The Recent Thymic Origin, Differentiation And Suppressive Mechanism Of Regulatory T Cells

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A dissertation submitted to the University of Adelaide in candidature for the degree of Doctor of Philosophy in the Faculty of Science
December 2013

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Nicholas Mabarrack

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Acknowledgements

I would like to acknowledge my supervisor, Graham Mayrhofer, who generously contributed his time, expertise and resources to this project. It has been a rewarding collaborative effort. It's been a privilege to work with you, and I value what I have learned. Graham really cares about students and their future, and was very genuinely forthcoming and honest to me about the career prospects of PhD graduates and employment opportunities that postdoctoral graduates can expect. He provided valuable and responsible career counselling to me and selflessly put aside many hours to give top priority to reading my thesis and providing rapid, positive, respectful and encouraging feedback to me to guide me on my way. It was an inspiring "world-class" experience that prompted me to change career to medicine.

I would like to acknowledge my friend and colleague Nicola for many interesting scientific discussions and morale boosting meetings that helped us deal with the problems of being a PhD student in the Bioscience field.

I would like to thank Mahin Moghaddami for her help in preparing synovium rich tissue samples from arthritic rats, and showing an interest in my work.

I would like to thank Sandy McIntyre for his professional and exceptional technical assistance in flow sorting cells for me and for the many long and interesting discussions we shared at the Detmold Facility.

I would also like to thank Alan Bishop for his expert technical assistance in flow sorting cells and for many amusing and entertaining conversations.

"Education is not always empowering. It can create barriers to rewarding careers, demand sacrifices without promise of reward, and present opportunities nobody would want."

February 2009

Abstract

Regulatory T cells are a purported lineage of $CD4^+$ cells that inhibit the proliferation and effector functions of other T cells to prevent the development of autoimmune disease. However, little is known about how they arise, their lifespan and their patterns of recirculation. Furthermore, the mechanisms through which they inhibit other T cells remain unclear. In order to address these issues, we investigated the relationship between regulatory T cells and recent thymic emigrants (RTE) which are newly formed T cells released into the periphery from the thymus. The $CD25^+$ $Foxp3^+$ regulatory T cell subset was found to be closely associated with RTE, and generated the $CD25^-$ $Foxp3^+$ T regulatory T cell subset by unidirectional differentiation. This process was exploited to mature flow sorted $CD4^+$ $CD25^{bright}Foxp3^+$ T cells into $CD25^-$ $Foxp3^+$ T cells and determine that they retain their functional suppressive activity. The phenotype and physiology of the $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ regulatory T cell subsets were characterised and compared to conventional T cell subsets, revealing the differential expression of numerous key molecules. The high expression of CD62L and LFA-1 by $CD25^+$ $Foxp3^+$ regulatory T cells was consistent with both their relative enrichment within secondary lymphoid tissues and their sessile nature. The profile of adhesion molecules on the surface of $CD25^-$ $Foxp3^+$ cells suggested they may tend to localise to sites of inflammation other than the lamina propria, as they have a low expression of CD103 and CD62L, but high expression of LFA-1. However, $CD25^-$ $Foxp3^+$ regulatory T cells were found to selectively migrate into the intestinal mucosa, where they were enriched, and they also returned back to the thymus, suggesting they may constitute a tissue homing subset of regulatory T cells.

We explored the mechanism of regulatory T cell suppression, and found that regulatory T cells condition APC to reduce their ability to activate other T cells. Following the application of this system to differential gene expression microarray analysis, we identified several putative molecular targets of regulation including 10 novel predicted serine/threonine kinases, a novel four point-1, ezrin, radixin and moesin domain containing signalling molecule, the E2F transcription factor 5, and a CD163-like molecule.

Aging was found to negatively affect the productivity of the thymus, although it was found to be still generating new T cells into old age. While the number of thymocytes decreased with aging, the number of $Foxp3^+$ cells in the thymus was unaffected, possibly their preferential recirculation back to the thymus. The size of the peripheral T cell pool decreased with aging, and the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells among $CD4^+$ T cells declined. However, the proportion of $CD25^-$ $Foxp3^+$ regulatory T cells increased with aging in the periphery. The conversion of $CD25^+$ $Foxp3^+$ regulatory T cells into $CD25^-$ $Foxp3^+$ T cells may compensate for the declining thymic output of $CD25^+$ $Foxp3^+$ regulatory T cells that occurs with aging, in order to maintain the regulatory T cell pool.

Abbreviations

APC	Antigen Presenting Cell
BrdU	Bromodeoxyuridine
BLAST	Basic Local Alignment Search Tool
CD	Cluster of differentiation
CFA	Complete Freund's Adjuvant
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FERM	Four point-1, ezrin, radixin and moesin domain
FITC	Fluorescein isothiocyanate
HEV	High endothelial venule
ICAM	Intercellular adhesion molecule
IDO	Indolaminde-2,3-dioxygenase
IFN	Interferon
IL	Interleukin
LAG	lymphocyte activation gene
LFA	Lymphocyte function antigen
MFI	Mean fluorescence intensity
MHC	Major histocompatibility
NCBI	National Centre for Biotechnology Information
PE	Phycoerythrin
PECy7	Phycoerythrin-Cy7
RAG	Recombination activation gene
RNA	Ribonucleic acid
RTE	Recent thymic emigrant
SRT	Synovium rich tissues
TCR	T cell receptor
TDL	Thoracic duct lymph
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor
TNFSF	Tumour necrosis factor superfamily
w/v	Weight per volume

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Publications

Scientific Publications

Mabarrrack, N., Turner, N., and G. Mayrhofer (2008) “The recent thymicorigin, differentiation and turnover of regulatory T cells” Journal of Leukocyte Biology 84 (5) 1287-1297

Mabarrrack, N., and G. Mayrhofer “The suppressive mechanism of regulatory T cells” (manuscript in preparation)

Mabarrrack, N., and G. Mayrhofer “ The effect of aging on the thymic output of regulatory T cells” (manuscript in preparation)

Abstracts and conference presentations

Oral presentation by N.Mabarrack at the Proceedings of the Annual Scientific Meeting of the Australian Society of Medical Research, Adelaide, 2007 “The origins, lifespan and turnover of regulatory T cell subsets in rats” Nicholas H.E. Mabarrack and Graham Mayrhofer.

Oral presentation by N. Mabarrack at the 37th Annual Conference of the Australasian Society for Immunology, Sydney, 2007. “The origins, lifespan and function of regulatory T cell subsets in rats” Nicholas H.E. Mabarrack and Graham Mayrhofer.

Oral presentation by N.Mabarrack at the Proceedings of the Annual Scientific Meeting of the Australian Society of Medical Research, Adelaide, 2008 “The recent thymic origin, differentiation and turnover of regulatory T cells” Nicholas H.E. Mabarrack and Graham Mayrhofer.

Oral Presentation by N. Mabarrack at the Society of Leukocyte Biology conference in Denver, Colorado 2008 “The recent thymic origin, differentiation and turnover of regulatory T cells. Nicholas H.E. Mabarrack and Graham Mayrhofer.

Chapter 1: Introduction

1.1 Introduction

The adaptive arm of the immune system provides exquisite specificity towards foreign antigens while possessing intrinsic self-tolerance, enabling the efficient initiation of immune responses against invading pathogenic organisms bearing foreign antigens while avoiding immune responses towards self antigens. The specificity of the adaptive immune system for foreign antigens is achieved through two different forms of antigen receptors, which are expressed by B and T lymphocytes. While B lymphocytes can directly recognise antigens through their antigen receptors, for most antigens they are dependent on T cells for the initiation of immune responses.

T cells recognise antigens indirectly through their clonally distributed T cell receptor (TCR) for antigen, which is generated through the processs of somatic TCR gene rearrangement and random chain pairing that can potentially produce up to 10^{13} unique receptors (Davis and Bjorkman 1988). While not all unique TCRs can be present in the T cell repertoire simultaneously due to the limited the size of the T cell pool, the ability of individual TCRs to recognise many different peptides is thought to enable responses to virtually all potential foreign antigens that may be encountered (Mason 1998). In addition, the production of new T cells by the thymus, at a rate that decreases with age (Murray *et al.* 2003), and the constant turnover of the T cell pool, means that the T cell repertoire is not only versatile but changes dynamically over time (Bell and Sparshott 1997).

The recognition of antigen by T cells requires a process known as antigen presentation, in which antigen presenting cells (APCs) display small peptide fragments that are loaded onto major histocompatibility complex (MHC) molecules (Bjorkman *et al.* 1987, Germain *et al.* 2004). These peptide fragments, which range in length from approximately 8 to 10 amino acids for MHC class I molecules and 13-17 amino acids for MHC class II molecules are derived through different mechanisms that reflect their different immunological roles (Chicz *et al.*1992).

1.2 Antigen presentation

MHC class I molecules display peptides derived from freshly translated proteins synthesised in the cytoplasm, enabling the immune system to monitor the proteome for viruses or aberrant cells (Agrawal *et al.* 2000). $CD8^+$ T cells recognise peptides in the context of MHC class I molecules. They are capable of mediating the destruction of cells that present foreign peptides through the targeted release of lytic enzymes and display of cytotoxic ligands that kill the abberant cells (Lechler *et al.* 2005). In contrast, MHC class II molecules are loaded with peptides acquired from the extracellular milieu by APCs, and are recognised by $CD4^+$ T cells that mediate the induction of immune responses through the recruitment and activation of other leukocytes, including antigen-specific B cells and components of the innate immune system (Wolf *et al.* 1995). The activation of T cells requires professional APCs known as dendritic cells (DCs), although epithelial cells, monocytes, macrophages and B cells are also capable of presenting antigen to T cells (Banchereau *et al.* 2000). The presentation of antigen to T cells first occurs during their development in the thymus, through a process referred to as thymic selection, which imbues T lymphocytes with an intrinsic tolerance to self-antigens.

1.3 T cell development and thymic selection

T cells develop in the thymus from lymphoid precursor cells through interactions with stromal cells including thymic epithelial cells (Jenkinson *et al.* 2005). Lymphoid precursor cells continually emigrate from the bone marrow to the thymus throughout life, where they replace existing precursors (Ford *et al.*1968). After emigrating to the thymus, lymphoid precursor cells remain quiescent for 2-3 days, before differentiating into exponentially proliferating thymocytes (Jotereau *et al.* 1987). These nascent thymocytes do not initially express either CD4 or CD8 molecules, and it is at this stage that genetic rearrangement of TCR chains commences.

There are 4 known TCR polypeptide chains, known as the α, β, γ, and δ chains, which form either $\alpha\beta$ or $\gamma\delta$ heterodimers. The majority of mature T cells express the $\alpha\beta$ heterodimer while the remainder (1-10%) are unconventional T cells that express the $\gamma\delta$ heterodimer (Davis and Bjorkmann 1988). Interestingly, $\gamma\delta$ thymocytes arise as a discrete population before the differentiation of $\alpha\beta$ thymocytes has commenced, and are capable of directly recognising antigens including proteins and also phosphate containing molecules in the absence of MHC molecules (Pardoll *et al.* 1987, Kaufmann 1996). The differentiation of $\alpha\beta$ T cells is more tightly regulated through the process of thymic selection, to restrict their recognition of antigen to peptides loaded onto MHC molecules.

The first stage of thymic selection begins with the random genetic rearrangement of the TCR β-chain by double negative thymocytes expressing the interleukin 2 (IL-2) receptor α chain (Levelt and Eichmann 1995). This process is facilitated by the recombination activating genes RAG1 and RAG2, which recognise and recombine the genes encoding antigen receptor chains to generate unique antigen receptor chains from V (variable), D (diversity), and J (joining) regions of TCR genes (Yannoutsos *et al.* 2001). The successful rearrangement and expression of the α and β-chains of the TCR along with the TCR coreceptor CD3 is thought to enable the proliferation of double negative thymocytes and their progression to the $CD4^+$ $CD8^+$ double positive stage (Groettrup and von Boehmer 1993, Godfrey *et al.*1993). The rearrangement of the α-chain is initiated during the transition to the double positive stage, and proceeds until moderate to high avidity interactions with MHC molecules expressed by the cortical epithelial cells of the thymus are achieved (Robey and Fowlkes 1994). The successful ligation of the rearranged $\alpha\beta$ TCR to MHC-peptide terminates RAG activity and delivers survival signals to the $CD4^+$ $CD8^+$ thymocytes (Turka *et al.* 1991, von Boehmer 1994). Interestingly, a comprehensive albeit incomplete TCR repertoire can be positively selected through interactions with a single peptide species, while the maximum the diversity of positively selected cells can be approximated with only a few thousand different peptides (Ignatowicz *et al.* 1996, Mason 1998). However, double positive thymocytes that fail to produce a rearranged TCR capable of interacting with MHC molecules after a certain period of time die by neglect as they enter a default apoptotic pathway (Watanabe *et al.* 2000). This process ensures that all $\alpha\beta$ T cells

are restricted to recognising their antigen in the context of MHC molecules. Following positive selection, the T cell repertoire is subjected to negative selection, which achieves self-tolerance through the deletion of T cells that recognise MHC-peptide complexes in the thymus with high affinity.

The process of negative selection is mediated only in part by cortical thymic epithelial cells, which are inefficient in mediating negative selection due to their low expression of MHC molecules (Robey and Fowlkes 1994). However, as $CD4^+$ $CD8^+$ thymocytes mature and migrate from the cortex and into the medulla, they encounter both medullary thymic epithelial cells and bone marrow derived APCs such as macrophages and DCs. These cells express higher levels of MHC molecules and are highly efficient in inducing negative selection (Sprent and Webb 1995, Faro *et al.* 2004). In order to ensure that developing thymocytes are exposed to a more complete range of self antigens, medullary thymic epithelial cells utilise a transcription factor known as AIRE (autoimmune regulator) to express proteins that are otherwise exclusively expressed in the periphery in a tissue-restricted manner (Anderson *et al.* 2002). The tendency of thymocytes to perish upon their recognition of MHC-peptide complexes with high affinity is thought to be a consequence of the nascent configuration of their TCR signal transduction pathways. While TCR stimulation triggers calcium influxes in both thymocytes and mature T cells, thymocytes experience a much smaller calcium influx and rapidly undergo apoptosis following TCR ligation (Finkel *et al.* 1989, Nossal 1994). The process of negative selection enables the immune system to purge itself of the majority of self-reactive T cells, which become enriched in the cohort following positive selection as many of them bind strongly to the main structure of MHC molecules, away from the peptide-loaded cleft that mature T cells interact with. The remaining cells display TCRs that bind MHC-self peptide complexes with low to moderate affinity, but many of which will be able to bind MHC-foreign peptide complexes with higher affinity, and may ultimately be used to recognise peptides derived from infiltrating pathogens (Huseby *et al.* 2008).

The final stage in the generation of new T cells by the thymus involves the differentiation of $CD4^+$ $CD8^+$ thymocytes into $CD4^+$ $CD8^-$ helper T cells and $CD4^-$ $CD8^+$ cytotoxic T cells. The CD4/CD8 lineage decision has hitherto been an unclear process, that was proposed either to occur through instructive signals

delivered to thymocytes or through a process of stochastic differentiation and selection (Robey and Fowlkes 1994). It is now thought that the quantity of TCR signal received determines the lineage commitment of thymocytes, such that low doses of antigen permit the differentiation of thymocytes towards $CD8^+$ T cells, and higher doses redirect thymocytes towards becoming $CD4^+$ T cells (Wantabe *et al.* 2000). Recent experimentation has identified a novel regulator of alternate lineage commitment known as Th-POK that when induced directs positively selected thymocytes away from their default differentiation to $CD8^+$ T cells and commits them to differentiate into $CD4^+$ T cells (He and Kappes 2006). Although Th-POK does not enhance CD4 expression on its own, it antagonises the repression of CD4 expression mediated by Runx3, which is normally expressed by $CD8^+$ T cells (Wildt *et al.* 2007). It is thought that strong signalling through the TCR promotes differentiation into CD4+ T cells, as this silences a distal regulatory element that suppresses Th-POK expression, leading to the derepression of CD4 expression and the commitment thymocytes to the $CD4^+$ lineage (He *et al.* 2008). Although the differentiation of thymocytes into either the $CD4^+$ or $CD8^+$ lineage is largely complete by the time they leave the thymus, their maturation into fully immunocompetent and phenotypically mature T cells continues in the periphery.

1.4 Recent thymic emigrants

The T cell pool is continually resupplied from the thymus through the production of new T cells that arrive in the periphery as recent thymic emigrants (RTE) (Uldrich *et al.* 2006). Although RTE cannot be distinguished from other T cells on the basis of known cell surface antigens in mice or humans, they possess a unique phenotype in the rat that has enabled their characterisation. The expression of Thy-1 (CD90) is restricted to thymocytes and RTE in rats, and is lost as RTE mature in the periphery (Hosseinzadeh and Goldschneider 1993). In addition, RTE do not initially express the CD45 isoform CD45RC, which is induced as they mature (Luettig *et al.* 2001). As RTE make the phenotypic transition to $Thy1^-$ $CD45RC^+$ naïve T cells, a functional maturation also occurs as the T cells become increasingly immunocompetent (Yang and Bell 1992). In contrast to mature T cells, RTE are uniquely susceptible to conversion to a functionally tolerant state. In murine models of skin and heart transplantation that normally lead to allogeneic

reactions and graft rejection, RTE exposed to the allogeneic grafts suppress allogeneic reactions towards the graft, while in separate animals mature T cells from the same strain as the RTE strongly stimulate allogeneic reactions and induce graft rejection (Modigliani *et al.* 1996). While the processes that facilitate the divergence of RTE into either a tolerant or immunocompetent state are unclear, the functional divergence seems to correlate with the differential expression of CD45 isoforms.

The CD45 gene contains a series of exons designated A, B and C, and these can be differentially spliced to produce several distinct CD45 isoforms. While all leukocytes express CD45, the particular isoform that is expressed is often shared within a cell subset, although there is substantial variation between animal species. For example while human naïve T cells express the CD45RA isoform, murine naïve T cells express the CD45RB isoform, and naïve rat T cells express the CD45RC isoform (Rodgers *et al.* 1992). Thymocytes and RTE express a low molecular weight isoform of CD45 that does not include the A, B, or C exons and is referred to as CD45RO (Powrie and Mason 1990A). However, memory T cells, which are long lived T cells generated following antigenic challenge, also express the CD45RO isoform. As RTE mature in the periphery in rats, they are thought to switch isoform expression to become $CD45RC^{+}$ naïve T cells. While the majority of RTE differentiate into $CD45RC^{+}$ T cells that can readily mount immune responses, some RTE either fail to upregulate CD45RC or revert back to the CD45RO isoform, and these cells are not able to initiate immune responses (Yang and Bell 1992). While the process through which these cells arrive at a tolerant state is unclear, the correlation of immunocompetence with CD45RC isoform expression may reflect the modulatory effects of different CD45 isoforms themselves on T cells.

Although no specific ligand has yet been identified for CD45, it is thought to modulate TCR signalling by relieving the inhibition of the Src-like protein tyrosine kinase Lck, which is responsible for transducing TCR signals through its activation of immunoreceptor tyrosine-based activation motifs (ITAMs). It is thought that the homodimerisation of CD45 may interfere with its ability to dephosphorylate Lck and thus enable more efficient TCR signalling (Irles *et al.* 2003). In support of this hypothesis, it has been demonstrated that the smaller extracellular domain (or

ectodomain) of the CD45RO isoform permits highly efficient dimerisation, resulting in decreased signalling via the TCR. In contrast, dimerisation of higher molecular weight isoforms is inefficient, and associated with stronger signalling through the TCR (Xu and Weiss 2002). While it is unclear whether the inability of RTE that remain $CD45RO^+$ to mount immune responses is a result of contact with their antigens (Yang and Bell 1992), contact with antigen in the absence of additional activating signals is a major mechanism through which T cells are rendered tolerant in the periphery.

1.5 Peripheral tolerance

Although the negative selection of T cells with a high affinity for self antigen-MHC complexes in the thymus minimises the generation of potentially pathogenic self-reactive T cells, it is thought that some self-reactive T cells still reach the periphery. Indeed, the naïve T cell pool of $CD4^+$ T cells contains many autoreactive T cells capable of causing autoimmune diseases when transferred into lymphopenic syngeneic hosts (Powrie and Mason 1990B, Powrie *et al.* 1993). The process through which the frequency of potentially pathogenic T cells is reduced in the periphery resembles that of negative selection, and involves the functional inactivation of T cells following antigen presentation by epithelial cells and other non-professional APCs (Field *et al.* 1997). The presentation of antigen to T cells in the absence of activating costimulatory signals either leads to apoptosis, or entry into an anergic state in which the cells are refractory to activation. Interestingly, the isoform of CD45 expressed by the T cells seems to predict the outcome of their interaction with antigen presented in the absence of costimulatory signals. Most $CD45RO^+$ T cells (including RTE) appear to be rendered anergic, while most $CD45RA^+$ naïve T cells undergo apoptosis (Marelli-Berg *et al.* 1997). The cytokine interleukin-15 (IL-15) however promotes the survival of $CD45RA^+$ naïve T cells, enabling them to survive as anergic T cells. Interestingly, while IL-2 does not rescue T cells from apoptosis following antigen presentation, anergised T cells retain their ability to proliferate in the presence of IL-2 (Dooms *et al.* 2000). Although T cells that become anergised following the presentation of self antigens are often regarded simply as cells whose function

neutralised, their inability to provide survival signals to the presenting APCs bestows upon them potent regulatory properties (Frasca *et al.* 2003).

1.6 Anergised T cells

T cells that encounter their antigen in the absence of activating, costimulatory signals enter an anergic state, in which they adopt a unique phenotype and remain permanently refractory to activation. The activation of T cells requires bi-directional costimulatory signals between APCs and T cells. Provided they are sufficiently strong, these signals form positive feedback loops that increase their magnitude past a threshold, resulting in the mutual activation of the interacting cells (Germain *et al.* 2001). However, upon antigen presentation, anergised T cells are unable to provide costimulation to APCs in the form of CD154, which is ordinarily induced upon TCR stimulation and serves to protect APCs from apoptosis (Bowen *et al.* 1995, Koppi *et al.* 1997). The interaction of anergic cells with immature DCs results in the arrest of DC maturation and the inhibition of costimulatory molecule expression (Frasca *et al.* 2003). Mature DCs also experience a downregulation of costimulatory molecules upon interactions with anergic T cells, and they also downregulate MHC II molecule expression, decreasing their capacity for antigen presentation (Vendetti *et al.* 2000). In addition, the production of the anti-inflammatory cytokine IL-10 by anergised cells further downregulates the expression of the potent costimulatory molecules CD80 and CD86 by APC (Ding *et al.* 1993), and strongly enhances APC apoptosis (Ludewig *et al.* 1995). Thus, anergised T cells reduce the longevity and stimulatory capacity of APCs, to deprive other T cells of stimulation.

1.7 T cell costimulation

The activation of $CD4^+$ T cells in response to infection results in the generation of qualitatively distinct immune responses depending on the magnitude of antigen presentation and the strength of accompanying costimulatory signals delivered by DCs. The costimulatory signals exchanged between DCs and T cells are transduced through costimulatory molecules that belong to either the

immunoglobulin-like superfamily or the tumour necrosis factor superfamily (TNFSF). The most critical costimulatory molecules for T cell activation are thought to be CD80 and CD86 (also known as B7.1 and B7.2) of the immunoglobulin-like superfamily, which are only expressed at high levels by DCs, and deliver activating signals through CD28 on the T cell surface (Inaba *et al.* 1994, Inaba *et al.* 1997, Banchereau *et al.* 2000). During T cell activation, costimulatory signals transduced through CD80 and CD86 via CD28 synergise with those transduced via the TCR and drive the transcription of the costimulatory molecule CD154 by T cells. CD154 is a member of the TNFSF, which ligates to CD40 and induces CD80 and CD86 expression by APCs. The latter interaction completes a positive feedback loop drives T cell activation (Germain *et al.* 2001). The activation of T cells induces an alternate ligand for CD80 and CD86, cytotoxic T lymphocyte antigen 4 (CTLA-4), which binds CD80 and CD86 with higher affinity and inhibits the transduction of costimulatory signals to regulate T cell activation (Sharpe and Freeman 2002).

1.8 Immunoglobulin-like superfamily costimulatory molecules

The costimulatory molecules CD80 and CD86 are mainly expressed by APCs, and are upregulated upon the detection of a number of characteristic molecular components of invading pathogens such as bacterial DNA, lipopolysaccharide (LPS) and Neisserial porins (McAdam *et al.* 1998). The main pattern recognition receptors responsible for detecting these “danger signals” are known as the toll-like receptors (TLRs), of which ten have been identified. The TLRs are a key component of the innate immune system, enabling rapid responses towards many bacteria, viruses, fungi and protozoa. In cooperation with TLR1 and TLR6, TLR2 is capable of recognising molecules derived from such diverse sources as Gram-negative bacteria, Mycoplasma, spirochetes, mycobacteria, and fungi (Takeda *et al.* 2003). TLR3 can recognise double stranded RNA (dsRNA), which is associated with viral infections, while TLR4 recognises LPS derived from Gram-negative bacteria, TLR5 recognises flagellin derived from motile bacteria, and TLR9 recognises unmethylated CpG motifs present in bacterial DNA (Kadowaki *et al.* 2001). However, no natural ligands have yet been identified for TLR7, TLR8 or TLR10. Although the majority of molecules recognised by TLRs are associated

with pathogenic microorganisms, both TLR2 and TLR4 also recognise members of the heat shock protein (HSP) family, which are released by dying cells and may provide an endogenous “danger signal” that can activate DCs and alert the immune system to a cryptic infection (Takeda *et al.* 2003). Upon the recognition of pathogens through TLRs expressed by immature DCs residing in the peripheral tissues, the cells translocate to lymph nodes, upregulating their expression of MHC molecules and costimulatory molecules to become mature DCs capable of activating T cells.

Although the most critical costimulatory molecules for $CD4^+$ T cell activation are CD80 and CD86 (Banchereau *et al.* 2000), the immunoglobulin-like superfamily contains several other costimulatory receptor-ligand pairs including ICOS (inducible T cell costimulator)/ ICOS ligand (ICOS-L); Programmed Death 1 (PD-1)/ PDL-1, PDL-2; and the newly discovered B7-H3, B7-H4, B7S1 and B7S3 molecules (Sharpe and Freeman 2002, Sica *et al.* 2003, Prasad *et al.* 2003, Yang *et al.* 2007). While many of these molecules play a key role in the activation of T cells, several act to inhibit T cell activation. ICOS is expressed by activated T cells and resting memory T cells, and it can bind to ICOS-L expressed by B cells, macrophages, and DCs, resulting in enhanced T cell proliferation (Yoshinaga *et al.* 1999). Although ICOS is not required for T cell activation, it sustains the activity of effector T cells through stimulation the production of several cytokines, and it promotes B cell responses through the induction of CD154 on the T cell surface (Gonzalo *et al*. 2001, McAdam *et al.* 2001). The cytokines produced by T cells after costimulation through ICOS are IL-4, IL-5, interferon-γ (IFN–γ), tumour necrosis factor-α (TNF-α) and granulocyte-macrophage colony stimulating factor (GMCSF), which are also produced upon costimulation through CD28. However, while costimulation delivered through ICOS does not enhance IL-2 production, it strongly enhances the production of the regulatory cytokine IL-10, which inhibits CD80 and CD86 expression by APCs (Ding *et al.* 1993, Hutloff *et al.* 1999).

The inhibitory costimulatory molecule Programmed Death 1 (PD-1) is expressed by activated T cells. Stimulation via this molecule induces the production of IL-10, which requires the co-production of small amounts of IL-2 (Dong *et al.* 1999). The activation of T cells is inhibited by ligation of Programmed Death ligand 1 (PD-L1) or PD-L2. This does not increase cell death, but inhibits the production of IL-2, IL-4

and IFN-γ. However, this inhibition of T cell proliferation and cytokine production can be overcome with stronger stimulation through the TCR (Freeman *et al.* 2000, Latchman *et al.* 2001). Despite this, the neutralisation of signalling through PD-1 can reduce the induction of T cell anergy, and mice genetically deficient in PD-1 (called knock out mice) suffer from spontaneous autoimmunity, indicating that inhibitory signals transduced through PD-1 play a critical role in maintaining tolerance to self antigens (Nurieva *et al.* 2006). In addition to PD-1, PD-L1 and PD-L2, several new inhibitory molecules have recently been discovered, including B7-H3, B7-H4, B7-S1 and B7-S3 (Chapoval *et al.* 2001, Sica *et al.* 2003, Prasad *et al.* 2003, Yang *et al.* 2007). Although B7-H3 can stimulate IFN-γ production and enhance T cell proliferation *in vitro*, it acts as a negative regulator of T cell proliferation *in vivo* (Chapoval *et al.* 2001, Nurieva *et al.* 2006). Although no ligand has yet been found for B7-H3, its homologue B7-H4 (which is similarly inhibitory) appears to bind a molecule expressed by T cells, and it is likely that this ligand may be shared between the two (Sica *et al.* 2003). The B7 superfamily member 1 (B7-S1), and B7-S3 are the most recently discovered inhibitory costimulatory molecules expressed by APCs. The molecules act to control the activation of autoreactive T cells *in vivo*, although little is yet known about their mechanism of action (Prasad *et al.* 2003, Yang *et al.* 2007).

1.9 TNF superfamily costimulatory molecules

In addition to costimulation provided by immunoglobulin-like superfamily costimulatory molecules, T cell activation is also regulated by members of the TNF superfamily, which not only function as costimulatory molecules but also regulate T cell proliferation and survival following activation. The TNF superfamily (TNFSF) members comprise a complex system of ligands and receptors that regulate T cell behaviour through several distinct mechanisms. Many TNFSF receptors contain death domains, which can initiate signalling cascades that activate caspases to trigger apoptosis. Alternatively, TNFSF receptors may contain TNF receptor-associated factor (TRAF) binding domains, which can recruit TRAF proteins that are then translocated to the nucleus to modulate gene expression and affect

activation, differentiation and survival (Aggarwal 2003). The costimulatory CD40-CD154 pathway is a critical regulator of T cell activation that can facilitate the potentiation of T cell activation by DCs. The presentation of antigen to T cells by DCs induces CD154 expression by the T cells, and this is ligated by CD40 on the DC plasma membrane (Grewal *et al.* 1996). The ligation of CD40 enables the recruitment and activation of TRAF2 and TRAF6, which initiate the transduction of signals that activate NFκ-B within DCs and drive the transcriptional upregulation of CD80 and CD86. This in turn enhances the induction of CD154, forming a positive feedback loop that can potentiate T cell activation (Germain 2001, Hostager *et al.* 2003).

The costimulatory molecule CD27 is another member of the TNFSF that is expressed by almost all naïve T cells and is expressed at higher levels following activation (Borst *et al.* 1989). The ligation of CD27 to CD70, which is expressed exclusively by activated DCs, is believed to enable the antigen-specific expansion of T cells through the provision of survival signals. The activation of TRAF2 and TRAF5 by CD27 culminates in the transduction of signals that activate NFκ-B and it is believed that this signalling pathway is responsible for enhancing the survival of T cells following activation (Hendriks *et al.* 2000). The expression of CD70 is transiently upregulated on B cells following their activation and occurs at sites where effector T cells are responding to antigen, thus facilitating their accumulation. Consistent with this observation, effector T cells fail to accumulate at sites of infection in CD27 knock out mice, impairing protective immune responses (Borst *et al.* 2005). The propensity of CD27 to promote T cell survival is key to the generation of T cell memory, which is profoundly delayed in the absence of the molecule (Hendriks *et al.* 2000).

The generation of T cell memory is also associated with the transduction of signals initiated through CD134 (OX40) and mediated by TRAF2 and TRAF5,. In contrast, TRAF3 inhibits these signals (Kawamata *et al.* 1998). The expression of OX40 by T cells is dependent on ligation of CD28 by either CD80 or CD86, and it follows T cell activation with a delay of 1-2 days. Similarly, the expression of OX40-L by APCs does not occur until several days after activation. CD40L may also be expressed by activated T cells and activated endothelium, where it is thought to sustain T cell responses (Gramaglia *et al.* 2000). The activation of T cells is

unaffected by signalling through OX40, but its ligation following T cell activation results in the sustained activation of protein kinase B (PKB), which is initially activated by signal transductions through CD28. It is thought that the sustained activation of PKB enables the long-term survival of T cells by inducing transcription of the anti-apoptotic proteins Bcl-2 and Bcl-xL (Song *et al.* 2004). In the absence of OX40 signalling, T cells are unable to maintain the transcription of Bcl-2 and Bcl-xL for more than 4-8 days following activation, and rapidly perish (Rogers *et al.* 2001). Thus OX40 costimulation is essential for the formation of T cell memory by enabling sufficient quantities of T cells to survive long enough to differentiate into memory T cells (Gramaglia *et al.* 2000).

The generation of T cell responses that drive antibody production is promoted by CD30, which is induced upon TCR signalling and functions as a costimulatory molecule by enhancing T cell activation (Watts 2005). In addition, the expression of CD30 is upregulated following costimulation through CD28 and in the presence of IL-4, a T cell derived cytokine that drives B cell responses. The expression of CD30 appears to be dependent on signal transducer and activator of transcription (STAT)-6, a critical mediator of IL-4-induced signal transduction (Toennies *et al.* 2004). The ligand for CD30 (CD30L) is mainly expressed by B cells, and also by antigen presenting cells that are found at sites where B cells interact with T cells (Kim *et al.* 2003). It is thought that signalling through CD30 is essential for the survival of T cells, especially those that participate in the formation of germinal centres, and in the absence of CD30 the ability of the immune system to produce antibody upon rechallenge is inhibited (Gaspal *et al.* 2005).

The glucocorticoid induced TNFR family related (GITR) molecule is a potent costimulatory molecule that is thought to facilitate T cell activation through mitogen activated protein (MAP) kinase signalling pathways, which lie downstream of costimulatory signals transduced through CD28 (Ronchetti *et al.* 2004). As GITR costimulates T cells, it protects them from apoptosis that follows TCR stimulation in the absence of costimulation, but not from other apoptotic stimuli (Nocentini *et al.* 1997). GITR ligand (GITRL) is expressed by B cells, macrophages and DCs, and following ligation to GITR on the surface of T cells activates NFκ-B to enhance their proliferation and cytokine production (Yu *et al.* 2003). While other TNFRs have been shown to activate NFκ-B through TRAF2, the activation of NFκ-B is

inhibited as a consequence of GITR induced signalling via TRAF2, and instead occurs through TRAF4 and TRAF5 (Esparza and Arch 2004, Esparza and Arch 2005, Esparza and Arch 2006).

1.10 Effector T cells

Following successful activation, it had been proposed that $CD4^+$ T cells can either differentiate into Th1 or Th2 type effector cells, which are distinguished by their mutually exclusive patterns of cytokine expression and induce either cell-mediated or humoral immune responses respectively (Abbas *et al.* 1996). The differentiation of T cells into proinflammatory Th1 cells that secrete IL-2 and interferon-γ (IFN-γ) was thought to be associated with the costimulatory molecule CD86, while CD80 costimulation was associated with Th2 cells that secrete IL-4, IL-5, and IL-13 and stimulate antibody production by B cells (Kuchroo *et al.* 1995, Bashian *et al.* 1997, Shortt *et al.* 1998). However, the differential effect of costimulation through CD80 and CD86 on the polarisation of T cells into Th1 or Th2 subsets does not indicate that they deliver qualitatively different signals. In fact, both molecules can induce the generation of either Th1 or Th2 responses, depending on their level of expression (Levine *et al.* 1995). Due to the integration of costimulatory and TCR signals, it is now thought that the differentiation of T cells is not regulated by individual costimulatory signals, but that it is dependent on the strength of the TCR/costimulatory signal received (Germain *et al.* 2001). Strong T cell priming generates Th1 responses and inhibits Th2 differentiation, while lower levels of priming induces differentiation to Th2 cells (Tao *et al.* 1997, Rogers and Croft 2000). The presence of cytokines in the extracellular milieu also influences this process, by directing the behaviour of lymphocytes following their activation.

The differentiation of T cells into Th1 effector cells results from their interactions with DCs primed with intracellular pathogens such as chlamydia, salmonella, and viruses (de Jong *et al.* 2004). The ligation of characteristic molecules derived from intracellular pathogens to specific TLRs induces the production of the cytokine IL-12 by immature DCs, which potently stimulates IFN-γ production by T cells to promote their differentiation into Th1 cells (Trincheri 2003). Furthermore, CD40

ligation on the surface of mature DCs potently induces IL-12 production, to promote Th1 differentiation (Kalinski *et al.* 1999). Upon the binding of IL-12 to its receptor on the surface of T cells, signals transduced by STAT4 result in the repression of the transcription factor GATA-3, which is associated with the differentiation of T cells into Th2 effector cells (Ouyang *et al.* 1998). Although IL-12 promotes the differentiation of naïve T cells to Th1 effector cells, it is not absolutely required for Th1 differentiation. Additional mechanisms exist in APCs that are activated downstream of TLR ligation and influence T cell differentiation. The signals transduced through TLRs in APCs utilise a scaffold protein known as MyD88, which is required for APC to induce the differentiation of T cells into Th1 effector cells (Jankovic *et al.* 2002, Faisal *et al.* 2008). The signals transduced through MyD88 induce the expression of Delta4, a member of the notch pathway that promotes Th1 responses (Amsen *et al.* 2004). Thus, following the detection of intracellular pathogens by TLRs, the expression of costimulatory molecules by DCs is upregulated, their production of proinflammatory cytokines is induced and the display of Delta notch ligands is altered to drive T cell differentiation toward Th1 effector cells (Moser and Murphy 2000, Worsley *et al.* 2008). Protective immune responses against intracellular pathogens are efficiently initiated by Th1 cells, which recruit and activate macrophages to phagocytose and kill microbes. Th1 cells may also develop cytotoxic activity to promote the lysis of infected cells and help limit infection (Romagnani *et al.* 1991).

The detection of extracellular pathogens such as mycoplasma, spirochetes, or fungi by specific TLRs on the surface of DCs typically leads to the generation of Th2 immune responses following interactions between the primed DC and naïve T cells (Netea *et al.* 2005). The stabilisation of T cell polarisation towards a Th2 associated cytokine profile has been associated with the autocrine production of IL-4, which efficiently induces Th2 differentiation in activated T cells (Le Gros *et al.* 1990, Schmitz *et al.* 1994). However, the initial differentiation of T cells into Th2 cells does not require IL-4 signalling, and appears to result from the sustained expression of the transcription factor GATA-3, which is downregulated in the presence of IL-12 to permit Th1 differentiation (Ouyang *et al.* 1998, Jankovic *et al.* 2000). In addition, CTLA-4 ligation inhibits GATA-3 expression, permitting the differentiation of Th1 effector cells but precluding Th2 development (Nasta *et al.* 2006). It is likely that this effect is a result of the stronger priming associated with

the generation of Th1 cells which necessitates CTLA-4 induction to dampen costimulatory signals during T cell activation. Thus GATA-3 promotes Th2 responses by inducing Th2 cytokine production, enhancing Th2 cell proliferation and preventing differentiation to Th1 effector cells (Zhu *et al.* 2006). In addition, the anti-inflammatory cytokine IL-10 produced by Th2 cells also inhibits the function of Th1 cells by suppressing secretion of proinflammatory cytokines and downregulating costimulatory molecules expressed by APCs (Enk *et al.* 1993). Recent findings suggest that signalling through the Jagged members of the Notch signalling pathway may also promote Th2 differentiation through their induction of GATA-3 and enhancement of IL-4 production (Amsen *et al*. 2004). However, while Jagged members can efficiently induce Th2 differentiation, they are not required for the generation of Th2 responses (Worsley *et al.* 2008). In summary, the generation of Th2 responses is associated with the moderate activation of T cells by APCs, and appears to be a default differentiation pathway that can be influenced by cytokines and promoted by Jagged Notch ligands (Liotta *et al.* 2008). Th2 effector cells activate B cells to produce antibodies that bind to invading microbes, and the synthesis of IL-4 by Th2 cells induces B cells to produce IgE. In addition, Th2 cytokines stimulate the proliferation of mast cells, which can load IgE onto their plasma membrane and utilise it as an antigen receptor to mediate protective immune responses against many extracellular pathogens (Del Prete *et al.* 1991).

Recent evidence has suggested that infection with certain extracellular pathogens such as Klebsiella or Candida species may require T cell responses distinct that are characterised by the production of IL-17 by $CD4^+$ T cells (Matsuzaki *et al.* 2007). These $CD4^+$ T cells, termed Th17 cells, are driven to produce IL-17 due to their expression of the orphan nuclear receptor RORγt transcription factor (Ivanov *et al.* 2006). The differentiation of Th17 cells from $CD4^+$ T cells is directed by IL-1β and IL-6 produced by DCs after detection of extracellular pathogens through lectin pattern recognition molecules (LeibundGut-Landmann *et al.* 2007). The synthesis of IL-17 by Th17 cells induces the local production of macrophage inflammatory protein 2 (MIP-2), which promotes neutrophil recruitment, and granulocyte colony stimulating factor (G-CSF), which enhances the proliferation and effector functions of neutrophils (Ye *et al.* 2001). In addition to the recruitment and activation of neutrophils, Th17 cells also enhance the production of anti-microbial peptides

(Liang *et al.* 2006), and induce the formation of tight junctions to strengthen the intestinal barrier and control enteric infections (Kinugasa *et al.* 2000). While Th17 cells play an important role in protective immunity, the ease with which they can differentiate from other $CD4^+$ T cell subsets in the presence of IL-1β and IL-6, and also IL-21 and IL-23, means they also feature prominently in the pathogenesis of many autoimmune diseases (Fouser *et al.* 2008, Oukka 2008). Strikingly, recent evidence suggests that these cytokines can compromise the intrinsic self-tolerance of the T cell compartment of the immune system, by converting cells of the critically important regulatory T cell lineage to express RORγt and produce IL-17 (Koenen *et al.* 2008, Osorio *et al.* 2008, Beriou *et al.* 2008).

1.11 Regulatory T cells

Regulatory T cells are purported to be a lineage of $CD4^+$ T cells that inhibit the proliferation and effector functions of other T cells, acting to prevent the development of autoimmune disease (Sakaguchi *et al.* 1995). While earlier research had suggested that an immunoregulatory $CD8^+$ suppressor T cell lineage may enable the T cell compartment to actively inhibit immune responses (Murphy *et al.* 1976), this work was found to be inconclusive and the concept of an immunoregulatory T cell population fell from favour (Steinmetz *et al.* 1982, Kronenberg *et al.* 1983). Despite this setback, the concept of a lineage of immunoregulatory T cells was later revived through functional investigations of $CD4^+$ T cells defined on the basis of their expression of CD45 isoforms. It was observed that the adoptive transfer of naïve rat $CD45RC^+$ T cells, separated from $CD45RC^-$ T cells (memory T cells and RTE), caused multiple autoimmune diseases when transferred into lymphopenic syngeneic hosts (Powrie and Mason 1990). However, when $CD4^+$ $CD45RC^-$ T cells were co-transferred, the induction of autoimmune disease was prevented, suggesting that a population of T cells with regulatory activity is present within the $CD45RC^-$ subset. Subsequently, an additional marker for this immunoregulatory population was found, when it was observed that the adoptive transfer of $CD4^+$ T cells depleted of cells expressing the high affinity IL-2 receptor-α chain (CD25) into athymic nude mice induced multiple autoimmune diseases (Sakaguchi *et al.* 1995). The co-transfer of $CD4^+$

$CD25^+$ cells was found to inhibit the induction of autoimmune disease in a dose dependent manner. These $CD25^+$ regulatory T cells were later shown to be identical to the $CD45RC^-$ immunoregulatory T cells (Sakaguchi *et al.* 1995, Takahashi *et al.* 1998). Although it was previously believed that all $CD4^+$ $CD25^+$ T cells represented activated T cells, a clear functional difference was established between $CD4^+$ $CD25^+$ T cells isolated from normal animals, which inhibit the proliferation of other T cells, and activated T cells derived from $CD4^+$ $CD25^-$ T cells, which possess no regulatory activity (Kuniyasu *et al.* 2000). So called "natural" $CD4^+$ $CD25^+$ regulatory T cells have a distinct phenotype that distinguishes them from activated T cells, as they also express the immunoregulatory molecules CTLA-4, TGF-β receptor 1 and IL-10 (Knoechel *et al.* 2006). However, the most reliable marker of regulatory T cells is the forkhead box p3 (Foxp3) transcription factor, which is believed to program their differentiation, and confers on $CD4^+$ T cells the regulatory T cell phenotype and suppressive activity (Hori *et al.* 2003).

1.12 The origins of regulatory T cells

Regulatory T cells are thought to differentiate in the thymus through their recognition self peptides displayed to them in the context of MHC II molecules (Bensinger *et al.* 2001, Apostolou *et al.* 2002, Fontenot and Rudensky 2004, Caton *et al.* 2004, Larkin *et al.* 2007). This thymic selection process has been demonstrated using transgenic RAG-2 knockout mice in which a model "self" peptide is expressed along with transgenic TCRs that are specific for the peptide. In the absence of the model self peptide, mice expressing the transgenic TCR contain a very low proportion of $CD25^+$ regulatory T cells in the thymus compared with wild-type mice. These few regulatory T cells appear to arise following rearrangement of the transgenic TCRs and may have different antigen specificities (Itoh *et al.* 1999). Strikingly, when mice express both the model self-peptide and cognate TCR that recognises the peptide with high avidity, a high proportion of the TCR transgenic thymocytes express CD25, and these cells have an immunoregulatory activity. It is therefore thought that high affinity interactions with self-peptides may select regulatory T cells through a process similar to positive selection (Jordan *et al.* 2001). The cells that mediate this selection process are

believed to be thymic cortical epithelial cells, which are known to mediate the positive selection of thymocytes (Bensinger *et al.* 2001). It has therefore been proposed that regulatory T cells represent the most self reactive T cells that avoid negative selection and that regulatory T cells have a high avidity for self antigens (Schwartz *et al.* 2005).

However, the thymic selection of regulatory T cells has been more thoroughly examined using a transgenic mouse model that enables the expression of the model self peptide to be modulated. While the proportion of $CD25^+$ immunoregulatory thymocytes bearing a transgenic TCR that recognises the model self peptide increases with increasing expression of the model self peptide, this effect is due to the negative selection of $CD4^+$ $CD25^-$ thymocytes (van Santen *et al.* 2004). In fact, the number of $CD25^+$ thymocytes is only slightly affected by the expression of the peptide and the extensive negative selection of $CD4^+$ $CD25^-$ thymocytes. While the numbers of $CD25^+$ thymocytes bearing a transgenic TCR marginally increases when the model self peptide is expressed at high levels, this is accompanied by the same increase in the number of $CD25^+$ thymocytes not bearing the transgenic TCR, and may reflect homeostatic proliferation induced by lymphopenia (Kawahata *et al.* 2002, van Santen *et al.* 2004). Thus, the level of expression of cognate antigen has only a minor, non-specific effect on the numbers of regulatory T cells that appear in the thymus, and the “thymic selection” of regulatory T cells that has been described is an artefact of the differential sensitivity of regulatory T cells and conventional T cells to negative selection. In fact, it appears that $CD25^+$ thymocytes are approximately 3-fold less sensitive to negative selection compared to their $CD4^+$ $CD25^-$ thymocyte counterparts (van Santen *et al.* 2004). This finding suggests that regulatory T cells are unlikely to recognise antigen with high avidity, since high avidity interactions with self peptide MHC complexes are thought to induce negative selection (Sprent and Webb 1995).

The induction of Foxp3 in the thymus is thought to occur predominantly in the medulla, where the most mature thymocytes are found. While some $CD4^+$ $CD8^-$ thymocytes express CD25, they do not possess regulatory activity until several days after they first appear. The acquisition of regulatory activity by these cells coincides with their expression of Foxp3, which is observed in the thymic medulla

several days after the appearance of mature $CD4^+$ $CD8^-$ thymocytes (Fontenot *et al.* 2005, Jiang *et al.* 2006). This suggests that T cells may need to reach a certain stage of maturation before they can differentiate into regulatory T cells. Interestingly, it has been observed that RTE can acquire regulatory functions after entering the periphery, a capacity that reduces as they mature (Modigliani *et al.* 1996). This suggests that T cells are only amenable to differentiation into regulatory T cells at a distinct stage of maturation. Furthermore, it is unclear whether Foxp3 induction occurs in the thymus, or whether the regulatory T cells that appear in the medulla several days following the generation of mature thymocytes represent T cells have recirculated back to the thymus from the periphery after acquiring regulatory functions there as RTE.

