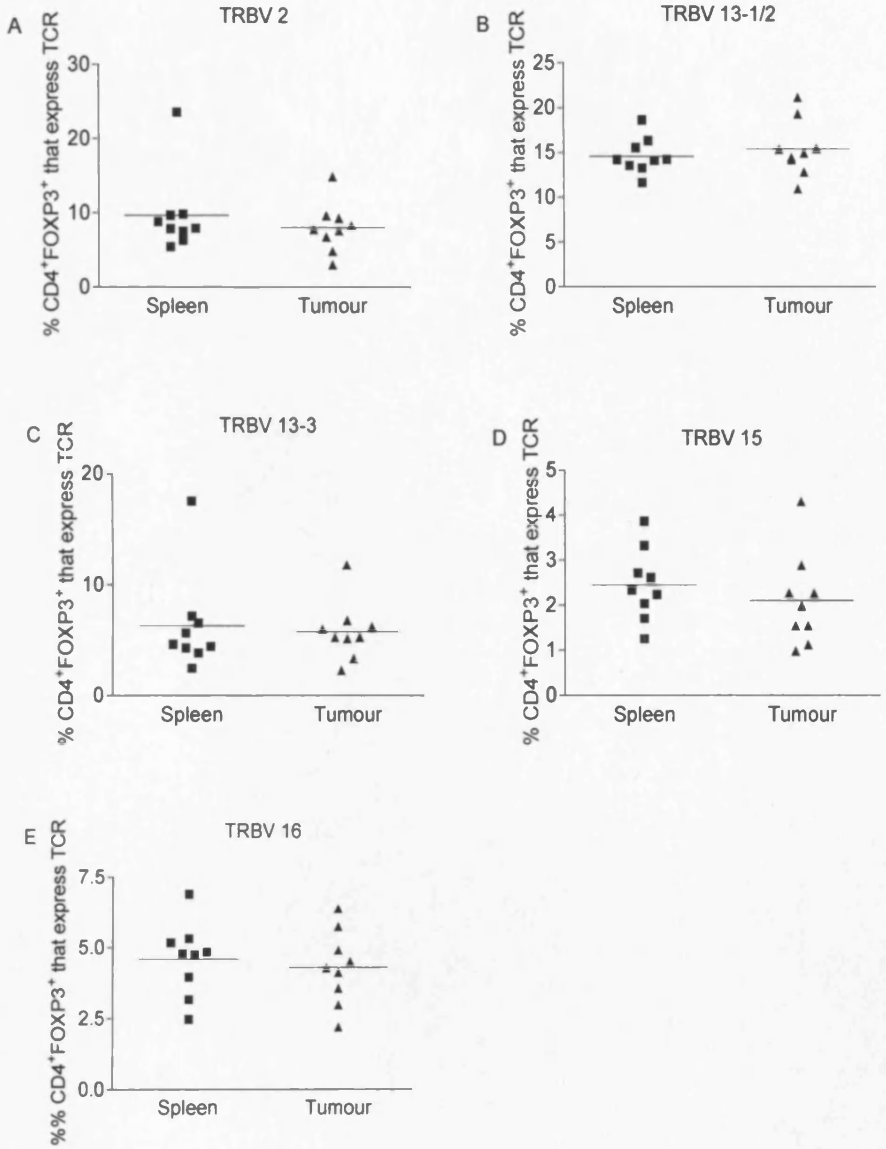

Figure A5. TRBV usage by $CD4^+FOXP3^+$ Treg cells from spleen and tumour.
Splenocytes and single cell suspensions of tumour tissue were stained with anti-CD4, -FOXP3 and a series of anti-TCRvβ antibodies specific to TRBV 2, 13-1/2, 13-3, 15 and 16. The percentage of $CD4^+Foxp3^+$ Treg cells that expressed TRBV 2 (A), 13-1/2 (B), 13-3 (C), 15 (D) and 16 (E) is shown for spleen and tumour.