Regulatory T cells proliferate rapidly *in vivo*, and appear to encounter their cognate antigens continuously (Fisson *et al.* 2003). Although the origins of regulatory T cells remain unclear, a recent report has suggested they represent rapidly turning over memory T cells. Most T cells in humans are $CD45RA^+$ naïve T cells, while $CD45RO^+$ T cells comprised of both memory T cells and RTE form a minority of T cells. Regulatory T cells are predominantly $CD45RO^+$, and the lifespan of $CD45RO^+$ $CD25^+$ regulatory T cells is considerably shorter than that of conventional T cells, with a half-life of around 8 days. In contrast, $CD45RO^+$ $CD25^-$ memory T cells have an approximate half-life of 24 days, while naïve T cells have a half-life of approximately 200 days (Vukmanovic-Stejic *et al.* 2006). While the $CD45RO^+$ $CD25^+$ regulatory T cell population investigated does not include all regulatory T cells, some of which are $CD45RA^+$ (Seddiki *et al.* 2006), and some of which are $CD25^-$ (Zelenay *et al.* 2005), this study suggests that regulatory T cells are unlikely to be self-sufficient in the periphery, and are probably derived from another T cell population. However, since memory T cells are previously activated T cells involved in protective immunity, it is unclear why these cells would differentiate into regulatory T cells. However, if regulatory T cells are indeed self-reactive, they may be derived from $CD45RO^+$ recent thymic emigrants that have been anergised through contact with self antigen, which would explain their rapid turnover *in vivo*. Consistent with this hypothesis, the continued production of T cells by the thymus has been shown to be vital for the maintenance of tolerance to allografts, suggesting that the thymus may play a key role in replenishing regulatory T cells or their immediate precursors (Niimi *et al.* 2001). While

regulatory T cells are widely regarded as self-reactive, it is unlikely that they recognise self with high avidity, due to their insensitivity to negative selection (van Santen *et al.* 2004). However, they may recognise self with low avidity. Even low avidity interactions between the TCRs on the surface of regulatory T cells and MHC-peptide complexes are sufficient to trigger their suppressive mechanism (Larkin *et al.* 2006). Recent studies have shown that regulatory T cells do not recognise self with high avidity, and probably recognise foreign antigens (Pacholczyk *et al.* 2007). Thus, regulatory T cells may recognise self peptides through low avidity interactions, which may explain why they bear many features of anergised T cells.

The induction of Foxp3 in the periphery has been observed following the repeated administration of peptides, indicating that regulatory T cells can be induced in the periphery following encounter with antigen (Dahlberg *et al.* 2007). Indeed, analyses of the Foxp3 promoter have revealed that nuclear factor of activated T cells (NFAT) binds to the Foxp3 promoter following TCR ligation, leading to induction of Foxp3 transcription (Mantel *et al.* 2006). Thus, it is plausible that regulatory T cells may represent T cells that have been anergised through the interaction of their TCR with cognate antigens. In support of this idea, regulatory T cells are anergic to TCR stimulation due to the inhibition of transcription factors NF-AT and NFκ-B by Foxp3, which prevents their activation and their synthesis of IL-2, IL-4 and IFN-γ (Bettelli *et al.* 2005). Regulatory T cells also bear the markers of failed activation that are characteristic of anergised T cells, including impaired protein kinase C activity and defects in Ras, MEK and ERK signal transduction pathways that lie downstream of the TCR (Li *et al.* 2005, Hickman *et al.* 2006). Although attempts have been made to explore the relationship between T cell anergy and regulatory T cell differentiation (Kuniyasu *et al.* 2000), the methods that were used to investigate anergised T cells did not replicate the established phenotype or functional properties of anergised T cells, most importantly their demonstrated regulatory activity (Marelli-Berg *et al.* 1997, Kubsch *et al.* 2003, Frasca *et al.* 2003).

1.13 The suppressive mechanism of regulatory T cells

The regulatory activity associated with regulatory T cells was initially attributed to their expression of CD25, which was thought to sequester IL-2 produced by activated T cells, thus inhibiting their proliferation (Thornton and Shevach 1998). However, the blocking of CD25 on the surface of regulatory T cells does not abolish regulatory T cell activity (Kohm *et al.* 2006). It is now thought that CD25 may be important for the expansion of regulatory T cells, as they are dependent on IL-2 for proliferation while being unable to produce it themselves (Setoguchi *et al.* 2005, Zhang *et al.* 2005, Malek 2008). Thus regulatory T cells may use IL-2 as a homeostatic factor, proliferating in parallel with effector cells that are producing the cytokine so that they are able to regulate them.

Conversely, the cytokines produced by regulatory T cells limit the proliferation of effector T cells to provide negative feedback, although this appears to occur indirectly (Miyara and Sakaguchi 2007). The production of IL-10 by regulatory T cells downregulates costimulatory molecules expressed by DCs and impairs their ability to stimulate T cells, effectively limiting the generation of new effector T cells (Kubsch *et al.* 2003). Regulatory T cells also secrete TGF-β, which is important for limiting inflammation and in promoting healing *in vivo*, and reducing the stimulatory capacity of DCs (Bonham *et al.* 1996). Although no direct mechanism of action for TGF-β on T cells has been demonstrated, the stimulation of T cells by APCs in the presence of TGF-β can induce Foxp3 expression in T cells to effectively convert them into regulatory T cells (Zheng *et al.* 2002, Chen *et al.* 2003). While the cytokines produced by regulatory T cells play a key role in limiting inflammation *in vivo,* the suppressive mechanism associated with regulatory T cells is cell contact-dependent, suggesting the involvement of molecules expressed on their plasma membrane (Miyara and Sakaguchi 2007).

It has been proposed that regulatory T cells may execute their mechanism of suppression through display of the inhibitory costimulatory molecule CTLA-4 to CD80 or CD86 on the surface of T cells. While CD80 and CD86 are usually expressed on the surface of APCs, they can be acquired by T cells through the

transfer of membrane segments onto the T cell surface following stimulation (Sabzevari *et al.* 2001). It has been hypothesised that the interaction of CTLA-4 expressed by regulatory T cells and CD80 or CD86 expressed by stimulated T cells may deliver an inhibitory signal that can curtail T cell activation (Miyara and Sakaguchi 2007). However, T cells deficient in CD80 and CD86 remain highly susceptible to suppression by regulatory T cells, indicating that this mechanism is unlikely to explain their suppressive activity (May *et al.* 2007). Alternatively, it may be that CTLA-4 on the surface of regulatory T cells mediates suppression by inducing the expression of the enzyme indolamine 2,3-dioxygenase (IDO) in DCs, upon ligation to CD80 or CD86. IDO is known to catalyse tryptophan catabolism, producing inhibitory soluble metabolites that may kill responder T cells (Fallarino *et al.* 2006). However, since regulatory T cells deficient in CTLA-4 expression still possess potent regulatory activity, this biological pathway may have little relevance to the suppressive mechanism of regulatory T cells (Tang *et al.* 2004).

It has been proposed that LAG-3, a molecule expressed by regulatory T cells may interact with the peptide-loaded MHC molecules being presented to other T cells, and weaken or modify the stimulation delivered (Huang *et al.* 2004). While this may seem like a plausible molecular mechanism, when one considers that the size of the interacting cells is much larger than the size of the molecules they express, it is likely regulatory T cells would be physically excluded from the interactions between the APC and other T cells, and thus this mechanism seems implausible. It has also been found that regulatory T cells may kill activated T cells and antigen presenting cells through a perforin-dependent mechanism (Grossman *et al.* 2004). However, this is unlikely to explain how they are able to inhibit T cell proliferation, since the majority of cells survive the following suppression mediated by regulatory T cells (Miyara and Sakaguchi 2007).

Although the mechanism of regulatory T cell suppression remains poorly defined, several studies have implicated costimulatory signals in ablating regulatory activity, suggesting that regulatory T cells may act in an identical manner to anergised T cells, by inhibiting the capacity of APC to stimulate responder T cells (Takahashi *et al.* 1998, McHugh *et al.* 2002, Ji *et al.* 2004, Shimizu *et al.* 2005). When strong costimulation is delivered to regulatory T cells through CD28, the anergic and suppressive state of regulatory T cells is disrupted (Takahashi *et al.*

1998). In addition, the delivery of costimulation through GITR promotes the proliferation of regulatory T cells (McHugh *et al.* 2002), and abrogates regulatory T cell suppression (Ji *et al.* 2004). This is not due to the delivery of a survival signal to effector cells, as pre-treatment of regulatory T cells with a GITR stimulating antibody prior to their adoptive transfer into models of GVHD is as effective in abrogating regulation as the *in vivo* administration of the antibody (Shimizu *et al.* 2002). In addition, anti-CD45 antibodies that costimulate T cell proliferation, potentially by neutralising the inhibitory effects of CD45 on T cell signalling, have been shown to reverse the anergic state of regulatory T cells and abrogate their suppressive activity (Shimizu *et al.* 2005).

1.14 Project hypotheses

Regulatory T cells perform an essential role in preventing the development of autoimmunity, but their origins, lifespan and mechanism of action remain unclear. I hypothesise that regulatory T cells may represent T cells that have been rendered anergic in the thymus or as RTE, and that characterising their cell surface molecules will determine whether they possess a phenotype characteristic of anergised T cells. I propose that regulatory T cells, or their immediate precursors, may be associated with RTE as they appear to have a short lifespan and would require continual replacement. Therefore, the project aims to measure the thymic output of regulatory T cells, explore their relationship with RTE in the periphery, and measure their lifespan. I further hypothesise that regulatory T cells may inhibit T cell proliferation in an identical manner to that of anergised T cells, by conditioning APC and reducing the ability of these cells to stimulate the proliferation of other T cells. An *in vitro* assay designed specifically to test this hypothesis will be used to determine whether this is the case.

1.15 Project aims

- To characterise the phenotype of regulatory T cells in the rat, with particular emphasis on the relationship between RTE and regulatory T cells, and the presence of markers of anergised T cells on regulatory T cells

- To directly investigate the relationship between RTE and regulatory T cells through adult thymectomy and intrathymic labelling techniques

- To measure the proliferation rate and lifespan of both $CD25^{+}$ $Foxp3^{+}$ and $CD25^{-}$ $Foxp3^{+}$ subsets of regulatory T cells *in vivo*

- To determine if regulatory T cells suppress T cell responses by conditioning APC to reduce their ability to stimulate other T cells

- To investigate changes in the thymic output, numbers and phenotype of regulatory T cells that occur with ageing

Chapter 2: Materials and Methods

2.1 Animals

2.1.1 Animal strains

Female inbred specific pathogen-free DA CD45.1 (wild-type) and DA CD45.2 congenic (Spargo *et al.* 2006) rats were obtained from the Central Animal House of the University of Adelaide. The rats used in each experiment were all from the same weaning. During the experimental period they were provided with standard rat chow and water *ad libitum* and housed in conventional conditions at the Laboratory Animal Services Facility, Adelaide University. All studies were approved by the Animal Ethics Committee of the University of Adelaide.

2.1.2 Bromodeoxyuridine (BrdU) administration

For studies of BrdU incorporation, animals were provided with fresh sterile drinking water containing 0.8 mg/ml BrdU (Sigma, USA) plus glucose (1%) to overcome taste aversion daily for 7 days (Jamieson *et al.* 2004). The intake of the 0.8 mg/ml BrdU 1% glucose solution by the animals was measured daily, and was similar to that of normal drinking water, thus verifying that no taste aversion was occurring. For experiments examining loss of BrdU-labelled cells, rats were provided with BrdU-containing drinking water for 7 days as above and then transferred to normal drinking water. For experiments examining the proliferation of a cohort of T cells, animals received an intraperitoneal (i.p.) injection of 1mg/ml BrdU in 1 ml of PBS every hour for three hours.

2.1.3 Thymectomy

Where indicated, animals underwent thymectomy or sham operation under isoflurane anaesthesia at 5-6 weeks of age (Mayrhofer 1979). After surgery, the rats were given drinking water containing 13 μg/ml polymyxin B sulphate (Sigma, USA) for one week and then allowed to recover for at least 1 month before being used experimentally.

2.1.4 Intra-thymic injection

The technique was performed essentially as described by Hosseinzadeh and Goldscheider (1993), under isoflurane anaesthesia. Briefly, 10μl of FITC (1mg/ml in PBS) was injected intra-thymically into 2 sites per lobe, using a 1ml insulin syringe fitted with a 29 G needle. Rats were killed at various intervals after the intrathymic injections and cell suspensions were prepared from the thymus and from the pooled cervical, mesenteric, celiac, inguinal and popliteal lymph nodes, as described below.

2.1.5 Thoracic duct cannulation

Rats were anaesthetised using isoflurane and shaved over the abdomen and to the left mid-axillary line. The tail vein was catheterised with a 23G needle and 3ml of PBS containing heparin (1 unit/ml) was injected over the period of the operation. The shaved area was cleaned with 70% ethanol an in incision made into the abdomen, parallel to the left costal margin. Using cotton buds, the left kidney was mobilised towards the midline and the retroperitoneal fat was cleared by blunt dissection to reveal the aorta and thoracic duct. The abdominal contents were displaced medially using a retractor, to allow a clear view of the field. The thoracic duct was separated from the aorta and a suture was passed between the two for later securing of the cannula into the duct. A 14 G needle was passed through the posterior abdominal wall lateral to the psoas muscle and at the level at which the cannula was to be inserted. The cannula was passed through the needle to the exterior and the needle was withdrawn over the cannula. The cannula was then filled with heparin solution. A small incision was made in the thoracic duct using iridectomy scissors, and the tip of the cannula was introduced and secured by tying the pre-placed suture. The cannula was anchored to the posterior wall of the abdomen at its exit using a drop of cyanacrylate (‘superglue’), the abdominal contents were returned to their usual positions and the abdominal cavity was closed with a silk suture. The area of incision was painted with lignocaine local anaesthetic and the skin then closed with a further silk suture. The rat was placed in a Bollman metabolic cage and provided with standard rat food pellets and

isotonic saline for drinking. Lymph was collected overnight into a flask containing 5ml of 25U/ml heparin in PBS.

2.1.6 Adoptive transfer

The adoptive transfer of donor (DA CD45.2) T cells into host (DA CD45.1) rats was performed under isoflurane anaesthesia by tail-vein injection. The cells were resuspended in 2 ml of PBS containing 1% heat-inactivated normal rat serum.

2.1.7 Induction of adjuvant induced arthritis

Adjuvant induced arthritis was induced through the injection of 100μl of Complete Freund's Adjuvant (CFA) into the tail base (Spargo *et al.* 1996). Essentially all DA rats develop polyarthritis using this protocol, with inflammation developing 9-10 days after inoculation.

2.1.8 Assessment of the severity of arthritis

Severity of polyarthritis was measured by allocating a score for each paw as follows: 0 (no evidence of arthritis), 1 (single focus of redness or swelling), 2 (two or more foci of redness or swelling), 3 (confluent but not global swelling) or 4 (severe global swelling). The "joint score" for each rat is the sum of the scores obtained from the four paws (maximum score = 16).

2.2 Preparation of cell suspensions from rat tissues

2.2.1 Preparation of $CD4^+$ T cells from lymph nodes and spleens

Lymph node cells were expressed from fragments of finely chopped cervical, mesenteric and/or celiac lymph nodes by use of a loosely fitting glass homogeniser. Purified $CD4^+$ T cells were obtained by depletion of B cells and $CD8^+$ T cells with immuno-magnetic beads. Briefly, cells were incubated with a mixture of neat hybridoma supernatants containing mAbs 341 ($CD8\beta^+$ T cells),

MARK-1 and OX33 (B cells) plus 0.1% sodium azide for 1 h on a rotator at 4°C. Cells were washed twice with PBS containing 1% FCS and 0.1% sodium azide, then incubated on a rotator at 4°C for 45 minutes with Pan Mouse IgG Dynabeads (Dynal, AS, Oslo, Norway) at a concentration of 1 bead per cell. After removal of cells bound to the beads using a Dynal MP6 magnet, the cells remaining were incubated with further Pan Mouse Dynabeads at a concentration of 1 bead per cell and the bound cells were removed. Analysis of the negatively selected cells showed that <1% of purified cells could be stained with the depleting antibodies and 97-99% of the purified cells could be stained with the PE-conjugated mAb OX38 (anti-CD4).

2.2.2 Preparation of Allogeneic responder T cells

Allogeneic $CD4^+$ $CD25^-$ T cells for use in MLRs were isolated from PVG/c lymph nodes as described above, using mAb OX39 (anti-CD25) in addition to mAbs 341, MARK-1 and OX33 supernatants to allow removal $CD4^+$ $CD25^+$ cells.

2.2.3 Isolation of $CD4^+$ $CD25^{bright}$ regulatory T cells

Lymph nodes from 2 rats were excised and chopped finely with curved scissors, then homogenised using a glass tissue homogeniser. The cell suspension, typically containing 2 x 10^8 cells, was washed twice in IF buffer before staining the $CD4^+$ T cells with 5ml of mAb W3/25 (anti-CD4) culture supernatant containing 10% NRS. After 45 minutes with constant agitation at 4ºC, cells were washed twice in 10ml IF buffer, then stained with a 1/100 dilution of allophycocyanin-conjugated goat anti-mouse IgG in 5ml containing 10% NRS, for 45 minutes at 4ºC. The cell suspension was then washed twice and incubated with 100μl of NMS for 20 minutes on ice to block the goat anti-mouse idiotopes of the secondary antibody. Without removal of the NMS, the $CD25^+$ cells were stained with a 1/100 dilution of OX39-FITC in 5ml containing 10% NRS, for 45 minutes at 4ºC. $CD4^+$ After washing, $CD25^{bright}$ cells were isolated by high speed flow sorting using a FACSAria cell sorter (BD Biosciences).

2.2.4 Preparation of $CD4^+CD8^-$ thymocytes

Thymocytes were prepared as described for lymph node cells. $CD4^+CD8^-$ (single-positive) and $CD4^-CD8^-$ (double-negative) thymocytes were isolated by negative selection, using mAbs 341 ($CD8\beta^+$ expressing cells), MARK-1 and OX33 (B cells), as described for preparation of $CD4^+$ lymph node cells. $CD4^+CD8^-$ single-positive cells were distinguished from $CD4^-CD8^-$ double negatives by staining with mAb OX38 (anti-CD4), as described below.

2.2.5 Preparation of $CD4^+$ T cells from blood

Blood mononuclear cells were isolated by Lymphoprep (Nycomed, Norway) density gradient centrifugation. Rats were anaesthetised using isoflurane and blood was obtained by cardiac puncture using a 23G needle fitted to a 5ml heparinised syringe.. The blood was diluted in an equal volume of PBS, overlayed onto 3ml of room temperature Lymphoprep and centrifuged at 850 x g for 20 minutes at room temperature (no brake). Following centrifugation, the mononuclear cell layer was removed to a fresh tube and washed twice in PBS plus 2% FCS to remove platelets. $CD4^+$ T cells and monocytes were isolated by depletion, using mAbs 341 ($CD8\beta^+$ expressing cells), MARK-1 and OX33 (B cells), as described for preparation of $CD4^+$ lymph node cells. $CD4^+$ T cells were distinguished from blood monocytes by staining with mAb R73 (anti-$\alpha\beta$TCR), as described below.

2.2.6 Thoracic duct lymphocytes obtained by thoracic duct cannulation

Thoracic duct (TD) lymphocytes, obtained from filtered lymph by centrifugation, were washed twice and $CD4^+$ T cells isolated by negative selection as described for lymph node cells.

2.2.7 Optiprep density gradient separation of small and large lymphocytes

Negatively selected $CD4^+$ T cells (5×10^7 cells) were washed twice at room temperature in PBS, then resuspended in a 10ml volume of PBS and mixed with 4ml of Optiprep, all at room temperature. A 1.084g/ml solution of Optiprep was prepared by diluting 2ml of Optiprep to 10ml using 0.85% saline (v/v) containing 0.5% BSA (w/v) and 10mM HEPES 1mM ethylenediaminetetraacetic acid (EDTA), while an 1.068 g/ml solution was prepared by dilution of 5ml of Optiprep to 20ml . The 14 ml cell suspensions were then overlayed with 7.5ml of 1.084 g/ml diluted Optiprep, followed by 20ml of 1.068 g/ml diluted Optiprep, and finally 0.5ml of HEPES-buffered saline. The gradients were then centrifuged at 700g for 25 minutes at room temperature (no brake) to separate small, high-density lymphocytes and large, low-density lymphocytes.

2.2.8 Preparation of splenic antigen presenting cells

Splenic antigen presenting cells were negatively selected from suspensions of spleen cells that were prepared as described above. The hybridoma supernatants MARK-1 and OX33 were used in the depletion of B cells, and the supernatants R73 and OX39 were used in the depletion of T cells and all $CD25^+$ cells (to ensure that there was no contamination with $CD4^+$ $CD25^+$ regulatory T cells). Following the addition of Pan Mouse Dynabeads as described previously, negatively selected non-B cell non-T cell antigen presenting cells remained, which would be expected to contain some contaminating NK cells.

2.2.9 Preparation of dendritic cells from lymph nodes

Pooled cervical, coeliac, mesenteric, inguinal and popliteal lymph nodes were excised from DA CD45.2 rats, chopped finely with curved scissors and then suspended in 10ml of room temperature RPMI + 10% FCS. The suspension of lymph node fragments was digested with 78 U of collagenase type I (Worthington Biochemical Corporation, USA) and 34 U of DNAse I (Worthington Biochemical Corporation, USA) for 25 minutes at room temperature with constant agitation. In

order to dissociate T cells from dendritic cells, EDTA was added (10mM final concentration) to the suspension and the cells were incubated with agitation for a further 5 minutes. The solution was then pipetted vigorously with a shortened Pasteur pipette and placed aside for 20 minutes to allow undigested matter to settle. The supernatant, then the undigested debris were filtered sequentially through a course stainless steel sieve of 500 μm mesh size, a fine stainless steel sieve of 250 μm mesh size, and a nylon mesh filter of 70 μm mesh size (BD Falcon, USA). The resulting cell suspension was washed in PBS containing 10mM EDTA and 5% FCS and subjected to Percoll density gradient separation to isolate a fraction enriched in dendritic cells Mayrhofer *et al.*, 1986). The gradient consisted of a series of Percoll solutions (35%, 40%, 45%, 50%, 55%), made from an isotonic stock solution of 90% Percoll containing 10% 10x PBS. This stock solution was diluted appropriately with PBS containing 10mM EDTA and 5% FCS. Each Percoll step gradient was created by layering 2.5ml volumes of these dilutions into a sterile 15ml tube (BD Falcon, USA) and overlayed with washed cells (approx. 1 x 10^8 per tube) resuspended in 2.5ml of 20% Percoll. After centrifugation at 400g for 30 minutes at room temperature using an fixed angle rotor, the interface between the 40 and 45% Percoll layers was harvested and the cells were washed with PBS containing 10mM EDTA and 5% FCS. Approximately 30-40% of the cells in the resulting preparation appeared to be lymphocytes by microscopic examination, with no obvious macrophages, which was confirmed by staining for the scavenger receptor CD163. Lymphocytes were depleted using immunomagnetic beads as described in 2.28.

2.2.10 Preparation of lamina propria cells from the small intestine

Lymphoid cells were prepared from the lamina propria of the small intestine by a modification of methods used to prepare mucosal mast cells (Befus *et al.* 1982, Lee *et al*. 1985). Divalent ion-containing and divalent ion-deficient Hanks buffered balanced salt solutions (HBBS), containing 25mM HEPES, were prepared as described. Unless mentioned, all procedures were carried out at room temperature. Each small intestine was removed intact from the exsanguinated rat, trimmed of mesenteric fat and rinsed by perfusion with isotonic saline. Peyer's patches were excised and discarded and the remaining gut was divided into 10 mm segments. These were opened longitudinally and washed for 5 min in 20 ml of

Ca-and Mg-containing HBBS supplemented with 5% FCS, using a magnetic stirrer. To remove epithelium, the solution was replaced with 50 ml of Ca- and Mg-free HBBS containing $1.3x10^{-4}$ M EDTA and stirred for 10 min. After two further washes in divalent ion-free medium, the segments were transferred to 25 ml of Ca- and Mg-containing HBBS containing 20% FCS and 25 units/ml of collagenase. The segments were then digested by stirring continuously at 37^{o}C for one hour and the residual tissue was removed by filtration through gauze. This first filtrate was saved and the tissue was transferred to Ca- and Mg-containing HBBS supplemented with 5% FCS and passed repeatedly through a wide-bored Pasteur pipette. Large fragments were removed by gauze filtration and the second filtrate was passed repeatedly through a narrow-bored Pasteur pipette to break up cell clumps.

The two filtrates were then combined and mucus and colloidal material was removed using the method of Mayrhofer and Whately (1983). In brief, the volume of the pooled filtrate was filled with teased wettable cotton wool, which was then removed and contained fluid was returned to the tube by spooling tightly using forceps. This extraction process was performed twice and the resulting cell suspension was centrifuged at 1200 rpm for 8 minutes at room temperature. The pellet was resuspended in 10 ml of 30% Percoll and filtered through a small loosely packed cotton wool column. After further centrifugation, the pellet was resuspended in 1 ml of 30% Percoll, to which was added 480µl of FCS and 3.6mg of solid dithiothreitol. The volume was made up to 18ml and aliquots were layered over six Percoll step gradients (2ml of 80%, 6ml of 35%, both containing 2% FCS). The gradients were centrifuged at 2,300rpm for 45 minutes at room temperature in an angle head rotor. Cells overlying the 80% Percoll were harvested, washed with divalent ion-containing HBBS (5% FCS) and resuspended in FACS buffer in preparation for labelling for flow cytometry.

2.2.11 Preparation of synovium rich tissue cell suspensions

Cells lining the synovium of the knee joint were obtained by lavage, using a solution of RPMI-1640 containing 10% (v/v) heat inactivated foetal bovine serum, 1% (v/v) glutamine, 67 µg/ml penicillin and 100 µg/ml streptomycin and 250 U/ml

collagenase I (Sigma). Lavage was accomplished by introducing a 26-gauge needle, attached to a 1ml syringe containing ~0.4ml of collagenase solution, into the joint space from the lateral border of the patella. The joint space was lavaged for ~2min by repeated injection and withdrawal of 50-100 μl of medium. The aggregate yield of cells from lavage of the knees of three rats was 2.5 x 10^5 cells.

2.3 Tissue culture procedures

2.3.1 Preparation of Tissue culture media

Media for the production of hybridoma supernatants consisted of Roswell Park Memorial Institute (RPMI) 1640 containing 10% (v/v) heat inactivated foetal bovine serum, 1% (v/v) glutamine, 67 μg/ml penicillin and 100 μg/ml streptomycin. Media for use in mixed lymphocyte reactions consisted of RPMI 1640 containing 25mM HEPES, 1mM sodium pyruvate, 10 μM β-mercaptoethanol, 10% (v/v) heat inactivated foetal bovine serum, 1% (v/v) glutamine, 67 μg/ml penicillin and 100 μg/ml streptomycin. The 2X tissue culture freezing mix used for the cryogenic storage of cell lines consisted of 30% (v/v) heat inactivated foetal bovine serum and 20% (v/v) dimethylsulfoxide (DMSO) in RPMI 1640 medium.

2.3.2 Thawing of hybridoma cell lines

Hybridoma cells lines were grown from frozen cryovials stored at -78ºC under liquid nitrogen. Vials (1 ml) were thawed using a 37ºC water bath and 1 ml of tissue culture medium was added over 5 minutes to dilute the DMSO. Cells were left to stand for 15 minutes in a 37ºC in a 5% CO_2 incubator and DMSO was then further diluted by the addition of 2ml of tissue culture medium over 5 minutes. Cells were allowed to stand for 15 minutes, were centrifuged at 1000rpm and then washed twice to remove residual DMSO.

2.3.3 Culture of hybridomas and production of supernatant

Cell lines were maintained in 5 ml volumes in T25 tissue culture flasks (BD Falcon , USA) initially, scaled up to 20 ml volumes in T75 flasks (BD Falcon , USA), and then 100 ml volumes in T175 flasks to enable the production of large volumes of supernatant. Hybridoma cultures were allowed to grow to exhaustion once sufficient culture volume was achieved, and the supernatant derived by centrifugation at 1200 rpm was passed through a 0.22 μm Millipore filter. The sterile supernatant was stored in 0.1% Sodium azide in 50 ml aliquots at 4ºC in the dark until use, and the antibody activity was tested by comparisons with previous batches before use.

2.3.4 Freezing of hybridoma cell lines

In order to maintain frozen stocks of hybridoma cell lines, cells were frozen down during the early stages of culture to replenish stocks in storage. Hybridomas were centrifuged at 1000 rpm and resuspended at a concentration of 1 x 10^7 cells/ml in their supernatant. Cells were then diluted 1:1 in tissue culture freezing solution and placed in a room temperature Nalgene 1ºC freezing chamber filled with fresh isopropanol. The freezing chamber was then placed at -80ºC and allowed to gradually cool to -80ºC for 24 h before the tubes were placed into liquid nitrogen storage.

2.4 Mixed lymphocyte reactions

2.4.1 Functional analyses of *in vivo* matured $CD25^-$ $Foxp3^+$ T cells, using a one-way allogeneic mixed lymphocyte reaction

$CD4^+$ $CD25^-$ allogeneic responder T cells (1 x 10^4) were isolated from pooled cervical, coeliac, mesenteric, inguinal and popliteal lymph nodes from a PVG/c donor by immuno-magnetic bead depletion of B cells and $CD4^+$ $CD25^+$ and $CD8^+$ T lymphocytes. Splenic APC (1 x 10^3) from DA CD45.2 rat spleens, isolated by

depletion of B and T lymphocytes as described above, were treated with 20 μg/ml mitomycin C (Sigma, USA) for 30 minutes at 37°C. FACS-sorted $CD4^+$ $CD25^{bright}$ T cells (1 x 10^4) from the pooled cervical, coeliac, mesenteric, inguinal and popliteal lymph nodes of DA CD45.2 rats were tested in parallel with $CD4^+$ $CD25^-$ $Foxp3^+$ T cells. The latter were obtained by "parking" FACS-sorted $CD4^+$ $CD25^{bright}$ T cells from RT7b donors in DA hosts for three weeks to allow maturation of approx. 50% of the cells into $CD4^+$ $CD25^-$ $Foxp3^+$ T cells (Mabarrack *et al,* 2008). The $CD4^+$ $CD25^-$ ($Foxp3^+$) subset of donor-derived cells was re-isolated by FACS on the basis of their expression of the CD45.2 donor marker. The purified Treg subsets were co-cultured for 4 days in 96 well round bottomed plates, then pulsed with 1 μCi per well of [^{3}H] TdR and harvested 18h later.

2.4.2 Two-stage mixed lymphocyte reaction for the analysis of the mechanism of regulatory T cell function

Dendritic cells were isolated from pooled cervical, coeliac, mesenteric, inguinal and popliteal lymph nodes of DA CD45.2 rats by enzymic digestion and Percoll density gradient separation, as previously described (2.2.9). The cells were used at a density of 1 x 10^5 cells per well. Syngeneic regulatory T cells were isolated from lymph node cells by FACS sorting $CD4^+$ $CD25^{bright}$ cells as previously described (2.2.3). In order to determine whether regulatory T cells act by "conditioning" DC, control cultures (in which regulatory T cells are added to DC and remained in culture when allogeneic responder T cells were added in the second step) were compared to cultures in which regulatory T cells are added to DC and then removed prior to the addition of responder T cells in step two ("conditioned" DC). In step one, DCs to be used in control cultures were treated with 20 μg/ml mitomycin C (Sigma, USA) for 30 minutes at 37°C prior to use. The washed cells were then plated at 5 x 10^4/well, either with or without 2 x 10^5 regulatory T cells, and incubated for 24 h. DC to be "conditioned" in step one were not treated with mitomycin C prior to plating at 5 x 10^4/well, either with or without 2 x 10^5 regulatory T cells, followed by incubation for 24 h. DCs were re-purified from the "conditioning" cultures by resuspending them in ice-cold PBS containing 5% FCS plus 0.01M EDTA to dissociate them from the regulatory T cells. The released T cells were then removed by labelling the cell suspension on ice with 0.5

ml of mAb R73 hybridoma supernatant containing 10% NRS, washing twice with ice-cold PBS containing 5% FCS and 0.01 M EDTA, and depleting the T cells with 50 μl of Pan Mouse IgG Dynabeads. These purified "conditioned" DCs (conditioned with or without regulatory T cells) were treated with 20 μg/ml mitomycin C (Sigma, USA) for 30 minutes at 37°C and returned to culture wells at 5 x 10^4 per well. In control wells, the DC and regulatory T cells were allowed to remain together in culture. In step two, allogeneic $CD4^+$ $CD25^-$ responder T cells were then added to each well (1 x 10^5) and the cells were co-cultured for 4 days before the addition of tritiated thymidine (1 μCi per well). Cultures were then harvested 18h later to assess T cell proliferation.

2.5 Antibodies

2.5.1 Primary antibodies

The mouse anti-rat monoclonal antibodies (mAbs) used in this study for flow cytometric and immunohistochemical analyses are described in Table 2.1. These monoclonal antibodies were either produced by hybridomas held by the Discipline of Microbiology and Immunology, obtained as gifts, or purchased from commercial sources as indicated. With the exception of the mAb MARK-1 (anti-rat κ chains), all antibodies were used in the presence of 10% heat-inactivated normal rat serum (NRS) to block non-specific binding through Fc receptors and to absorb any residual cross reactivity by secondary antibodies for rat Ig.

2.5.2 Conjugated antibodies

Allophycocyanin conjugated goat anti-mouse immunoglobulin was purchased from BD Pharmingen (San Diego, CA, USA) and used at a concentration of 2 μg/ml. Fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (mAb OX39), Phycoerthyrin (PE)-conjugated anti-CD4 (mAb OX38), PE conjugated anti-αβTCR (mAb R73), FITC-conjugated anti-BRDU (clone 3D4), and the isotype controls IgG1-FITC and IgG1-PE purchased from BD Pharmingen

(San Diego, CA, USA) were used at concentrations of 2 μg/ml. Allophycocyanin-conjugated anti-BRDU was obtained from eBiosciences (San Diego, CA, USA) and used at a concentration of 2 μg/ml. PE-anti-rat Foxp3 (FJK-16s) was obtained from eBiosciences (San Diego, CA, USA) and used at 5 μg/ml. Biotinylated anti-CD25 was obtained from Serotec (Oxford, UK) and used at a concentration of 2 μg/ml. Biotinylated anti-Thy1, biotinylated anti-a/b TCR, a biotinylated IgG isotype control, and Strepavidin-conjugated PE-Cy7 were obtained from BD Pharmingen (San Diego, CA, USA) and used at a concentration of 2 μg/ml.

2.6 Preparation of normal rat serum for use in flow cytometry

Normal rat serum was prepared from healthy DA CD45.1 or CD45.2 rats that had not been subjected to any procedure and were used to obtain normal tissues for phenotypic or functional analyses of T cells. Animals were anaesthetised using isoflurane and cardiac puncture, using a 23G needle fitted onto a 5 ml syringe, was performed through the rib cage. This yielded 4-6 ml blood, which was allowed to clot for 2 h at room temperature. The serum was collected and centrifuged at 8 000 g for 5 minutes in an eppendorf 5417R centrifuge to pellet residual red blood cells. It was then collected and incubated in a 56ºC water bath for 30 minutes to inactivate complement, before storage at -20ºC until use.

Table 2.1Mouse anti rat monoclonal antibodies used for flow cytometry and immunohistochemistry

The clone designation, specificity, staining patterns on leukocytes, sources and concentrations used are displayed.

Abbreviations:

S/N: supernatant

Mϕ:monocytes and macrophages

APC: antigen presenting cells

RTE: Recent thymic emigrants

DC:Dendritic cells

NK cell: natural killer cell

Ascites containing mAb V65 was kindly provided by Dr T. Hunig, Wurzburg, Germany.

Culture supernatant containing mAb 1D4.5 was kindly provided by Dr. L.K. Askman, Adelaide Australia

Table 2.1: Mouse anti-rat monoclonal antibodies used for flow cytometry and immunohistochemistry

Ab clone	Specificity	Distribution on leukocytes	Form	Concentration	Isotype	Source/ reference
R73	α/β TCR	α/β T cells	S/N	neat	IgG_1	hybridoma/ Hunig *et al.* 1989
V65	γ/δ TCR	γ/δ T cells	S/N	neat	IgG_1	supplied by Dr T. Hunig/ Kuhnlein *et al.* 1994
OX34	CD2 (LFA-2)	peripheral T cells, thymocytes	S/N	neat	IgG_{2a}	hybridoma/ Jefferies *et al.* 1985
W3/25	CD4	T cell subset, thymocytes, mϕ, DC	S/N	neat	IgG_1	hybridoma/ Williams *et al.* 1977
OX19	CD5	peripheral T cells, thymocytes	S/N	neat	IgG_1	hybridoma/ Dallman *et al.* 1984
OX8	CD8α-chain	T cell subset, thymocytes, NK cells	S/N	1/2	IgG_1	hybridoma/ Brideau *et al.* 1980
341	CD8β-chain	T cell subset,thymocytes	S/N	neat	IgG_{2a}	hybridoma/ Torres-Nagel *et al.* 1992
8A2	CD11c	Dendritic cells and γ/δ T cells	S/N	neat	IgG_1	hybridoma/ Kovach *et al.* 1992
OX39	CD25 (IL-2R α chain)	activated T cells,activated B cells	S/N	neat	IgG_1	hybridoma/ Paterson *et al.* 1987
W3/13	CD43 (leukosialin	T cells, plasma cells, NK cells, granulocytes	S/N	neat	IgG_1	hybridoma/ Kroese *et al.* 1985
OX50	CD44 (hyaluronate R)	most leukocytes except some B cells	S/N	neat	IgG_{2a}	hybridoma/ Paterson et al. 1987
OX1	CD45 (all isoforms)	All leukocytes	S/N	neat	IgG_1	hybridoma/ Woolett et al. 1985
His41	CD45.2	Leukocytes from the $RT7^b$ strain	purified	2 μg/ml	IgG_1	eBiosciences/Butcher *et al.* 1987
OX33	CD45RA	B cells	S/N	neat	IgG_1	hybridoma/ Woolett *et al.* 1985
OX22	CD45RC	$CD8^+$ T cells, NK cells, naïve $CD4^+$ T cells	S/N	neat	IgG_1	hybridoma/ Woolett et al. 1985
1A29	CD54 (ICAM-1)	APC, subset of T cells	S/N	neat	IgG_1	hybridoma/ Tamatani and Miyasaka 1990
OX85	CD62L (L-selectin)	B cells, subset of T cells, neutrophils	S/N	neat	IgG_1	hybridoma/Lasky *et al.* 1995
OX26	CD71 (transferrin receptor)	dividing/proliferating cells	S/N	neat	IgG_1	hybridoma/ Jefferies et al. 1985
OX7	CD90 (Thy-1)	thymocytes, RTE, immature B cells	S/N	neat	IgG_1	hybridoma/ Mason and Williams 1990
OX40	CD134	antigen-experienced T cells	S/N	neat	IgG_{2a}	hybridoma/ Paterson *et al.* 1987
WCH203	CD152 (CTLA-4)	some T cells	S/N	neat	IgG_1	Provided by the WCH, Adelaide
OX62	α_{E2}-integrin	DC, intraepithelial T cells	S/N	neat	IgG_1	hybridoma/ Brenan *et al.*1982
B56	Ki67 histone antigens	detectable in cells that are in cell cycle	purified	2 μg/ml	IgG_1	Serotec/ Gerdes *et al*.1983
MARK-1	rat Ig κ-chain	B cells	S/N	neat	IgG_1	hybridoma/ Bazin *et al.* 1984
OX6	MHC class II	B cells, activated T cells, mϕ, DC	S/N	neat	IgG_1	hybridoma/ McMaster and Williams 1979
1B5	Giardia surface antigen	anti-*Giardia intestinalis* (negative control)	S/N	neat	IgG_1	hybridoma/Dr G. Mayrhofer (unpublished)
1D4.5	Salmonella surface antigen	anti-*Salmonella typhimurium* (negative control)	S/N	neat	IgG_{2a}	Provided by Dr. L. K. Ashman

2.7 Labelling of cells for flow cytometric analyses

2.7.1 Flow cytometry buffers

FACS wash consisted of phosphate buffered saline (PBS) containing 2% (v/v) heat inactivated foetal bovine serum (FBS) and 0.1% (w/v) sodium azide. FACS fix consisted of PBS containing 0.1% (w/v) formalin, 4% (w/v) glucose, and 0.1% (w/v) sodium azide.

2.7.2 Indirect labelling of cells with monoclonal antibodies (mAbs)

All labelling steps were performed at 4ºC. Cells were dispensed in aliquots of 1 x 10^6 cells per FACS tube (Falcon, Becton Dickinson Labware, USA) and centrifuged for 10 minutes at 200g in a refrigerated Eppendorf 5403 centrifuge (Eppendorf, USA). Pellets were resuspended in 50μl aliquots of either culture supernatant, or purified mAb, each containing 0.01% (w/v) sodium azide and 10% (v/v) heat inactivated NRS. The cells were then incubated on ice for 45 minutes with regular agitation. Following this incubation, the cells were washed twice in 3 ml FACS wash and then resuspended in 50 μl of allophycocyanin-conjugated goat anti-mouse secondary antibody (1/100 dilution) (BD Biosciences, USA). After incubation for 45 minutes on ice in the dark with gentle resuspension, cells were washed twice in 3 mls FACS wash. Cells not to be analysed for Foxp3 expression were fixed with 1 ml FACS fix, and incubated for 30 minutes on ice in the dark before being washed in 3 ml FACS wash and resuspended in 500μl FACS wash. Cells to be analysed for Foxp3 expression were fixed using the Foxp3 staining set fixation/permeablisation reagent according to the manufacturer's instructions (eBiosciences). Cells were stored at 4ºC in the dark prior to flow cytometric analysis.

2.7.3 Direct labelling of cells with fluorochrome-conjugated mAbs

Labelling was performed at 4ºC. Cell suspensions were prepared and cells aliquoted at 1 x 10^6 per FACS tube. After centrifugation, cells were resuspended in

50μl of conjugated antibody diluted to a concentration of 2 μg/ml (1:100) and incubated for 45 minutes on ice in the dark with regular agitation. Cells were then washed twice in 3 ml FACS wash. Cells not to be analysed for Foxp3 expression were fixed with 1 ml FACS fix, and incubated for 30 minutes on ice in the dark before being washed in 3 ml FACS wash and resuspended in 500μl FACS wash. Cells were stored at 4ºC in the dark prior to flow cytometric analysis. Cells to be also analysed for Foxp3 expression were fixed with 1ml Foxp3 staining set Fix/Perm solution (eBiosciences) and then stained for Foxp3 as described in 2.75.

2.7.4 Multi-fluorochrome labelling of cells

Cells were initially stained according to the indirect labelling technique outlined above, then resuspended in 20μl NMS and incubated for 20 minutes in the dark on ice to block free anti-mouse IgG valances. Without washing, cells were then labelled either with directly conjugated mAbs or with biotin-conjugated mAbs (each diluted 1/100 in FACS wash containing 10% NRS) for 45 minutes in the dark on ice. For direct conjugates, cells were then washed twice following staining before resuspension in 1 ml Foxp3 staining set fixation/permeablisation reagent (eBiosciences) per tube. For cells that were stained with biotin-conjugated mAbs, cells were washed twice and then resuspended in 20 μl NMS for 20 minutes in the dark on ice. Without washing, cells were then resuspended in 50 μl of a 1/100 dilution of streptavidin-conjugated PECy7 in FACS wash containing 10% NRS and incubated for 45 minutes in the dark on ice. Cells not to be analysed for Foxp3 expression were fixed as described above, while cells that were analysed for Foxp3 expression were fixed using the Foxp3 staining set fixation/permeablisation reagent (eBiosciences).

2.7.5 Detection of the intracellular transcription factor Foxp3

The detection of the intracellular transcription factor Foxp3 was performed using a Foxp3 staining set (eBiosciences) according to the manufacturer's instructions. Following the staining of cell surface molecules, cells to be analysed for Foxp3 expression were fixed in 1 ml of freshly prepared fixation/permeablisation reagent. The fixation/permeablisation reagent was prepared by the dilution of 1 part

fixation/permeablisation concentrate in 3 parts fixation/permeablisation diluent. Cells were then held in the fixation/permeablisation reagent overnight in the dark at 4ºC, and then washed in 3 ml FACS wash. Cells were then washed twice in 2 ml permeablisation reagent, which was prepared by dilution of 10X permeablisation reagent in ice cold Milli Q water. They were then resuspended in a total volume of 100 μl permeablisation reagent containing 20% NMS to block free anti-mouse IgG sites and incubated in the dark for 20 minutes. Foxp3 was stained by the addition of 2.5 μl of 0.125 μg/ml PE-conjugated anti-Foxp3 antibody. Cells were then incubated in the dark on ice for 30 minutes, washed twice in permeablisation reagent followed by a wash with 3 ml FACS wash before resuspension in 500 μl FACS wash for storage in the dark at 4ºC prior to flow cytometric analysis.

2.7.6 Protocol for the detection of the Ki67 proliferation-associated antigen

All labelling was performed at 4ºC. Cells were resuspended at a concentration of 1 x 10^6 per ml in a solution of 3% paraformaldehyde in PBS and kept in suspension on a tube spinner for 20 minutes at 4ºC in the dark. They were then washed twice in 3 ml FACS wash and resuspended in a 1/100 dilution of purified anti-Ki67 in 100μl of PBS containing 1% saponin and 5% Bovine serum albumin (saponin buffer) plus 10% NRS. After incubation for 45 minutes on ice with regular resuspension, the cells were washed in 3 ml of saponin buffer, followed by two washes in 3 ml aliquots of FACS wash. Cells were then resuspended in a 1/100 dilution of allophycocyanin-conjugated anti-mouse IgG mAb in 100 μl of saponin buffer containing 10% NRS, and incubated for 45 minutes on ice with regular resuspension. After two washes in 3 ml aliquots of FACS wash, cells that were not to be stained for cell surface antigens were fixed with FACS fix as previously described. Cells that were to be stained for cell surface markers were resuspended in 20 μl NMS and incubated for 20 minutes in the dark on ice. They were then resuspended in 50 μl of a 1/100 dilution of directly conjugated antibody and incubated on ice for 45-60 minutes in the dark. The stained cells were then washed with 3 ml FACS wash and resuspended in 500 μl FACS wash for storage in the dark at 4ºC prior to flow cytometric analysis.

2.7.7 Protocol for the simultaneous detection of the intracellular proliferation associated antigen Ki67 and intracellular Foxp3

A novel assay was developed that allowed simultaneous detection of Ki67 antigen and Foxp3. All labelling was performed at 4ºC. Cells were aliquoted at 1 x 10^6 per FACS tube, washed in 3 ml FACS wash and resuspended in 1 ml Foxp3 staining set fixation/permeablisation reagent prepared as previously mentioned. Cells were incubated overnight at 4ºC and then washed in 3 ml FACS wash. After resuspension in a 1/100 dilution of purified Ki67 in 100 μl of saponin buffer containing 10% NRS, the cells were incubated for 45 minutes on ice with regular resuspension. They were then washed in 3 ml of saponin buffer, followed by two washes in 3 ml aliquots of FACS wash. Cells were then resuspended in a 1/100 dilution of allophycocyanin -conjugated anti-mouse IgG mAb in 100 μl of saponin buffer containing 10% NRS, and incubated for 45 minutes on ice with regular resuspension. The surface labelled cells were then washed twice in 3 ml FACS wash, and then twice in 2 ml Foxp3 kit permeablisation buffer. They were resuspended in 100 μl Foxp3 kit permeablisation buffer containing 20% NMS, and incubated for 20 minutes on ice in the dark. Foxp3 was then stained by the addition of 2.5 μl of 0.125 μg/ml PE-conjugated anti-Foxp3. Cells were then washed twice in 2 ml Foxp3 kit permeablisation buffer, twice in FACS wash, and then resuspended in 20 μl NMS. After resuspendion in 50 μl of a 1/100 dilution of FITC-conjugated anti-CD25 (OX39), the cells were incubated on ice for 45 minutes in the dark. Finally, the cells were washed with 3 ml FACS wash and resuspended in 500 μl for storage in the dark at 4ºC prior to flow cytometric analysis.

2.7.8 Protocol for the detection of incorporated BrdU

All labelling was performed at 4ºC. Cells were resuspended in 500 μl of 0.15 M NaCl and 1 ml ethanol was added dropwise to each tube with gentle vortexing. Cells were allowed to incubate for 30 minutes in ice, and then washed with FACS wash. Cell pellets were resuspended in 1 ml of freshly prepared fixation/permeablisation buffer, consisting of 1% paraformaldehyde plus 0.05% Tween-20 in PBS, and incubated for 30 minutes at room temperature, followed by

30 minutes on ice. After centrifugation, incorporated BrdU in the DNA of the cells was exposed using Bovine pancreas DNAse I (Sigma, USA). Cells were treated for 30 minutes at room temperature with 100U DNAse I in a 1 ml volume of 150 mM NaCl solution containing 4 mM $MgCl_2$. Cells were then washed twice in FACS wash and free anti-mouse IgG sites were blocked with 20 μl NMS in 100 μl FACS wash for 20 minutes at room temperature. BrdU was then stained by the addition of 5μl anti-BrdU-FITC or 1 μl anti-BrdU-allophycocyanin antibody for 30 minutes at room temperature. Cells were then washed with 3 ml FACS wash and resuspended in 500 μl for storage in the dark at 4ºC prior to flow cytometric analysis.

2.7.9 Protocol for the simultaneous detection of incorporated BrdU and intracellular Foxp3

A second novel assay was developed to allow simultaneous detection of incorporated BrdU and Foxp3. All labelling was performed at 4ºC. Cells were stained for cell surface antigens as previously described. They were then fixed in 1 ml Foxp3 staining set fixation/permeablisation reagent (prepared as mentioned above) and then washed twice in 3 ml aliquots of FACS wash, followed by twice in 2 ml aliquots of permeablisation reagent. The permeabilised cells were then resuspended in a total volume of 100 μl permeablisation reagent containing 20% NMS to block free anti-mouse IgG sites and incubated in the dark for 20 minutes before staining for 30 minutes on ice with 2.5 μl of 0.125 μg/ml PE-conjugated anti-Foxp3. After washing twice in permeablisation reagent as before, the cells were washed in FACS wash and then fixed in 1 ml Foxp3 staining set fixation/ permeablisation reagent for 30 minutes in the dark on ice, resuspending regularly. The fixed cells were then washed in 3 ml FACS wash and resuspended in 1 ml of room temperature 150 mM NaCl 4 mM $MgCl_2$ buffer containing 100 U DNAse I (Sigma), followed by incubation at room temperature in the dark for 30 minutes. Cells were then washed with FACS wash and resuspended in 100 μl FACS wash containing 20% NMS for 20 minutes in the dark at room temperature. Incorporated BrdU was detected by addition of 10 μl of FITC-conjugated anti-BrdU (BD Biosciences) or 1 μl anti-BrdU-allophycocyanin antibody and incubation at room temperature for 30 minutes in the dark. Finally, the cells were washed twice with

FACS wash, resuspended in 500 μl FACS wash and stored in the dark at 4ºC prior to flow cytometric analysis.

2.8 Flow cytometry

Labelled cells were analysed using a FACSCanto flow cytometer (Becton Dickinson, USA) interfaced with the FACSDiva acquisition and analysis software package (version 4.1). Lymphocyte populations were gated on the basis of their forward (FSC) and side scatter (SSC) characteristics and at least 30,000 events were collected per sample. For three and four colour analyses and analysis of Ki67, at least 100,000 events were collected. For BrdU incorporation and intrathymic labelling with FITC, at least 1,000,000 events were collected.

2.8.1 One colour flow cytometry

An isotype control was used to calibrate fluorescence channel voltages and record background fluorescence for each sample analysed. Intact lymphocytes were gated on the basis of their forward and side scatter properties to exclude debris. Background fluorescence was defined as the part of the negative peak that contained 98-99% of recorded events falling within the lymphocyte gate.

2.8.2 Multi-fluorochrome flow cytometry

An isotype control was used to calibrate fluorescence channel voltages and record background fluorescence for each sample. The lymphocyte population was gated as described above and background fluorescence adjusted. Overlap of fluorescence signals between channels was compensated using appropriately labelled brightly stained positive controls for each channel. In each case, the dot plots of the channel with the positive control sample were plotted against all other channels, thus allowing adjustment until all signal overlaps were correctly compensated.

2.9 Immunohistochemistry

2.9.1 Preparing tissue blocks for sections

Samples of thymuses, lymph nodes, and spleens were removed from anaesthetised animals, embedded in OCT compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA), and snap-frozen in iso-pentane (Ajax chemical company, Australia) chilled over liquid nitrogen. All tissue samples were stored at -80°C until usage.

2.9.2 Preparation of solutions for immunohistochemistry and counterstaining

Indirect staining of molecules of interest by horseradish peroxidase conjugates was visualised using solution of substrate containing 17 mg/ml Urea hydrogen peroxide plus 0.7 mg/ml 3-3' diaminobenzidine (DAB) in 0.6 M Tris buffer, prepared immediately before use in 15 ml of Milli Q water using the Sigma Fast 3,3'-diaminobenzidine tablet set, according to the manufacturer's instructions (Sigma, USA).Sections were counterstained using Gill's haematoxylin, consisting of 0.2% Haematoxylin (monohydrate) (w/v), 935 μM $NaIO_4$, 26.4 mM $Al_2(SO_4)_3$.18 H_2O, 25% ethylene glycol (v/v) and 2% glacial acetic acid. They were de-stained using of 0.05% (w/v) hydrochloric acid and then differentiated in Scott's tap water (3.5 g $NaHCO_3$, 20 g $MgSO_4$ and 0.05 g sodium azide made up to one litre using Milli Q water).

2.9.3 Indirect immunoperoxidase

Tissue sections were cut using a Bright cryostat (Bright Instrument Company, Huntingdon, Cambridgeshire, England) equilibrated to -20°C. Frozen 7 μm sections were collected on clean glass slides and air dried. A circle was drawn around the section using a PAP pen (Zymed, San Francisco, CA, USA). Sections were then fixed in 95% ethanol for 10 minutes, washed 3 times in cold PBS over 6 minutes and then incubated with 50 μl of a solution of ice cold primary antibody containing 10% NRS in a humid chamber at 4°C for 1 h. After washing three times

in ice-cold PBS as previously described, the sections were incubated with 50 μl of the affinity purified $F(ab')_2$ sheep anti-mouse Ig conjugated to horseradish peroxidase (1/20) for 1 h at 4°C in a humid chamber. After three more washes in ice-cold PBS, the slides were moved to room temperature and incubated in the H_2O_2/DAB solution (described in 2.8.2) for 30 minutes. They were then counterstained with Gill's haematoxylin (see Section 2.9.5) before mounting for microscopic examination.

2.9.4 Indirect immunoperoxidase staining for Foxp3

Air-dried frozen sections (7 μm) of thymus were fixed for 5 minutes in ice cold acetone, then washed three times in an ice cold PBS containing 0.1% Tween 20 and 1% normal rat serum(2 minutes per wash). A circle was then drawn around each tissue section using a PAP pen, and the sections were then stained in a humidified box at 4°C for 1 h with 50 μl of a solution containing 25 μg/ml anti-Foxp3-biotin (FJK-16s, eBiosciences), 10% NRS and 0.1% Tween 20 in PBS. Slides were then washed 3 times in a wash buffer containing 0.1% Tween 20 and 1% normal rat serum in PBS, for 2 minutes per wash. Each slide was then stained with 50 μl of a 1:20 dilution of Strepavidin-horseradish peroxidase conjugate (Millipore Australia) in PBS containing 10% NRS and 0.1% Tween 20. After three more washes in the wash buffer , the slides were moved to room temperature and incubated in the H_2O_2/DAB solution (described in 2.8.2) for 30 minutes. The sections were counterstained with Gill's haematoxylin prior to mounting for microscopic examination.

2.9.5 Counterstaining with Haematoxylin

Slides were counterstained by immersion in Gill's haematoxylin for 1 minute and then rinsed briefly in PBS. The slides were then rinsed in dilute hydrochloric acid solution and washed in Scott's tap water for 2 minutes. Following a final rinse in Milli Q water, sections were dehydrated by passage through a series of ethanol baths: 70% ethanol for 2 minutes; absolute ethanol for 2 minutes; absolute ethanol for 2 minutes. Following dehydration, the slides were passed through as series of 3 baths of Safsolvent (Ajax chemical company, Australia) for 2 minutes each, then

mounted with Depex neutral mounting medium (Ajax Chemical company, Australia). The sections were viewed under an Olympus BX-40 light microscope and photographed using an integrated Olympus DP-70 digital camera and DP controller software (Olympus) at a resolution of 4080 x 3072 pixels.

2.10 Preparing tissue samples for histological analysis

Tissue samples of thymuses, lymph nodes, and spleens were removed from anaesthetised animals and placed overnight in 10% neutral buffered formalin. The fixed samples were dehydrated in ethanol, cleared in xylene and embedded into paraffin. Sections of 7 μm were cut and stained with haematoxylin and eosin, then mounted with Depex neutral mounting medium (Ajax Chemical company, Australia). Slides were viewed under an Olympus BX-40 light microscope and photographed using an integrated Olympus DP-70 digital camera using DP controller software (Olympus) at a resolution of 4080 x 3072 pixels.

2.11 Isolation of RNA for microarray analysis

In order to obtain RNA for microarray analyses, approximately 2 x 10^5 APC, either untreated, "conditioned" with flow sorted $CD4^+$ $CD25^{bright}$ regulatory T cells, or "conditioned" with $CD4^+$ $CD25^-$ conventional T cells, were solubilised in 800μl TRIzol. The solubilised cells were then passed through a 26-gauge needle twice, and allowed to stand for 5 minutes at room temperature. Each sample was then shaken with 160 μl chloroform for 15 seconds before being allowed to stand for 15 mins on ice to allow the partitioning of the aqueous and organic phases. The samples were then centrifuged at 6,500 g for 30 mins at 4ºC and the upper, aqueous phase removed to a nuclease-free tube. In order to minimise the loss of RNA, 10 μg glycogen was added to each tube to serve as a carrier molecule for the RNA. An equal volume of ice cold 70% ethanol was added, and the sample thoroughly mixed before it was applied to an RNeasy spin column (Qiagen). The column was loaded into a clean nuclease-free tube and spun at 6,500 g for 1 minute at 4ºC. The eluent was discarded and 700 μl of RW1 buffer (Qiagen) applied to the column, which was centrifuged at 6,500 g for 1 minute at 4ºC. The eluent was again discarded and the column washed twice more with 500 μl RPE

buffer (Qiagen). The spin column was then applied to a fresh tube and centrifuged at 6,500 g for 1 minute at 4ºC to remove residual liquid. A fresh collection tube was then substituted and the RNA eluted by applying 30 μl of nuclease-free water to the column, which was centrifuged at 6,500 g for 1 minute at 4ºC to obtain RNA in the eluent. The RNA was then taken to the Adelaide Microarray Centre on ice for quantitation, quality evaluation and application to microarray chips.

2.12 Reverse transcription of RNA, dye conjugation and hybridisation to GeneChip Microarray chips

The following procedures were carried out at the Adelaide Microarray Centre. For reverse transcription, 2 μl of anchored polyT(V)N (2 μg/ml) was added to 20 μl DEPC water containing the RNA, and the mixture was then incubated at 70°C for 10 minutes. The samples were then cooled on ice and mixed with 6 μl of 5X Superscript II buffer, 2 μl of 0.1M dithiothreitol, 2 μl of Superscript II reverse transcriptase, 0.6 μl of a solution of 25mM dATP, 25mM dGTP, 25mM dCTP, 10mM dTTP and 15mM dUTP, and the complete mixture was incubated at 42°C for 2.5 h. The RNA was then hydrolysed by adding 10 μl of 0.25M NaOH and10 μl of 0.5M EDTA, followed by incubation at 65°C for 15 minutes. The reactions were then neutralised by adding 15 μl of 0.2M acetic acid. The prepared cDNA was isolated using a QIAquick PCR purification kit (Qiagen), by mixing the samples with 300 μl buffer PB before application to the Qiagen spin column and centrifugation at 6500 x g for 1 minute. The column was then washed twice with 600 μl buffer PE and pure cDNA eluted in 90 μl Milli Q water. The purified cDNA was then dried in a partial vacuum, dissolved in 9 μl of 0.1M $NaHCO_3$ (pH 9), and incubated with Cy dye for 60 minutes at room temperature in the dark. The cDNA was then reisolated as previously described. GeneChip Gene 1.0 ST Microarray chips (Affymetrix) were immersed in 50 μl of hot Milli Q water (~90°C) for 5 minutes and then allowed to dry. The labelled cDNA was mixed with 0.64 μl of 25 mg/ml yeast tRNA, 4 μl of 2 mg/ml poly A and 20 μl of 1 mg/ml Cot-1 DNA, dried in a partial vacuum and then dissolved in 16 μl of 6.25 X saline sodium citrate buffer. The mixture was heated to 100°C for 3 minutes, chilled on ice, and 0.5 μl of 10% sodium dodecyl sulphate was then added before the mixture was transferred onto a glass coverslip. The arrays were then lowered onto the coverslips and incubated

at 42°C overnight in a humidified chamber to enable hybridisation. Unbound cDNA was then washed away with 0.5 X saline sodium citrate 0.01% sodium dodecyl sulphate for 5 minutes, 0.5 X saline sodium citrate for 5 minutes, then 0.2 X saline sodium citrate for 3 minutes. The microarrays were then allowed to dry in the dark before scanning on a GSC3000 7G scanner (Affymetrix).

Chapter 3: The phenotype and distribution of regulatory T cells in rats

3.1 Introduction

Regulatory T cells are a lineage of $CD4^+$ T cells that suppress the proliferation and effector functions of other T cells to prevent the development of autoimmune disease. The first description of a population of T cells, known as suppressor T cells that could inhibit the proliferation of other T cells was made more than 30 years ago (Murphy *et al.* 1976). Suppressor T cells were thought to reside within the $CD8^+$ lineage, but researchers experienced great difficulties in determining a specific phenotype associated with these cells. When it transpired that the proposed molecular determinants of suppressor T cells, known as I-J genes were unlikely to exist, the concept of an immunoregulatory T cell population fell from favour (Steinmetz *et al.* 1982, Kronenberg *et al.* 1983). However, the phenomenon of T cell regulation began its revival when it was found that $CD45RC^-$ $CD4^+$ T cells, which include both newly formed T cells that have arrived in the periphery known as recent thymic emigrants (RTE) and antigen experienced memory T cells, could inhibit the induction of autoimmune disease (Powrie and Mason, 1990). A more specific phenotype that identified T cells with a regulatory activity was obtained when it was observed that $CD4^+$ $CD25^+$ T cells could inhibit autoimmune disease resulting from the adoptive transfer of $CD4^+$ $CD25^-$ T cells (Sakaguchi *et al.* 1995, Takahashi *et al.* 1998). The finding that $CD4^+$ $CD25^+$ T cells could inhibit T cell proliferation was unexpected, as it was widely believed that these cells represented activated T cells. However, further experimentation revealed that the *in vitro* activation of $CD4^+$ $CD25^-$ T cells does not reproduce either the phenotype or regulatory properties of naturally occurring $CD4^+$ $CD25^+$ T cells, indicating that there cells are likely to comprise a novel T cell lineage (Kuniyasu *et al.* 2000).

A specific marker for regulatory T cells that could distinguish them from activated T cells was made when it was discovered that mutations in a specific transcription factor were responsible for the rare autoimmune disease known as immune dysregulation, polyendocrinopathy, enteropathy and X linked inheritance (IPEX) (Bennett *et al*. 2001). IPEX is caused by mutations in the transcription factor Forkhead box p3 (Foxp3), and manifests itself in a variety of autoimmune diseases including diarrhoea, insulin-dependent diabetes mellitus, thyroid disorders and eczema (Gambineri *et al.* 2003). When transfected into normal $CD4^+$ T cells, Foxp3 confers both the phenotype and regulatory activity associated with

regulatory T cells (Hori *et al.* 2003), and is believed to be responsible for programming the development of the regulatory T cell lineage *in vivo* (Sakaguchi *et al.* 2003).

The transcription factor Foxp3 acts to inhibit the activation and cytokine production by T cells that expressed it by directly binding the transcription factors Nuclear factor of activated T cells (NFAT) and Nuclear factor κB (NF-κB). These transcription factors are targets of signal transductions originating at the TCR and are required for T cell activation and the production of the cytokines IL-2, IL-4 and IFN-γ (Bettelli *et al.* 2005). Recent analyses of the Foxp3 promoter have revealed the presence of 6 NFAT binding sites, which suggests that TCR engagement or other signals that activate NFAT may induce Foxp3 expression (Mantel *et al.* 2006). Supporting this idea, it has been observed that Foxp3 is transiently induced upon TCR ligation in human $CD4^+$ T cells (Wang *et al.* 2007). Although the mechanisms responsible for directing the differentiation of regulatory T cells remain unclear, some have suggested that they may arise from self-reactive T cells that have encountered their antigen in the thymus (Caton *et al.* 2004) or in the periphery (Bacchetta *et al.* 2005). Such interactions would not be expected to activate the T cells, which like other T cells would require additional, costimulatory signals. Rather, it is more likely that these T cells would become anergised after recognising their antigen.

The expression of cell surface markers by regulatory T cells will be evaluated, in order to obtain information about their past. To investigate the possibility that regulatory T cells represent T cells that have been anergised following contact with self antigens, their expression of the TCR and its co-receptor CD4 will be assessed by flow cytometry, which are characteristically downregulated by anergised T cells (Taams *et al.*1999). The TCR is downregulated by T cells in response to stimulation through a process that involves the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ (D'Oro *et al.* 1997, von Essen *et al.* 2004). This process involves the dephosphorylation of a regulatory tyrosine on the C-terminus of $p56^{lck}$ by CD45, probably as a result of the serial engagement of TCRs, and leads to their degradation via the lysosomes (Lauritsen *et al.* 1998, von Essen *et al.* 2004). However, TCR molecules are not downregulated by activated T cells, as the phosphorylation of the CD3γ chain and activation of protein kinase C redirects

internalised TCR signalling complexes away from the lysosomes to be recycled (Salio *et al.* 1997, Dietrich *et al.* 1998). It is thought that this recycling process facilitates sustained signal accumulation during T cell activation (Menne *et al.* 2002, Utzny *et al.* 2006). The loss of TCR expression and its coreceptor CD4 from the surface of anergised T cells is likely to be the result of the degradation of the entire TCR signalling complex, which is not separated into its individual components as it is internalised and enters the lysosomal degradation pathway (Viola *et al.*1997, von Essen *et al.* 2004). In addition to assessing regulatory T cells for characteristic features of anergised T cells, the expression of activation-associated markers by regulatory T cells will be measured to determine if there are any indications that they have been stimulated by APC.

The expression of CD5, a molecule that is induced during the positive selection of thymocytes in proportion to the avidity of the TCR MHC-peptide interaction (Azzam *et al.* 1998) will also be assessed to determine the likely avidity of regulatory T cells for their cognate antigen. Although the molecular mechanism utilised by CD5 to regulate TCR signalling is unclear, it is recruited into the immunological synapse where it co-localises with CD3 and reduces the extent of tyrosine phosphorylation at the immunological synapse (Brossard *et al.* 2003). CD5 is thus thought to effectively tune the strength of TCR signal transductions. T cells maturing in the thymus that have a low avidity for antigen are only mildly affected by the absence of CD5, while T cells with higher avidity for antigen experience a profound shift towards negative selection when CD5 is absent (Azzam *et al.* 2001).

The expression of costimulatory molecules by regulatory T cells will be assessed, to determine to what extent the differential expression of these molecules may play a role in the functional differences regulatory T cells have with other subsets of $CD4^+$ T cells. The profile of adhesion molecules expressed by regulatory T cell subsets will also be analysed, to determine whether regulatory T cells differ from other T cell subsets in their expression of these molecules, which could indicate they have different migration characteristics. In order to correlate these findings with the normal behaviour of regulatory T cells *in vivo*, recirculating regulatory T cells will be obtained and their destinations under normal and inflammatory conditions determined.

3.2 Results

3.2.1 Expression of Foxp3 by $CD4^+$ T cells in the thymus and the peripheral lymphoid tissues.

In order to investigate the distribution of regulatory T cells in the thymus and in secondary lymphoid tissues, $CD4^+$ T cells in the thymus, lymph nodes, spleen, blood and thoracic duct lymph were isolated by negative selection through the immunomagnetic depletion of $CD8^+$ and B cells. In order to distinguish $CD4^+$ $CD8^-$ thymocytes from $CD4^-$ $CD8^-$ thymocytes, the expression of the CD4 molecule was included in flow cytometric analyses of thymocytes. The expression of Foxp3 by $CD4^+$ T cells in tissue samples was determined by flow cytometry, indicating that the thymus contains a significantly lower proportion of $Foxp3^+$ cells among $CD4^+$ single positive T cells compared to that found in any of the peripheral compartments ($p<0.03$) as determined using a two tailed paired Student's t test (Figure 3.2.1). The celiac lymph nodes, which drain the liver contained a consistently higher proportion of regulatory T cells compared with the proportions found in other lymph nodes and the spleen, although this difference was not statistically significant. Interestingly, thoracic duct lymph contained a significantly lower proportion of regulatory T cells compared with the proportion observed in lymph nodes, suggesting that regulatory T cells may be relatively sessile compared to other T cells ($p<0.001$).

3.2.2 Identification of regulatory T cells and activated T cells in rats

In order to identify $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^+$ $Foxp3^-$ activated T cells, $CD4^+$ T cells were negatively selected and their expression of CD25 and Foxp3 determined by flow cytometry. It was found that $CD4^+$ T cells from pooled lymph nodes were typically ~10% $CD25^+$ (Figure 3.2.2) and 10-15% $Foxp3^+$. Analysis of Foxp3 and CD25 co-expression by $CD4^+$ T cells reveals a discrete $Foxp3^+$ population, which is mostly $CD25^+$ in the lymph nodes of young adult rats

but a population of $CD25^-$ $Foxp3^+$ cells is also present. The majority (85-90%) of $CD4^+$ $CD25^+$ cells expressed Foxp3 and $CD4^+$ $CD25^{bright}$ cells were virtually all (95-99%) $Foxp3^+$. The expression of CD25 by regulatory T cells was found to vary between each compartment (Table 3.1). The smallest proportion of $CD25^-$ $Foxp3^+$ cells was observed in the thymus (1.2% of $CD4^+$ T cells), while the proportion in the peripheral blood was two-fold higher (2.5% of $CD4^+$ T cells). Interestingly, even greater proportions of $CD25^-$ $Foxp3^+$ cells were detected in the lymph nodes (4.5% of $CD4^+$ T cells), and spleen (5.4% of $CD4^+$ T cells), and in the case of the spleen, almost half of $CD4^+$ $Foxp3^+$ cells do not express CD25.

3.2.3 Expression of the activation associated markers by regulatory T cells

The transferrin receptor (CD71) is induced following T cell activation, and facilitates the delivery of the ionic cofactor iron (Fe^{3+}), complexed with the transferrin carrier protein into activated T cells to enable their proliferation (Kemp et al. 1987, Daniels *et al.* 2006). It was found that CD71 was only expressed by a small proportion of resting ($CD25^-$ $Foxp3^-$) T cells, and in agreement with previous reports (Daniels *et al.* 2006) a higher proportion of activated ($CD25^+$ $Foxp3^-$) T cells expressed CD71. However, the large variation in CD71 expression by activated T cells meant that this difference was not statistically significant using a two tailed paired Student's t test ($p=0.099$) (Figure 3.2.3). It was found that a significantly greater proportion of both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed CD71 relative to resting T cells ($p=0.00002$ and $p= 0.003$ respectively) using a two tailed paired Student's t test.

In humans and rats, the presence of major histocompatibility class II **(**MHC II) molecules on the T cell surface is associated with T cell activation. MHC II molecules can be acquired by activated T cells through both the transfer of membrane

Figure 3.2.1 The expression of Foxp3 by $CD4^+$ T cells

Lymphocytes were prepared from the thymus, peripheral blood, spleen, and a pool of cervical, celiac and mesenteric lymph nodes obtained from each normal 7 week old female RT7b rat. Separately, lymphocytes were prepared also from thoracic duct lymph (TDL) collected from each of three female RT7b rat donors.$CD4^+$ T cells were selected by immunomagnetic depletion of $CD8^+$ T cells and B cells. The depleted thymocytes were then stained with anti-CD4 antibody to distinguish $CD4^+$ single positive thymocytes from $CD4^-$ $CD8^-$ double positive thymocytes. The depleted cells were then stained with anti-Foxp3 antibody and the relative proportions of $Foxp3^+$ cells were determined by flow cytometry. Data represent the means ±SD of 3 independent experiments. The statistical significance of differences was determined using a two-tailed paired Students t test.

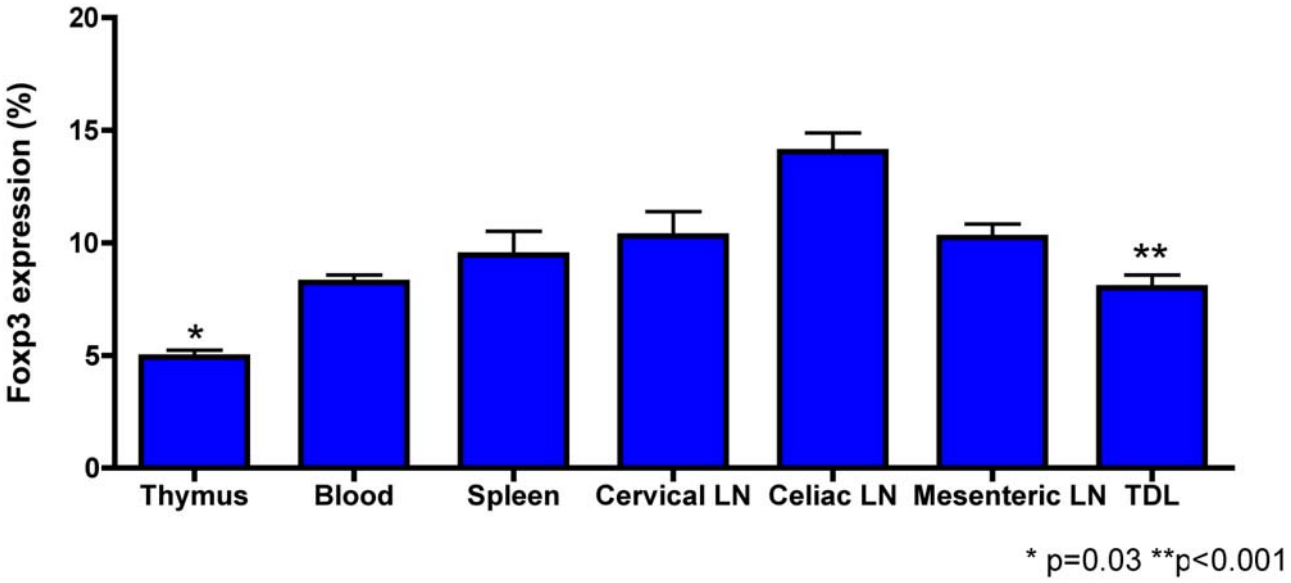
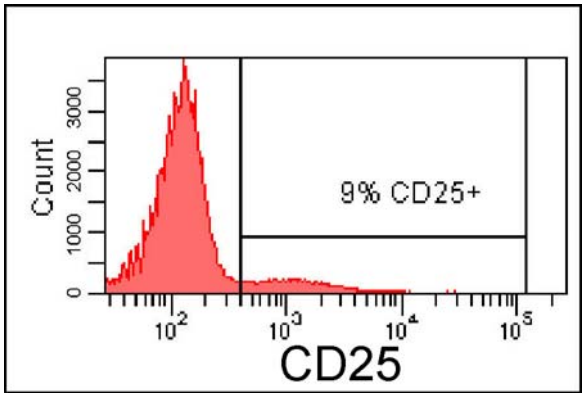

Foxp3 expression (%)
20
15
10
5
0
*
**
Thymus
Blood
Spleen
Cervical LN
Celiac LN
Mesenteric LN
TDL
* p=0.03 **p<0.001

Figure 3.2.2 Levels of the high affinity IL-2 receptor α -chain (CD25) can identify regulatory T cells and activated T cells in rats

$CD4^+$ T cells were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes by immunomagnetic depletion of B and $CD8^+$ T lymphocytes, and stained with antibodies against CD25 and Foxp3. The proportion of $CD4^+$ T cells expressing of CD25 (A) and Foxp3 (B) is expressed as a percentage. The coexpression of CD25 and Foxp3 by $CD4^+$ T lymph node cells is displayed as a dot plot (C), which shows the proportions of resting ($CD25^-$ $Foxp3^-$) T cells, activated ($CD25^+$ $Foxp3^-$) T cells, $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells within lymph node $CD4^+$ T cells. Also indicated is the gate that defines the brightest half ($CD25^{bright}$) of the $CD25^+$ cells. The expression of Foxp3 by all $CD4^+$ $CD25^+$ T cells (D), and by $CD25^{bright}$ $CD4^+$ T cells (E) is displayed. Representative of 3 experiments.

A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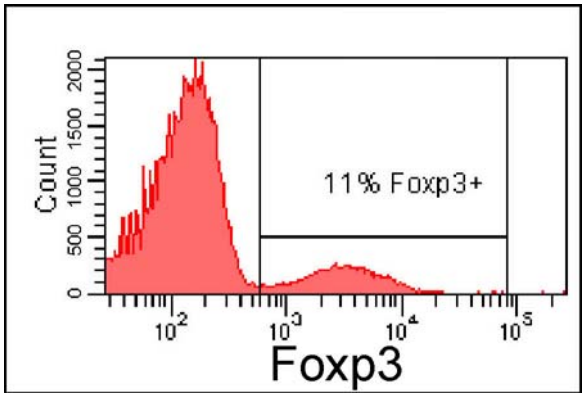
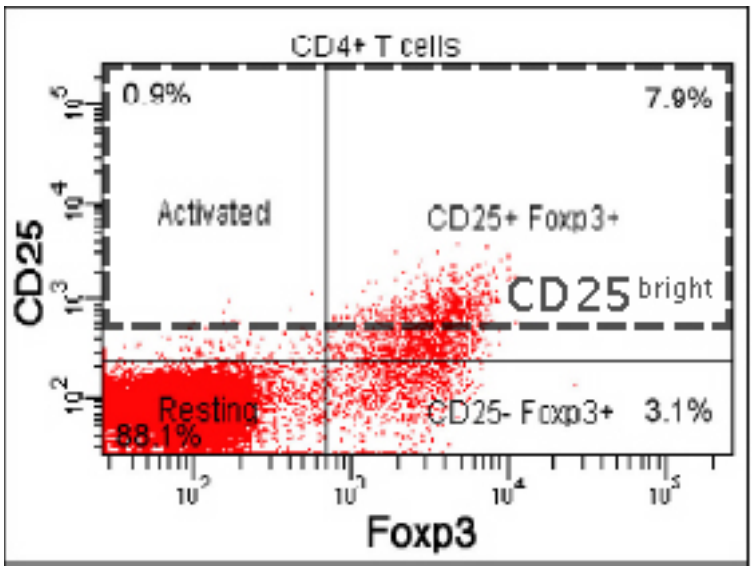
9% CD25+
CD25

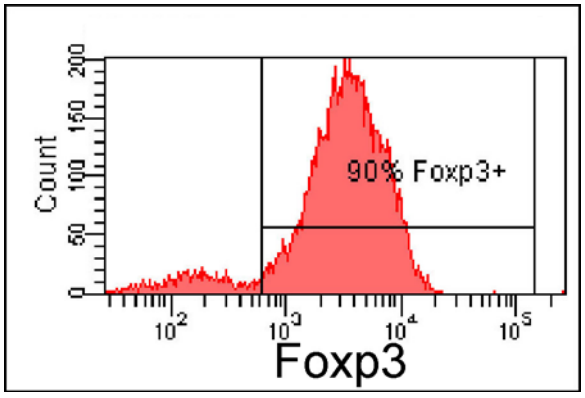
B


Count
11% Foxp3+
Foxp3

C


CD4+ T cells
0.9%
7.9%
Activated
CD25+ Foxp3+
CD25 bright
CD25- Foxp3+ 3.1%
Resting
88.1%
CD25
Foxp3

D


Count
90% Foxp3+
Foxp3

E

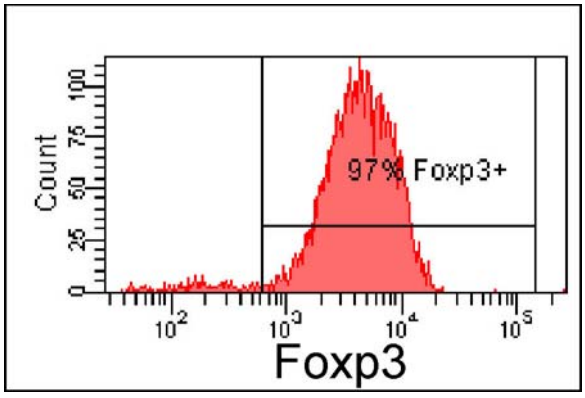

Count
97% Foxp3+
Foxp3

Table 3.1

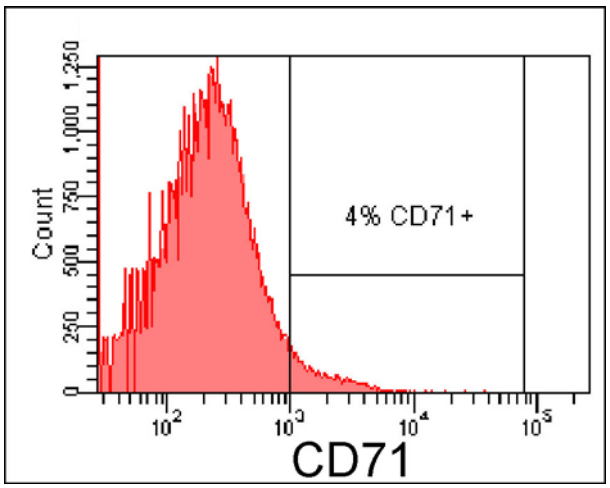
The co-expression of CD25 and Foxp3 by $CD4^+$ T cells negatively selected from suspensions of thymus, blood, spleen, lymph node (LN) and thoracic duct lymph (TDL) lymphocytes as determined by flow cytometry. The proportions of activated T cells ($CD25^+$ $Foxp3^-$), "resting" T cells ($CD25^-$ $Foxp3^-$), $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells within each compartment are indicated as percentages. The proportions of $CD4^+$ T cells that express Foxp3, $CD25^+$ cells that express Foxp3, and $Foxp3^+$ cells that express CD25 are also shown as percentages. Data represent means, ±SD, n=3.

Cells	Thymus	Blood	Spleen	LN	TD
$CD25^+$ $Foxp3^-$ activated T cells	3.6	0.9	1.1	0.9	1.3
$CD25^-$ $Foxp3^-$ resting T cells	91.2	90.5	88.0	83.4	93.7
$CD25^+$ $Foxp3^+$ T_{reg}	4.1	6.1	5.4	11.2	3.5
$CD25^-$ $Foxp3^+$ T_{reg}	1.2	2.5	5.4	4.5	1.5
Total $Foxp3^+$ T_{reg} (% of $CD4^+$ T cells)	5.3	8.6	10.8	15.7	5.0
$CD25^+$ T_{reg} (% of $CD25^+$ T cells)	59.6	90.5	90.8	94.6	79.4
$CD25^+$ T_{reg} (% of $Foxp3^+$ T cells)	77.3	71.0	50.0	71.3	70.0
$CD25^+$ T_{reg} : $CD25^-$ T_{reg} ratio	3.4	2.4	1.0	2.5	2.3

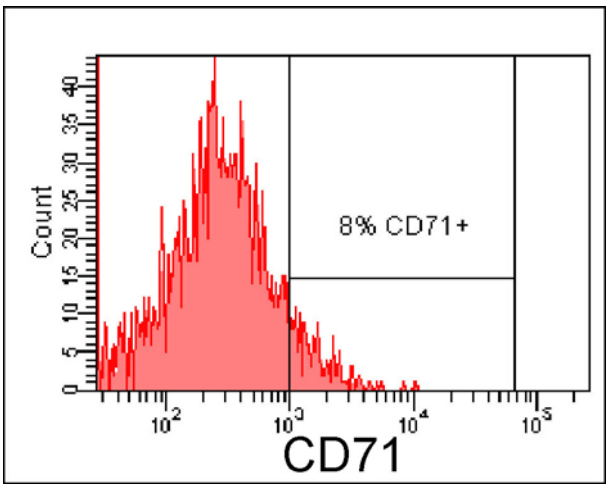
Figure 3.2.3 Some regulatory T cells express the transferrin receptor (CD71)

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The purified cells were then stained with antibodies against CD71, CD25 and Foxp3. The proportions of resting ($CD25^-$ $Foxp3^-$) T cells (A), activated ($CD25^+$ $Foxp3^-$) T cells (B), $CD25^+$ $Foxp3^+$ regulatory T cells (C) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) expressing CD71 are expressed as percentages. Representative of 3 experiments.

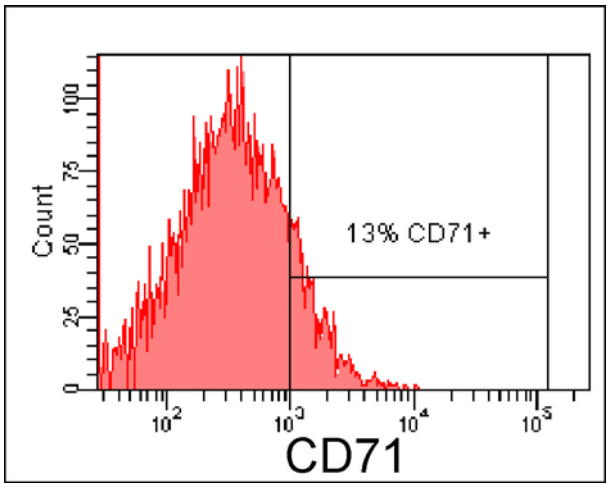
A Resting $CD4^+$ T cells



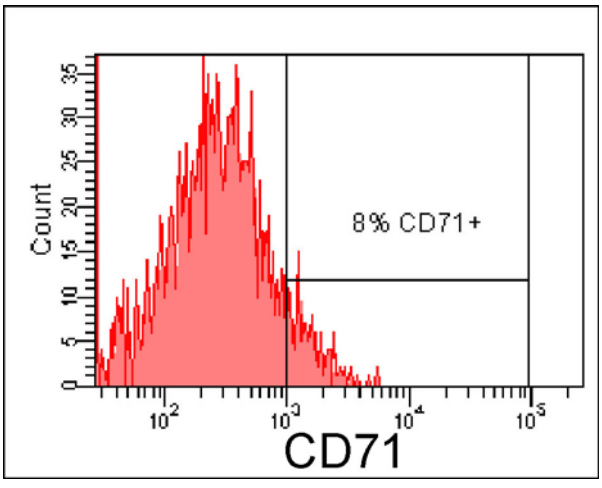
B Activated $CD4^+$ T cells



C $CD25^+$ $Foxp3^+$ T cells



D $CD25^-$ $Foxp3^+$ T cells



containing MHC II molecules from antigen presenting cells (APC), or by endogenous synthesis within the activated T cell (Patel *et al.* 2001). Analysis of MHC II expression by flow cytometry, revealed that a consistently greater proportion of activated T cells express MHC II compared with resting cells, although this was not significant (p=0.13) (Figure 3.2.4). Both resting and activated T cells contained an MHCII^{bright} population, (MFI>10^4 units), that was not observed in either the CD25$^+$ Foxp3$^+$ regulatory T cell or the CD25$^-$ Foxp3$^+$ putative regulatory T cell populations. The proportion of CD25$^+$ Foxp3$^+$ regulatory T cells that expressed MHC II (mean 8.0%, SD=1.0) was consistently lower than for activated T cells (mean 11.3%, SD=1.52) but higher than that of resting T cells (mean 7%, SD=2.0), and most of the MHC II$^+$ CD25$^+$ Foxp3$^+$ cells were MHC II^{dull}. Interestingly, CD25$^+$ Foxp3$^+$ regulatory T cells contained a significantly lower proportion of MHC II^{bright} cells (mean 0.3%, SD=0.1) compared with resting cells (mean 1.27%, SD=0.15) (p=0.0005). Furthermore, CD25$^-$ Foxp3$^+$ putative regulatory T cells had both a significantly lower proportion of MHC II$^+$ (mean 2.7%, SD=0.58) and MHCII^{bright} cells (mean 0.2%, SD=0.1) compared with resting cells (mean 7% SD=2.0, mean 3.0%, SD=0.1 respectively), and these differences were statistically significant (p=0.015 and p=0.0004 respectively).

3.2.4 Evaluation of regulatory T cells for markers of anergy

In order to assess regulatory T cells for markers associated with T cell anergy, their expression of the TCR for antigen and the CD4 co-receptor was measured, as they are both characteristically downregulated by anergised T cells (Taams *et al.* 1999, Jordan *et al.* 2000). Firstly, the expression of both the α/βTCR and γ/δTCR forms by regulatory T cells was determined. All regulatory T cells were found to express the α/βTCR, the most commonly expressed form, while none expressed the rarer γ/δTCR (data not shown). It was found that while activated T cells had a slightly higher expression of the α/βTCR (mean 39185, SD=2918) relative to resting T cells (mean 35996, SD =1042),

the difference was not statistically significant (p=0.06) (Figure 3.2.5). In contrast, both $CD25^+$ $Foxp3^+$ regulatory T cells (mean 29043, SD=1885) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 26097, SD=2535) expressed significantly lower levels of the α/βTCR compared with resting T cells (p= 0.007 and p= 0.004 respectively). Strikingly, a remarkably similar pattern of CD4 expression was observed to the pattern of TCR expression among the subsets of $CD4^+$ T cells examined (Figure 3.2.6). Activated T cells (mean 41088, SD=8293) expressed higher levels of CD4 compared with resting T cells (mean 36970, SD=10598), and this was statistically significant (p=0.02). In addition, both $CD25^+$ $Foxp3^+$ regulatory T cells (mean 32495, SD=8628) and $CD25^-$ $Foxp3^+$ putative regulatory cells (mean 31823, SD=8054) had a significantly lower expression of CD4 compared to resting T cells (p= 0.04 and p= 0.03 respectively).

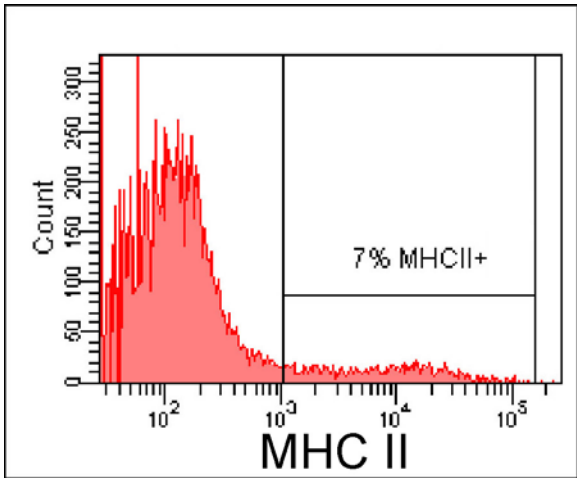
3.2.5 Expression of the CD5 molecule by regulatory T cells

The CD5 molecule is induced during the positive selection of T cells in the thymus in proportion to the avidity of the TCR-MHC interaction, and thus CD5 expression levels may indicate the relative avidity of regulatory T cell TCRs for their corresponding MHC molecules (Azzam *et al.* 1998). Interestingly, the expression of CD5 was significantly lower on both $CD25^+$ $Foxp3^+$ regulatory T cells (mean 9691, SD=1788) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 10464, SD=2076) compared with resting T cells (mean 11918, SD=2172) (p=0.001 and p=0.002 respectively), with $CD25^+$ $Foxp3^+$ regulatory T cells consistently having the lowest expression of CD5 (Figure 3.2.7). Activated T cells had a similar expression of CD5 compared to resting T cells (mean 12104, SD=2561).

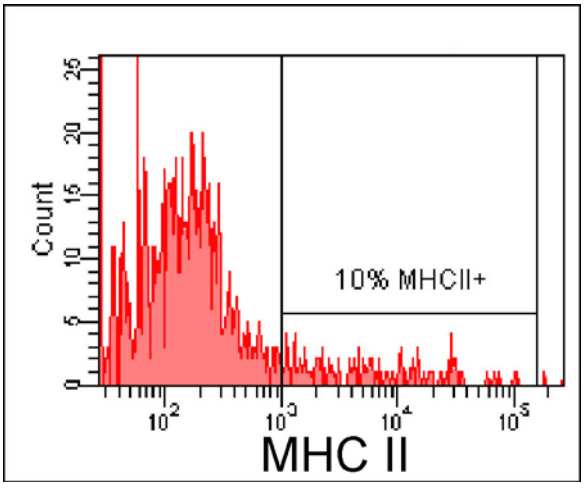
Figure 3.2.4 Expression of MHC II by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The cells were then stained with antibodies against MHC II, CD25 and Foxp3. The proportions of $Foxp3^-$ $CD25^-$ resting T cells (A), $Foxp3^-$ $CD25^+$ activated T cells (B), $CD25^+$ $Foxp3^+$ regulatory T cells (C) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) that express MHC II are expressed as percentages. Representative of 3 experiments.

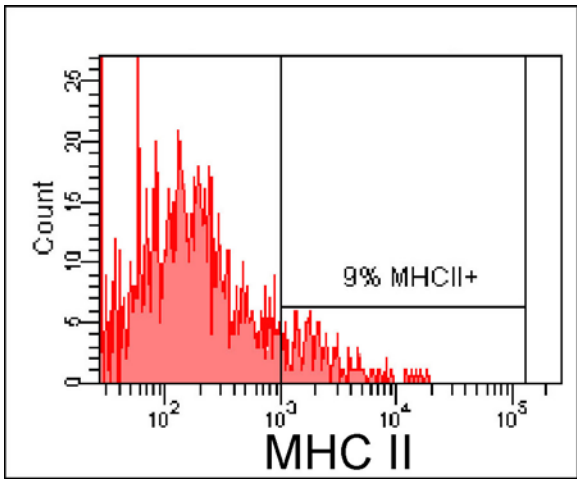
A Resting $CD4^+$ T cells



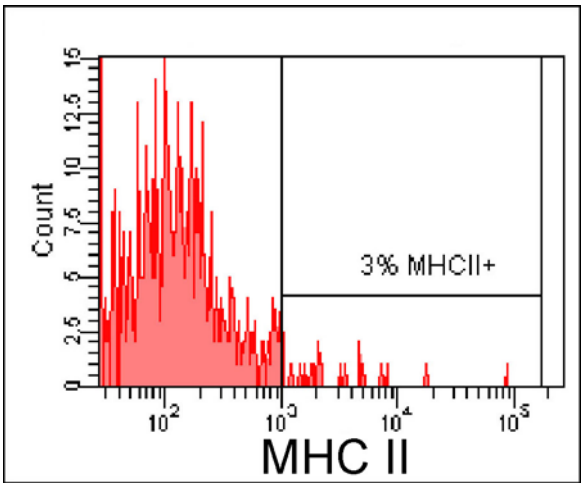
B Activated $CD4^+$ T cells



C $CD25^+$ $Foxp3^+$ T cells



D $CD25^-$ $Foxp3^+$ T cells



3.2.6 Expression of adhesion molecules by regulatory T cells

Adhesion molecules are expressed by lymphocytes to enable their migration out of the bloodstream and into either inflamed sites or into secondary lymphoid tissue where local immune responses can be initiated. In addition, many adhesion molecules are also used for the binding of T cells to APC to enable antigen presentation, which may lead to T cell activation in the presence of sufficient costimulatory signals. Therefore, we have undertaken to examine the expression of adhesion molecules by regulatory T cells, in order to better understand their interactions with other cells and gain insights into their biology.