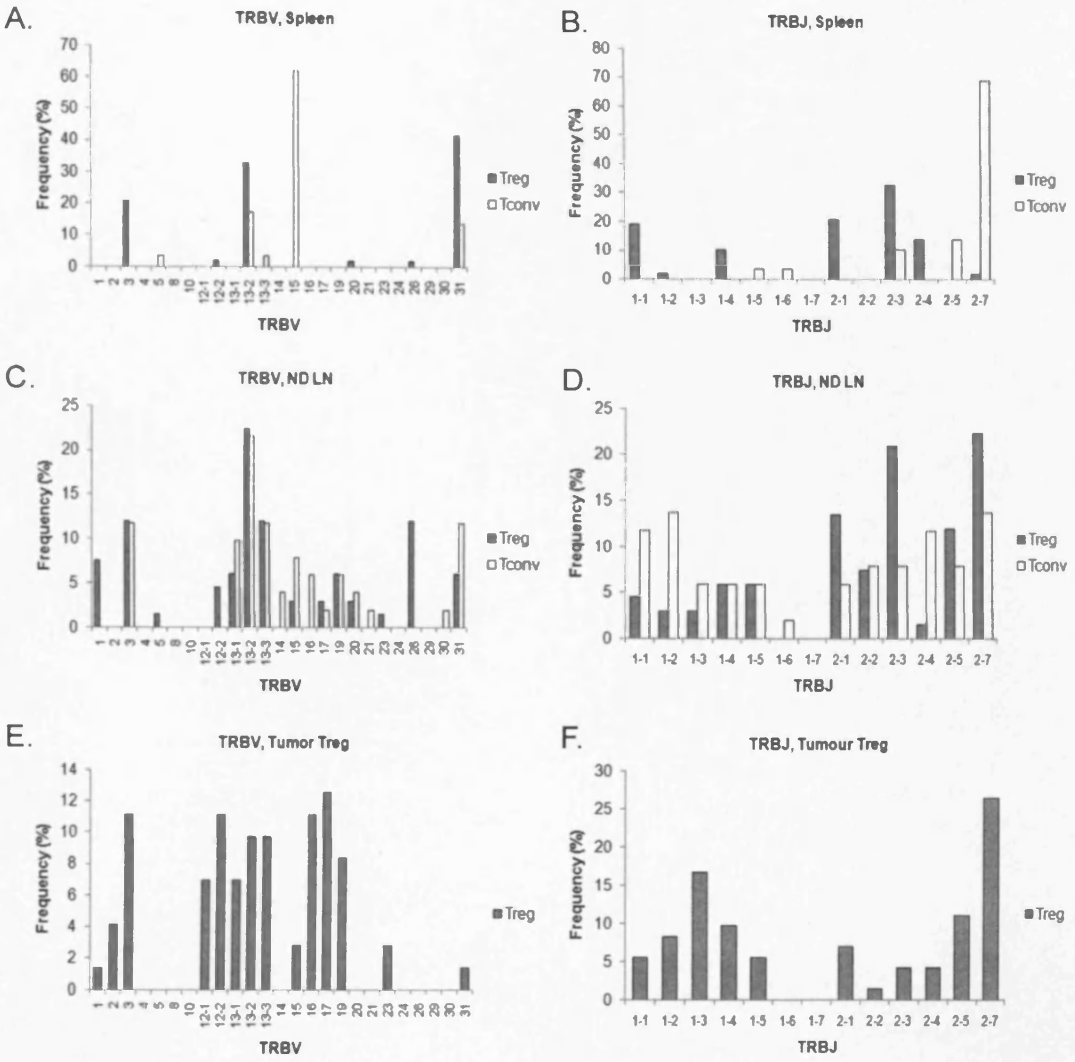

Figure A6. TRBV and TRBJ usage of $CD4^+FOXP3^+$ Treg and $CD4^+FOXP3^-$ Tconv usage in tumour bearing mice.

Tconv ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) cells were sorted from single cell suspensions of spleen, non draining lymph node and tumour tissue. TCR repertoire analysis was performed using 5'RACE. Graphs show the TRBV (A, C, & E) and TRBJ (B, D, & F) usage from the TCR sequence data obtained from Tconv (open bars) and Treg (filled bars).

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