The predominant form of the adhesion molecule CD44 on leukocytes is CD44H, which binds to hyalurnoic acid, a key component of the extracellular matrix (Peach *et al.* 1993). The expression of CD44H is thought to contribute to the adhesion of lymphocytes to the HEV in lymph nodes and Peyer's patches and thus facilitate the migration of recirculating cells into secondary lymphoid tissues (Huet *et al.* 1989). Although CD44 was found to be expressed by all $CD4^+$ T cells, the amount of CD44 expression differed between $CD4^+$ T cell subsets (Figure 3.2.8). While activated T cells expressed higher levels of CD44 compared to resting T cells on average, this was not statistically significant. Interestingly, both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory cells were found to express significantly more CD44 compared with resting T cells ($p=0.03$ and $p=0.04$).

The adhesion molecule leukosialin (CD43), is a highly glycosylated protein that is to regulate and coordinate T cell adhesion, and it also appears to inhibit pro-apoptotic signalling (Mattioli *et al.* 2004). It was found that activated T cells (mean 3225, SD=36) expressed lower amount of CD43 compared to resting T cells (mean 3603, SD=124), although this was not significant ($p=0.054$). The expression of CD43 however was found to be significantly lower on both

$CD25^+$ $Foxp3^+$ regulatory T cells (mean 2781,SD=115) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 2296, SD=158) relative to resting T cells (p=0.003 and p=0.02 respectively).

Intercellular adhesion molecule 1 (ICAM-1) is an adhesion molecule that binds to lymphocyte function associated protein 1 (LFA-1) and facilitates interactions of T cells with APC. ICAM-1 plays a key role on the surface of APC in stabilising the structure of the immunological synapse during T cell activation, to ensure efficient signalling (Bromley *et al.* 2002). The expression of ICAM-1 by activated T cells, $CD25^+$ $Foxp3^+$ regulatory T cells, $CD25^-$ $Foxp3^+$ putative regulatory T cells and resting T cells was compared by three-colour flow cytometry. It was found that a low proportion (mean 6.3%, SD=1.5) of resting $CD4^+$ T cells expressed ICAM-1, while a significantly higher proportion of activated T cells expressed ICAM-1 (mean 17%, SD=4.58) as assessed using a two tailed paired Student's t test (p=0.03) (Figure 3.2.10). Interestingly, it was found that an ICAM-1^{high} subpopulation was present only in the activated subset of $CD4^+$ T cells. While a significantly greater proportion of both $CD25^+$ $Foxp3^+$ regulatory T cells (mean 31%, SD=7.54) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 19%, SD=4.0) expressed ICAM-1 compared with resting T cells (p= 0.02 and p= 0.01 respectively), these subsets did not contain detectable numbers of ICAM-1^{high} cells.

LFA-1 is an adhesion molecule that is expressed at higher levels on memory T cells compared with naïve T cells (Dustin and Springer 1991). Its ligand, ICAM-1 is expressed by APCs and also by HEV, the endothelium of the lamina propria in the gut, and on vascular endothelium in inflamed tissues. The expression of LFA-1 by activated T cells, $CD25^+$ $Foxp3^+$ regulatory T cells, $CD25^-$ $Foxp3^+$ putative regulatory T cells and resting T cells was determined by three-colour flow cytometry. LFA-1 was expressed on all subsets of $CD4^+$ T cells, however it was noted that activated, $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ T cells expressed significantly higher levels of LFA-1 compared with resting T

Figure 3.2.5 TCR expression by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against the α/βTCR, CD25 and Foxp3 and examined by three colour flow cytometry. On the basis of expression of CD25 and/or Foxp3, cells in the $CD25^-$ $Foxp3^-$ (resting), $CD25^+$ $Foxp3^-$ (activated), $CD25^+$ $Foxp3^+$ (regulatory) and $CD25^-$ $Foxp3^+$ (putative regulatory) subsets were examined to determine relative levels of expression of the α/βTCR. Activated T cells had a slightly higher expression of the α/βTCR compared with resting T cells, although this was not statistically significant. In contrast, both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed significantly lower levels of TCR compared with resting T cells as determined using a two tailed paired Student's t test ($p= 0.007$ and $p= 0.004$ respectively). Data represent the means ±SD of 3 independent experiments.

TCR expression (MFI) by $CD4^+$ T cells

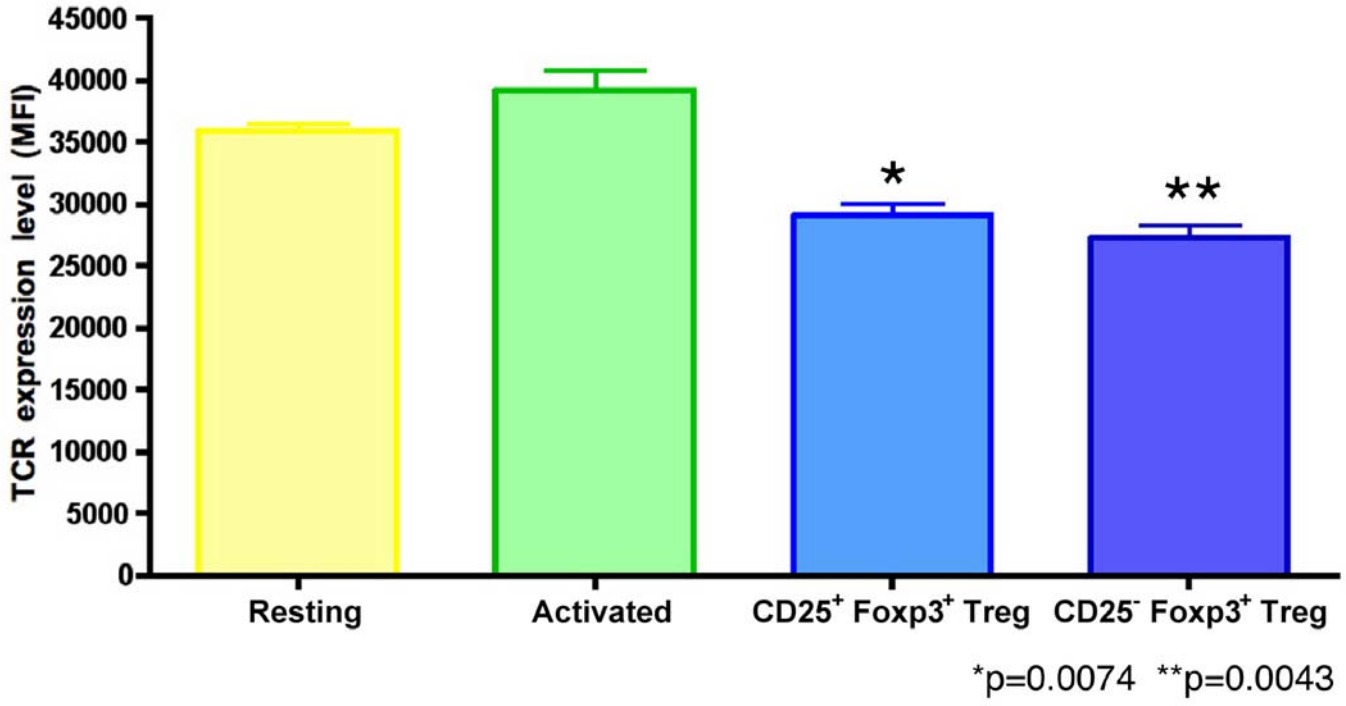


Figure 3.2.6 Expression of CD4 by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against CD4, CD25 and Foxp3 and examined by three colour flow cytometry. On the basis of expression of CD25 and/or Foxp3, cells in the $CD25^-$ $Foxp3^-$ (resting), $CD25^+$ $Foxp3^-$ (activated), $CD25^+$ $Foxp3^+$ (regulatory) and $CD25^-$ $Foxp3^+$ (putative regulatory) subsets were examined to determine relative levels of expression of CD4. It was found that while activated T cells had a slightly higher expression of CD4 relative to resting T cells, this was not statistically significant. In contrast, both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed significantly lower levels of CD4 compared with resting T cells as determined using a two tailed paired Student's t test ($p= 0.04$ and $p= 0.03$ respectively). Data represent the means ±SD of 3 independent experiments.

CD4 expression (MFI) by $CD4^+$ T cells

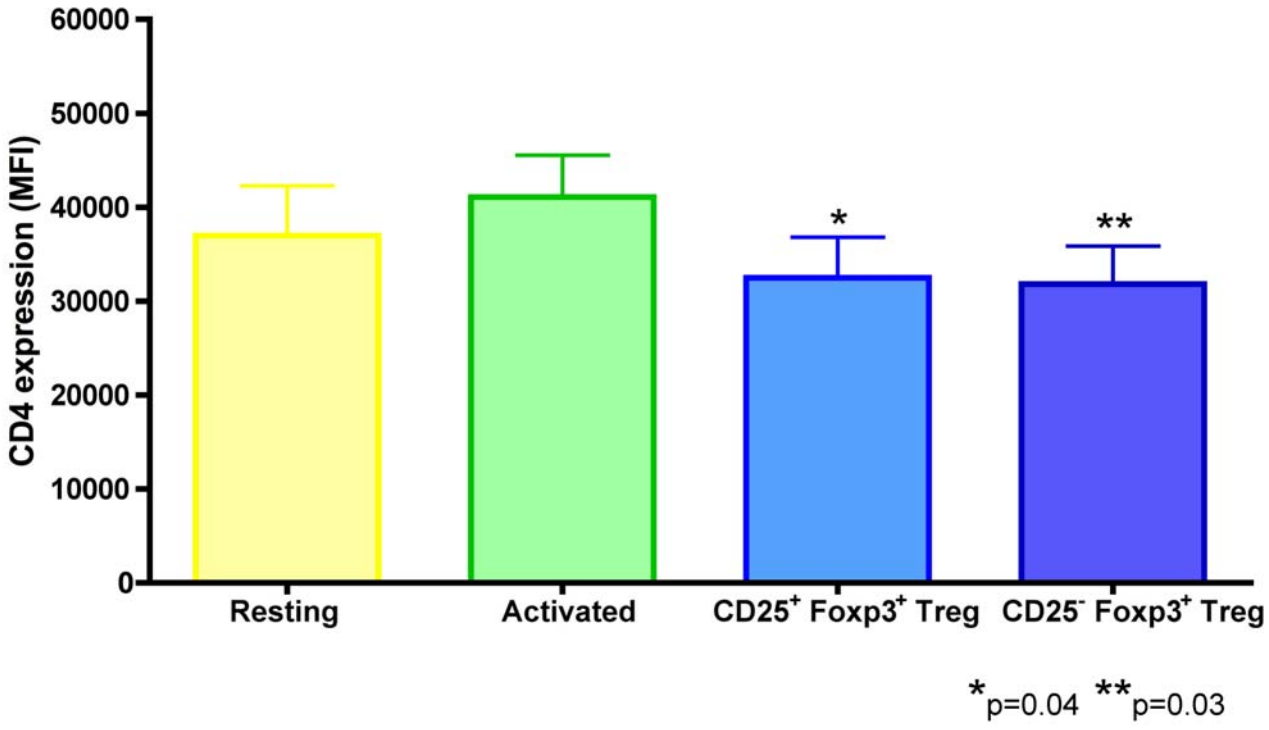


Figure 3.2.7 Expression of CD5 by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against CD5, CD25 and Foxp3 and examined by three colour flow cytometry. On the basis of expression of CD25 and/or Foxp3, cells in the $CD25^-$ $Foxp3^-$ (resting), $CD25^+$ $Foxp3^-$ (activated), $CD25^+$ $Foxp3^+$ (regulatory) and $CD25^-$ $Foxp3^+$ (putative regulatory) subsets were examined to determine relative levels of expression of CD5. While activated and resting T cells expressed similar amounts of CD5, both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells had a significantly lower expression of CD5 compared with resting T cells ($p=0.001$ and $p=0.002$ respectively) as determined using a two tailed paired Student's t test. Data represent the means ±SD of 3 independent experiments.

CD5 expression (MFI) by $CD4^+$ T cells

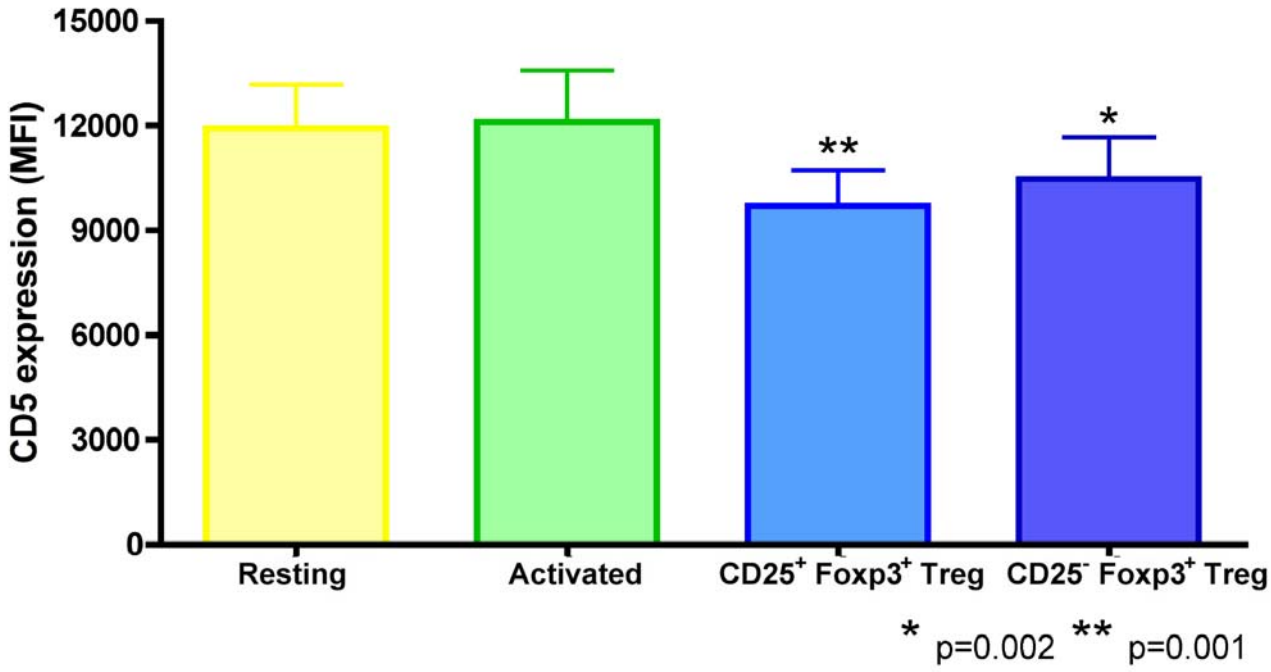


Figure 3.2.8 Some regulatory T cells express high levels of CD44

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against CD5, CD25 and Foxp3 and examined by three colour flow cytometry. On the basis of expression of CD25 and/or Foxp3, cells in the $CD25^-$ $Foxp3^-$ (resting), $CD25^+$ $Foxp3^-$ (activated), $CD25^+$ $Foxp3^+$ (regulatory) and $CD25^-$ $Foxp3^+$ (putative regulatory) subsets were examined to determine relative levels of expression of CD44. While activated T cells expressed higher levels of CD44 than resting cells, thee differences were not statistically significant when analysed using a two tailed paired Student's t test (NS). However, both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells were found to express significantly more CD44 ($p=0.03$ and $p=0.04$ respectively) than resting T cells using a two tailed paired Student's t test. Data represent the means ±SD of 3 independent experiments.

CD44 expression (MFI) by $CD4^+$ T cells

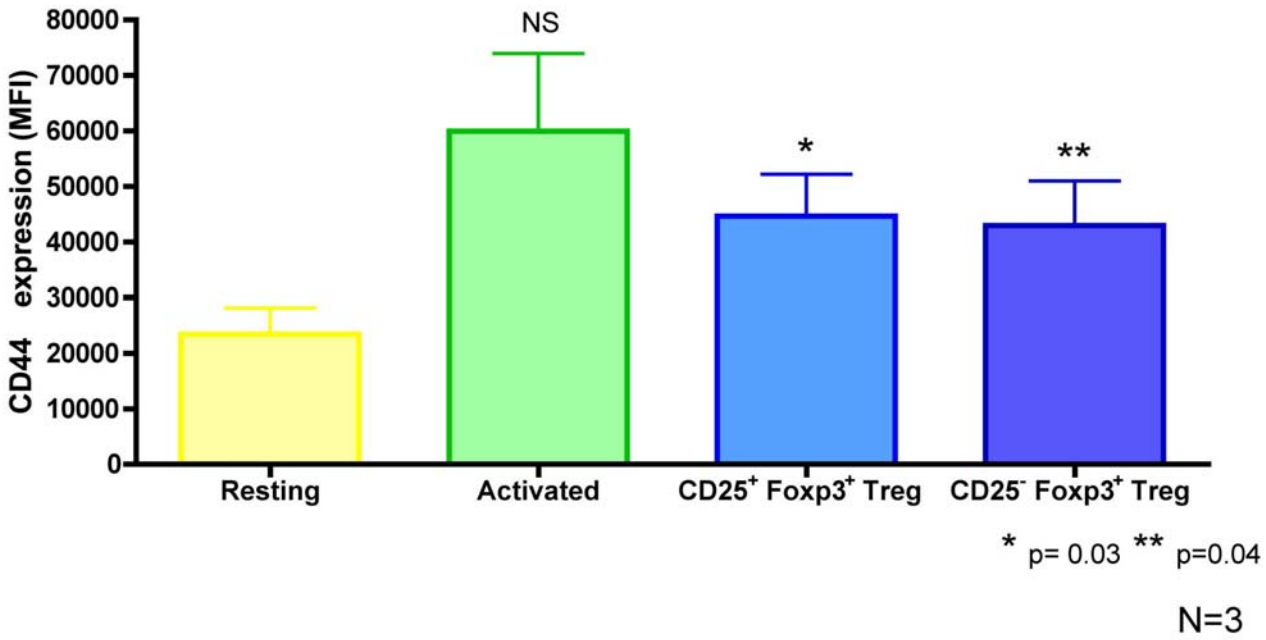


Figure 3.2.9 Expression of leukosialin (CD43) by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against CD43, CD25 and Foxp3 and examined by three colour flow cytometry. On the basis of expression of CD25 and/or Foxp3, cells in the $CD25^-$ $Foxp3^-$ (resting), $CD25^+$ $Foxp3^-$ (activated), $CD25^+$ $Foxp3^+$ (regulatory) and $CD25^-$ $Foxp3^+$ (putative regulatory) subsets were examined to determine relative levels of expression of CD43. Activated T cells expressed less CD43 than resting cells as assessed by the MFI, but this difference was not significant. Expression of CD43 was found to be significantly lower on both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells relative to resting T cells using a two tailed paired Student's t test ($p=0.003$ and $p=0.02$ respectively). Data represent the means ±SD of 3 independent experiments.

CD44 expression (MFI) by $CD4^+$ T cells

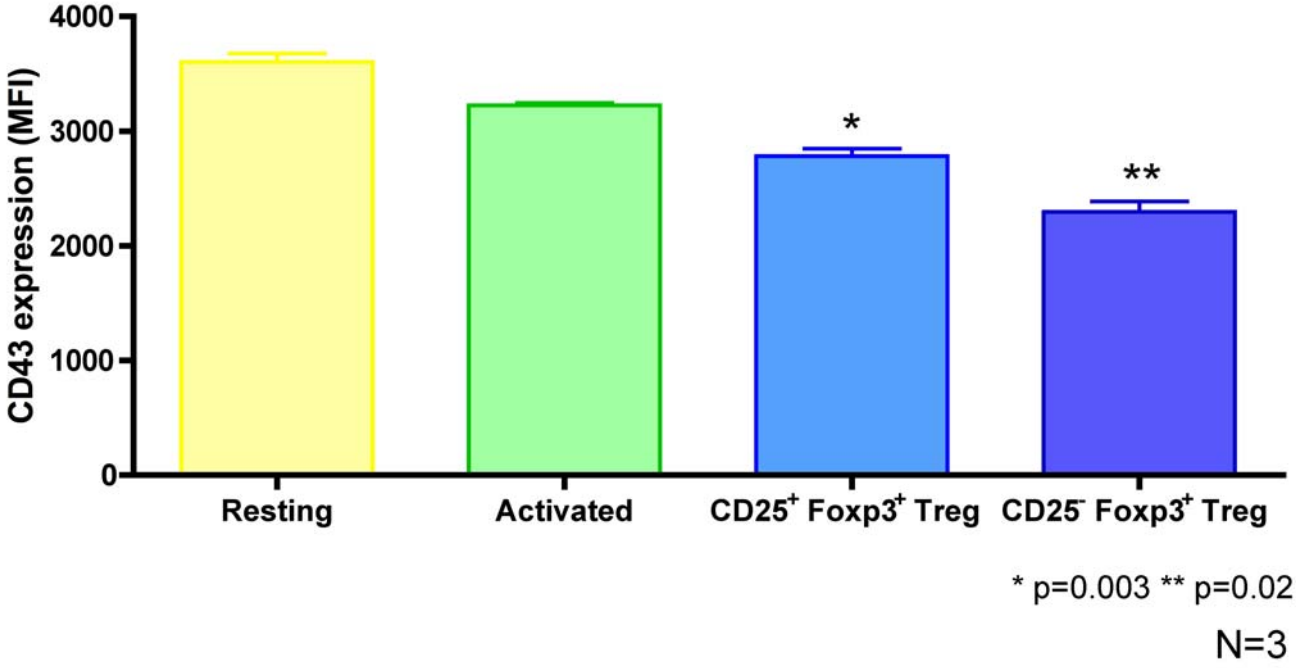
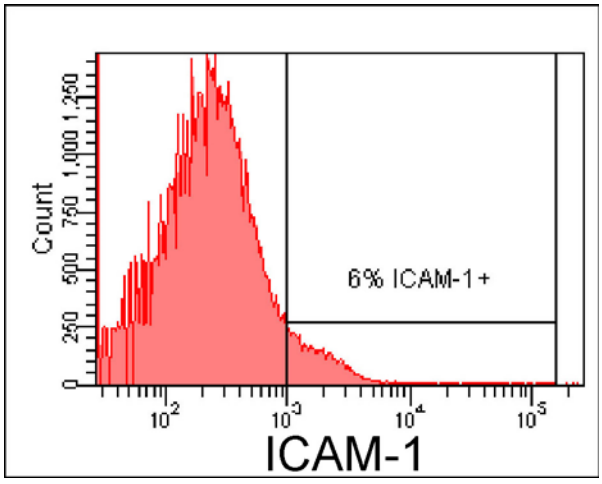


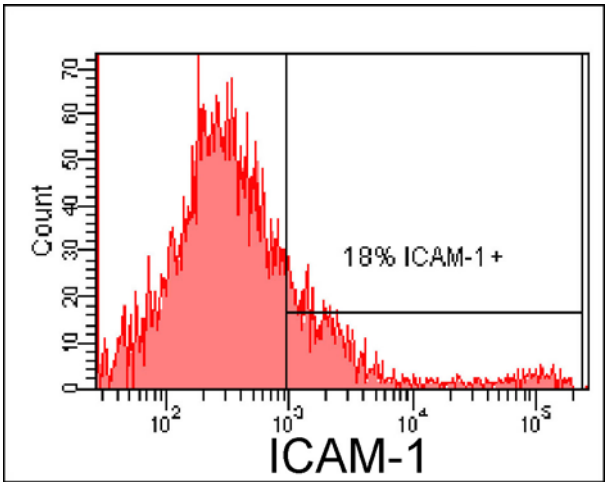
Figure 3.2.10 Expression of ICAM-1 (CD54) by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against ICAM-1, CD25 and Foxp3 and examined by three colour flow cytometry. The proportions of $Foxp3^-$ $CD25^-$ resting T cells (A), $Foxp3^-$ $CD25^+$ activated T cells (B), $CD25^+$ $Foxp3^+$ regulatory T cells (C) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) that expressed ICAM-1 are shown as percentages. Representative of 3 experiments.

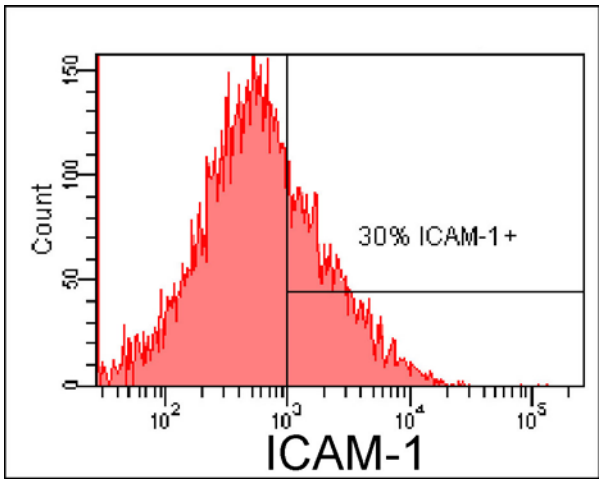
A Resting $CD4^+$ T cells



B Activated $CD4^+$ T cells



C $CD25^+$ $Foxp3^+$ T cells



D $CD25^-$ $Foxp3^+$ T cells

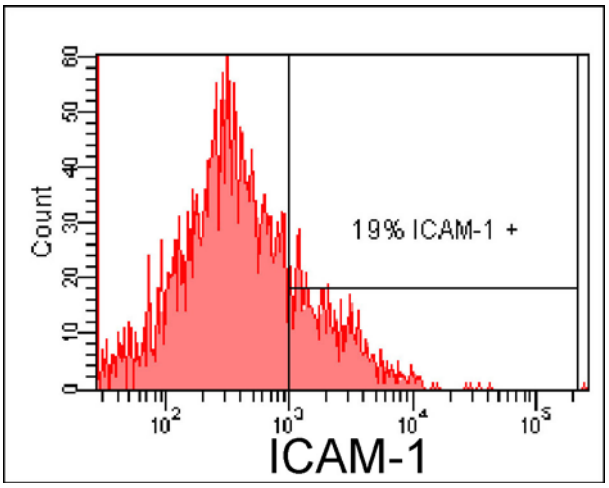
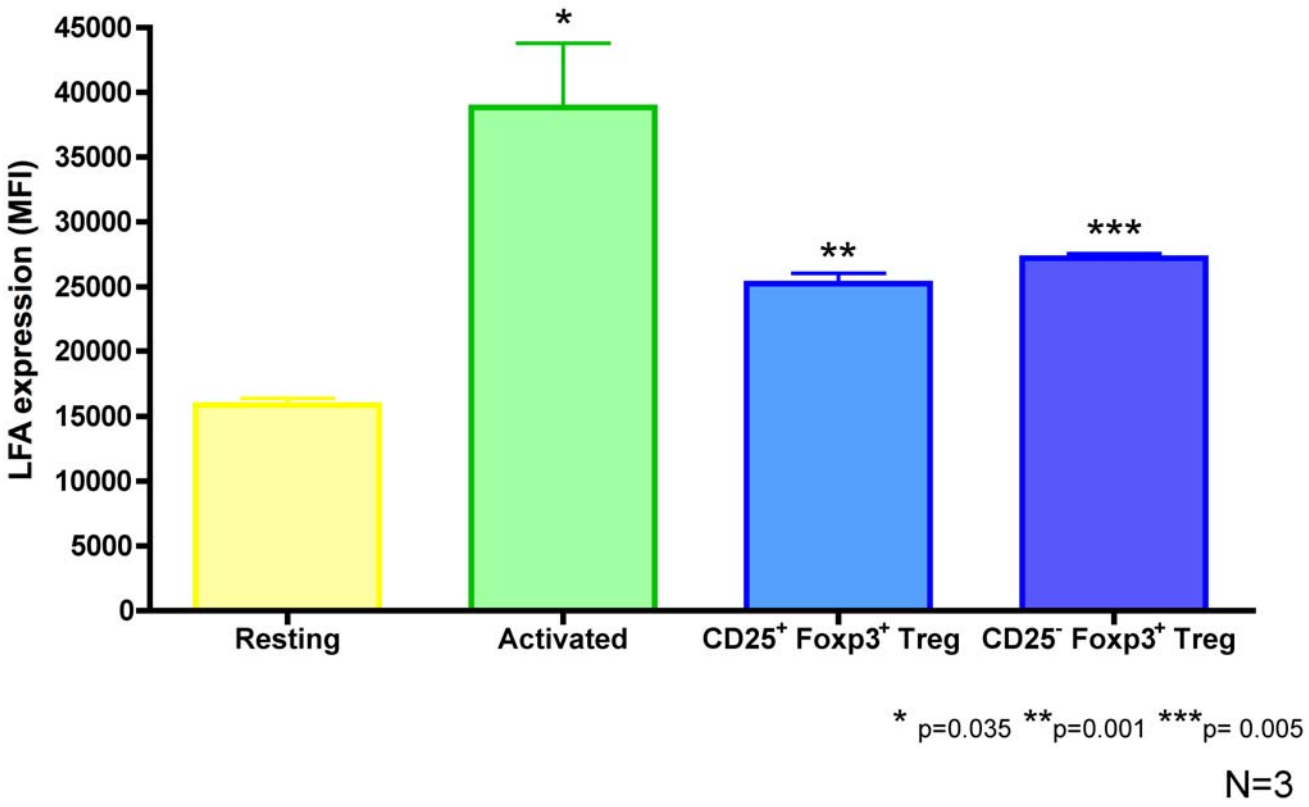


Figure 3.2.11 Expression of LFA-1 (CD11a, CD18) by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against LFA-1, CD25 and Foxp3 and examined by three colour flow cytometry. On the basis of expression of CD25 and/or Foxp3, cells in the $CD25^-$ $Foxp3^-$ (resting), $CD25^+$ $Foxp3^-$ (activated), $CD25^+$ $Foxp3^+$ (regulatory) and $CD25^-$ $Foxp3^+$ (putative regulatory) subsets were examined to determine relative levels of expression of LFA-1. Activated T cells, $CD25^+$ $Foxp3^+$regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed significantly higher levels of LFA-1 than resting T cells as determined using a two tailed paired Student's t test ($p= 0.04$, $p= 0.001$ and $p= 0.005$ respectively). Data represent the means ±SD of 3 independent experiments.

LFA-1 expression (MFI) by $CD4^+$ T cells



* p=0.035 **p=0.001 ***p= 0.005

N=3

cells as determined using a two tailed paired Student's t test (p= 0.04, p= 0.001 and p= 0.005 respectively) (Figure 3.2.11). Activated T cells were consistently found to have the highest expression of LFA-1 compared to other $CD4^+$ T cell subsets.

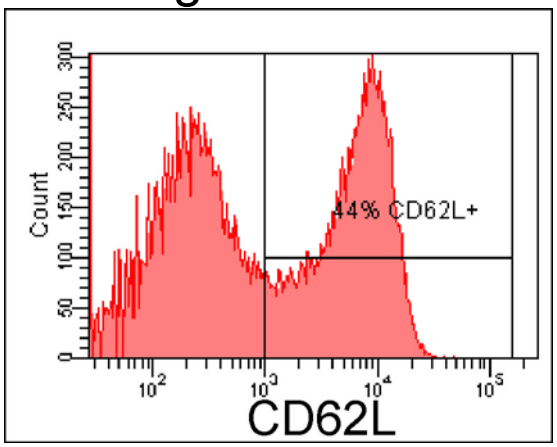
The expression of the adhesion molecule CD62L (L-selectin), which mediates the binding of lymphocytes to peripheral node addressin molecules expressed on the surface of HEV enables their extravasation into the lymph nodes (Rainer 2002). CD62L expression was therefore determined for subsets of $CD4^+$ T cells by three-colour flow cytometry, in order to gauge their relative abilities to migrate through the HEV (Figure 3.2.12). The proportions of resting (mean 45%, SD=1.2), activated (mean 48% SD=3.4), and $CD25^+$ $Foxp3^+$ T cells (mean 39%, SD=4.3) expressing CD62L were similar. However, a significantly lower proportion of $CD25^-$ $Foxp3^+$ T cells (mean 53%, SD=4.5) expressed CD62L compared with resting T cells (p=0.025). In contrast, the expression of CD62L by activated T cells as determined by mean fluorescence intensity was significantly higher than the expression of CD62L by resting T cells (p=0.05). $CD25^-$ $Foxp3^+$ putative regulatory T cells were found to express CD62L at significantly lower levels relative to resting T cells (p=0.02).

CD2 (LFA-2) is an adhesion molecule expressed by T cells that binds LFA-3 molecules expressed by endothelial cells, APC and lymphocytes. CD2 appears to be associated with antigen experience in T cells, as it is upregulated following contact with antigen and *in vitro* activation, and memory T cells have higher expression of CD2 compared with naïve T cells (Cantrell *et al*.1988, Wallace *et al*.1990). The expression of CD2 by activated T cells, $CD25^+$ $Foxp3^+$ regulatory T cells, $CD25^-$ $Foxp3^+$ putative regulatory T cells and resting T cells was determined. CD2 was expressed on all subsets of $CD4^+$ T cells, however activated T cells and $CD25^+$ $Foxp3^+$ T cells expressed significantly higher levels of CD2 compared with resting T cells as determined using a two tailed paired Student's t test (p= 0.01, and p= 0.01 respectively) (Figure 3.2.13).

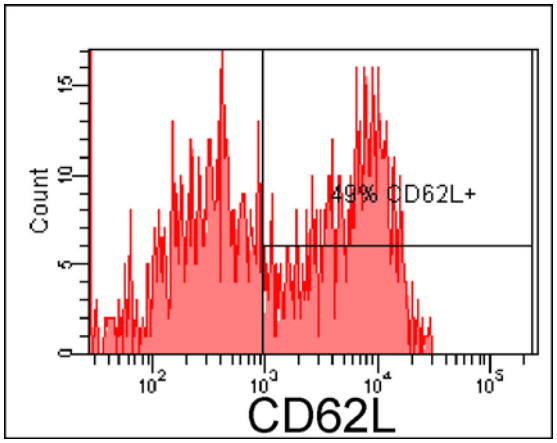
Figure 3.2.12 Expression of L-selectin (CD62L) by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against CD62L, CD25 and Foxp3 and examined by three colour flow cytometry. On the basis of expression of CD25 and/or Foxp3, cells in the $CD25^-$ $Foxp3^-$ (resting), $CD25^+$ $Foxp3^-$ (activated), $CD25^+$ $Foxp3^+$ (regulatory) and $CD25^-$ $Foxp3^+$ (putative regulatory) subsets were examined to determine relative levels of expression of LFA-1. The proportions of CD62L expressing resting T cells (A) activated T cells (B), and $CD25^+$ $Foxp3^+$ regulatory T cells (C) were similar. The proportion of $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) expressing CD62L was significantly lower than for resting T cells as determined using a two tailed paired students t test ($p=0.025$). The expression of CD62L by activated T cells as determined by mean fluorescence intensity (E) was significantly higher than the expression of CD62L by resting T cells ($p=0.05$). $CD25^-$ $Foxp3^+$ putative regulatory T cells were found to express CD62L at significantly lower levels relative to resting T cells ($p=0.02$). Data represent the means ±SD of 3 independent experiments.

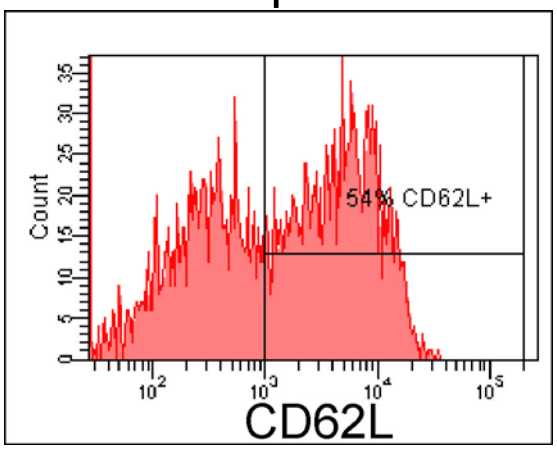
A Resting CD4+ T cells



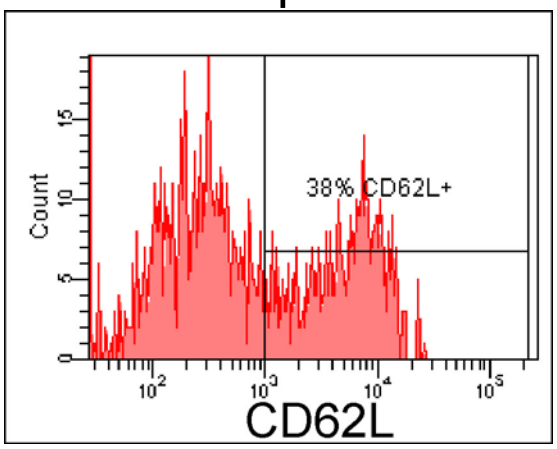
B Activated CD4+ T cells



C CD25+ Foxp3+ T cells

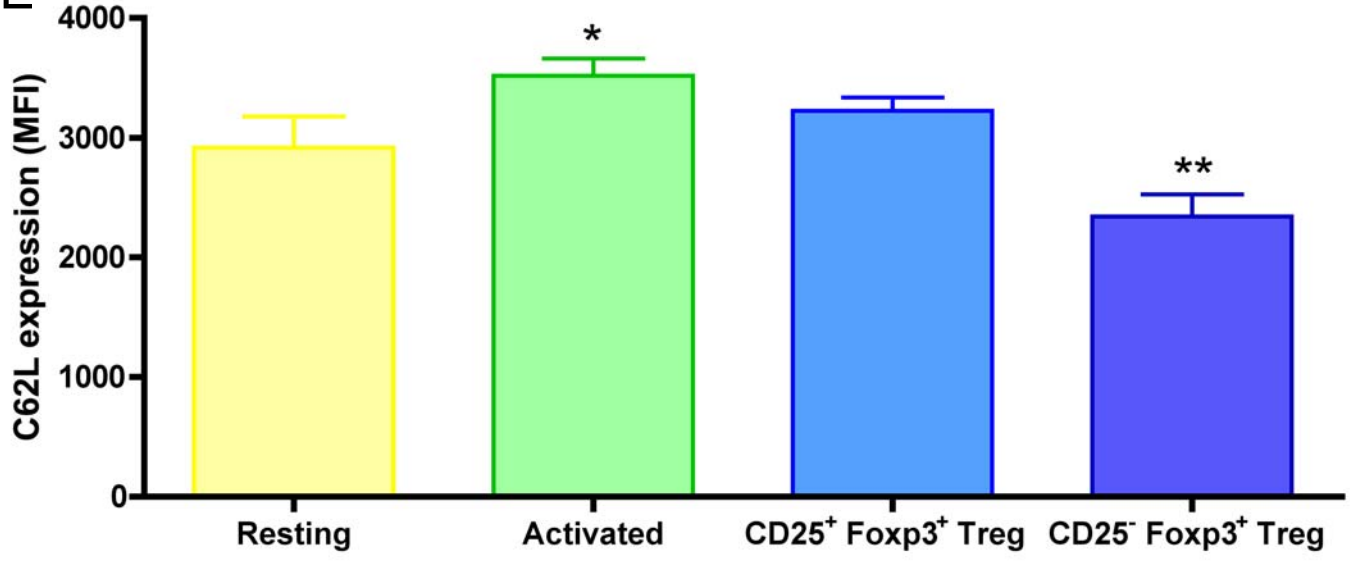


D CD25- Foxp3+ T cells



LFA-1 expression (MFI) by CD4+ T cells

E



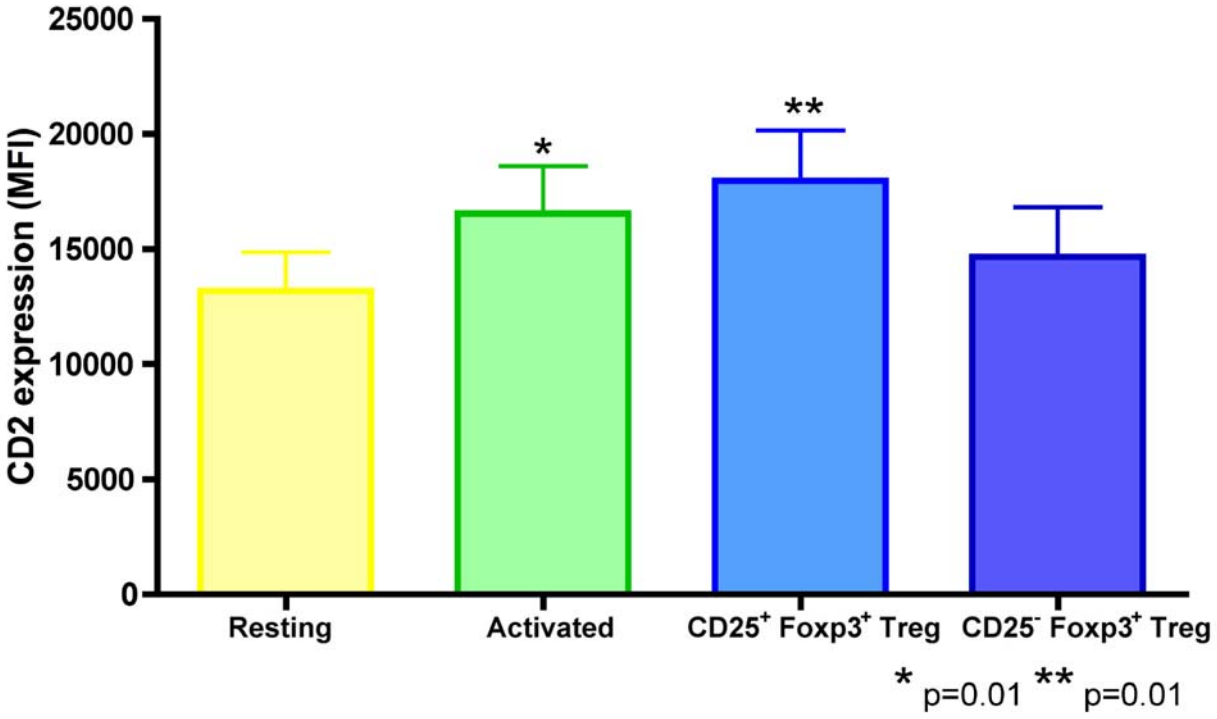
* p=0.05 ** p=0.02

N=3

Figure 3.2.13 Expression of CD2 by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against CD2, CD25 and Foxp3 and examined by three colour flow cytometry. On the basis of expression of CD25 and/or Foxp3, cells in the $CD25^-$ $Foxp3^-$ (resting), $CD25^+$ $Foxp3^-$ (activated), $CD25^+$ $Foxp3^+$ (regulatory) and $CD25^-$ $Foxp3^+$ (putative regulatory) subsets were examined to determine relative levels of expression of CD2. It was found that activated T cells and $CD25^+$ $Foxp3^+$ regulatory T cells expressed significantly higher levels of CD2 compared with resting T cells as determined using a two tailed paired Student's t test ($p= 0.01$, and $p= 0.01$ respectively). Data represent the means ±SD of 3 independent experiments.

CD2 expression (MFI) by $CD4^+$ T cells



Interestingly, $CD25^-$ $Foxp3^+$ T cells expressed the same amount of CD2 compared with resting T cells, suggesting they may not have recently contacted their antigen.

The adhesion molecule $\alpha_E\beta_7$ integrin (CD103), is thought to facilitate the localisation of T cells to mucosal tissues. E-cadherin, the only identified ligand for CD103, is mainly expressed in the gut epithelium and by gut-residing DC and is thought to contribute to the homing of the T regulatory cells to the mucosal tissues, where they localise to the epithelium and induce tolerance to antigens passing through the gut (Schon *et al.* 1999). The expression of CD103 by activated T cells, $CD25^+$ $Foxp3^+$ regulatory T cells, $CD25^-$ $Foxp3^+$ putative regulatory T cells and resting T cells was determined by three-colour flow cytometry (Figure 3.2.14). It was found that a low proportion of resting (A) T cells expressed CD103 (mean 1.3%, SD=0.6), while a significantly higher expression of CD103 was observed on activated T cells (mean 10.8%, SD=3) ($p=0.006$) (B). The proportions of $CD25^+$ $Foxp3^+$ regulatory T cells (mean 4.7%, SD= 1.4) (C), and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 4.1%, SD=0.6) (D) that expressed CD103 were also significantly higher than for resting T cells ($p=0.02$ and $p=0.005$ respectively). Interestingly, only the $CD25^+$ activated T cell and $CD25^+$ $Foxp3^+$ regulatory T cell subsets contained $CD103^{bright}$ cells (MFI>10^4 units).

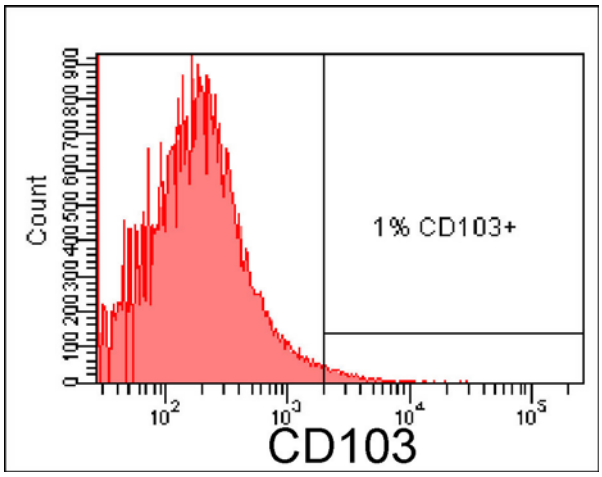
3.2.7 Expression of costimulatory molecules by regulatory T cells

Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) is a negative costimulatory molecule that acts to inhibit costimulatory signals and has been implicated in the suppressive mechanism associated with regulatory T cells (Sansom and Walker 2006). The expression of CTLA-4 by activated $CD4^+$ $CD25^+$ T cells, $CD25^+$ $Foxp3^+$ regulatory T cells, $CD25^-$ $Foxp3^+$ putative

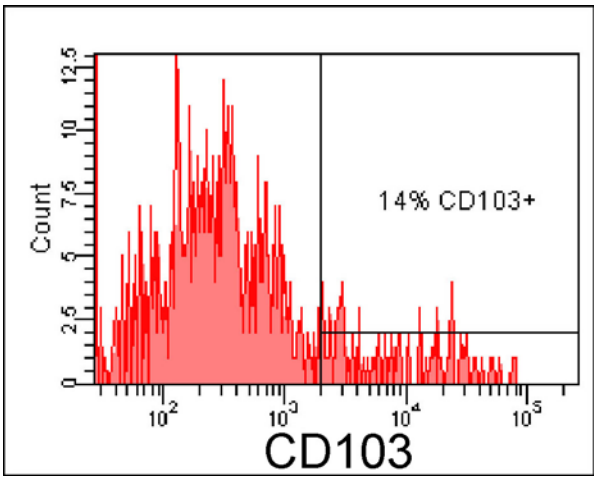
Figure 3.2.14 Expression of CD103 by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against CD103, CD25 and Foxp3. The proportions of resting T cells (A), activated T cells (B), $CD25^+$ $Foxp3^+$ regulatory T cells (C) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) expressing CD103 are expressed as percentages. Representative of 3 experiments.

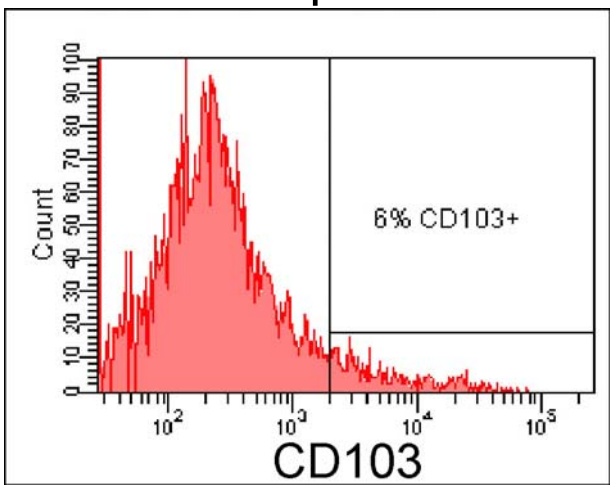
A Resting $CD4^+$ T cells



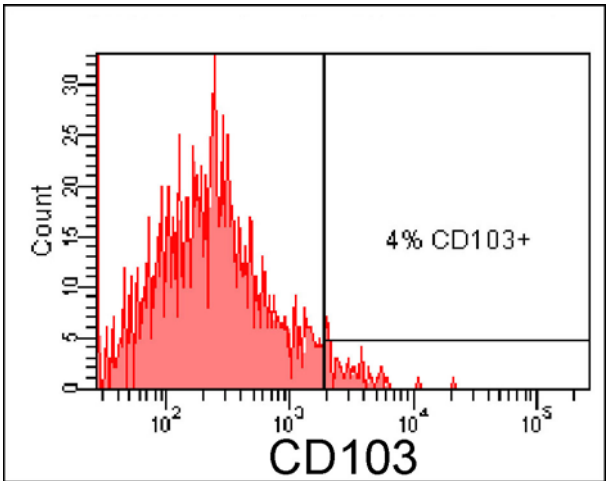
B Activated $CD4^+$ T cells



C $CD25^+$ $Foxp3^+$ T cells



D $CD25^+$ $Foxp3^+$ T cells



regulatory T cells and resting $CD25^-$ $Foxp3^-$ $CD4^+$ T cells was determined by three-colour flow cytometry. It was found that les than 1% of resting (mean 0.8%, SD=0.15) and activated (mean 0.57%, SD=0.2) $CD4^+$ T cells expressed CTLA-4 (Figure 3.2.15). Interestingly, a significantly greater proportion of $CD25^+$ $Foxp3^+$ regulatory T cells (mean 10%, SD= 1.3) expressed CTLA-4 compared with resting T cells (p= 0.008). However, although a consistently greater proportion of $CD25^-$ $Foxp3^+$ cells (mean 2.7%, SD=0.75) expressed CTLA-4 compared with resting T cells, this was not found to be statistically significant (p=0.06).

CD134 is a costimulatory molecule induced following T cell activation that acts to maintain antigen experienced T cells by providing survival signals (Weinberg *et al.* 2004). The expression of CD134 by activated T cells, $CD25^+$ $Foxp3^+$ regulatory T cells, $CD25^-$ $Foxp3^+$ putative regulatory T cells and resting T cells was determined by three-colour flow cytometry. Approximately 5% of resting $CD4^+$ T cells (mean 5.1%, SD=0.85) expressed CD134 (Figure 3.2.16). However, a significantly higher proportion of activated T cells (mean 39.0%, SD=5.6) expressed CD134 as assessed using a two tailed paired Student's t test (p=0.006). It was found that the majority of both $CD25^+$ $Foxp3^+$ regulatory T cells (mean 59%, SD-3.1) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 57%, SD=6.2) expressed CD134, significantly more than resting cells (p= 0.001 and p= 0.005 respectively).

A summary of the phenotypic characterisation of regulatory T cells is presented in Table 3.2.

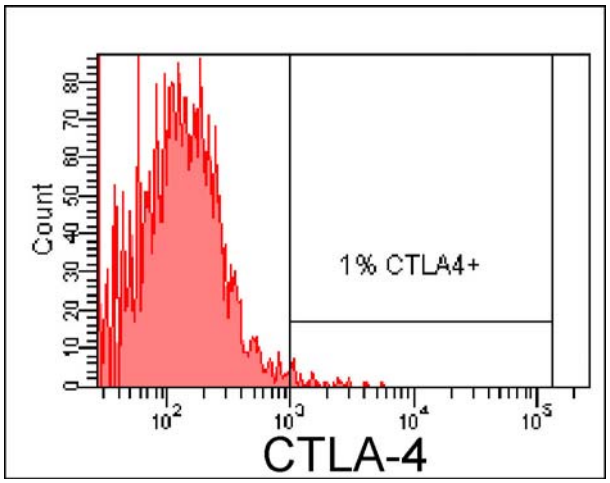
3.2.8 Investigating the entry of regulatory T cells into the thoracic duct and determining their subsequent destinations

To compare the propensities of regulatory and conventional $CD4^+$ T cell subsets to enter the thoracic duct, recirculating T cells and relatively sessile T

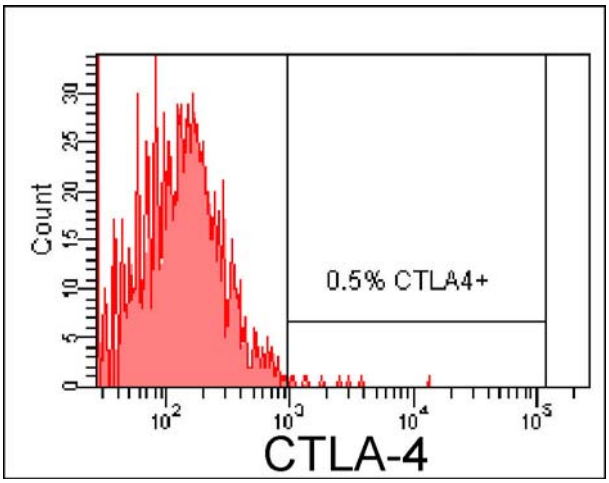
Figure 3.2.15 Expression of CTLA-4 by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against CTLA-4, CD25 and Foxp3 The proportions of resting T cells (A), activated T cells (B), $CD25^+$ $Foxp3^+$ regulatory T cells (C) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) expressing CTLA-4 are expressed as percentages. Representative of 3 experiments.

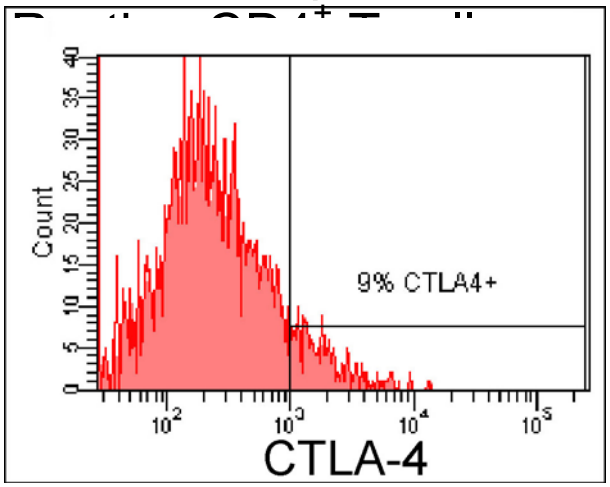
A Resting $CD4^+$ T cells



B Activated $CD4^+$ T cells



C $CD25^+$ $Foxp3^+$ T cells



D $CD25^-$ $Foxp3^+$ T cells

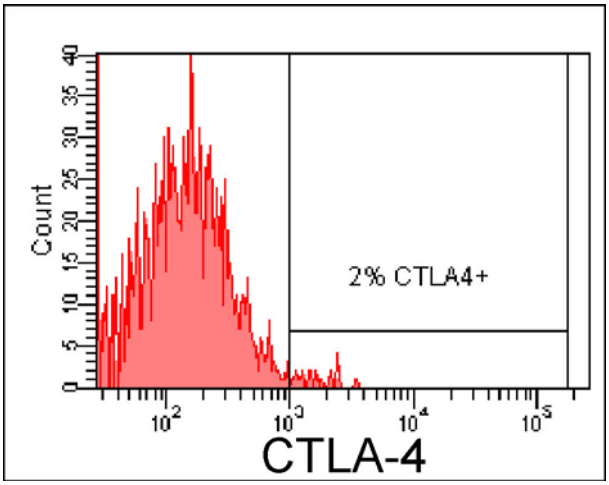
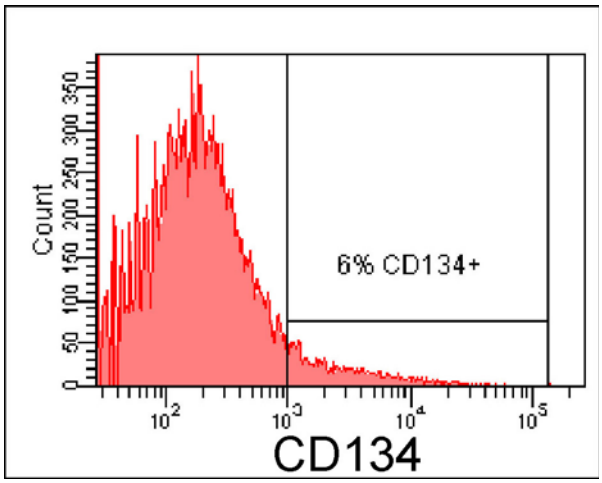


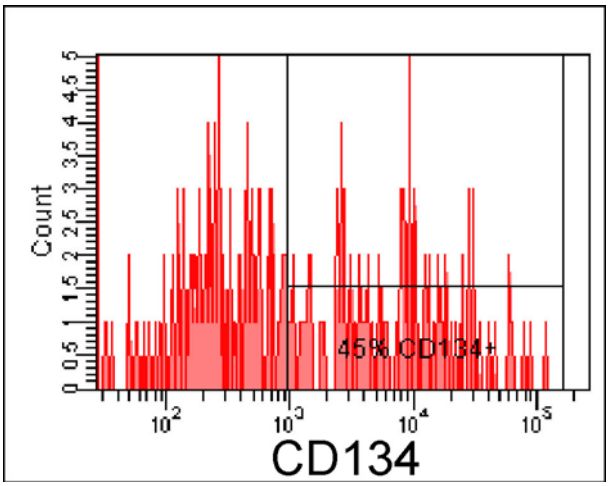
Figure 3.2.16 Expression of CD134 by regulatory T cells

Lymphocytes were isolated from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes of each rat and $CD4^+$ T cells were purified by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The negatively selected cells were stained with antibodies against CD134, CD25 and Foxp3. The proportion of resting T cells (A), activated T cells (B), $CD25^+$ $Foxp3^+$ regulatory T cells (C) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) expressing CD134 is expressed as a percentage. Representative of 3 experiments.

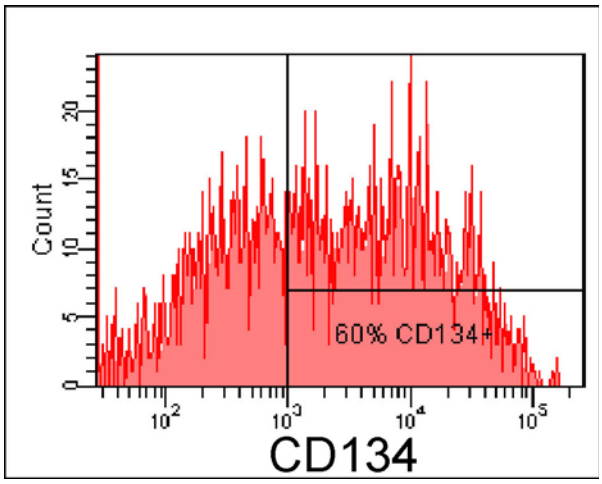
A Resting $CD4^+$ T cells



B Activated $CD4^+$ T cells



C $CD25^+$ $Foxp3^+$ T cells



D $CD25^-$ $Foxp3^+$ T cells

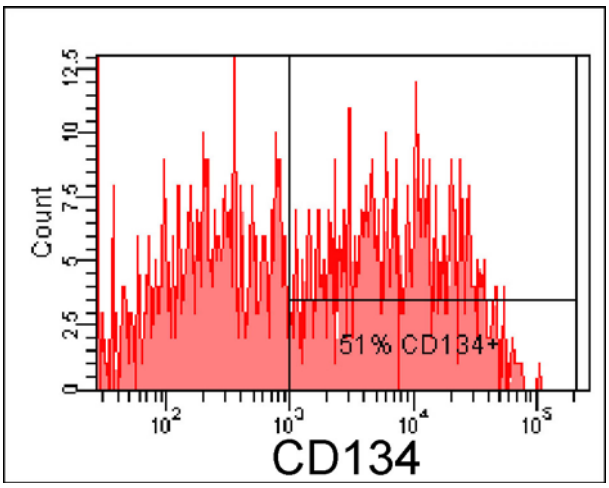


Table 3.2

The expression of cell surface molecules by resting, activated, $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells was determined by flow cytometry. The results are expressed either as the percentage of cells within each subpopulation that expresses the marker, or as the mean fluorescence intensity of the staining associated the molecule of interest. At least three independent experiments were performed per molecule.

Molecule expression (%$^{+}$)	Resting	Activated	CD25^{+} Foxp3^{+}	CD25^{-} Foxp3^{+}
CD71	4	8	13	8
MHCII	7	10	9	3
ICAM-1	6	18	30	19
CD103	1	14	6	4
CTLA-4	1	0.5	9	2
CD134	6	45	60	51

Molecule expression (MFI)	Resting	Activated	CD25^{+} Foxp3^{+}	CD25^{-} Foxp3^{+}
TCR	35996	39185	29043	26097
CD4	36970	41088	32495	31823
CD5	11918	12104	9691	10464
CD44H	23421	59944	44655	42975
CD43	3603	3225	2781	2296
LFA-1	15876	38847	25234	27209
CD62L	2918	3519	3224	2343
CD2	13186	16554	17973	14665

cells were partitioned by thoracic duct cannulation. The proportion of regulatory T cells remaining in the lymph nodes of the cannulated animals (mean 30.7%, SD=1.5) was more than 2 fold higher than in lymph nodes from intact animals (mean 11.5%, SD=2.3), and this was statistically significant (p=0.0002). In addition, the recirculatory T cell pool that was intercepted by thoracic duct cannulation over a 24h period was found to contain a significantly lower proportion of regulatory T cells (mean 8.0%, SD=1) compared with the proportion of $CD4^+$ T cells that remained in the lymph nodes (mean 30.7%, SD=1.5) (p= 0.0002) (Figure 3.2.17).

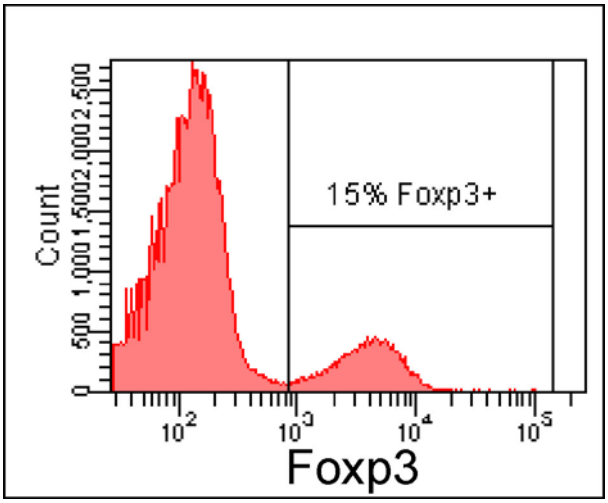
In order to investigate the recruitment of regulatory T cell subsets present in the thoracic duct lymph into the thymus, lymph nodes, spleen and intestinal mucosa, thoracic duct lymphocytes from normal CD45.2 DA rats were obtained and transferred into normal congenic CD45.1 DA rats. The phenotype of the $CD4^+$ T cells in the thoracic duct lymph was determined by flow cytometry, revealing that 0.4% were $CD25^+$ $Foxp3^-$ "activated" T cells, 5% were $CD25^+$ $Foxp3^+$, 3.2% were $CD25^-$ $Foxp3^+$ and 91.4% were $CD25^-$ $Foxp3^-$ "resting" T cells (see Table 3.3). After 24 h following the adoptive transfer, the thymuses, lymph nodes, spleen and intestinal mucosa were collected from the recipient and the proportions of donor and recipient conventional and regulatory T cell subsets were quantified by flow cytometry.

It was found that the proportions of host regulatory and conventional $CD4^+$ T cells in each compartment following the adoptive transfer were different to those found in normal animals (Table 3.3). In the thymus, a lower proportion of recipient $CD4^+$ $CD8^-$ T cells 2.4% (SD=0.06) were "activated" $CD25^+$ $Foxp3^-$ T cells, compared to 3.6% in normal unmanipulated animals (see Table 3.1), a difference that was statistically significant as assessed using a two tailed paired Student's t test (p=0.013). This decrease in the proportion of "activated" $CD4^+$ $CD8^-$ $CD25^+$ $Foxp3^-$ host cells in the thymus was accompanied by an increase in the proportion of resting $CD4^+$ $CD8^-$ $CD25^-$ $Foxp3^-$ host cells (mean 95.4%, SD=0.2)

Figure 3.2.17 Regulatory T cells are relatively sessile compared with other $CD4^+$ T cells

Thoracic duct lymph was collected over a 24 hour period and at the end of this period, cervical, celiac, mesenteric, inguinal and popliteal lymph nodes were removed and pooled. $CD4^+$ T cells were isolated immuno-magnetically from the lymphocytes as described previously, and then stained with antibodies against Foxp3. Lymph nodes were also taken from an age-matched animal and pooled in order to assess the proportion of regulatory T cells in the $CD4^+$ T cell population found in lymph nodes in an unmanipulated animal (A). It was found that $CD4^+$ T cells collected as thoracic duct lymph (B), contained proportionately less regulatory T cells than the more sessile $CD4^+$ T cells that remained within the lymph nodes (C). The proportion of $Foxp3^+$ cells in the sessile population compared with those in the efferent lymph was found to be significantly increased using a two tailed paired Student's t test ($p=0.0002$). Representative of 3 experiments.

A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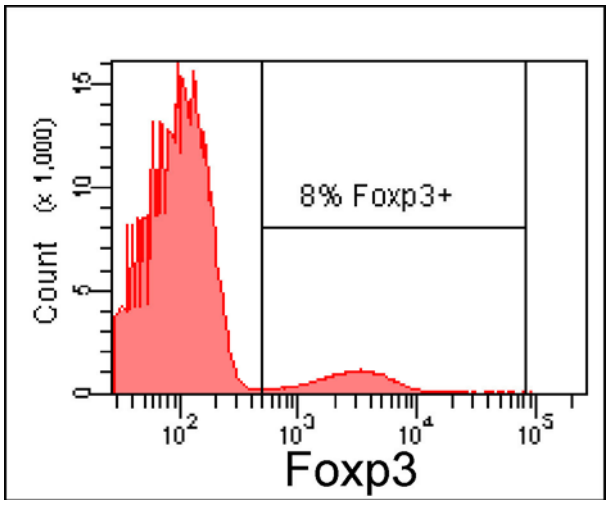
500
1,000
1,500
2,000
2,500
10^2
10^3
10^4
10^5
Foxp3

B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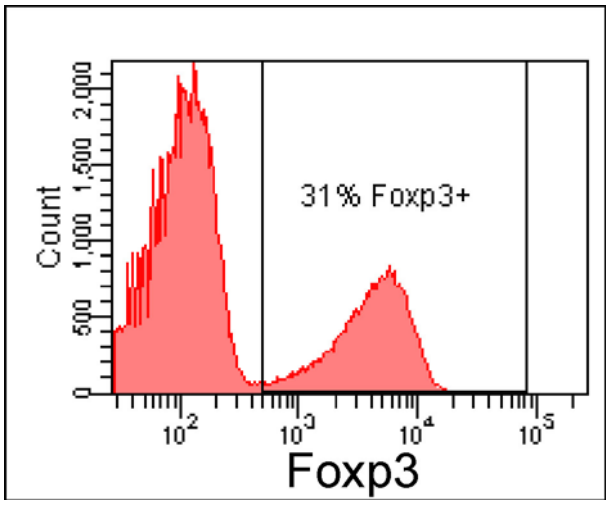
5
10
15
10^2
10^3
10^4
10^5
Foxp3

C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1,000
1,500
2,000
10^2
10^3
10^4
10^5
Foxp3

Table 3.3 The recruitment of $CD4^+$ T cell subsets from thoracic duct lymph into primary and secondary lymphoid tissue and the intestinal mucosa

Whole, unfractionated thoracic duct lymphocytes from normal DA CD45.2 donors were adoptively transferred into normal DA CD45.1 rats, and after 24 hours the host and donor cells were detected by flow cytometry in the thymus, lymph nodes, spleen and intestinal mucosa. The phenotype of host cells in the recipients, the proportion of donor cells and their phenotype were investigated by flow cytometry. Data represent results from four animals. Samples from 2 animals were pooled to enable the analysis of intestinal mucosa T cells (n=2).

Host T cells				
	Thymus	Spleen	Lymph node	Gut
$CD25^+$ $Foxp3^-$ activated T cells	1.4	1.9	1.7	21.1
$CD25^+$ $Foxp3^+$ T_{reg}	2.4	5.2	8.6	0.7
$CD25^-$ $Foxp3^+$ T_{reg}	0.8	2.1	3.2	1.2
$CD25^-$ $Foxp3^-$ resting T cells	95.4	88.0	86.5	77.1
Proportion of $CD4^+$ T cells that are donor derived	0.1	11.4	12.7	0.6
Donor T cells				
$CD25^+$ $Foxp3^-$ activated T cells	2.0	0.2	0.2	4.5
$CD25^+$ $Foxp3^+$ T_{reg}	17.3	4.4	3.5	4.6
$CD25^-$ $Foxp3^+$ T_{reg}	12.3	2.9	2.1	5.0
$CD25^-$ $Foxp3^-$ resting T cells	68.3	92.4	94.3	86.0
Phenotype of adoptively transferred $CD4^+$ TDL				
$CD25^+$ $Foxp3^-$ activated T cells	0.4			
$CD25^+$ $Foxp3^+$ T_{reg}	5			
$CD25^-$ $Foxp3^+$ T_{reg}	3.2			
$CD25^-$ $Foxp3^-$ resting T cells	91.4			

compared to the proportion found in normal animals (91.2%, see Table 3.1), a difference that was also statistically significant (p=0.007). The proportion of host $CD25^+$ $Foxp3^+$ regulatory T cells amongst $CD4^+$ $CD8^-$ cells following the adoptive transfer of thoracic duct lymphocytes was also lower (2.4%, SD=0.058) than that found in normal unmanipulated animals (4.1%, see Table 3.1) (p=0.03). However, the proportion of host $CD25^-$ $Foxp3^+$ putative regulatory T cells following the adoptive transfer (mean 0.8%, SD=0.1) was not significantly different from the proportion found in normal animals (1.2%, see Table 3.1) (p=0.07).

In the lymph nodes, the proportions of host activated, resting, and $CD25^+$ $Foxp3^+$ regulatory T cells (1.7%, 86.5%, and 8.6% respectively) were not significantly different from those found in normal animals (see Table 3.1) (p=0.11, p=0.07, and p=0.097 respectively). However, the proportion of host $CD25^-$ $Foxp3^+$ putative regulatory T cells in the lymph nodes (3.2%, SD=0.2) was significantly lower than that found in normal animals (4.5%) (p=0.025). In the spleen, the proportion of activated host $CD4^+$ T cells (1.93%, SD=0.058) was significantly higher than that found in normal animals (1.1%, see Table 3.1) (p=0.034). In contrast, the proportion of host $CD25^-$ $Foxp3^+$ putative regulatory T cells was much lower (2.1%, SD=0.15) compared with that found in normal animals (5.4%, see Table 3.1) (p=0.016). However, the proportions of host $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^-$ resting T cells in the spleen (5.2%, SD=3.5 and 90.7%, SD=0.5) respectively were no different to those found in normal animals (p=0.6 and p=0.8 respectively). T cell suspensions from the intestinal mucosa were also obtained (n=2) by pooling samples from 2 animals each. It was found that 21.1% (SD=2.8) of host $CD4^+$ T cells in the gut were $CD25^+$ $Foxp3^-$ activated cells, and strikingly, the proportion of $CD25^-$ $Foxp3^+$ putative regulatory T cells (1.15%, SD=0.07) was higher than the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells (0.65%, SD=0.07). The proportion of resting T cells was 77.1% (SD=2.8) amongst host $CD4^+$ T cells in the intestinal mucosa (Table 3.3).

The proportion of donor T cells in each compartment and their phenotype was also characterised, revealing that 0.15% (SD=0.057) of $CD4^+$ $CD8^-$ T cells in the thymus were donor derived, 12.65% (SD=0.83) of $CD4^+$ T cells in the lymph nodes were donor derived, 11.43% (SD=0.76) of splenic $CD4^+$ T cells were donor derived, and 0.65% (SD=0.23) of $CD4^+$ T cells in the intestinal mucosa were of donor origin (Table 3.3).

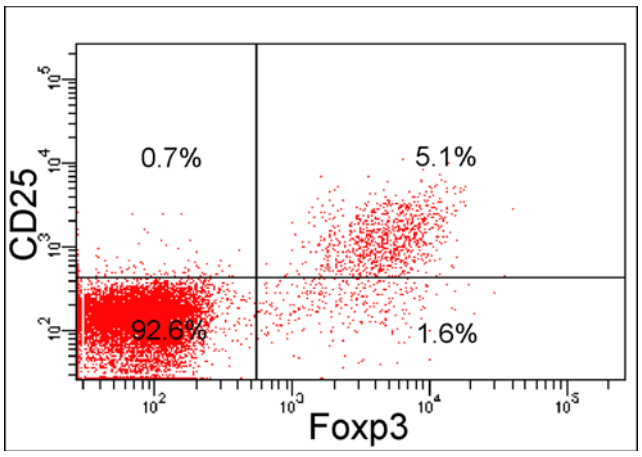
The phenotype of donor $CD4^+$ $CD8^-$ T cells that had localised to the thymus was examined (Figure 3.2.18), revealing that the proportion of activated T cells (mean 2%, SD=1) was comparable to the proportion found amongst thymic $CD4^+$ $CD8^-$ T cells from normal animals (1.13%, see Table 3.1) (p=0.2). While the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells amongst donor $CD4^+$ $CD8^-$ T cells in the thymus (mean 17.3%, SD=6.1) was consistently higher than the proportion found amongst $CD4^+$ $CD8^-$ T cells in normal animals (4.1%, see Table 3.1), this was not statistically significant (p=0.054). However, the frequency of $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 12.3%, SD=3.8) amongst donor $CD4^+$ $CD8^-$ T cells was significantly higher in the thymus compared to their frequency in normal thymic tissue (p=0.033). The proportion of resting $CD4^+$ T cells amongst donor cells in the thymus (mean 68%, SD=10) was not significantly different from that observed in normal thymic tissue (90.3%, see Table 3.1) (p=0.067).

In the lymph nodes, the proportion of donor $CD25^+$ $Foxp3^-$ “activated” T cells (mean 0.23%, SD=0.058) was not significantly different to those found in normal animals (11.2%, see Table 3.1) (p=0.08). However, the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells (mean 4.1%, SD=0.72) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 2.9%) amongst the donor $CD4^+$ T cells in the lymph nodes were significantly lower in comparison to their respective proportions in the lymph nodes of normal animals (10.6% and 4.8%, see Table 3.1) (p=0.022 and 0.0004 respectively). In addition, the proportion of resting $CD4^+$ T cells among the donor cells in the lymph nodes (mean 92.4%, SD= 0.59) was much higher

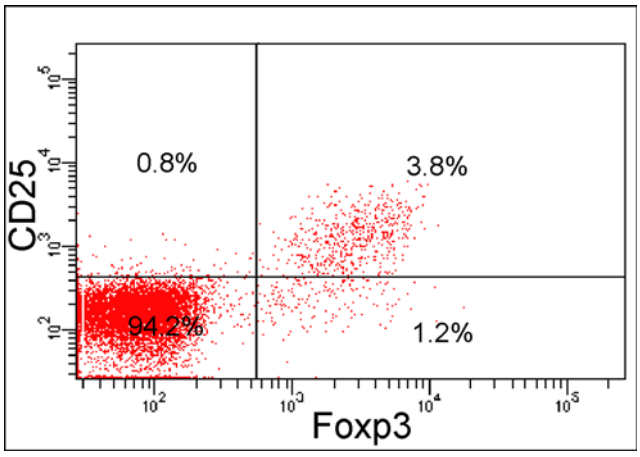
Figure 3.2.18 Recruitment of the putative tissue homing $CD25^-$ $Foxp3^+$ regulatory T cell subset

Unfractionated thoracic duct lymphocytes from RT7b CD45.2 donors were adoptively transferred intravenously to normal DA CD45.1 rats. Twenty four hours later, cell suspensions were prepared from the thymus, lymph nodes, spleen and the lamina propria of the small intestine of the recipients. The cells were stained with antibodies against CD4, CD25, Foxp3 and CD45.2 and analysed by flow cytometry to estimate the proportions of conventional $CD4^+$ T cells, $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells and $CD4^+$ $CD25^-$ $Foxp3^+$ regulatory T cells in the lymph nodes (A), spleen (B), intestinal lamina propria (C) and thymus (D). Data represent results from four animals. Samples from 2 animals were pooled to enable the analysis of intestinal mucosa T cells (n=2).

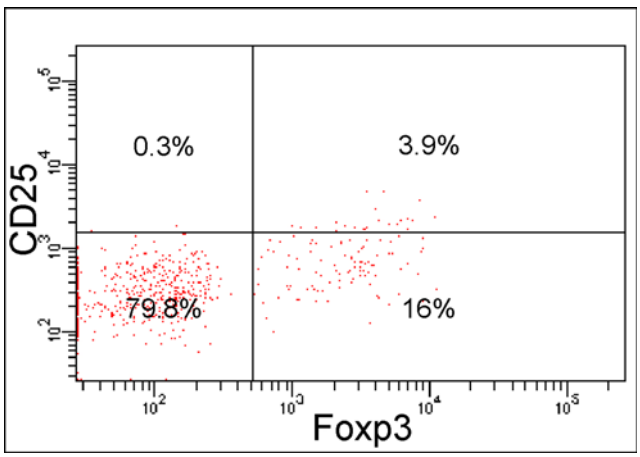
A


0.7%
5.1%
92.6%
1.6%
CD25
Foxp3

B


0.8%
3.8%
94.2%
1.2%
CD25
Foxp3

C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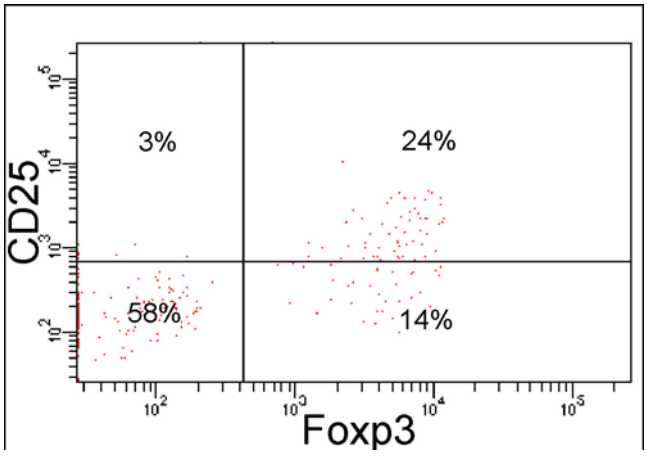
3.9%
79.8%
16%
CD25
Foxp3

D


3%
24%
58%
14%
CD25
Foxp3

than the proportion of resting $CD4^+$ T cells in the lymph nodes of normal animals (84%, see Table 3.1) (p=0.015).

When the phenotype of donor $CD4^+$ T cells in the spleen was analysed, it was found that a significantly lower proportion of both activated (mean 0.15%, SD=0.06), $CD25^+$ $Foxp3^+$ (mean 3.47%, SD=0.15), and $CD25^-$ $Foxp3^+$ (mean 2.1%, SD=0.26) donor T cells were present in the spleen compared to their proportions in normal animals (1.25%, 5.2%, and 5.1% respectively, see Table 3.1) (p=0.01, p=0.07 and p=0.02 respectively). The decreases in the proportions of activated, $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells among donor cells were accompanied by an increase in the proportion of resting T cells (mean 94.3%, SD=0.25) relative to the proportion found in normal animals (mean 87.9%, p=0.01).

In the intestinal tissues, the proportion of activated T cells was lower among donor $CD4^+$ T cells (mean 4.45%, SD=1.9) than host $CD4^+$ T cells (mean 21.1%, see Table 3.3), while the proportions of both donor $CD25^+$ $Foxp3^+$ regulatory T cells (mean 4.55%, SD=0.07) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 5.0%, SD=1.13) were higher than the proportions amongst host cells (mean 0.65% and 1.15% respectively, see Table 3.3). The enrichment of the $CD25^-$ $Foxp3^+$ putative regulatory T cell population within the donor cells that localised to the intestine, and the numerical superiority of the host $CD25^-$ $Foxp3^+$ putative regulatory T cells over their $CD25^+$ $Foxp3^+$ regulatory T cell counterparts, suggested they may represent a tissue homing subset of regulatory T cells. In order to determine whether $CD25^-$ $Foxp3^+$putative regulatory T cells are capable of localising to sites of inflammation, we adoptively transferred thoracic duct lymph from normal DA CD45.2 rats into DA CD45.1 rats that had received ~1 x 10^8 thoracic duct lymphocytes obtained from DA CD45.1 rats in the late prodomal stage (Spargo *et al.* 2001) of adjuvant-induced arthritis (on day 11 after the administration of CFA) 10 days earlier, and were experiencing adoptively transferred T cell mediated inflammation in their paws.

3.2.9 Determination of the destinations of regulatory T cell subsets found in thoracic duct lymph in an inflammatory setting

The adoptively transferred T-cell mediated inflammation that affected the paws of the DA CD45.1 recipients was scored as described in the Materials and Methods section, with total scores of 6, 5, 4 and 2 (out of a maximum of 16) observed at day 10 following the adoptive transfer. In order to analyse the inflamed synovium-rich tissues (SRT) of the arthritic recipients for the presence of donor cells, the hind paws were disarticulated, digested and the resulting cell suspension analysed along with cells recovered from the thymus, lymph nodes, spleens and intestinal mucosa. The phenotype of the $CD4^+$ T cells in each cell suspension was determined by flow cytometry. Additionally, the normal thoracic duct lymphocytes that were transferred to the recipient animals 10 days after they received thoracic duct lymphocytes from arthritic animals were also characterised. Approximately 1% of the adoptively transferred $CD4^+$ T cells were activated T cells, 5% were $CD25^+$ $Foxp3^+$ cells, 3% were $CD25^-$ $Foxp3^+$ cells, and 91% were resting T cells (see Table 3.4).

The phenotype of host T cells was examined by flow cytometry, revealing that in the thymus the proportions of activated (mean 1.8%, SD=0.58), $CD25^+$ $Foxp3^+$ (mean 0.73%, SD=0.06) and $CD25^-$ $Foxp3^+$ (mean 0.43%, SD=0.15) T cells were significantly lower than those found in normal animals (3.6%, 4.1% and 1.2% respectively) ($p=0.03$, $p=0.01$ and $p=0.02$ respectively) (see Table 3.4, Figure 3.2.19). The proportion of $CD25^-$ $Foxp3^-$ resting T cells however (mean 97%,

Table 3.4 The location of $CD4^+$ T cell subsets after the adoptive transfer of normal thoracic duct lymph into arthritic recipients

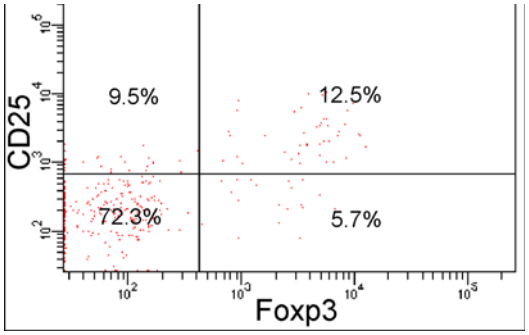
Whole, unfractionated thoracic duct lymphocytes from normal DA CD45.2 donors were adoptively transferred into arthritic DA CD45.1 rats on day 10 of adoptively transferred arthritis. After 24 hours, the host and donor cells were identified by flow cytometry in the thymus, lymph nodes, spleen, intestinal mucosa, and synovium rich tissues. The phenotype of host cells in the recipients, the proportion of donor cells and their phenotype were investigated by flow cytometry. Data represent results from four animals. Samples from 2 animals were pooled to enable the analysis of intestinal mucosa T cells and SRT T cells (n=2).

Host T cells					
	Thymus	Spleen	Lymph node	Gut	SRT
$CD25^+$ $Foxp3^-$ activated T cells	1.8	2.4	1.4	28.9	6.7
$CD25^+$ $Foxp3^+$ T_{reg}	0.7	2.1	4.0	0.6	0.55
$CD25^-$ $Foxp3^+$ T_{reg}	0.4	0.6	1.1	1.4	0.2
$CD25^-$ $Foxp3^-$ resting T cells	97.0	88.0	93.4	69.3	92.55
Proportion of $CD4^+$ T cells that are donor derived	0.2	15.4	17.2	0.6	0.21
Donor T cells					
$CD25^+$ $Foxp3^-$ activated T cells	7.7	0.2	0.4	9.3	14.3
$CD25^+$ $Foxp3^+$ T_{reg}	24.7	4.1	4.2	2.4	10.7
$CD25^-$ $Foxp3^+$ T_{reg}	10.0	2.2	1.5	2.2	2.7
$CD25^-$ $Foxp3^-$ resting T cells	57.7	93.4	93.6	86.2	72.35
Phenotype of adoptively transferred $CD4^+$ TDL					
$CD25^+$ $Foxp3^-$ activated T cells	0.4				
$CD25^+$ $Foxp3^+$ T_{reg}	5				
$CD25^-$ $Foxp3^+$ T_{reg}	3.2				
$CD25^-$ $Foxp3^-$ resting T cells	91.4				

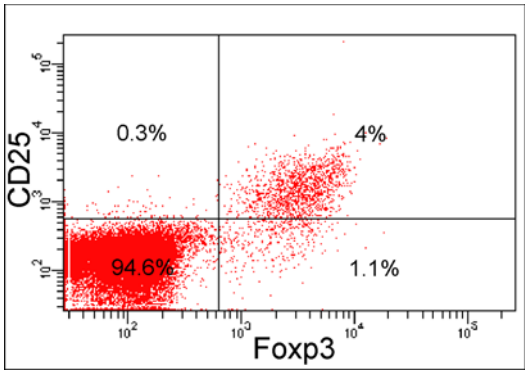
Figure 3.2.19 Recruitment of the putative tissue homing $CD25^-$ $Foxp3^+$ regulatory T cell subset to inflamed synovial tissues during adoptively transferred adjuvant-induced arthritis

Unfractionated thoracic duct lymphocytes from RT7b CD45.2 donors were adoptively transferred intravenously to DA CD45.1 rats 10 days after adoptive transfer of arthritis. Twenty four hours later, cell suspensions were prepared from the thymus, lymph nodes, spleen, intestinal mucosa and synovium rich tissues. The cells were stained with antibodies against CD4, CD25, Foxp3 and CD45.2 and analysed by flow cytometry to estimate the proportions of donor conventional $CD4^+$ T cells, $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells and $CD4^+$ $CD25^-$ $Foxp3^+$ regulatory T cells in the thymus (A), lymph nodes (B), spleen (C) intestinal lamina propria (D), and synovium rich tissues (E) were evaluated by flow cytometry. Data represent results from 4 animals. SRT and gut samples contained material pooled from 2 animals (n=2).

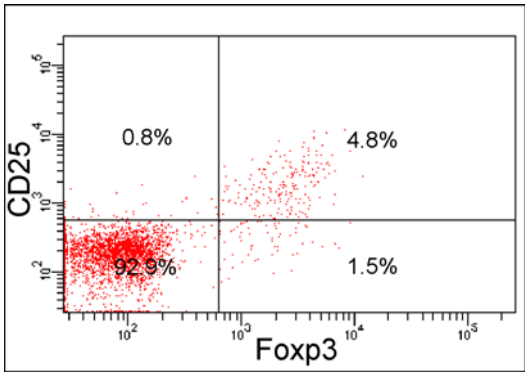
A


9.5%
12.5%
72.3%
5.7%
CD25
Foxp3

B


0.3%
4%
94.6%
1.1%
CD25
Foxp3

C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CD25
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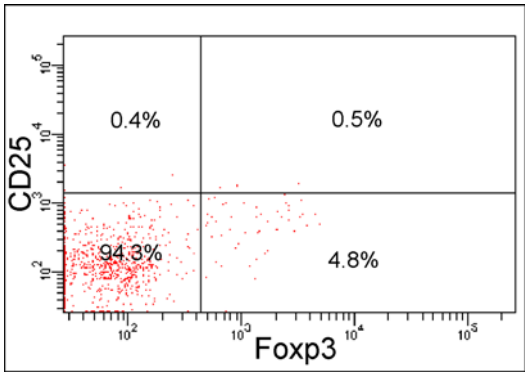

0.4%
0.5%
94.3%
4.8%
CD25
Foxp3

SD=0.46) was significantly higher than that found in normal animals (90.9%, see Table 3.1) (p=0.01).

In the lymph nodes, the proportion of activated (mean 0.22%, SD=0.03) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 2.9, SD=0.46) amongst host $CD4^+$ T cells were similar to those found in normal animals (0.6% and 4.8% respectively) (p=0.17 and p=0.08 respectively) (Table 3.4, Figure 3.2.19). However, the proportion of $CD25^+$ $Foxp3^+$ T cells (mean 4.43%, SD=0.23) was significantly lower than in normal animals (10.6%, see Table 3.1, p=0.01), and the proportion of resting T cells (mean 92.4, SD=0.67) was significantly higher (compared with 84.1%, see Table 3.1, p=0.004). In the spleen, the proportion of activated T host $CD4^+$ cells (mean 2.4%, SD=0.6) was no different to that found in normal animals (1.25%, see Table 3.1, p=0.11). However, the frequency of both $CD25^+$ $Foxp3^+$ regulatory (mean 2.1%, SD=0.26) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 0.63%, SD=0.06) was lower amongst host cells relative to their frequencies in normal animals (5.2%, p=0.03 and 5.75%, p=0.01 respectively, see Table 3.1). A corresponding increase in the frequency of resting T cells amongst host $CD4^+$ T cells (mean 94.9%, SD=0.38) compared to their frequency in normal animals (87.9%, see Table 3.1) was observed (p=0.006).

In the intestinal tissue, it was found that 9.3% (SD=0.7) of $CD4^+$ host T cells were of the activated phenotype, 2.4% (SD=2.2) were $CD25^+$ $Foxp3^+$ cells, 2.15% (SD=0.35) were $CD25^-$ $Foxp3^+$ cells, and 86.15% (SD=1.2) were resting T cells.
However, in the SRT 6.7% (SD=1.7) of $CD4^+$ host T cells were activated T cells, 0.55% (SD= 0.35) were $CD25^+$ $Foxp3^+$ cells, 0.2% (SD=0.14) were $CD25^-$ $Foxp3^+$ cells, and 92.6% (SD=1.9) were resting T cells. Thus little recruitment of host regulatory T cells into the site of inflammation was observed, while activated T cells were readily recruited.

Subsequently, the proportion of donor cells in each compartment was measured, revealing that in the recipient thymus 0.19% (SD=0.26) of $CD4^+$ $CD8^-$ cells were of donor origin, while 15.35% (SD=1.02) of splenic $CD4^+$ T cells and 17.15% (SD=1.19)

of lymph node $CD4^+$ T cells were donor derived (see Table 3.4). In the intestinal tissue, 0.56% (SD=0.04) of $CD4^+$ T cells were donor derived, while in the SRT 0.21% (SD=0.0002) of cells were of donor origin.

The phenotype of the normal donor cells recruited into the thymuses, lymph nodes, spleens, intestines and synovium rich tissues of arthritic rats was then evaluated. Interestingly, it was found that higher proportions of activated (mean 7.67%, SD=6.8), $CD25^+$ $Foxp3^+$ regulatory T cells (24.7%, SD=13.6), and $CD25^-$ $Foxp3^+$ putative regulatory T cells (10%, SD=8.89) were present among donor cells in the thymus relative to those found in normal animals (3.9%, 4.05% and 1.15% respectively, see Table 3.1) although these differences were not significant (p=0.42, p=0.13 and p=0.23 respectively). The proportion of donor $CD25^-$ $Foxp3^-$ "resting" T cells in the thymus was 57.7% (SD=15.3) (see Table 3.4).

In the lymph nodes, the proportions of activated (mean 0.23%, SD=0.06) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 4.13%, SD=0.15) amongst donor $CD4^+$ T cells were not significantly different to those found in normal animals (0.6% and 4.8% respectively) (p=0.15 and p=0.07 respectively). However, the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells (4.13%, SD=0.15) amongst donor cells was significantly lower than in the proportion seen in normal animals (10.6%, p=0.01). The proportion of resting cells within the donor $CD4^+$ T cell population in the lymph nodes (93.4%, SD=1.07) was significantly higher compared to that in normal animals (84%, p=0.004). In the spleen, the proportion of activated T cells (0.4%, SD=0.1) in the donor $CD4^+$ T cell population was less than the proportion observed in normal animals (1.25%, see Table 3.1), although this was not statistically significant (p=0.053). However, a significantly lower proportion of both $CD25^+$ $Foxp3^+$ regulatory (mean 4.17%, SD=0.085) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (mean 1.47%, SD=0.32) within the donor population was observed when compared to their respective proportions in normal animals (5.2% and 4.7%) (p=0.005 and p=0.003). An increase in the proportion of resting T cells (93.6%, SD=1.63) occurred relative to resting T cells in the spleens of normal animals (87.9%, p=0.008). The population of donor cells that had infiltrated into the intestinal tissues of arthritic recipients was

composed of 9.3% (SD=0.7) activated T cells, 2.4% (SD=2.3) $CD25^+$ $Foxp3^+$ T cells, 2.15% (SD=0.35) $CD25^-$ $Foxp3^+$ T cells, and 86.15% (SD=1.2) resting T cells (Table 3.4), thus containing a lower proportion of activated T cells compared to the donor cells that infiltrated into the intestinal tissues of normal animals, and a higher proportion of $CD25^+$ $Foxp3^+$ regulatory T cells. In the SRT, it was found that 14.25% (SD=5.6) of donor cells were activated, 10.7% (SD=6.7) were $CD25^+$ $Foxp3^+$ cells, 2.7% (SD=2.26) were $CD25^-$ $Foxp3^+$ cells, and 72.35% (SD=14.6) were resting cells. Thus, the preferential recruitment of activated donor cells into the SRT of arthritic recipients may account for the lower proportion of activated cells homing to their intestinal tissues relative to the proportion observed in the intestinal tissues of normal recipients.

3.3 Discussion

3.3.1 The distribution of regulatory T cells in lymphoid tissues

The relatively low expression of Foxp3 in the thymus of adult rats (Figure 3.2.1) compared with the periphery suggests that thymic regulatory T cells may expand upon arrival in the periphery, or that their conventional T cell counterparts are subjected to further elimination of self reactive cells once they reach peripheral self antigens. Alternatively, it may be that some recent thymic emigrants (RTE) that do not express Foxp3 may be converted into regulatory T cells upon their encounter of self antigens as they arrive in the periphery. Indeed, it has been shown that RTE can be converted into functionally suppressive cells (Modigliani *et al.*1996), which may be the result of this process. It was found that amongst the secondary lymphoid organs, the celiac lymph nodes that drain the liver contained a consistently higher proportion of $Foxp3^+$ T cells. The higher proportion of these regulatory T cells in the celiac lymph node may reflect the immunosuppressive environment of the liver, which contains large numbers of DC and macrophages and that secrete IL-10, TGF-β and prostaglandin (Lu *et al.* 2001, Knolle 2000), creating a cytokine environment that inhibits costimulatory molecule expression and maturation of APC. Interestingly,

despite the fact that regulatory T cells comprise 10-15% of $CD4^+$ T cells in the lymph nodes, a much smaller proportion of $CD4^+$ T cells in thoracic duct lymph were regulatory T cells. This behaviour may result from antigen recognition, which may delay the exit of regulatory T cells from the lymph nodes. Alternatively, the expression of chemokine receptors and adhesion molecules by regulatory T cells may be different from other T cells, resulting in different recirculatory patterns.

3.3.2 Expression of activation-associated markers by regulatory T cells

The expression of the high affinity IL-2 receptor α chain (CD25) was the first characterised phenotypic feature of regulatory T cells (Sakaguchi *et al.* 1995), but since it is also associated with activated T cells (Cantrell and Smith 1983), it cannot specifically identify regulatory T cells. In order evaluate CD25 as a marker of both regulatory T cells and activated T cells in the rat, we investigated the coexpression of CD25 and Foxp3 by $CD4^+$ T cells. Strikingly, it was found that approximately 90% of $CD25^+$ cells are $Foxp3^+$ in the rat, with the remaining minority consisting of $CD25^+$ $Foxp3^-$ activated T cells (Figure 3.2.2). The brightest half of $CD25^+$cells ($CD25^{bright}$) were found to be almost all regulatory T cells (98-99%), indicating that it may be possible to purify rat regulatory T cells by flow sorting $CD4^+$ $CD25^{bright}$ cells. The expression of CD25 is transiently induced upon contact with cognate antigen (Cantrell and Smith 1983), and so its expression by regulatory T cells may arise from repetitive interactions with self-antigen. In this case, $CD25^-$ $Foxp3^+$ cells may represent regulatory T cells that have not seen their antigen for some time. Alternatively, if CD25 is only transiently induced in regulatory T cells, albeit for a longer duration, $CD25^+$ $Foxp3^+$ may represent freshly anergised T cells while $CD25^-$ $Foxp3^+$ cells may represent much older regulatory T cells.

The transferrin receptor CD71 mediates iron uptake into cells, where it is required as a cofactor for the efficient function of enzymes (Daniels *et al.* 2006). The use of antibodies that block the transferrin receptor have been shown to inhibit T cell

proliferation, presumably due to iron deprivation (Kemp *et al.* 1987). Upon activation, T lymphocytes express CD71 soon after the induction of CD25, and require the expression of the transferrin receptor for their subsequent DNA synthesis and division (Neckers and Cossman 1983). CD71 can therefore be loosely regarded as a marker of activation in T cells, and its expression may be indicative of the capacity of a population of T cells to proliferate. The expression of the transferrin receptor by activated $CD25^+$ $Foxp3^-$ T cells was observed higher relative to resting cells (Figure 3.2.3). Interestingly, a greater proportion of both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed CD71 compared with resting T cells, suggesting that they have a greater capacity for proliferation that resting T cells. This is an aspect of their biology that will be investigated in more detail in Chapter 5.

The presence of MHC II on $CD4^+$ T cells is thought to be an indicator of recent activation, as MHC II molecules are transferred from APC to T cells through an activation-dependent process (Patel *et al.* 2001). Additionally, $CD4^+$ T cells may also be able to synthesise MHC II endogenously, although this activity is also restricted to activated T cells (Mannie *et al.* 2004). In these studies it was found that while a consistently greater proportion of activated T cells expressed MHC II compared with resting cells, this was not statistically significant (Figure 3.2.4). However, both resting and activated T cells contained an MHC II^{bright} population (MFI>10^4 units), which was not present in either the $CD25^+$ $Foxp3^+$ regulatory T cell or $CD25^-$ $Foxp3^+$ putative regulatory T cell populations. The MHC II^{bright} population may represent recently activated T cells, which would be expected to lose MHC II expression by dilution through mitosis, if it is not lost through a more active process. While a consistently lower proportion of $CD25^+$ $Foxp3^+$ cells expressed MHC II compared with activated cells, the proportion was consistently higher than for resting cells. However, a significantly lower proportion of $CD25^+$ $Foxp3^+$ cells were $MHCII^{bright}$ compared with resting cells. The dull expression of MHC II by $CD25^+$ $Foxp3^+$ regulatory T cells observed indicates that these cells are unlikely to have been activated by APC, and may have acquired MHC II molecules following unproductive encounters with them that have not culminated in T cell activation. Interestingly, $CD25^-$ $Foxp3^+$ putative regulatory T cells had both a significantly lower proportion of MHC II^+ and $MHCII^{bright}$

cells compared with resting cells, suggesting they are unlikely to be the transiently activated T cell subset described in humans (Allan *et al.* 2007).

3.3.3 Regulatory T cells display characteristic features of failed activation

The concept of regulatory T cells as a lineage, distinct from T cells that have failed to activate upon antigen recognition and have entered an anergic state, is based on the contentious interpretation of observations. While T cells that are anergised i*n vitro* strongly resemble regulatory T cells, and share both their dependency on IL-2 for proliferation, and their propensity to inhibit T cell activation, the possibility that regulatory T cells are T cells that have been anergised *in vivo* has been ruled out by some groups (Kuniyashi *et al.* 2000).

The principal reason for why Kuniyashi and colleagues concluded that regulatory T cells could not be T cells that have been anergised *in vivo*, is that the preparations of "anergised" T cells they produced did not bear suppressor activity and were not dependent on IL-2 for proliferation, which are two well established features of T cells that have been anergised through antigen presentation in the absence of sufficient costimulation (Marelli-Berg *et al.* 1997, Kubsch *et al.* 2003, Frasca *et al.* 2003). Furthermore, their protocol for testing anergised T cells *in vivo* involved removing the cells that have become $CD25^+$, and testing the cells that have remained $CD25^-$. This approach is problematic as anergised T cells are in fact $CD25^+$ (Taams *et al.* 1998), and thus Kuniyasu and colleagues were not investigating the correct population of cells.

T cells that have been anergised share many phenotypic markers with regulatory T cells, including programmed death 1, CTLA-4, TGF-β receptor 1, and IL-10 (Taams *et al.*1998, Lechner *et al.* 2001). Anergised T cells also express CD25, and like regulatory T cells, some of them are also $CD25^-$ (Taams *et al.* 1998, Zelaney *et al.* 2005). However, the lack of CD25 expression by a proportion of T cells anergised *in*

vitro has been given as a basis to exclude the possibility that regulatory T cells may represent T cells that have been anergised *in vivo* (Kubsch *et al.* 2003).

Therefore, we have undertaken to determine whether regulatory T cells, have a low expression of the TCR and CD4 coreceptor, which is a characteristic feature of anergised T cells (Jordan *et al.* 2000). The degradation of the TCR signalling complex following antigen stimulation in anergised T cells occurs due to a lack of protein kinase C activation following TCR ligation, which redirects internalised TCR complexes through a recycling pathway back to the cell surface in activated T cells (Utzny *et al.* 2006). When we examined regulatory T cells frothier expression of CD4 and the TCR, we found that both $CD25^{+}$ $Foxp3^{+}$ regulatory T cells and $CD25^{-}$ $Foxp3^{+}$ putative regulatory T cells expressed significantly lower levels of both the TCR and CD4 compared with resting T cells as determined using a two tailed paired Student's t test (Figures 3.2.5 and 3.2.6 respectively). This finding indicates that regulatory T cells bear features characteristic of anergised T cells in addition to those that have already been described (Lechner *et al.* 2001, Hickman *et al.* 2006). Therefore, these findings will be extended upon in future experimentation that will include observations of regulatory T proliferation and their relationship with RTE, some which may be rendered anergic as they enter the periphery and encounter self antigen. Furthermore, in Chapter 6 I will endeavour to elucidate the suppressive mechanisms of regulatory T cells armed with the information that is already known about the regulatory mechanisms of anergised T cells, to determine how the process of regulation occurs.

3.3.4 Expression of adhesion molecules by regulatory T cells

The expression of several adhesion molecules by regulatory T cells and conventional T cells was measured to assess whether differences exist between the two subsets that may be reflected in their migration characteristics *in vivo*. CD44 is a key adhesion molecule that binds to hyalurnoic acid, a key component of the extracellular matrix (Peach *et al.* 1993). CD44 is thought to mediate lymphocyte adhesion to the hyalurnoic acid coated surface of HEV (Huet *et al.* 1989) and has been shown to be

required for the homing of lymphocytes to the Peyer's patches (Jalkanen *et al.* 1987). It was found that a significantly greater amount of CD44 could be detected on the surface of both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells compared to resting T cells (Figure 3.2.7). This finding would suggest that regulatory T cells are relatively sessile, as they may tend to adhere more efficiently to the hyalurnoic acid found on HEV and traffic out of the recirculating pool into the lymph nodes. Indeed, regulatory T cells were found to be relatively sessile compared with their conventional T cell counterparts (Figure 3.2.17). Future studies in which the efficacy of ablating regulatory T cell adhesion to the HEV by disrupting the binding of CD44 to hyalurnoic acid could determine the contribution of CD44 to the adhesion of regulatory T cells to HEV, which if disrupted may affect their ability to suppress T cell responses *in vivo*.

The adhesion molecule leukosialin (CD43), is a highly glycosylated protein that appears to regulate T cell adhesion, activation and apoptosis (Mattoili *et al.* 2004). Although initial investigations indicated that T cells have an enhanced ability to undergo homotypic adhesion and to bind fibronectin and ICAM-1 when they lack CD43 (Manjunath *et al.* 1995), further work demonstrated that in normal animals CD43 promotes integrin-mediated adhesion and enhances the binding of T cells to fibronectin (Sanchez-Mateos *et al.* 1995). The expression of CD43 has been shown to inhibit apoptosis by blocking Fas-Fas ligand signal transductions (He and Bevan 1999), and it is also actively downregulated by apoptotic cells during apoptosis such that T cells with a low expression of CD43 are either susceptible to apoptosis, or have already entered an apoptotic pathway (Nusbaum *et al.* 2004). The enhanced adhesion of T cells lacking CD43 to fibronectin and ICAM-1 that has been observed in knock out mice (Manjunath *et al.* 1995) may be complicated by the constitutive role of CD43 in preventing apoptosis, as it is likely that the population of T cells that survive in CD43 knock out mice represent only the T cells most resistant to apoptosis in normal animals, (Mattoili *et al.* 2004). Our findings indicated that almost all resting T cells express CD43, while activated T cells contained a greater proportion of $CD43^-$ cells (Figure 3.2.8). $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells had an even greater proportion of $CD43^-$ cells, and those cells that

did express CD43 expressed it at a lower level. The low expression of CD43 expression by some regulatory T cells that we measured may permit their spontaneous apoptosis, and is consistent with reports that regulatory T cells are an apoptosis-prone subset of T cells (Taams *et al.* 2001).

ICAM-1 is an adhesion molecule predominantly expressed by APC that binds to LFA-1 to facilitate interactions of T cells with APC. ICAM-1 plays a key role in stabilising the structure of the immunological synapse during T cell activation, to ensure efficient signalling (Bromley *et al.* 2002). Its expression is also induced upon T cell activation, where it may either serve as an adhesion molecule to enable the attachment of lymphocytes to sites of inflammation, or to compete with ICAM-1 on the surface of APC to enable disengagement (Dougherty *et al.*1988). It was found a significantly higher proportion of activated T cells expressed ICAM-1 compared with resting T cells (Figure 3.2.9), and furthermore, an ICAM-1^{high} subpopulation was present in the activated subset of $CD4^+$ T cells.
Although a greater proportion of both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed ICAM-1 compared with resting T cells, no ICAM-1^{high} subpopulation was present. Thus, the ICAM-1^{high} cells that were contained within the activated T cell subset may represent cells that have recently been activated, as ICAM-1 is strongly induced during T cell activation (Dougherty *et al.* 1988, Bromley *et al.* 2002). The reason for this strong induction of ICAM-1 is not clear, but may enable the disengagement of activated T cells bound to APC through ICAM-1 by competing for LFA-1 on surface of T cells. Alternatively, ICAM-1 may be induced to allow the adhesion of T cells to inflamed endothelium at sites of inflammation, so that they may extravasate and participate on local immune responses (Stanciu and Djukanovic 1998).

LFA-1 is an adhesion molecule that binds to ICAM-1, CD43, and several other adhesion molecules (Khunkaewla et al. 2007). Thus it can facilitate the recruitment of T cells to HEV, the endothelium of the lamina propria in the gut, and on other endothelium where ICAM-1 is expressed (Dougherty *et al.*1988). In addition LFA-1 on the surface of T cells enables the formation of immunological synapses with antigen

presenting cells and thus is important fort cell activation (Bromley *et al.* 2002). LFA-1 was found to be expressed by all $CD4^+$ T cells, although it was noted that activated, $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed significantly higher levels of LFA-1 compared with resting T cells as determined using a two tailed paired Student's t test (Figure 3.2.10). The higher expression of LFA-1 by regulatory T cells and activated T cells compared with resting T cells might indicate that regulatory T cells and activated T cells have recently contacted their antigen, as it is known that LFA-1 is expressed at higher levels on antigen experienced T cells (Dustin and Springer 1991). These findings also suggest that regulatory T cells may be able to localise to sites of inflammation through the interaction of the LFA-1 molecules on their plasma membrane with ICAM-1 molecules expressed in inflamed tissues.

L-selectin (CD62L) is an adhesion molecule that facilitates the localisation of lymphocytes to secondary lymphoid tissue (Rainer 2002). While naïve T cells are known to express CD62L, it is downregulated after encounter with antigen, which may then enable recirculating T cells to avoid recruitment into quiescent secondary lymphoid tissue and instead localise to sites of inflammation in the periphery (Bradley *et al.* 1994). The expression of CD62L by activated T cells as determined by mean fluorescence intensity was significantly higher than the expression of CD62L by resting T cells (Figure 3.2.11). $CD25^-$ $Foxp3^+$ putative regulatory T cells were found to express CD62L at significantly lower levels relative to resting T cells. This may suggest that $CD25^-$ $Foxp3^+$ putative regulatory T cells are antigen experienced cells and may be less likely to localise to the lymph nodes. Combined with their high expression of LFA-1, the low expression of CD62L suggests that perhaps $CD25^-$ $Foxp3^+$ putative regulatory T cells may continue to recirculate past the HEV of secondary lymphoid tissue and localise to the lamina propria or other sites of inflammation. In contrast, a higher proportion of $CD25^+$ $Foxp3^+$ cells expressed CD62L compared with resting T cells, suggesting that they may be more likely to enter secondary lymphoid tissue through HEV.

CD2 is an adhesion molecule expressed by $CD4^+$ T cells that binds to LFA-3, which is expressed by endothelial cells, APC and lymphocytes (Wilkins *et al*. 2003). The CD2-LFA-3 interaction is thought to contribute to antigen recognition by positioning the interacting TCR and MHC molecules on the opposing T cell and APC membranes (van der Merwe *et al.*1995). The disruption of the CD2-LFA-3 interaction results in a reduction in T cell activation, and recent evidence suggests CD2 may play a key role in T cell activation by aiding the internalisation of TCR signalling complexes to effectively reset TCR signalling soon after the establishment of contact with APC (Singleton *et al.* 2006). It is known that CD2 is expressed at higher concentrations on the surface of antigen-experienced memory T cells (Wallace *et al.* 1990), and it is upregulated upon activation (Cantrell *et al.* 1988), suggesting that CD2 upregulation may signify antigen experience. CD2 was expressed on all subsets of $CD4^+$ T cells, although activated T cells and $CD25^+$ $Foxp3^+$ T cells expressed significantly higher levels of CD2 compared with resting T cells (Figure 3.2.12). Interestingly, $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed the same amount of CD2 compared with resting T cells. The higher levels of CD2 on activated and $CD25^+$ $Foxp3^+$ regulatory T cells may indicate they have recently encountered their antigen, while the lower levels of CD2 on $CD25^-$ $Foxp3^+$ putative regulatory T cells may indicate either that they have not contacted their antigen, or that they have not recently encountered their antigen.

The adhesion molecule $\alpha_E\beta_7$ integrin (CD103) binds to E-cadherin, which is exclusively expressed in the gut epithelium and by gut-residing DC. CD103 is thought to facilitate the localisation of T cells to mucosal tissues and this process is thought to be important for the maintenance of tolerance to dietary antigens passing through the gut through the recruitment of regulatory T cells (Schon *et al.* 1999). However, studies have shown that the absence of CD103 expression on regulatory T cells does not prevent their migration to the gut or their ability to suppress colitis (Annacker *et al*. 2005). It was found that a low proportion of resting T cells expressed CD103, while a much higher expression of CD103 was observed on activated T cells (Figure 3.2.14). The proportion of $CD25^+$ $Foxp3^+$ regulatory T cells that expressed CD103 was less than half that of activated T cells, while $CD25^-$ $Foxp3^+$ putative regulatory T cells had an even lower expression of CD103. Interestingly, $CD103^{bright}$ cells (MFI>10^4 units)

were only observed in $CD25^+$ activated T cell and $CD25^+$ $Foxp3^+$ regulatory T cell subsets, which may represent T cells in which CD103 has recently been induced. The relatively low expression of CD103 on regulatory T cells may indicate that only a low proportion of regulatory T cells are recruited into mucosal tissues, however, since it appears to be functionally redundant on regulatory T cells, $CD103^-$ regulatory T cells also may home into the gut to maintain tolerance to mucosal antigens (Annacker *et al*. 2005).

3.3.5 Expression of costimulatory and inhibitory molecules by regulatory T cells

Regulatory T cells have been reported to constitutively express CTLA-4, an inhibitory molecule normally induced during antigen presentation to modulate T cell activation (Takahashi *et al.* 2000). Although the mechanisms through which regulatory T cells inhibit the proliferation of other T cells remains unclear, CTLA-4 has been implicated in mediating the suppressive activity of regulatory T cells (Sansom and Walker 2006), although it is not absolutely required for suppression to be observed (Tang *et al.* 2004). It was found that a low proportion of resting and activated $CD4^+$ T cells expressed CTLA-4 (Figure 3.2.15). Although a greater proportion of $CD25^+$ $Foxp3^+$ regulatory T cells expressed CTLA-4 compared to resting cells, this amounted to only 10% of the population expressing CTLA-4. Interestingly, the expression of CTLA-4 by $CD25^-$ $Foxp3^+$ putative regulatory T cells was lower than their $CD25^+$ $Foxp3^+$ counterparts, suggesting that the two subsets may have different capacities to suppress T cell proliferation. In Chapter 5 this issue will be addressed through a direct comparison of the suppressive capacities of the $CD25^+$ $Foxp3^+$and $CD25^-$ $Foxp3^+$ subsets.

CD134 is a costimulatory molecule induced following T cell activation that is believed to maintain antigen-experienced cells by providing survival signals to them (Weinberg *et al.* 2004). Our findings indicate that while a low proportion of resting $CD4^+$ T cells express CD134, a significantly higher proportion of activated T cells express CD134, which would be expected to include some cells transitioning to a memory T cell role

(Figure 3.2.16). Strikingly, the majority of both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative T cells express CD134, suggesting they may be antigen experienced cells. The finding that regulatory T cells express high levels of CD134 is interesting when one considers that they are reportedly more susceptible to apoptosis than other T cell subsets (Kasprowicz *et al.* 2005). Since the major function of CD134 in T cells appears to be the provision of survival signals, CD134 may play a prominent role in enabling the survival of regulatory T cells. Thus, future studies could examine the role of CD134 in the maintenence of regulatory T cells *in vivo*, as it may ultimately provide a therapeutic target in situations where the manipulation of regulatory T cell survival is desired.

CD5 is a glycoprotein expressed by both thymocytes and peripheral T cells (Berney *et al.* 2001) that is induced during the maturation of T cells directly in proportion to the avidity of the TCR-MHC interaction (Azzam *et al.* 1998). It is thought that CD5 inhibits TCR signal transduction and thus effectively tunes the strength of TCR signal transductions by reducing their strength within T cells that bind MHC molecules more strongly (Azzam *et al.* 2001). It has recently been shown that mice lacking CD5 experience an accumulation of apoptotic activated T cells in their spleens and lymph nodes, which is thought to result excessively strong TCR signalling that occurs in the absence of CD5 (Axtell *et al.* 2004).
Interestingly, while activated T cells had a higher expression of CD5 compared with resting T cells, both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells had a much lower expression of CD5 (Figure 3.2.7).

This finding contradicts the expectation that regulatory T cells would have a higher expression of CD5, since they are believed to be represent T cells with a high avidity for self antigens (Caton *et al.* 2004). However, their affinity for self-antigens may still be high, provided they recognise prevalent antigens, since affinity is a function of the sum of interactions between molecules of a certain avidity. This is consistent with reports that suggest that regulatory T cells are maintained in the periphery through continuous interactions with the antigens they recognise (Fisson *et al.* 2003).

3.3.6 The destinations of regulatory T cells in thoracic duct lymph

The significantly lower proportion of Foxp3$^+$ cells in thoracic duct lymph compared with the proportion of Foxp3$^+$ cells in the lymph nodes would suggest that regulatory T cells may be relatively sessile (Figure 3.2.1). In order to determine if regulatory T cells are relatively sessile, the recirculating and sessile pools of T cells were partitioned through thoracic duct cannulation. It was found that the recirculating pool of CD4$^+$ T cells contained a significantly lower proportion of Foxp3$^+$ regulatory T cells compared with the sessile pool remaining within the lymph nodes of the cannulated animal, indicating that regulatory T cells are more sessile compared with other CD4$^+$ T cells (Figure 3.2.17). This tendency may result from the recognition of self-antigens on the surface of HEV or on APC within the lymph nodes by regulatory T cells, or their pattern of adhesion molecule expression. In any case their sessile lifestyle may reflect their need for exogenously produced IL-2 for proliferation that they acquire from other T cells within the lymph nodes.

In order to investigate the destinations of regulatory T cells returning back into the bloodstream, we adoptively transferred thoracic duct lymphocytes into congenic hosts and recovered donor cells from the thymus, secondary lymphoid tissues and also the lamina propria to determine if any localise to peripheral tissue.

Following adoptive transfer, we determined that donor CD25$^-$ Foxp3$^+$ putative regulatory T cells were present at a higher frequency in the thymus among donor CD4$^+$ (CD8$^-$) T cells than in normal animals, although other subsets of donor CD4$^+$ T cells were present in proportions comparable to those seen in normal animals. Thus, CD25$^-$ Foxp3$^+$ putative regulatory T cells appear to enter the thymus in greater numbers than other CD4$^+$ T cells. This tendency may even be underestimated by our experimental approach as CD4$^+$ thoracic duct lymphocytes contain lower proportions of CD25$^-$ Foxp3$^+$ putative T cells than CD4$^+$ T cells in peripheral blood do, and thus

an even greater entry of $CD25^-$ $Foxp3^+$ putative T cells into the thymus may occur under normal physiological conditions. Similarly, the entry of $CD25^+$ $Foxp3^+$ regulatory T cells into the thymus may be underestimated by our experimental approach. In order to measure the entry of T cells from the blood into the thymus more accurately, it would be necessary to use a different experimental approach, as the adoptive transfer of peripheral blood $CD4^+$ T cells is constrained by the limited volume of blood that could be collected from donor animals. The limited numbers of blood $CD4^+$ T cells that could be used for an adoptive transfer would be unlikely to yield useful results. Alternatively, it may be possible to use a thymic grafting approach similar to that reported for examining thymic output (Berzins *et al.*1999), using DA CD45.1 donor thymuses and DA CD45.2 recipients. The CD45.1 thymic grafts could then be examined to measure the entry of host (CD45.2) $CD4^+$ T cell subsets once the animals have recovered from surgery.

Following adoptive transfer, it was observed that both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells were present at lower frequencies in the spleen and lymph nodes among donor cells compared with their respective frequencies in normal animals. This finding likely reflects the relatively sessile behaviour of regulatory T cells under normal physiological conditions (Figure 3.2.17), which may explain their low frequencies in thoracic duct lymph (Table 3.1), and thus the low proportion of regulatory T cells amongst the donor cells in the lymph nodes and spleen.

Interestingly, we found that $CD25^-$ $Foxp3^+$ putative regulatory T cells were enriched within the donor cells that had entered the intestinal tissues, where they had also selectively migrated despite their low expression of CD103 (Table 3.3). Strikingly, less donor $CD25^+$ $Foxp3^+$ T cells had entered the intestinal tissues than their $CD25^-$ $Foxp3^+$counterparts, despite being present in greater numbers among the adoptively transferred T cells (Table 3.3), and having a greater expression of CD103 (Table 3.2), which is associated with gut-homing T cells (Schon *et al.* 1999). Furthermore, amongst the host $CD4^+$ T cells in the gut, $CD25^-$ $Foxp3^+$ putative regulatory T cells

outnumbered their $CD25^+$ $Foxp3^+$ counterparts, despite being present in smaller numbers in almost every other compartment (Table 3.1)

The adoptive transfer of thoracic duct lymphocytes was found to perturb recipient $CD4^+$ ($CD8^-$) T cells in the thymus, with lower numbers of host $CD25^+$ $Foxp3^+$ and $CD25^+$ $Foxp3^-$ "activated" T cells observed in the thymuses of recipient animals compared with their proportions in normal animals (Table 3.3). In order to determine if these changes reflect an inhibition of new T cell production in the thymus, it would be necessary to measure the thymic output of T cells in these animals, by quantifying the numbers of recent thymic emigrants produced in this period, using an approach such as intrathymic labelling (Hosseinzadeh and Goldschnieder 1993), or the use of transgenic animals that enable the identification of recent thymic emigrants (Bousilan *et al.* 2004). Interestingly, host activated T cells were found to be present in elevated numbers in the spleens of recipients, while they were present in numbers similar to those observed in normal animals in the lymph nodes. It is unclear whether this observation is the result of the large numbers of donor activated T cells that were received from the adoptive transfer affecting the distribution of host activated T cells, but it is unlikely to signify the initiation of an allogeneic reaction to the CD45.2 antigen, which does occur after the transfer of DA $CD45.2^+$ T cells into DA $CD45.1^+$ hosts (Spargo *et al.* 2006).

In order to examine the recruitment of $CD4^+$ T cell subsets into the thymus, lymph nodes, spleen, intestinal tissues and SRT under conditions in which inflammation is affecting the paws, normal thoracic duct lymphocytes were adoptively transferred into animals experiencing adoptively transferred arthritis.
The normal donor cells that had entered the thymuses of arthritic recipients contained higher proportions of activated T cells and $CD25^+$ $Foxp3^+$ regulatory T cells compared with those that had entered the thymuses of normal recipients. This finding suggests that inflammation in the peripheral tissues can affect the entry of T cells into the thymus, potentially by inducing the expression of molecules such as integrin alpha 4, which has been previously shown to facilitate the entry of antigen experienced T cells into the thymus (Bell *et al.*1995).

The arthritic recipients of thoracic duct lymphocytes contained less host $CD25^+$ $Foxp3^+$ regulatory T cells in their lymph nodes and spleens compared with normal recipients, but higher proportions of host $CD25^+$ $Foxp3^+$ regulatory T cells in their intestinal tissues. However, these host $CD25^+$ $Foxp3^+$ regulatory T cells do not appear to enter the SRT in any great numbers, despite the robust infiltration of host activated T cells (Table 3.4). The proportion of host activated T cells in the intestinal tissues was lower for arthritic recipients of normal lymph than normal recipients, which may be attributed to their infiltration into the SRT.

In contrast to our findings in normal animals, the donor regulatory T cells that were observed to localise to the SRT of arthritic joints were predominantly $CD25^+$ $Foxp3^+$ cells (Figure 3.2.19). Thus it is not clear whether these cells represent tissue homing $CD25^-$ $Foxp3^+$ putative regulatory T cells that have regained CD25 expression after entering the inflamed environment of the SRT, where they are likely to encounter cognate antigen, or if they were indeed originally $CD25^+$ $Foxp3^+$ regulatory T cells that have homed directly into the SRT.

3.3.7 Summary

In conclusion, we have shown that regulatory T cells are found at significantly lower concentrations within the thymus compared to the periphery, and are present at high concentrations in lymph nodes. Regulatory T cells express several activation-associated markers including CD25 and CD71, but do not express MHC II, indicating they have not been activated. Furthermore, regulatory T cells display indicators of failed activation, including downregulated TCR expression and lower expression of the CD4 coreceptor relative to resting T cells. Regulatory T cells had a significantly lower expression of CD5, indicating they may have a lower avidity for MHC-peptide than their conventional T cell counterparts, suggesting that if regulatory T cells do represent anergised T cells, they might be recognising prevalent self antigens with low avidity.

When the expression of costimulatory molecules was examined, it was found that a greater proportion of $CD25^+$ $Foxp3^+$ regulatory T cells expressed CTLA-4 compared with resting cells. Interestingly, $CD25^-$ $Foxp3^+$ putative regulatory T cells had a lower expression of CTLA-4 compared to their $CD25^+$ $Foxp3^+$ counterparts, although it is not clear whether this difference translates into differences in the potencies of these subsets in suppressing T cell proliferation, which will be evaluated in Chapter 5. Strikingly, the majority of both
$CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed the costimulatory molecule CD134, suggesting that this molecule may be important for their maintenance of regulatory T cells through the provision of survival signals. Further work will be required to determine the role of CD134 in maintaining the survival of regulatory T cells.

We have found that regulatory T cells are relatively sessile compared to other T cells, and comprise approximately one third of the sessile pool of $CD4^+$ T cells, while less than a tenth of the recirculating pool of $CD4^+$ T cells are regulatory T cells. The expression of adhesion molecules on regulatory T cells was similar to their expression on activated T cells, but activated T cells typically expressed higher levels of adhesion molecules that are reportedly induced upon activation. The high expression of CD62L and LFA-1 by $CD25^+$ $Foxp3^+$ regulatory T cells was congruent with both their relative enrichment within secondary lymphoid tissues and their sessile nature. The profile of adhesion molecules on the surface of $CD25^-$ $Foxp3^+$ cells suggested they may tend to localise to sites of inflammation other than the lamina propria, as they have a low expression of CD103 and CD62L, but high expression of LFA-1. However, $CD25^-$ $Foxp3^+$ putative regulatory T cells were found to selectively migrate into the intestinal mucosa, where they were enriched. In addition, $CD25^-$ $Foxp3^+$ regulatory T cells are enriched within the population of cells that recirculate back to the thymus, where they may act to regulate thymic function. Unexpectedly, the regulatory T cells that enter the SRT were found to express CD25, and it is unclear whether these cells represent $CD25^+$ $Foxp3^+$ cells that have migrated into the SRT, or whether they belong to the tissue homing $CD25^-$ $Foxp3^+$ putative regulatory T cell subset and have re-acquired CD25 expression following their entry into the SRT.

The relationship between these two regulatory T cell subsets will be explored in Chapter 5, following an investigation into their relationship to the thymus presented in Chapter 4.

Chapter 4: The recent thymic origin of regulatory T cells

4.1 Introduction

Regulatory T cells are thought to constitute a unique lineage of $CD4^+$ T cells that develop in the thymus along a differentiation program controlled by Foxp3 (Hori *et al.* 2003, Sakaguchi 2005). They are thought to emigrate from the thymus early in life, as the removal of the thymus in certain strains of mice on day 3 after birth results in multiple organ autoimmunity, but thymectomy after day 7 results in little or no autoimmunity (Sakaguchi and Sakaguchi 1990, Maggi *et al.* 2005). While no Foxp3 expression is detectable in the neonatal mouse thymus until day 3 after birth, regulatory T cells are present at a very low frequency in the thymus during first week after birth, but increase in absolute number 6-fold by the time adulthood has been reached (Jiang *et al.* 2006). It has been postulated that high-affinity interactions in the thymus between thymocytes bearing self-reactive TCRs and MHC II molecules on the surface of thymic stromal cells, induce these thymocytes to become regulatory T cells (Maggi *et al.* 2005). However, this model has been based on a series of experimental results that have been widely misinterpreted, that when thoroughly examined do not support this proposed mechanism of regulatory T cell differentiation (van Santen *et al.* 2004).

The experimental observations that led to the contemporary model of regulatory T cell selection were obtained using transgenic mice, which were designed such that they both express a foreign peptide and contain T cells that recognise that peptide (Jordan *et al.* 2001). In these animals, the proportion of $CD25^+$ cells increases in the thymus with increasing expression of the foreign peptides, which was originally interpreted to mean that regulatory T cells were being induced through their recognition of the foreign peptides in the thymus (Jordan *et al*. 2001, Kawahata *et al.* 2002). However, the numbers of $CD25^+$ thymocytes in these systems remain unchanged with increasing expression of transgenic peptides, while the absolute number of $CD25^-$ T cells declines in a dosage dependent manner, due to their negative selection (van Santen *et al.* 2004). Thus it appear this artefact is responsible for the increased proportion of $CD25^+$ cells observed in the thymus and periphery of animals co-expressing transgenic TCRs and their corresponding transgenic peptide

antigens (Jordan *et al.* 2001). The observation that regulatory T cells are insensitive to negative selection also suggests they have a relatively low avidity for self antigens (Schwartz *et al.* 2005), which is supported by the finding that peptides only weakly cross reactive to the TCR of regulatory T cells can activate their suppressive mechanism (Larkin et al. 2007). This would also be consistent with our finding that regulatory T cells express low levels of CD5, a molecule induced in T cells in proportion to the affinity of the TCR for MHC molecules (Figure 3.2.8). Thus, the thymic selection of regulatory T cells through their high affinity recognition of self antigens in the thymus seems unlikely. Rather, regulatory T cells may recognise prevalent self antigens with low avidity, and become regulatory T cells as they anergised through the serial engagement of their TCR. This process may be complete by the time the T cells leave the thymus, or it may continue in the periphery and affect recent thymic emigrants (RTE), which are newly formed T cells released by the thymus into the periphery.

Interestingly, it is known that while RTE can acquire regulatory properties, mature T cells are refractory to this functional conversion (Modigliani *et al.* 1996). RTE may be at a stage in their development that enables them to survive being presented with their antigen in the absence of activating, costimulatory signals, which induces apoptosis in mature T cells (Marelli-Berg *et al.* 1997), and also immature thymocytes (Finkel *et al.* 1989, Nossal 1994). Investigations into the relationship between regulatory T cells and RTE are difficult to perform in humans, as RTE possess no unique cell surface phenotypic markers that distinguish them from other T cells (Hassan *et al.* 2001). However, these investigations can be more easily undertaken in mice and rats using intrathymic labelling techniques (Scollay *et al.* 1982). This approach involves the injection of the thymus with a detectable label, such as FITC, which can then be used to identify RTE as $FITC^+$ cells in the periphery (Hosseinzadeh and Goldscheider 1993). While no useful markers of RTE have been identified in mice, it has been established that a unique phenotype does occur in rats, enabling more extensive studies and making this model arguably the best available for a thorough investigation of RTE.

Rat RTE exclusively express the glycoprotein molecule Thy-1 (CD90), a member of the immunoglobulin-like superfamily that is expressed by thymocytes and RTE and is downregulated as RTE mature in the periphery (Douglas 1972, Kroczek *et al.* 1986, Hosseinzadeh and Goldscheider 1993). Although the function of Thy-1 is unknown, it is capable of supporting the adhesion of thymocytes to thymic epithelial cells, and may function as a signal transduction molecule (He *et al.* 1991). The expression of Thy-1 is not restricted to thymocytes and RTE in mice, which also express Thy-1 on mature T cells, while in humans it is only found on a minority of thymocytes (Kroczek *et al.* 1986). However, intrathymic labelling experiments have confirmed that Thy-1 is expressed by all RTE in the rat, which downregulate their expression of the molecule in the first week following their arrival in the periphery. Interestingly, as Thy-1 expression decreases, RTE upregulate the CD45RC isoform, signifying their maturation towards a naïve T cell phenotype (Hosseinzadeh and Goldscheider 1993).

The CD45 molecule is a glycoprotein expressed by all leukocytes that exists in different isoforms due to the differential splicing of its A, B, and C variable exons (Powrie and Mason 1990). Thymocytes and memory T cells express a low molecular weight isoform of CD45 that does not include the A, B, or C exons and is referred to as CD45RO. As rat RTE mature in the periphery, they begin to express the CD45RC isoform, which is associated with naïve T cells and equivalent to the CD45RA isoform in humans, and the CD45RB isoform in mice (Rodgers *et al.* 1992). However, memory T cells can also revert back to this phenotype, such that some $CD45RC^{+}$ cells may have previously encountered antigen (Yang and Bell, 1992). Interestingly, a functional maturation occurs concomitantly with the transition of RTE to the $Thy1^{-}$ $CD45RC^{+}$ naïve T cell phenotype as the T cells become increasingly immunocompetent (Yang and Bell 1992). It remains possible that this phenomenon may occur due to the modulatory effect of CD45 on signal transduction and lymphocyte activation, rendering cells more sensitive to activation signals as they mature in the periphery (Powrie and Mason 1990).

In a recently published edition of the Journal of Clinical Investigation, Vukamanivic-Stejic *et al.* (2006) investigated the turnover of $CD45RO^{+}$ $CD25^{high}$ regulatory T cells

in human subjects. In adults, regulatory T cells are found predominantly within the $CD45RO^+$ subset, although a functionally suppressive $CD45RA^+$ population persists throughout adult life (Seddiki *et al.* 2006). The $CD45RO^+$ $CD25^{high}$ regulatory T cells were hypothesised to originate from rapidly turning over memory T cells (Vukmanovic-Stejic *et al.* 2006). However, this interpretation does not account for the presence of $CD45RA^+$ regulatory T cells, the equivalent to $CD45RC^+$ regulatory T cells in the rat. An alternative explanation is that the $CD45RO^+$ regulatory T cells are derived from $CD45RO^+$ RTE, some of which may switch isoform expression as they mature in the periphery to become $CD45RA^+$.

An additional molecule that appeared to be potentially useful in the identification of RTE was the CD8 a-chain. At 14 hours following their egress from the thymus, ~20% of RTE in the lymph nodes have the phenotype $CD4^+$ $CD8\alpha^+$ ($CD8\beta^-$), and their expression of CD8a is lost during maturation to mature $CD4^+$ T cells (Jimenez *et al.* 2002). However, while most $CD8\alpha^+$ T cells are RTE,and their numbers are reduced following thymectomy and during ageing, T cells activated *in vitro* can also express $CD8\alpha$ (Kenny *et al.* 2004). Thus, $CD8\alpha$ is unlikely to be an exclusive marker of RTE.

The relationship of RTE to regulatory T cells was evaluated initially by examining the expression of Foxp3, CD45RC and CD8a by $CD4^+$ $Thy1^+$ T cells (presumptive RTE) in secondary lymphoid tissues of rats. This was followed by an examination of the effects of adult thymectomy on the number and phenotype of regulatory T cells and RTE. Finally, possible associations between regulatory T cells and RTE were investigated by employing intra-thymic labelling to identify RTE directly.

4.2 Results

4.2.1 Regulatory T cells express some markers associated with RTE

In order to examine possible associations between regulatory T cells and RTE, the expression Thy-1, $CD45RC^-$ and $CD8\alpha$ by regulatory T cells was assessed by flow

cytometric analysis of negatively selected CD4+ T cells. Negatively selected $CD4^+$ T cells from 7-8 week old adult female rats were stained to detect CD25 and Foxp3, plus either Thy-1, CD45RC or CD8α. This allowed expression of the latter markers to be examined in resting ($Foxp3^-$ $CD25^-$) T cells, activated ($Foxp3^-$ $CD25^+$) T cells, $CD25^+$ $Foxp3^+$ regulatory T cells, and $CD25^-$ $Foxp3^+$ putative regulatory T cells. Interestingly, it was found that in the lymph nodes approximately 20% of resting T cells expressed Thy-1, suggesting that a fifth of the resting $CD4^+$ T cell pool may represent recent emigrants from the thymus (Figure 4.2.1). While a slightly higher proportion of activated T cells in the lymph nodes (~22%) expressed Thy-1, more than 25% of $CD25^+$ $Foxp3^+$ regulatory T cells in the lymph nodes were Thy-1^+, suggesting that these cells could be RTE. In contrast, a smaller proportion (~15%) of $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed Thy-1 relative to resting T cells, indicating that they are not as closely associated with RTE. Similar results were obtained with $CD4^+$ T cells isolated from the spleen (data not shown).

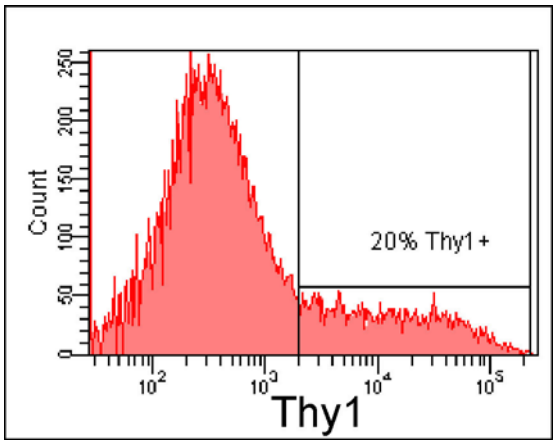
The CD45RC isoform was expressed at high levels by most of the resting $CD4^+$ T cells, with only ~25% having the $CD45RC^-$ phenotype (Figure 4.2.2). In contrast, most $CD25^+$ $Foxp3^+$ regulatory T cells (~70%) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (>70%) did not express CD45RC. The low proportion of CD45RC- cells could signify their recent emigration from the thymus, or that they have encountered cognate antigens. Nevertheless, some $CD45RC^+$ regulatory T cells were present, and these could represent “naïve” regulatory T cells, or more likely, antigen-experienced cells that have reverted to a naïve phenotype (Yang and Bell 1992).

As discussed above, the CD8α homodimer is known to be expressed by some RTE, and also by T cells following activation (Kenny *et al.* 2004). CD8α was expressed by only a minority (~2%) of resting $CD4^+$ T cells, and by a small proportion (<10%) of activated T cells (Figure 4.2.3). It seems, therefore, that there may be qualitative or quantitative differences in the activating signals that lead to the expression of CD25 or CD8α on activated $CD4^+$ T cells. Only a small proportion (~5%) of $CD25^-$ $Foxp3^+$ putative regulatory T cells expressed the CD8α homodimer, but a much higher proportion (>30%) of $CD25^+$ $Foxp3^+$ regulatory T cells expressed the molecule.

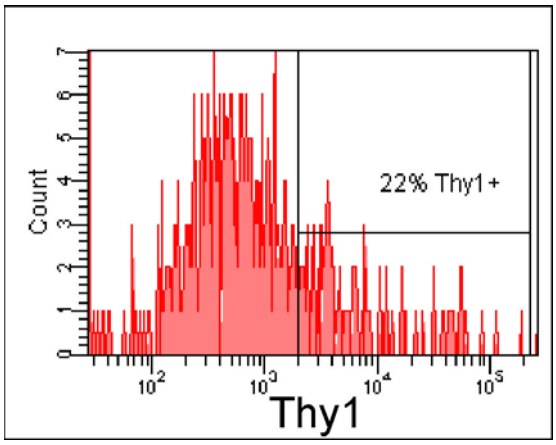
Figure 4.2.1 Expression of Thy-1 by regulatory T cells

Cells from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph node cells were depleted of B and $CD8^+$ T lymphocytes. The purified $CD4^+$ T cells were stained with antibodies against Thy-1, CD25 and Foxp3 and analysed by flow cytometry. The proportions of $Foxp3^-$ $CD25^-$ resting T cells (A), $Foxp3^-$ $CD25^+$ activated T cells (B), $CD25^+$ $Foxp3^+$ regulatory T cells (C) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) that expressed Thy1 are shown as percentages. Representative of 3 experiments.

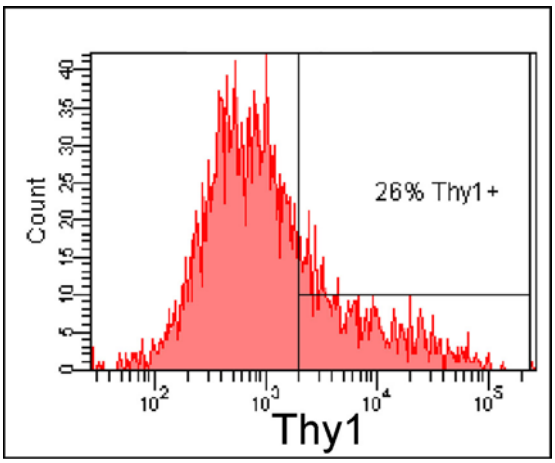
A Resting $CD4^+$ T cells



B Activated $CD4^+$ T cells



C $CD25^+$ $Foxp3^+$ T cells



D $CD25^-$ $Foxp3^+$ T cells

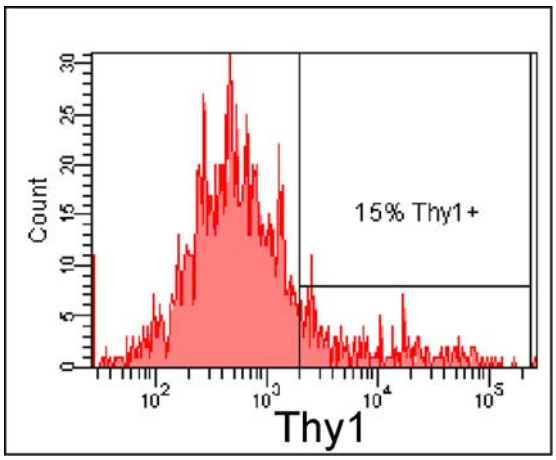
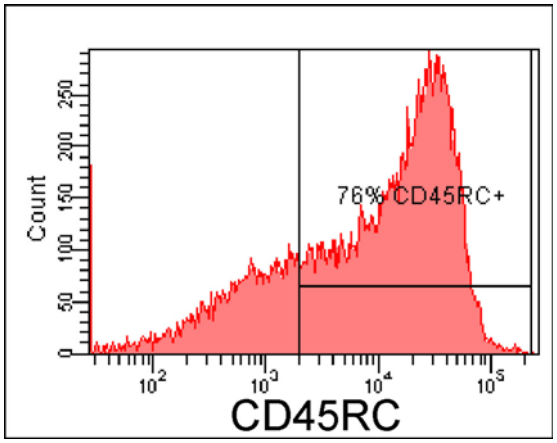


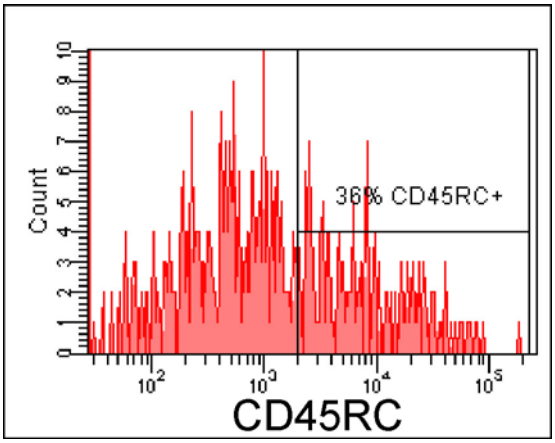
Figure 4.2.2 Expression of CD45RC by regulatory T cells

Cells from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph node cells were depleted of B and $CD8^+$ T lymphocytes. The purified $CD4^+$ T cells were stained with antibodies against CD45RC CD25 and Foxp3. The proportions of $Foxp3^-$ $CD25^-$ resting T cells (A), $Foxp3^-$ $CD25^+$ activated T cells (B), $CD25^+$ $Foxp3^+$ regulatory T cells (C) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) that expressed CD45RC are shown as percentages. Representative of 3 experiments.

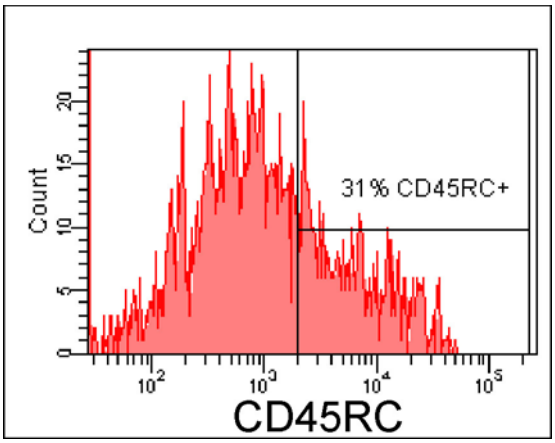
A Resting $CD4^+$ T cells



B Activated $CD4^+$ T cells



C $CD25^+$ $Foxp3^+$ T cells



D $CD25^-$ $Foxp3^+$ T cells

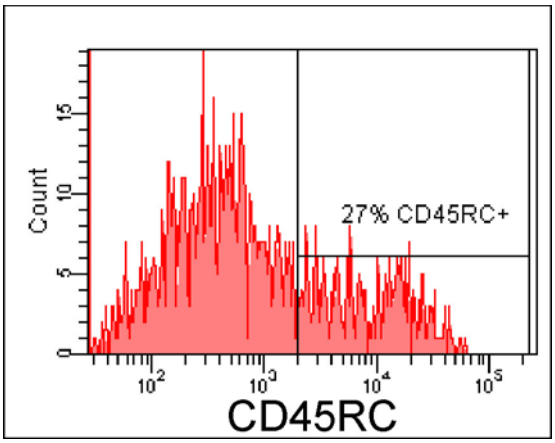
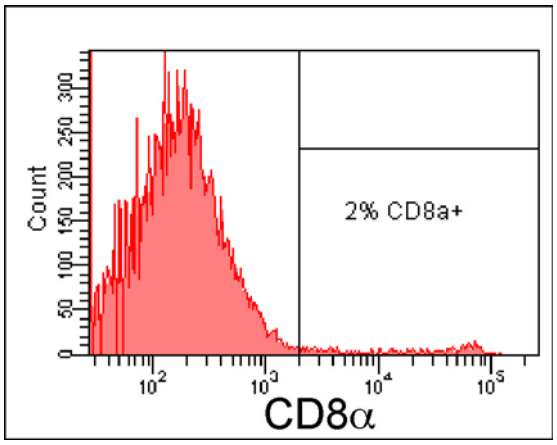


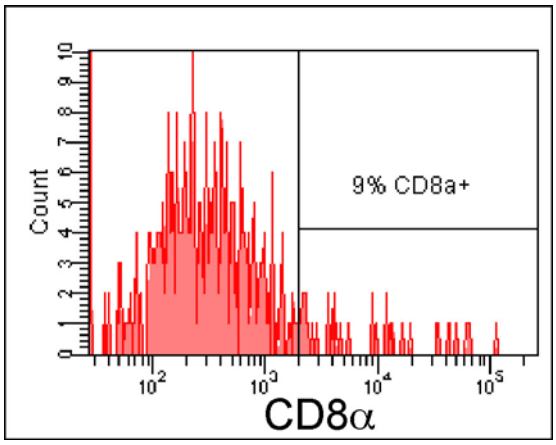
Figure 4.2.3 Regulatory T cells coexpress the CD8α homodimer

Cells from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph node cells were depleted of B and $CD8^+$ T lymphocytes. The purified $CD4^+$ T cells were stained with antibodies against CD8α, CD25 and Foxp3. The proportions of $Foxp3^-$ $CD25^-$ resting T cells (A), $Foxp3^-$ $CD25^+$ activated T cells (B), $CD25^+$ $Foxp3^+$ regulatory T cells (C) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (D) that express the CD8α chain are expressed as percentages. Representative of 3 experiments.

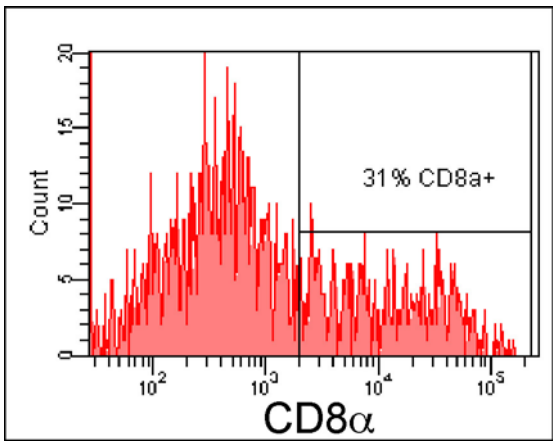
A Resting $CD4^+$ T cells



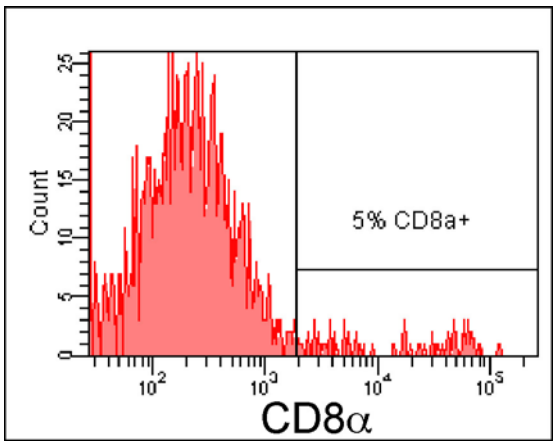
B Activated $CD4^+$ T cells



C $CD25^+$ $Foxp3^+$ T cells



D $CD25^-$ $Foxp3^+$ T cells



Furthermore, it was found that CD8α^+ T cells were enriched for Foxp3$^+$ T cells (Figure 4.2.4). Therefore, expression of CD8α correlates more closely with regulatory T cells than with conventional T cells, although it is unclear whether this is due to their putative recent thymic origin or whether it indicates recent encounter with antigen.

4.2.2 Effect of thymectomy on the number and phenotype of regulatory T cells

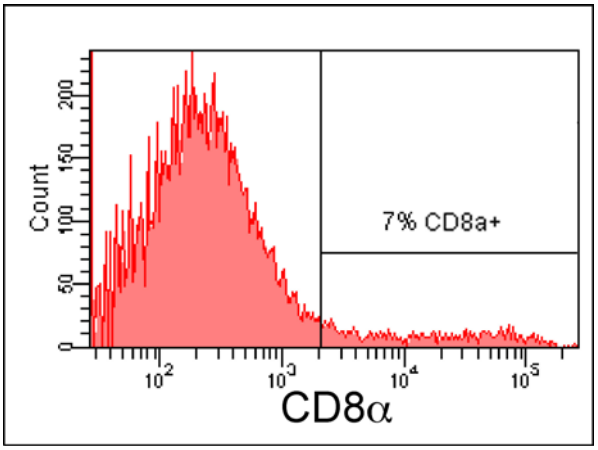
In order to further explore the relationship between regulatory T cells and RTE, numbers of regulatory and conventional T cells and their expression of markers associated with RTE was assessed following thymectomy. Female DA CD45.2 rats were either thymectomised or sham-thymectomised at 6 weeks of age, placed on antibiotics (see Methods) and allowed to recover for at least 3 weeks before being used experimentally. At either 0, 30 or 60 days following thymectomy or sham thymectomy, cells were prepared from pooled mesenteric, celiac, cervical, inguinal and popliteal lymph nodes and CD4$^+$ T cells were negatively selected by immunomagentic depletion of B cells and CD8β^+ T cells. The CD4$^+$ T cells were then stained to detect CD25 and Foxp3, plus either Thy-1, CD45RC or CD8α.

The absolute number of CD25$^-$ Foxp3$^-$ resting T cells that expressed Thy-1 remained steady in sham-thymectomised animals, but decreased rapidly in thymectomised animals (Figure 4.2.5). Numbers of CD25$^+$ Foxp3$^+$ regulatory T cells that expressed Thy1 decreased in a similar manner, but experienced a small increase in sham-thymectomised animals. In contrast, numbers of CD25$^-$ Foxp3$^+$ putative regulatory T cells decreased only marginally in thymectomised animals, while numbers increased approximately 4-fold in sham thymectomised animals. These findings indicated that numbers of Thy1$^+$ CD25$^+$ Foxp3$^+$ regulatory T cells are indeed dependent on the presence of an intact thymus, while the Thy-1$^+$ CD25$^-$ Foxp3$^+$ putative regulatory T cell subset can persist for considerable periods of time after thymectomy and are either long lived or generated in the periphery.

Figure 4.2.4 Expression of Foxp3 by $CD4^+$ $CD8\alpha^+$ T cells

Cells from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph node cells were depleted of B and $CD8^+$ T lymphocytes. The purified $CD4^+$ T cells were stained with antibodies against $CD8\alpha$, CD25 and Foxp3. The proportions of expression of $CD4^+$ T cells that express $CD8\alpha$ (A), and $CD4^+$ $CD8\alpha^+$ T cells that express Foxp3 (B) are shown as percentages. Representative of 3 experiments.

A CD4$^+$ T cells



B CD4$^+$ CD8α$^+$ T cells

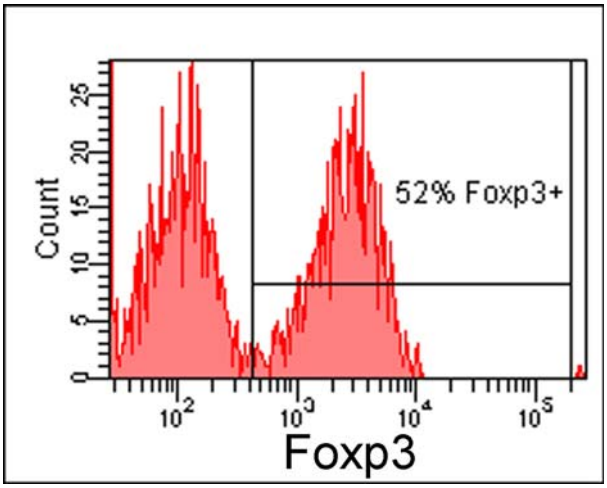
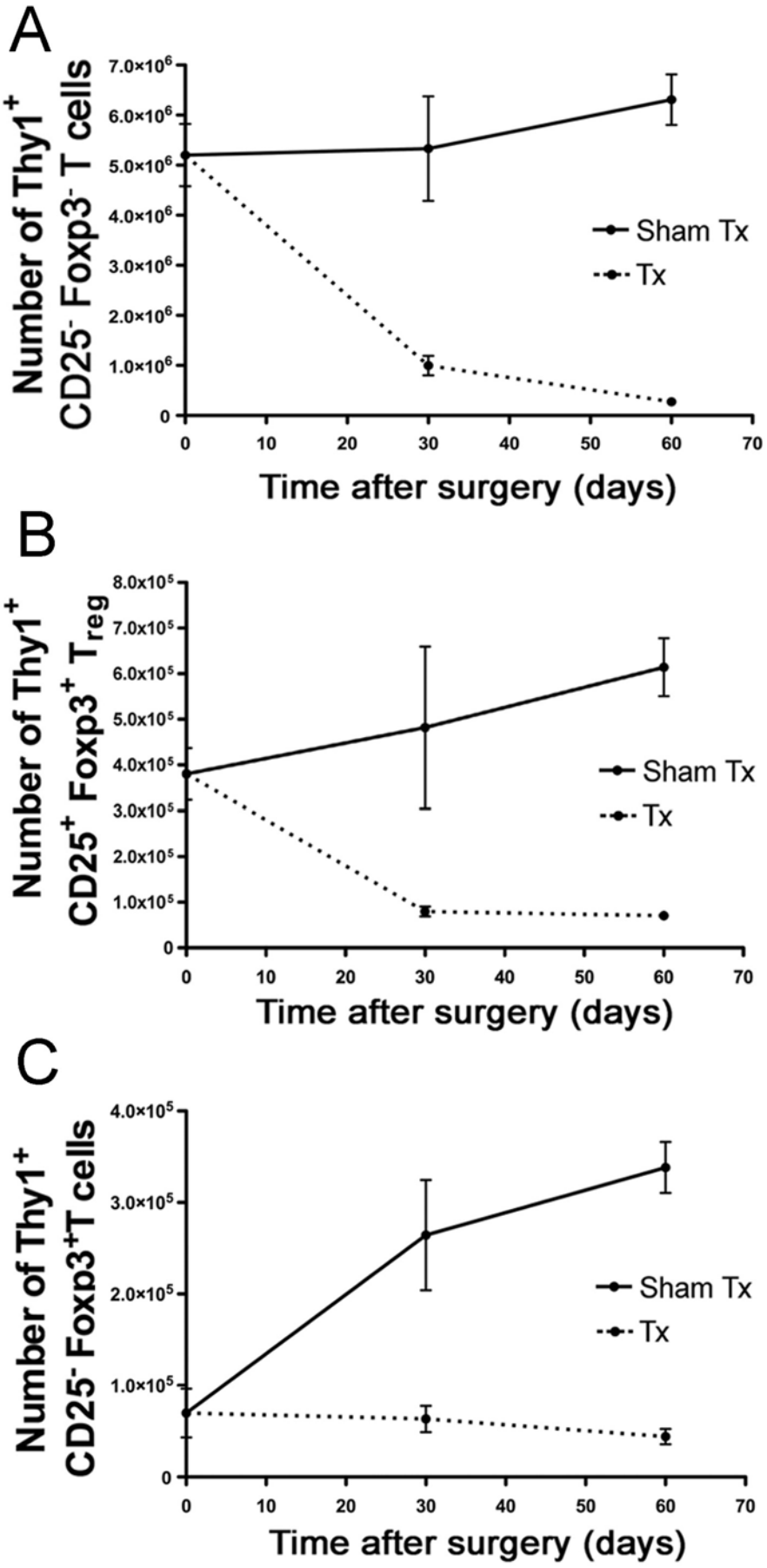


Figure 4.2.5 The effect of thymectomy on the absolute numbers of conventional and regulatory T cells that express Thy-1

Lymphocytes were prepared from pooled mesenteric, celiac, cervical, inguinal and popliteal lymph nodes of sham thymectomised and thymectomised rats at 0, 30 and 60 days following surgery (n=3 per group). A cell count was performed to estimate total lymphocyte numbers and a sample was labelled with anti-CD4 to assess the total numbers of $CD4^+$ T cells by flow cytometry. $CD4^+$ T cells were purified from the remainder by negative selection. The purified $CD4^+$ T cells were stained with antibodies against Thy-1, CD25 and Foxp3, and subsets of $CD25^-$ $Foxp3^-$ resting T cells, $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells were identified by flow cytometry and each was examined for expression of Thy-1. This allowed calculation of the mean (± SD) absolute number of Thy-1^+ resting T cells (A), $CD25^+$ $Foxp3^+$ regulatory T cells (B) and $CD25^-$ $Foxp3^+$ putative regulatory T cells (C). Representative of 3 experiments.


A
Number of Thy1+ CD25- Foxp3- T cells
7.0x10^6
6.0x10^6
5.0x10^6
4.0x10^6
3.0x10^6
2.0x10^6
1.0x10^6
0
0 10 20 30 40 50 60 70
Time after surgery (days)
Sham Tx
Tx
B
Number of Thy1+ CD25+ Foxp3+ Treg
8.0x10^5
7.0x10^5
6.0x10^5
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
0 10 20 30 40 50 60 70
Time after surgery (days)
Sham Tx
Tx
C
Number of Thy1+ CD25- Foxp3+T cells
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
0 10 20 30 40 50 60 70
Time after surgery (days)
Sham Tx
Tx

Absolute numbers of CD45RC$^-$ resting T cells, which include both memory T cells and most RTE, decreased following thymectomy, while the absolute numbers of the same subset increased over the same period in sham-thymectomised controls (Figure 4.2.6). There was a similar decrease in the numbers of CD45RC$^-$ CD25$^+$ Foxp3$^+$ regulatory T cells in the thymectomised animals, while numbers of CD45RC$^-$ CD25$^-$ Foxp3$^+$ cells remained stable. Numbers of both regulatory subsets increased during the observation period in sham-thymectomised control rats, with a 6-fold increase in the CD45RC$^-$ CD25$^-$ Foxp3$^+$ subset. There was no significant change in the absolute number of CD8α^+ resting T cells after thymectomy, but the numbers increased in sham thymectomised animals (Figure 4.2.7). This indicates a partial dependence of this subset on continuing thymic function, consistent with expression of CD8α by some RTE. Thymectomy was followed by a more pronounced decrease in numbers of CD8α^+ CD25$^+$ Foxp3$^+$ regulatory T cells consistent with the greater proportion of CD8α cells in this subset and indicating a dependence on the thymus to maintain numbers in the periphery. However, the number of CD25$^-$ Foxp3$^+$ cells expressing CD8α remained steady following thymectomy. The increases in numbers of the regulatory T cell subsets during the same period in sham-thymectomised rats, especially in the case of the CD25$^-$ Foxp3$^+$ subset (5-fold), suggests that both are dependent on continuing thymic function for their maintenance.

In order to assess whether thymectomy had induced lymphopenia, the number of CD4$^+$ T cells in lymph nodes of thymectomised or sham thymectomised rats was determined seven days after surgery. Indeed, a significantly lower number of CD4$^+$ T cells was present in thymectomised rats compared to sham thymectomised controls ($p=0.022$) (Figure 4.2.8). The proliferation of CD4$^+$ T cell subsets in these rats was then investigated to determine whether the smaller size of peripheral pool had induced homeostatic proliferation. The animals were thymectomised or sham-thymectomised at 6 weeks of age, as described above. After three weeks of recovery, they were provided with drinking water containing the thymidine analogue BrdU (0.8mg/ml) for one week. Homeostatic proliferation of the CD25$^-$ Foxp3$^-$ resting T cells as assessed by the proportion of cells that had incorporated BrdU was approximately 10-fold higher in thymectomised animals than in sham thymectomised

Figure 4.2.6 The effect of thymectomy on the absolute number of CD45RC$^-$ conventional and regulatory T cells

Aliquots of the purified CD4$^+$ T cells described in Figure 4.2.5 were labelled with antibodies against CD45RC, CD25 and Foxp3 and the expression of CD45RC was examined in the CD25$^-$ Foxp3$^-$ resting T cell, CD25$^+$ Foxp3$^+$ regulatory T cell and CD25$^-$ Foxp3$^+$ putative regulatory T cell subsets. The absolute numbers (mean ±SD) of CD45RC$^-$ cells in the resting T cell (A), CD25$^+$ Foxp3$^+$ regulatory T cell (B) and CD25$^-$ Foxp3$^+$ putative regulatory T cell (C) subsets were calculated for the pooled lymph nodes from sham thymectomised and thymectomised animals at 0, 30 and 60 days following surgery (n=3 per group).

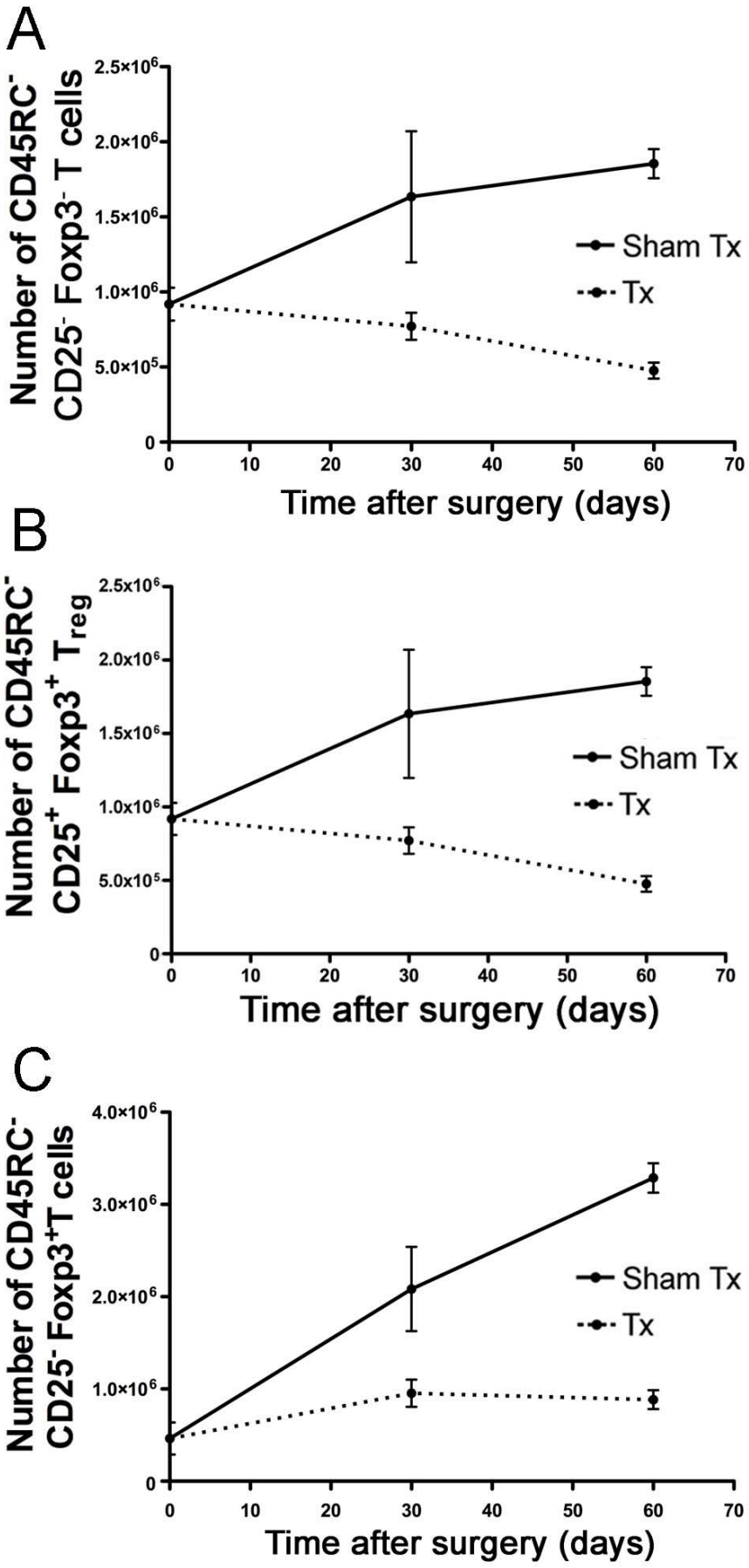

A
Number of CD45RC- CD25- Foxp3- T cells
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0
0 10 20 30 40 50 60 70
Time after surgery (days)
Sham Tx
Tx
B
Number of CD45RC- CD25+ Foxp3+ Treg
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0
0 10 20 30 40 50 60 70
Time after surgery (days)
Sham Tx
Tx
C
Number of CD45RC- CD25- Foxp3+T cells
4.0×10^6
3.0×10^6
2.0×10^6
1.0×10^6
0
0 10 20 30 40 50 60 70
Time after surgery (days)
Sham Tx
Tx

Figure 4.2.7 The effect of thymectomy on the absolute number of CD8a$^+$ conventional and regulatory T cells

Aliquots of the purified CD4$^+$ T cells described in Figure 4.2.5 were labelled with antibodies against CD8α, CD25 and Foxp3 and the expression of CD8α was examined in the CD25$^-$ Foxp3$^-$ resting T cell, CD25$^+$ Foxp3$^+$ regulatory T cell and CD25$^-$ Foxp3$^+$ putative regulatory T cell subsets. The absolute numbers (mean ±SD) of CD8α cells in the resting T cell (A), CD25$^+$ Foxp3$^+$ regulatory T cell (B) and CD25$^-$ Foxp3$^+$ putative regulatory T cell (C) subsets were calculated for the pooled lymph nodes from sham thymectomised and thymectomised animals at 0, 30 and 60 days following surgery (n=3 per group).

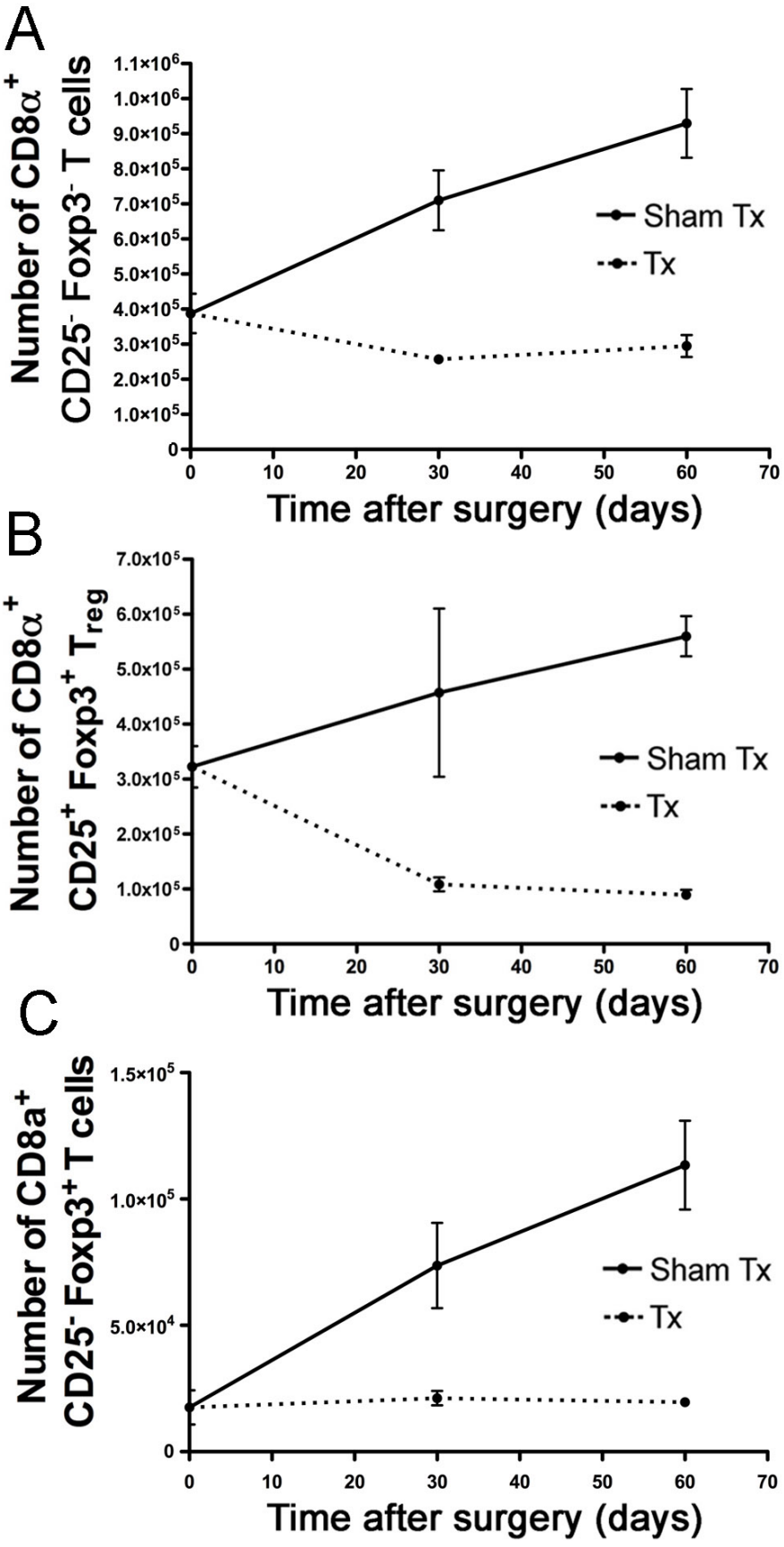
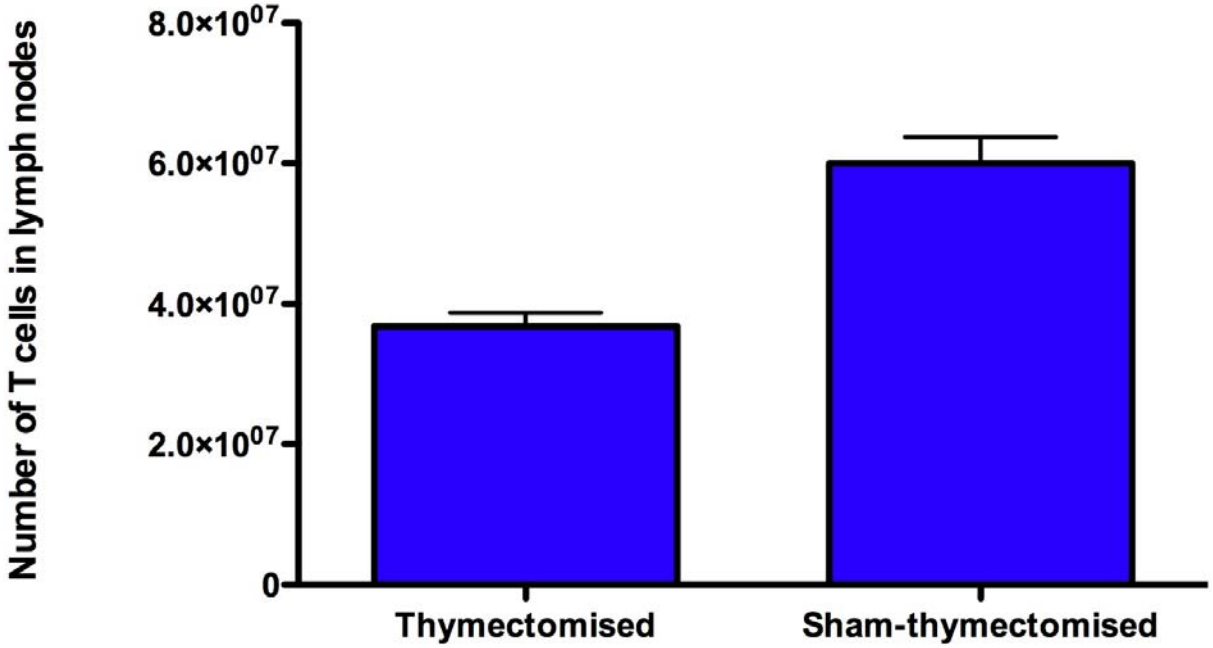

A
Number of CD8α+ CD25- Foxp3- T cells
1.1×10^6
1.0×10^6
9.0×10^5
8.0×10^5
7.0×10^5
6.0×10^5
5.0×10^5
4.0×10^5
3.0×10^5
2.0×10^5
1.0×10^5
0
0 10 20 30 40 50 60 70
Time after surgery (days)
Sham Tx
Tx
B
Number of CD8α+ CD25+ Foxp3+ Treg
7.0×10^5
6.0×10^5
5.0×10^5
4.0×10^5
3.0×10^5
2.0×10^5
1.0×10^5
0
0 10 20 30 40 50 60 70
Time after surgery (days)
Sham Tx
Tx
C
Number of CD8a+ CD25- Foxp3+ T cells
1.5×10^5
1.0×10^5
5.0×10^4
0
0 10 20 30 40 50 60 70
Time after surgery (days)
Sham Tx
Tx

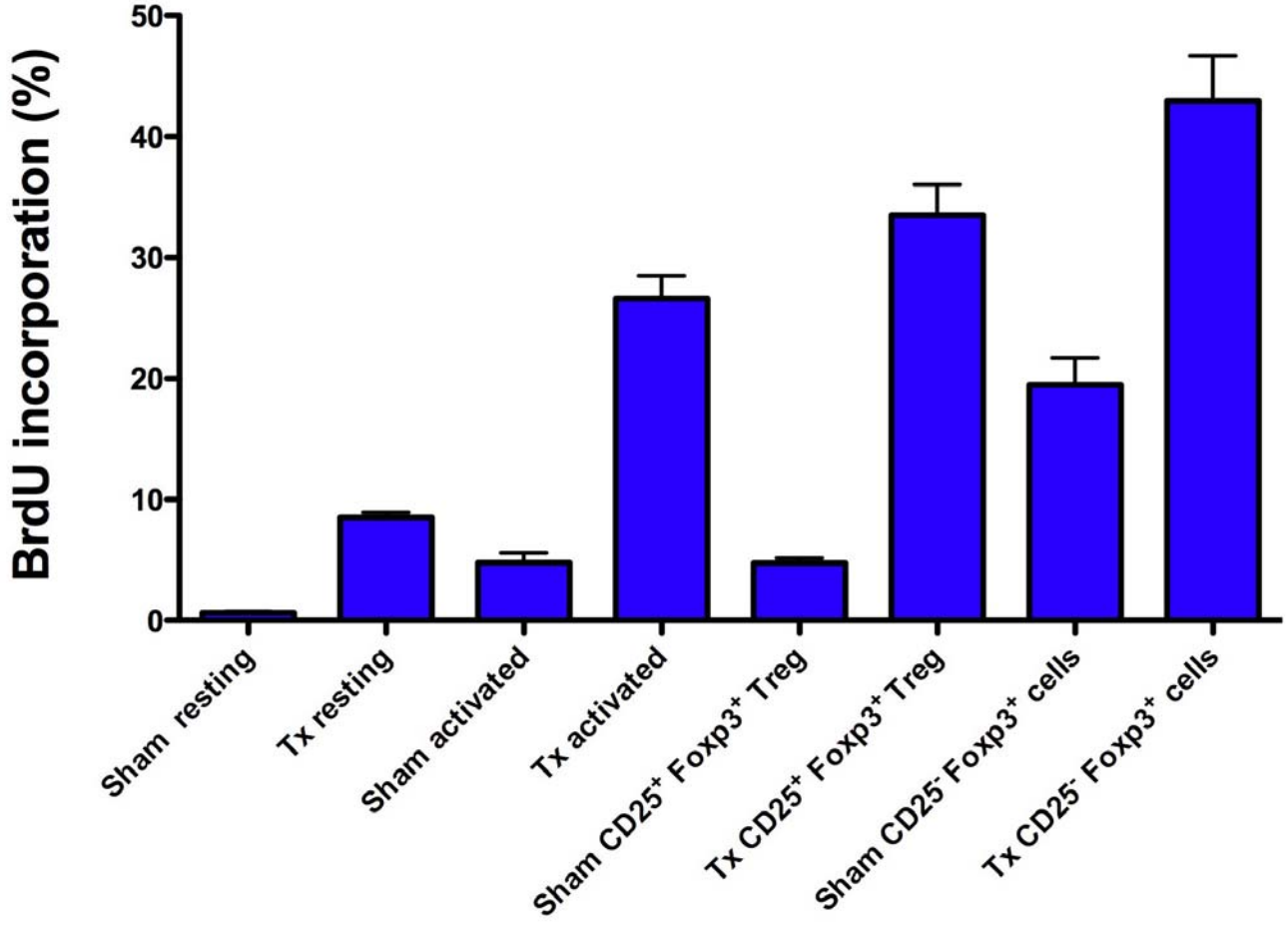
Figure 4.2.8 The effect of thymectomy on homeostatic proliferation of conventional and regulatory T cells

Female 6 week old DA CD45.2 rats were either thymectomised or sham-thymectomised, and were then allowed to recover for 3 weeks. At this time they received 0.8mg/ml BrdU in their drinking water for 7 days, and at the end of this period (30 days after surgery), they were killed to collect mesenteric, celiac, cervical, inguinal and popliteal lymph nodes. Lymphocytes were prepared from the pooled lymph nodes. $CD4^+$ T cells were purified by negative selection and cells stained for CD25, Foxp3 and BrdU (See Methods section 2.7.9). The proportions of $Foxp3^-$ $CD25^-$ resting T cells, $Foxp3^-$ $CD25^+$ activated T cells, $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells that had incorporated BrdU were determined by flow cytometry and are expressed as percentages (B). Data represent means ±SD of 3 animals per group.

A


Number of T cells in lymph nodes
8.0×10^07
6.0×10^07
4.0×10^07
2.0×10^07
0
Thymectomised
Sham-thymectomised

B


BrdU incorporation (%)
50
40
30
20
10
0
Sham resting
Tx resting
Sham activated
Tx activated
Sham CD25+ Foxp3+ Treg
Tx CD25+ Foxp3+ Treg
Sham CD25- Foxp3+ cells
Tx CD25- Foxp3+ cells

animals, and there was also a 5-fold higher proportion of labelled $CD25^+$ $Foxp3^-$ activated T cells (Figure 4.2.8). The proportions of labelled $Foxp3^+$ T cells were also higher in thymectomised animals, but interestingly, while the proportion of labelled $CD25^+$ $Foxp3^+$ regulatory T cell was six times higher than in sham thymectomised controls, the labelling of $CD25^-$ $Foxp3^+$ cells was only 2 times greater. This difference was due mainly to the surprisingly high proportion of labelled $CD25^-$ $Foxp3^+$cells in the sham-thymectomised controls. Because thymectomy perturbed the proliferation rates of $CD4^+$ T cell subsets, presumably by homeostatic control mechanisms, it is difficult to make a quantitative estimate of the contribution that the thymus makes to the maintenance of peripheral T cell subsets in the steady state.

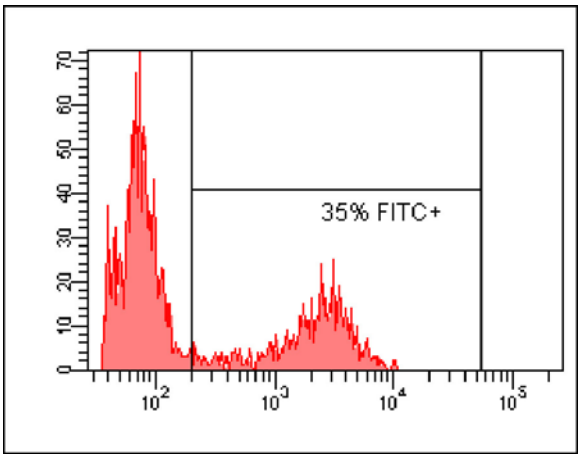
4.2.3 Characterisation of recent thymic emigrants identified in the periphery following intrathymic labelling

An intra-thymic labelling approach was used to allow direct identification of RTE in the periphery.This technique involves injecting a concentrated solution of FITC into the thymus, where it labels thymocytes non-specifically by binding covalently to cell-surface proteins. Labelled RTE that emerge into the periphery can be identified as $FITC^+$ cells by flow cytometry (Hosseinzadeh and Goldschnieder 1993). Intrathymic injections of FITC (1 mg/ml in PBS; 2 x 10 μl per lobe) routinely labelled 30-40% of thymocytes (Figure 4.2.9). RTE were detected as $FITC^+$ cells in the lymph nodes and spleen collected 2 hours to 178 hours (seven days) following intrathymic injection. In order to assess the expression of markers associated with the $FITC^+$ RTE, $CD4^+$ T cells were purified by negative selection from preparations of lymph node and spleen cells collected 24 hours after intra-thymic injection of FITC. The cells were then stained to detect either Thy-1, CD45RC or CD8α. Of the $FITC^+$ RTE, 95% expressed Thy-1, while 17% expressed CD45RC at low levels (Figure 4.2.10). Interestingly, the expression of CD8α was found to be bimodal, revealing discrete populations of $CD8\alpha^-$ (~67%) and $CD8\alpha^+$ (~33%) RTE.

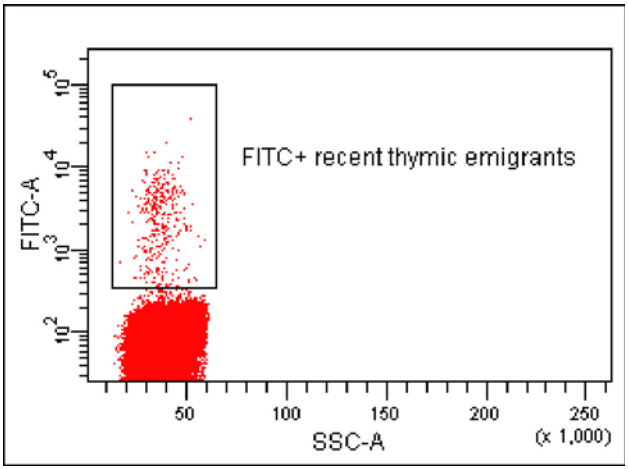
Figure 4.2.9 Identification of labelled recent thymic emigrants (RTE) in the periphery following intrathymic injection of FITC

Thymocytes in 7-8 week old animals were labelled *in situ* by intrathymic injection of FITC (2 x 10μl injections of 1mg/ml FITC in PBS per thymic lobe). Twenty four hours later, cell suspensions were prepared from the thymus, pooled mesenteric, celiac, cervical, inguinal and popliteal lymph nodes and the spleen. Labelling by FITC was detected by flow cytometry. At this time, 30-40% of thymocytes were labelled (A), and $FITC^+$ RTE were identified in the lymph nodes (B) and spleen (C) at 24 h post injection. Representative of 6 experiments.

A


35% FITC+

B


FITC+ recent thymic emigrants
FITC-A
SSC-A
(x 1,000)

C

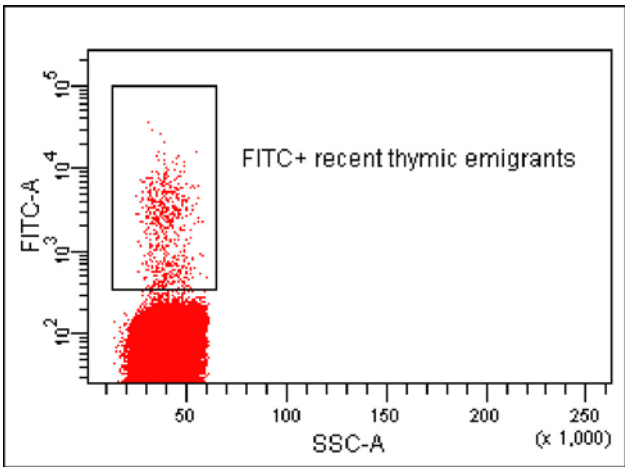
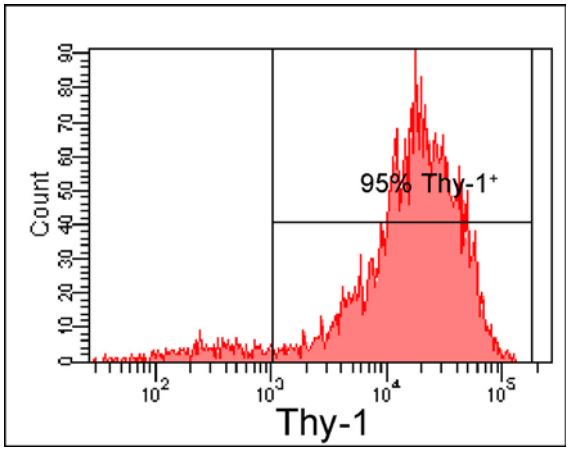

FITC+ recent thymic emigrants
FITC-A
SSC-A
(x 1,000)

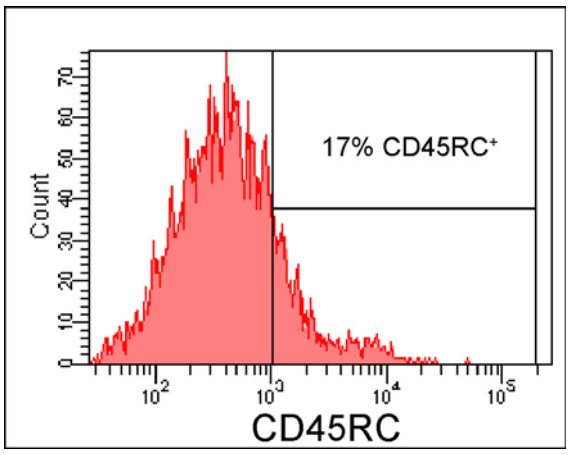
Figure 4.2.10 Surface antigen phenotype of FITC$^+$ recent thymic emigrants

Thymocytes in 7-8 week old animals were labelled *in situ* by intrathymic injection of FITC (2 x 10μl injections of 1mg/ml FITC in PBS per thymic lobe). Twenty four hours later, cell suspensions were prepared from pooled mesenteric, celiac, cervical, inguinal and popliteal lymph nodes and CD4$^+$ T cells were purified by negative selection. Samples were stained by indirect immunofluorescence (APC) with antibodies against Thy-1, CD45RC or CD8α and expression of Thy-1 (A), CD45RC (B) or CD8α (C)by FITC$^+$ RTE was then assessed by flow cytometry. Representative of 3 experiments.

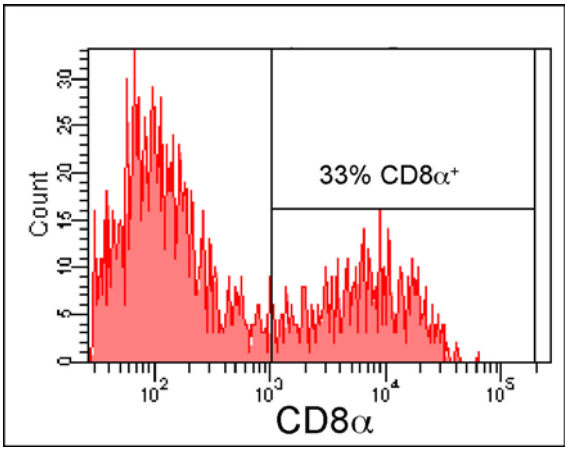
A



B



C



Expression of Foxp3 by $FITC^+$ RTE was examined in lymph node and spleen cells prepared from rats at regular intervals following intrathymic injection. At both sites, the proportion of RTE expressing Foxp3 increased rapidly in the first 24 h, reaching 20-25% of all $FITC^+$ RTE in the spleen, and 30-35% of all $FITC^+$ RTE in the lymph nodes (Figure 4.2.11). At this time, ~15% of $FITC^+$ RTE in blood expressed Foxp3. Subsequently, the proportions of $FITC^+$ RTE expressing Foxp3 decreased to levels comparable to the proportion of $CD4^+$ T cells that express Foxp3 in normal animals. To determine whether this phenomenon could be attributed to the preferential proliferation of regulatory T cells over other RTE following their arrival in the periphery, animals received intrathymic injections with FITC and an intraperitoneal injection of BrdU (1 mg) simultaneously and this was followed by further injections of BrdU at 1 hour and 2 hours later. Twenty four hours later, incorporation of BrdU by $FITC^+$ RTE and $FITC^-$ $CD4^+$ T cells was assessed by flow cytometry. The proportions of $Foxp3^+$ and $Foxp3^-$ $FITC^+$ RTE were identical to those of corresponding $Foxp3^+$ and $Foxp3^-$ subsets of $FITC^-$ $CD4^+$ T cells (Figure 4.2.12). It appears, therefore, that $Foxp3^+$ RTE label in no greater proportions than other $Foxp3^+$ cells. However, in both cases, the $Foxp3^+$ subsets contained a higher proportion of labelled cells than their $Foxp3^-$ conventional counterparts.

Conversion of $Foxp3^-$ RTE into $Foxp3^+$ regulatory T cells is another explanation for the high proportion of regulatory cells amongst RTE. Because $Foxp3^-$ RTE cannot be purified in sufficient numbers, we elected to use $Foxp3^-$ thymocytes as an alternative to test this hypothesis. Thymocytes obtained from a DA.CD45.2 donor were depleted of $CD25^+$ cells, resulting in a preparation containing only 0.3-0.8% $Foxp3^+$ cells. Approximately 1×10^9 of these cells were adoptively transferred into congenic recipients, and the proportion of $CD4^+$ donor cells expressing Foxp3 determined on the sample prior to injection, and either 1 day or 2 weeks following the adoptive transfer (Figure 4.2.13). Control rats received 1×10^9 unfractionated thymocytes, of which approximately 4.5% were $Foxp3^+$ cells. In the lymph nodes of recipients that had received thymocytes depleted of regulatory T cells, the proportion of regulatory T cells among donor cells had increased almost 6-fold between day one and two weeks after transfer. Since the proportion of donor cells in the lymph nodes was

Figure 4.2.11 Expression of Foxp3 by FITC$^+$ RTE

In order to assess the expression of Foxp3 by RTE, rats received intrathymic injections of FITC (See Figure 4.2.9), and cell suspensions were prepared from pooled lymph nodes and the spleen at either 2, 12, 24, 48, 72 or 120 hours later. Blood samples were taken at 2 and 24 hours after intra-thymic labelling and mononuclear cells were prepared by lymphoprep density gradient centrifugation. $CD4^+$ T cells were purified by negative selection and stained with antibodies against Foxp3 (PE). Expression of Foxp3 by FITC$^+$ RTE was determined by flow cytometry. As a comparison, thymocytes prepared from normal rats were depleted of $CD8^+$ T cells and B cells and stained with antibodies against CD4 and Foxp3. This allowed identification of $CD4^+$ single positive thymocytes, and assessment of their expression of Foxp3 by flow cytometry. Data represent mean ±SD of 4 animals per time point

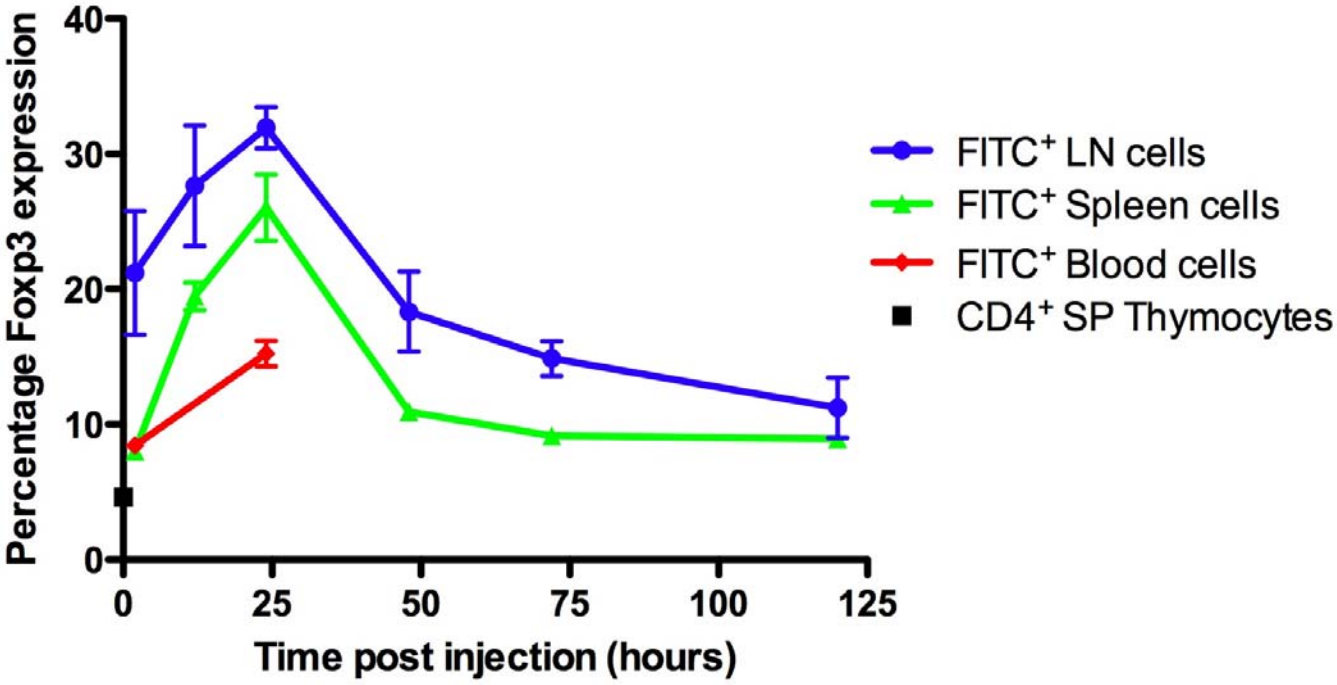
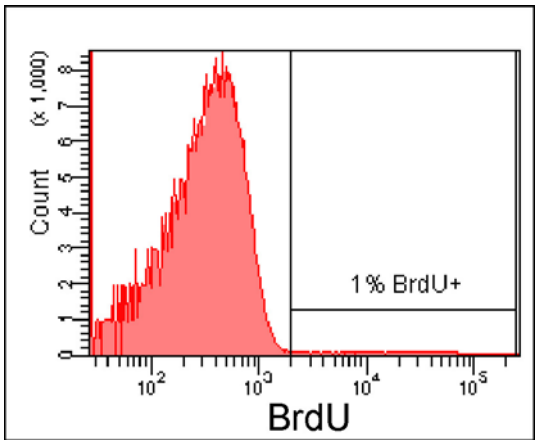

Percentage Foxp3 expression
40
30
20
10
0
0
25
50
75
100
125
Time post injection (hours)
FITC+ LN cells
FITC+ Spleen cells
FITC+ Blood cells
CD4+ SP Thymocytes

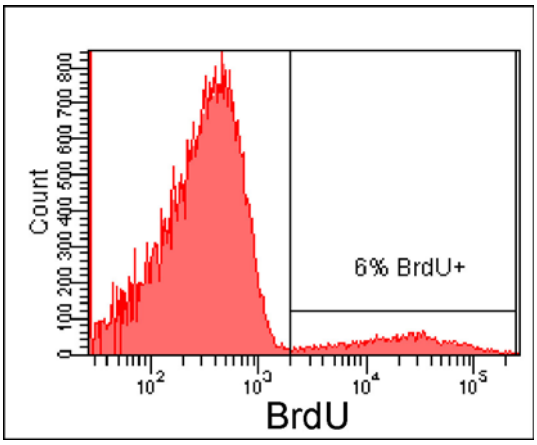
Figure 4.2.12 Proliferation of RTE

In order to determine the relative proliferation rates of $FITC^+$ RTE and other $CD4^+$ T cells, rats received intrathymic injections of FITC (See Figure 4.2.9) followed closely by an intraperitoneal injection of BrdU (1mg). The animals then received further injections of BrdU at 1 hour and 2 hours following intrathymic injection. Twenty four hours after intra-thymic injection, lymphocyte suspensions were prepared from the mesenteric, celiac, cervical, inguinal and popliteal lymph nodes and $CD4^+$ T cells were purified by negative selection. Samples were stained to detect Foxp3, treated with DNAse to expose incorporated BrdU and stained with anti-BrdU antibody. Among the $FITC^-$ cells, BrdU had been incorporated by very few conventional $Foxp3^-$ $CD4^+$ T cells (A), but by a larger proportion of $Foxp3^+$ regulatory T cells (B). In the $FITC^+$ cells (RTE), the proportion of BrdU labelled cells was higher in the $Foxp3^+$ RTE (D) than in the $Foxp3^-$ RTE (C). However, the proportions of BrdU-labelled $Foxp3^-$ $FITC^+$ RTE (C) and $Foxp3^-$ $FITC^-$ conventional T cells (A) were similar, as were the proportions of BrdU labelled $Foxp3^+$ RTE (D) and $Foxp3^+$ $FITC^-$ regulatory T cells (B). Data are representative of 3 animals.

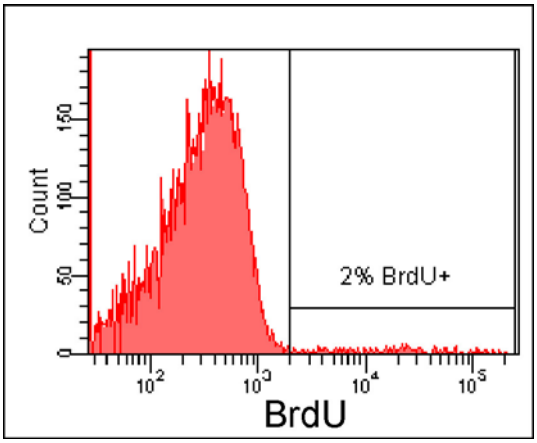
A


Count
(x 1,000)
1% BrdU+
BrdU

B


Count
6% BrdU+
BrdU

C


Count
2% BrdU+
BrdU

D

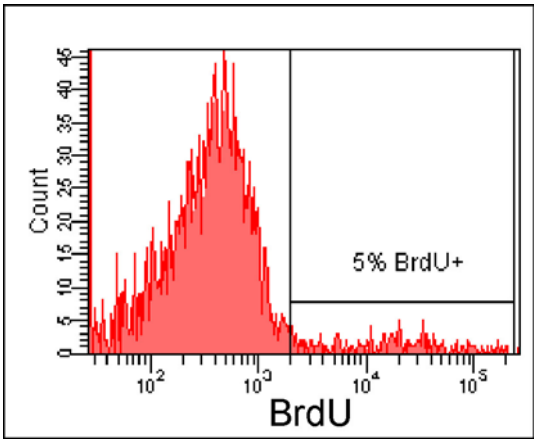
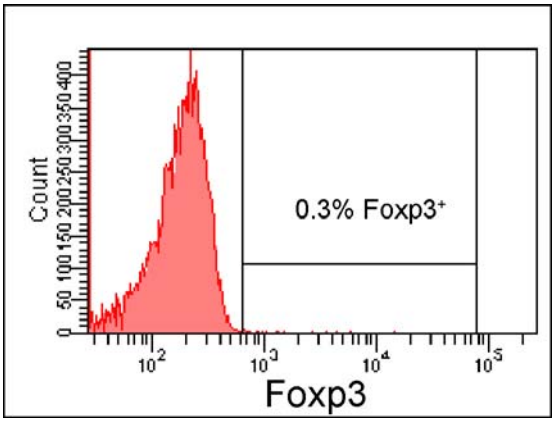

Count
5% BrdU+
BrdU

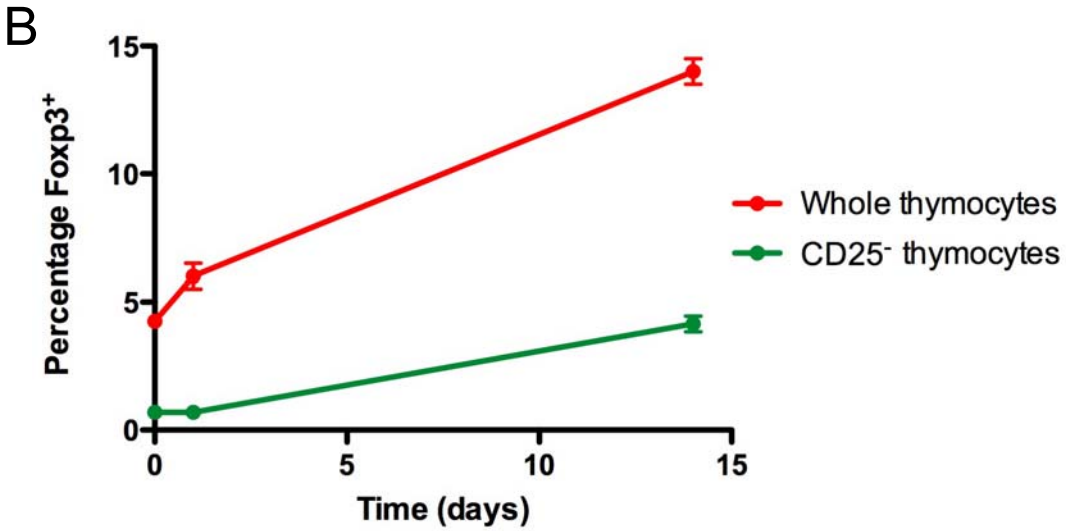
Figure 4.2.13 Conversion of Foxp3$^-$ thymocytes to Foxp3$^+$ regulatory T cells in the periphery

Thymocytes were prepared from DA CD45.2 rats and half were labelled with antibodies against CD25 then depleted using immunomagnetic beads. After depletion, the remaining cells were labelled with antibodies against CD8β, CD4 and Foxp3, and flow cytometric analysis showed that only 0.3-0.8% of the remaining CD4$^+$ single positive cells expressed Foxp3 (A). A second control sample of thymocytes remained untreated. In order to determine whether Foxp3$^-$ thymocytes can convert into Foxp3$^+$ regulatory T cells when introduced into the periphery, CD25 depleted or untreated thymocytes were transferred intravenously into DA CD45.1 recipients. The proportion of regulatory T cells was estimated in the donor cells at the time of transfer, and by use of the CD45.2 marker in pooled mesenteric, celiac, cervical, inguinal and popliteal lymph nodes of recipients killed at either day 1 or 14 days after the transfer (B). Similar results were obtained by analysis of spleen cells from the recipients. Results shown mean (± SD). Number of recipients per group was four for the transfers of CD25 depleted thymocytes and two for the transfers of untreated thymocytes.

A


Count
0.3% Foxp3+
Foxp3

B


Percentage Foxp3+
Time (days)
Whole thymocytes
CD25- thymocytes

approximately 1% of the total lymphocytes at both one day and at two weeks following the adoptive transfer, this change is not likely to be due to the death of large numbers of Foxp3$^-$ thymocytes. In recipients of unseparated thymocytes, regulatory T cells as a proportion of donor CD4$^+$ T cells had increased almost 2.5 fold between day one and two weeks later. The rate of increase in the proportion of Foxp3$^+$ cells, indicated by the slope of the graph was only slightly lower in animals that received thymocytes depleted of regulatory T cells. The slightly faster rate of increase in the proportion of regulatory T cells observed in recipients of unseparated thymocytes could be due either to conversion of some of the CD25$^+$ Foxp3$^-$ cells into regulatory T cells, or to proliferation of the CD25$^+$ Foxp3$^+$ in the original inoculum over the 2 week period. The minimal effect of that depletion of CD25$^+$ cells had on the rate of increase in the proportion of Foxp3$^+$ donor-derived cells in the recipient suggests that some conversion of Foxp3$^-$ cells into regulatory T cells had occurred. This can be considered as “proof of concept” evidence that RTE can be converted to regulatory T cells in the periphery, presumably by contact with appropriately presented cognate self antigens.

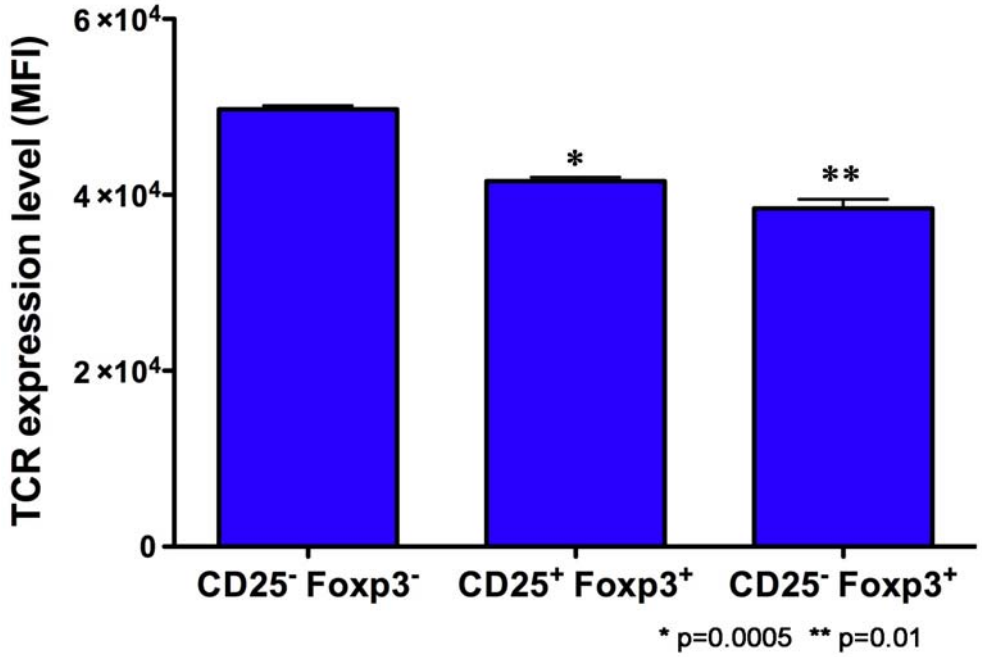
The level of expression of the TCR was then investigated in Foxp3$^+$ thymocytes and Foxp3$^+$ RTE to determine whether reduced TCR expression is already apparent in these cells, as it is in mature regulatory T cells and anergised T cells. It was found that the level of TCR expression in both the CD25$^+$ Foxp3$^+$ and CD25$^-$ Foxp3$^+$ subsets of CD4$^+$ single positive thymocytes was significantly lower than in the CD25$^-$ Foxp3$^-$ subset (Figure 4.2.14) ($p=0.0005$ and $p=0.01$ respectively). Furthermore, TCR expression by RTE was consistently lower on the Foxp3$^+$ subset compared with the Foxp3$^-$ subset from as soon as 2 h after intra-thymic injection of FITC, and remained lower over following the seven day period. It is assumed that this phenotype is established by contact with cognate antigens within the thymus and/or during conversion of RTE to regulatory T cells in the periphery.

In order to observe the phenotypic maturation of freshly released RTE in the periphery, rats received intra-thymic FITC and were killed from 2 hour post injection to 178 hours (7 days) post injection. The observations were made in intact animals in

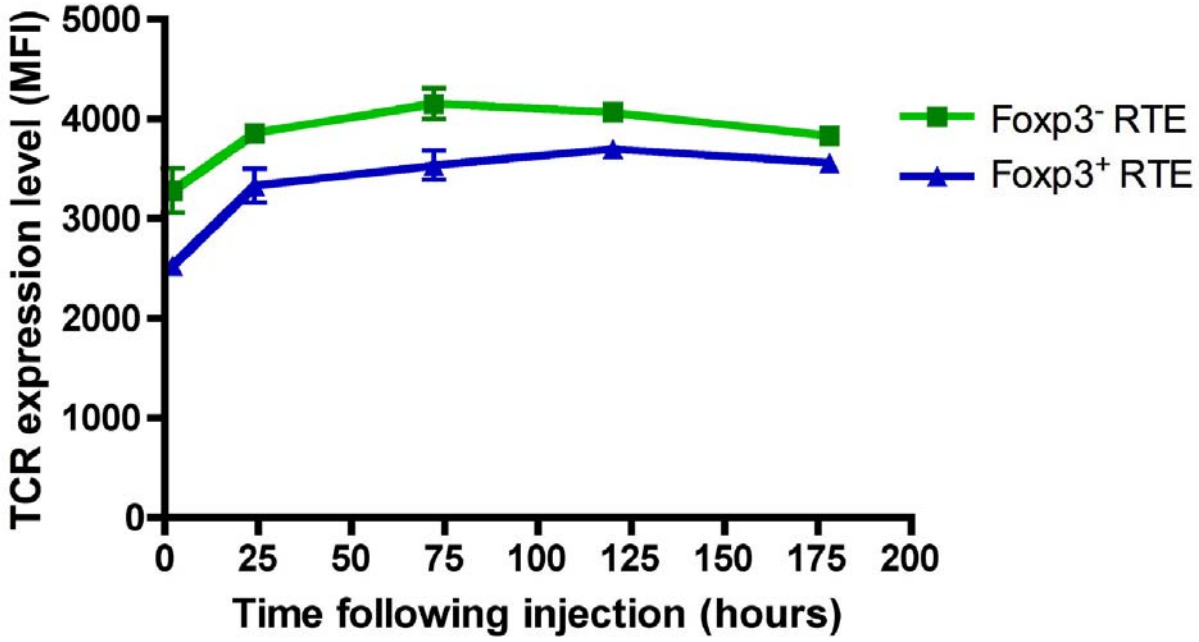
Figure 4.2.14 Levels of TCR expression by thymocytes and FITC$^+$ RTE

In order to assess the levels at which the TCR is expressed by Foxp3$^-$ conventional thymocytes and Foxp3$^+$ thymocytes, CD4$^+$ single positive thymocytes were separated from CD8$^+$ CD4$^-$ thymocytes and CD8$^+$ CD4$^+$ cells thymocytes by depletion of all cells expressing CD8. The CD4$^+$ single positive thymocytes were then distinguished from double negative cells by staining the remaining thymocytes with anti-CD4 antibody, and they were also stained with antibodies against the TCR and Foxp3. The levels of TCR expression level by the Foxp3$^+$ CD4$^+$ single positive thymocytes and Foxp3$^-$ CD4$^+$ single positive thymocytes were assessed (A). Both CD25$^+$ Foxp3$^+$ and CD25$^-$ Foxp3$^+$ thymocytes expressed significantly lower levels of TCR than the CD25$^-$ Foxp3$^-$ thymocytes ($p=0.0005$ and $p=0.01$ respectively). CD4$^+$ T cells purified from peripheral lymph nodes at 2, 24, 48, 72, 120 or 178 hours after intrathymic injection of FITC were stained with antibodies against the TCR and Foxp3. FITC$^+$ RTE at each time point were assessed for expression of Foxp3 and the Foxp3$^+$ and Foxp3$^-$ subsets were compared for the level of their expression of the TCR (B). Data represent the means ±SD of 3 animals per time point.

A


6 ×10⁴
4 ×10⁴
2 ×10⁴
0
TCR expression level (MFI)
*
**
CD25⁻ Foxp3⁻
CD25⁺ Foxp3⁺
CD25⁻ Foxp3⁺
* p=0.0005 ** p=0.01

B


5000
4000
3000
2000
1000
0
TCR expression level (MFI)
0
25
50
75
100
125
150
175
200
Time following injection (hours)
Foxp3⁻ RTE
Foxp3⁺ RTE

order to avoid any potential homeostatic disruption of the maturation of RTE that might be caused by thymectomy. At the specified times, suspensions of spleen and lymph node cells were prepared and $CD4^+$ T cell purified by negative selection and labelled to detect Foxp3 plus either Thy-1, CD45RC or CD8α by flow cytometry. When $FITC^+$ RTE were examined in both lymph nodes and spleen, it was found that levels of expression of Thy-1 by the $Foxp3^+$ subset declined rapidly over the first 24 hours after intra-thymic injection and then declined more slowly over the remainder of the observation period (Figure 4.2.15). In contrast, there was a much more gradual decline in Thy-1 expression by the $Foxp3^-$ subset. Additionally, the proportion of $FITC^+$ RTE that expressed CD45RC increased progressively in the $Foxp3^-$ subset in both cells taken from the lymph nodes and the spleen (Figure 4.2.16), as described for RTE in general by Hosseinzadeh and Goldscheider (1993). In contrast, there was little change overall in the proportion of $CD45RC^+$ cells in the $Foxp3^+$ subset in lymph nodes, and a delayed increase in cells taken from the spleen. In the case of the RTE-associated marker CD8α, there was a striking increase in the proportion of cells expressing this molecule in the $Foxp3^+$ subset of $FITC^+$ RTE in cells taken from both the lymph nodes and spleen, reaching ~60% in lymph nodes by 48 hours. Over the same period, expression of CD8a declined in the Foxp3- subpopulation in lymph nodes and increased only slightly in the spleen (Figure 4.2.17).

4.3 Discussion

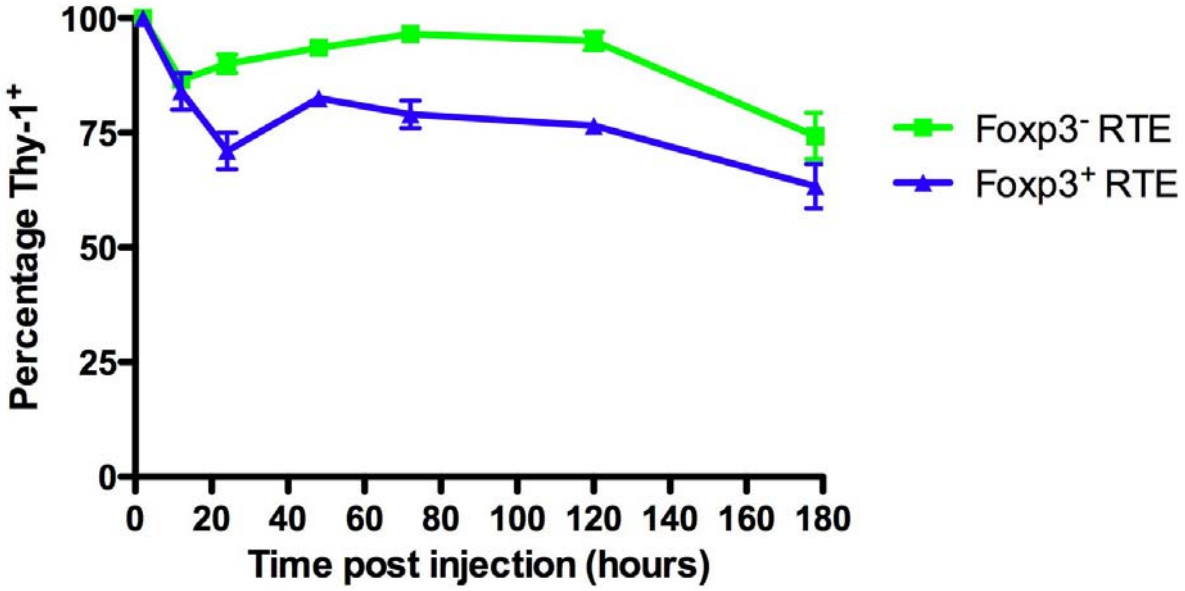
4.3.1 Expression of markers associated with RTE by regulatory T cells

It is thought that regulatory T cells may be selected in the thymus through high avidity interactions with self-peptides (Schwartz 2005), where they differentiate along a program controlled by Foxp3 (Maggi *et al.* 2005). Although this view is widely accepted in the literature (Sakaguchi *et al.* 2004), it arises from a set of observations made by Jordan and colleagues (2001) in double transgenic mice, which express both a specific inducible neo-self antigen and a TCR that recognises a specific

Figure 4.2.15 The expression of the Thy-1 by RTE as they mature in the periphery

Thymocytes were labelled *in situ* by intrathymic injection of FITC (See Figure 4.2.9), and at regular intervals animals were killed to obtain lymph nodes (pool of mesenteric, celiac, cervical, inguinal and popliteal lymph nodes) and spleens. $CD4^+$ T cells were purified by negative selection and stained with antibodies against Thy-1 and Foxp3. Proportions of cells expressing Thy-1 in the $Foxp3^+$ and $Foxp3^-$ subsets of $FITC^+$ RTE were examined for both lymph node cells (A) and spleen cells (B). Data represent the means ±SD of 3 animals per time point.

A


Percentage Thy-1+
100
75
50
25
0
0 20 40 60 80 100 120 140 160 180
Time post injection (hours)
Foxp3- RTE
Foxp3+ RTE

B

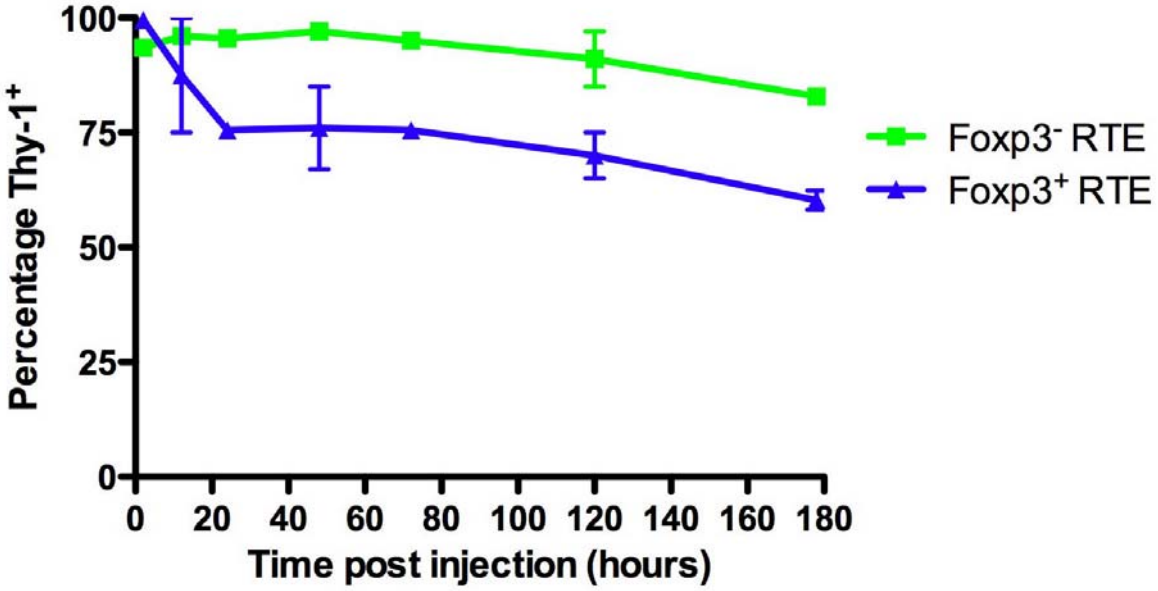

Percentage Thy-1+
100
75
50
25
0
0 20 40 60 80 100 120 140 160 180
Time post injection (hours)
Foxp3- RTE
Foxp3+ RTE

Figure 4.2.16 Expression of CD45RC by RTE as they mature in the periphery

Thymocytes were labelled *in situ* by intrathymic injection of FITC (See Figure 4.2.9), and at regular intervals animals were killed to obtain lymph nodes (pool of mesenteric, celiac, cervical, inguinal and popliteal lymph nodes) and spleens. $CD4^+$ T cells were purified by negative selection and stained with antibodies against CD45RC and Foxp3. Proportions of cells expressing CD45RC in the $Foxp3^+$ and $Foxp3^-$ subsets of $FITC^+$ RTE were examined for both lymph node cells (A) and spleen cells (B). Data represent the means ±SD of 3 animals per time point.

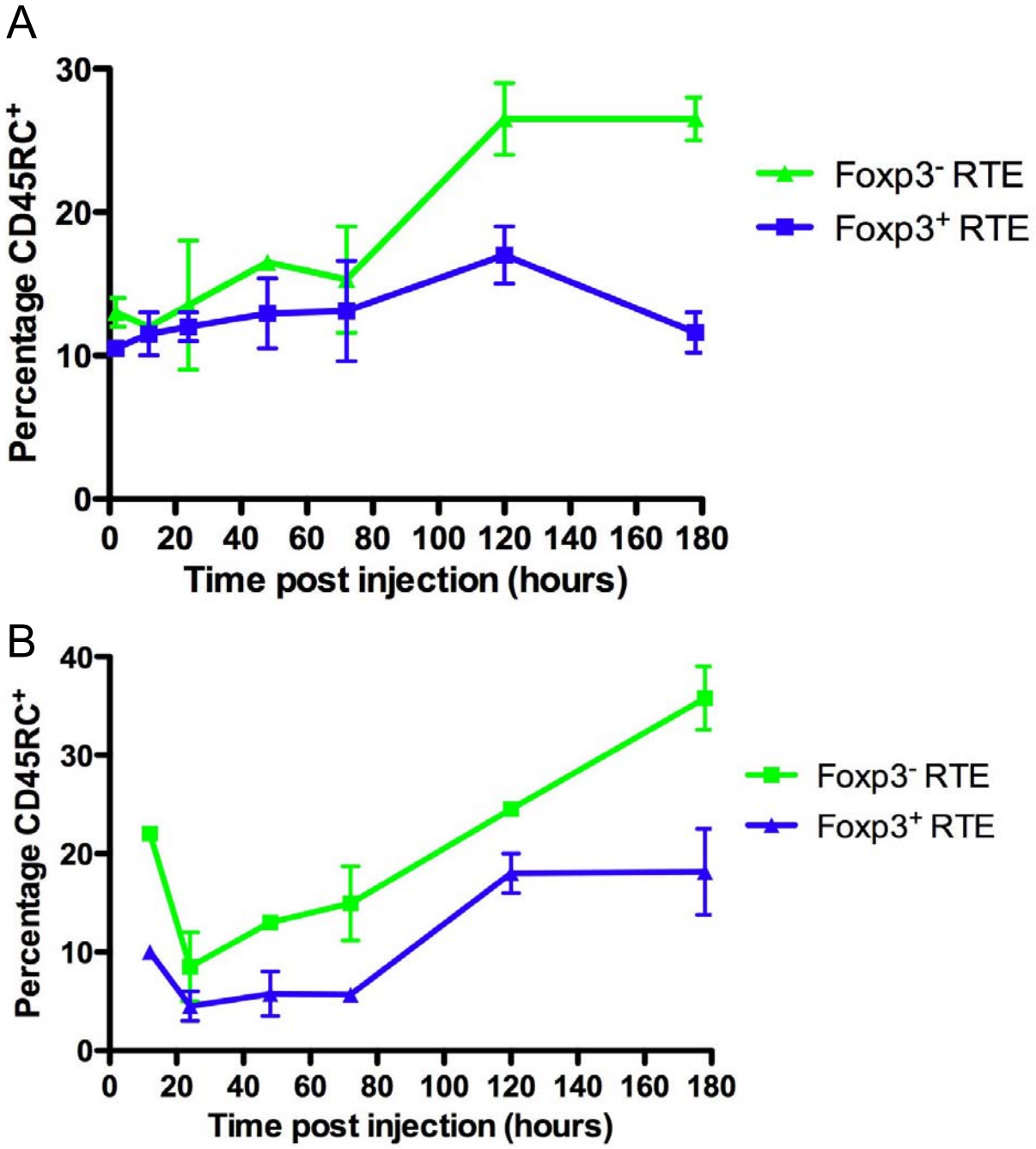
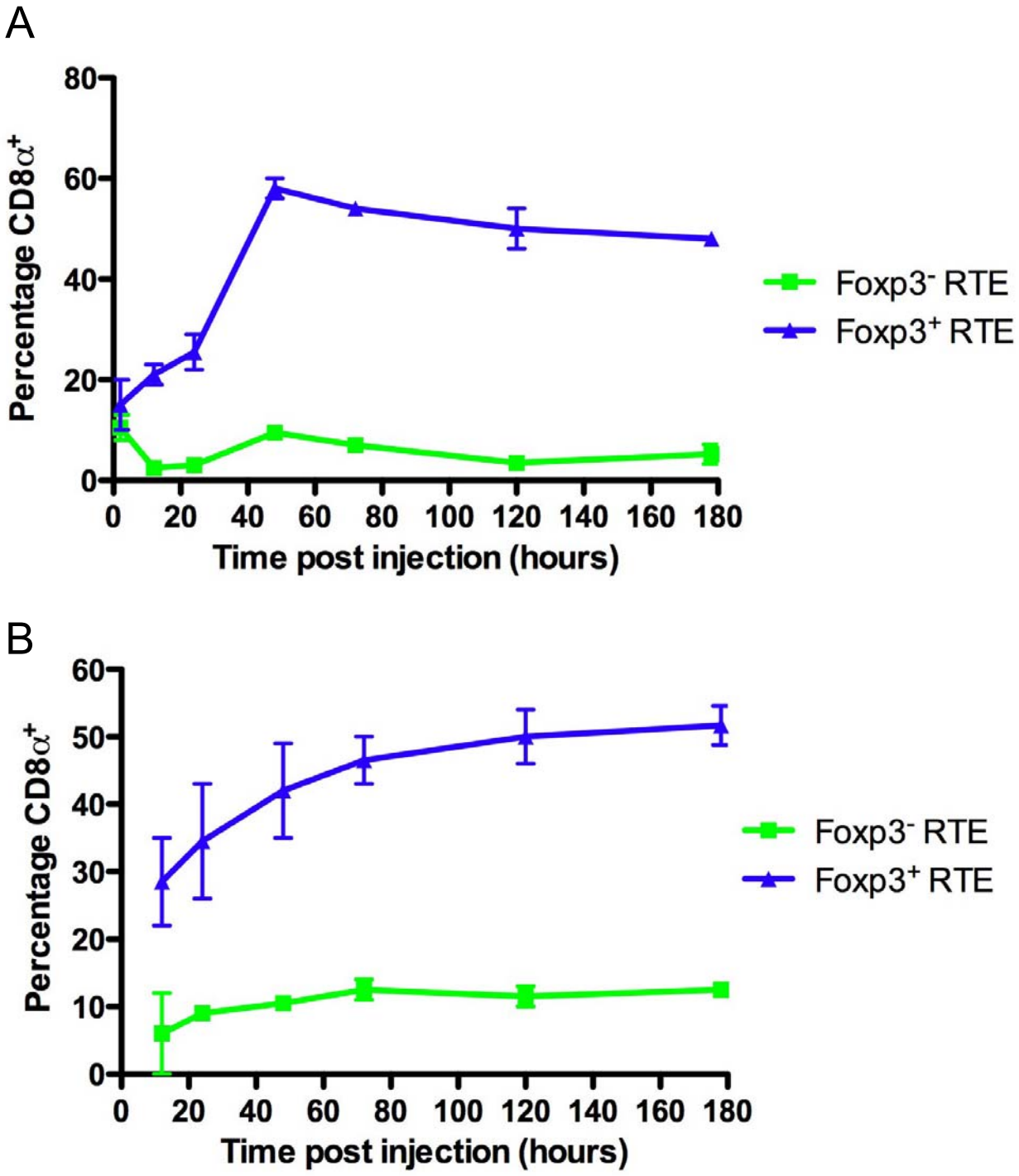

A
Percentage CD45RC+
30
20
10
0
0 20 40 60 80 100 120 140 160 180
Time post injection (hours)
Foxp3- RTE
Foxp3+ RTE
B
Percentage CD45RC+
40
30
20
10
0
0 20 40 60 80 100 120 140 160 180
Time post injection (hours)
Foxp3- RTE
Foxp3+ RTE

Figure 4.2.17 Expression of the CD8α homodimer by RTE as they mature in the periphery

Thymocytes were labelled *in situ* by intrathymic injection of FITC (See Figure 4.2.9), and at regular intervals animals were killed to obtain lymph nodes (pool of mesenteric, celiac, cervical, inguinal and popliteal lymph nodes) and spleens. $CD4^+$ T cells were purified by negative selection and stained with antibodies against CD8α and Foxp3. Proportions of cells expressing CD8α in the $Foxp3^+$ and $Foxp3^-$ subsets of $FITC^+$ RTE were examined for both lymph node cells (A) and spleen cells (B). Data represent the means ±SD of 3 animals per time point.


A
Percentage CD8α+
80
60
40
20
0
0 20 40 60 80 100 120 140 160 180
Time post injection (hours)
Foxp3- RTE
Foxp3+ RTE
B
Percentage CD8α+
60
50
40
30
20
10
0
0 20 40 60 80 100 120 140 160 180
Time post injection (hours)
Foxp3- RTE
Foxp3+ RTE

epitope of the antigen. Because the transgenes were crossed onto a RAG knockout background, all of the T cells recognise the neo-self epitope. While the proportion of $CD4^+$ T cells that expressed CD25 in the thymus increased with higher expression of the transgenic peptide (Jordan *et al*. 2001, Kawahata *et al.* 2002), no direct evidence was presented that this was due to the selection of regulatory T cells in the thymus. Because the number of regulatory T cells in the thymus was unaffected by increasing expression of the transgenic antigen, and the absolute number of $CD25^-$ T cells declined in a dosage dependent manner, it seems likely that this phenomenon actually reflects the negative selection of $CD25^-$ cells (van Santen *et al.* 2004). Although this interpretation contradicts the hypothesis that regulatory T cells are generated by encountering their antigen in the thymus, it has been established that regulatory T cells recognise MHC-presented self peptides more efficiently than other T cell subsets (Hsieh *et al.* 2004). A possibility exists, therefore, that regulatory T cells represent T cells that have been anergised by cognate antigen in the periphery (Taams and Akbar 2005).

This work was undertaken to examine whether conversion of RTE is a significant source of regulatory T cells. The experiments of Modigliani *et al.* (1996) have indeed suggested that RTE have a unique ability to acquire immunosuppressive properties. While the phenotype of RTE in humans and mice is indistinguishable from that of mature T cells using available surface antigens, Thy-1 is expressed by rat RTE and lost as cells mature in the periphery (Hosseinzadeh and Goldschneider 1993). Additionally markers that are useful in the characterisation of $CD4^+$ RTE in rats are the level of expression of CD45RC (Yang and Bell 1992), and expression of the $CD8\alpha$ homodimer (Kenny *et al.* 2004).

Among $CD4^+$ T cells, Thy-1 was expressed by ~30% of $CD25^+$ $Foxp3^+$ regulatory T cells, compared with ~20% for each of the conventional activated ($CD25^+$ $Foxp3^-$) and resting ($CD25^-$ $Foxp3^-$) subsets (Figure 4.2.1). Interestingly, the $CD25^-$ $Foxp3^+$ putative regulatory T cell population had the lowest proportion (~15%) of $Thy\text{-}1^+$ cells. On this basis, the data suggest that $CD25^+$ $Foxp3^+$ regulatory T cells are more closely related to RTE than are other $CD4^+$ T cell subsets. Additionally, the expression of the

CD8α was enriched in the CD25$^+$ Foxp3$^+$ subset (~30%), compared with 1-10% of other T cell subsets (Figure 4.2.3). Furthermore, ~50% of all CD4$^+$ T cells that express the CD8α homodimer are CD25$^+$ Foxp3$^+$ regulatory T cells (Figure 4.2.4). However, expression of CD45RC was found to be low in both the CD25$^+$ Foxp3$^+$ and CD25$^-$ Foxp3$^+$ subsets (Figure 4.2.2). Interpretation of this finding is complicated by the sequence of CD45 isoform expression by RTE (Hosseinzadeh and Goldschneider 1993) and following antigen experience by conventional T cells (Yang and Bell 1992). Most thymocytes do not express CD45RC and RTE have the CD45^{low} phenotype during the first few days in the peripheral circulation. However, expression of CD45RC is upregulated by the majority of RTE, which acquire the phenotype of naïve CD4$^+$ T cells (Hosseinzadeh and Goldschneider 1993). Subsequently, some of these cells revert to the CD45^{low} phenotype and this is thought to reflect entry into the memory T cell pool (Yang and Bell 1992). On this basis, low expression of the CD45RC phenotype by regulatory T cells is consistent with either retention of the early RTE phenotype, or a more rapid transition to the memory phenotype than occurs in conventional RTE. To further clarify the relationship between RTE and regulatory T cells, the cells were observed during the period following adult thymectomy.

The absolute numbers of CD4$^+$ Thy1$^+$ cells with the phenotypes CD25$^+$ Foxp3$^+$ (regulatory T cells) and CD25$^-$ Foxp3$^-$ (resting T cells) decreased rapidly following thymectomy, indicating that the maintenance of both subsets depends on continuing thymic function (Figure 4.2.5). However, thymectomy caused little change in the number of Thy1$^+$ CD4$^+$ cells with the CD25$^-$ Foxp3$^+$ (putative regulatory T cell) phenotype. This finding indicates that this population has a less direct dependence on continuing thymic function, although the fact this subset increased 4-fold over the observation period in sham-thymectomised rats suggests that thymectomy had compromised this increase. The latter interpretation would be consistent with an indirect requirement for newly formed RTE for the longer term maintenance of the CD25$^-$ Foxp3$^+$ subset.

The absolute numbers of $CD4^+$ $Thy\text{-}1^+$ cells with the phenotypes $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^-$ that express CD45RC decreased following thymectomy, albeit not to the same extent as the numbers expressing Thy-1 (Figure 4.2.6). As discussed above, this probably reflects the presence of $CD45RC^-$ memory T cells in the conventional subset. In the case of the regulatory subset, this is less clear but it suggests that these cells have encountered cognate self antigen in the periphery ands switched to the memory phenotype of CD45 ($CD45RC^-$). Interestingly, the numbers of $CD45RC^+$ $CD25^-$ $Foxp3^+$ putative regulatory T cells remained stable following thymectomy. This observation suggests a less direct relationship to RTE. However, the observation that numbers of CD45RC+ cells of this subset increased by 6-fold over the same period in sham-thymectomised controls indicates that thymectomy prevented this increase and suggests that the subset may have an indirect requirement for RTE to maintain normal numbers in the periphery. It is likely, therefore, that low expression of CD45RC under steady state conditions by some conventional $CD4^+$ T cells and by most $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ T cells is due to experience with antigen.

The CD8α homodimer is expressed mainly by RTE but also by some T cells that have been activated (Kenny *et al.* 2004) Since the majority of CD8α expressing cells also express Foxp3, it was hypothesised that many CD8α^+ RTE may be regulatory T cells (Figure 4.2.4). Therefore, the effects of thymectomy on the numbers of CD4+ T cells expressing CD8a were investigated (Figure 4.2.6).

The absolute numbers of resting T cells expressing CD8α homodimer experienced a modest decrease, while the numbers of $CD25^+$ $Foxp3^+$ cells expressing CD8α experienced a rapid decrease (Figure 4.2.7). In contrast, numbers of $CD25^-$ $Foxp3^+$ T cells expressing CD8α remained stable. Numbers of each subset increased in sham-thymectomised controls, especially in the $CD25^-$ $Foxp3^+$ subset, which increased 5-fold during the period of observation. These findings suggest, therefore, that maintenance of numbers in each subset is dependent either directly or indirectly on continuing thymic function. It appears significant that although there were only modest decreases in the numbers of $CD25^-$ $Foxp3^+$ cells that express either Thy-1, CD8α or

low levels of CD45RC following thymectomy, there was nevertheless no dramatic increase, as was observed in sham-thymectomised controls. The findings suggest, therefore, that this subset might be maintained by differentiation from an immediate precursor that is itself closely related to RTE. The most likely candidate for this precursor is the $CD25^+$ $Foxp3^+$ subset. Interpretation of the effects of thymectomy could be affected by undesired consequences of thymectomy on the behaviour of the peripheral T cell pool. In particular ,the size of the peripheral T cell pool might be affected in these young adult rats, leading to compensatory homeostatic proliferation.

In order to determine whether the thymectomised rats exhibited lymphopenia and homeostatic proliferation, numbers of T cells were compared in the lymph nodes of thymectomised and sham-thymectomised rats, where it was revealed that significantly lower numbers of $CD4^+$ T cells were present in thymectomised rate ($p=0.022$) (Figure 4.2.8). The proliferation of $CD4^+$ T cell subsets was assessed by incorporation of BrdU by each of the subsets of $CD4^+$ T cells *in vivo*. Following 7 days of BrdU administration, proportions of labelled resting, activated, $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells were higher in thymectomised rats than in sham thymectomised controls (Figure 4.2.8). This finding indicates that thymectomy had the effect of increasing homeostatic proliferation in all subsets. However, homeostatic proliferation was not sufficient to sustain the increase of regulatory T cells that was observed in the intact controls, emphasising further the importance of the thymus in maintaining numbers of both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells. An alternative approach, that avoids the complication of thymectomy-induced lymphopenia, is required to determine the physiological contribution of the thymus to the maintenance of regulatory T cells.

4.3.2 The recent thymic origin of regulatory T cells

Intrathymic injection of FITC has been used previously to label thymocytes, which can be detected later as $FITC^+$ RTE when they emerge into the periphery (Hosseinzadeh and Goldschneider 1993). In these studies, thymectomy was performed after the

intrathymic injection of FITC, allowing the maturation of phenotype to be observed in a cohort of labelled RTE. However, in the studies described herein, intrathymic injection was employed in intact animals to monitor directly the contribution made by the thymus to the maintenance of the regulatory T cell pool under physiological conditions. It was found that the injection of 1mg/ml FITC into two sites per lobe (4 x 10μl injections) consistently labelled 20-40% of thymocytes, as assessed 24 h post injection, at which time RTE could be identified in the spleen and lymph nodes as $FITC^+$ cells (Figure 4.2.9).

At 24 hours after intra-thymic injection, essentially all the $FITC^+$ RTE had the phenotype Thy-1^+, and $CD45RC^{low}$, confirming the work of others (Hosseinzadeh and Goldschneider 1993). Furthermore, 33% of RTE expressed the CD8α homodimer (Figure 4.2.10). The expression of Foxp3 by RTE was then assessed, revealing that while only ~5% of $CD4^+$ single positive thymocytes express Foxp3, the proportion of RTE expressing Foxp3 increased in the lymph nodes, blood and spleen over the first 24 hours following intrathymic injection (Figure 4.2.11). Subsequently, the proportion of $Foxp3^+$ T cells decreased over the next 4 days to reach proportions comparable to those in the general pool of $CD4^+$ T cells. The increased proportions of $Foxp3^+$ T cells observed over the first 24 h are unlikely due to the differential localisation of regulatory and conventional T cells, as the increases were observed in all compartments. In order to assess the possibility that $Foxp3^+$ T cells newly released might proliferate rapidly in the periphery after encountering cognate self antigens, rats received intra-thymic FITC and 1 mg of BrdU i.p. simultaneously, followed by two further i.p. injections of BrdU at 1 hour and at 2 hours after the initial injection. Labelling of $Foxp3^+$ $FITC^+$ RTE over the first 24 hours was then assessed, revealing that only a minority of $Foxp3^+$ RTE (~5%) had incorporated BrdU (Figure 4.2.12). Therefore, it is unlikely that the proliferation of $Foxp3^+$ RTE following their entry into the periphery can account for the high frequency of $Foxp3^+$ cells amongst $FITC^+$ RTE within the first 24 hours after their entry into the periphery.

An alternative possibility is that the increase in the proportion of $Foxp3^+$ $FITC^+$ RTE during the first 24 hours after intra-thymic injection is due to the conversion of self-

reactive cells into regulatory T cells by encounter with cognate antigens in the periphery. The asymptotic decline in the frequency of $Foxp3^+$ cells amongst RTE that occurs after their first 24 hours in the periphery may indicate a more rapid turnover of regulatory T cells compared to their conventional T cell counterparts, a possibility that will be explored in Chapter 5.A possible difficulty in this interpretation is that an incidence of self-reactive cells amongst RTE of >30% would be much higher than expected, based on the theoretical size of the TCR repertoire. However, as the process of positive selection in the thymus ensures that all T cells are only able to survive if they are at least weakly self reactive (von Boehmer 1994, Ignatowicz *et al.* 1996), such a scenario seems entirely plausible. Alternatively, the high proportion of regulatory T cells amongst RTE in the first 24 hours following their arrival in the periphery may be the result of a more rapid emigration of $Foxp3^+$ cells from the thymus compared with their $Foxp3^-$ counterparts. The importance of understanding the relationship between RTE and regulatory T cells and in particular the role of peripheral conversion, made modelling of the behaviour of naïve conventional RTE in the periphery an important objective. This was achieved by use of single positive thymocytes as a source of "naïve RTE" as described by Yang and Bell (1992).

Thymocytes prepared from DA.CD45.2 thymus were depleted of $CD25^+$ cells to remove $CD25^+$ $Foxp3^+$ regulatory T cells. Only 0.3-0.8% of the remaining $CD4^+$ single positive cells expressed Foxp3, compared to ~4.5% in un-depleted thymocytes. One day after the cells were transferred intravenously to congenic recipients, there was no difference in the proportion of donor cells expressing Foxp3 recovered from recipient lymph nodes compared to what was injected the day before (Figure 4.2.13). However, by 2 weeks after transfer, the percentage of $Foxp3^+$ cells had increased 2.5-fold in recipients of un-depleted thymocytes and 6-fold in recipients receiving depleted thymocytes. The results did not mirror the rapid increase in $Foxp3^+$ cells observed amongst $FITC^+$ RTE in the first 24 hours after intra-thymic injection of FITC. Strikingly however, there was an increase in the percentage of $Foxp3^+$ donor-derived cells by two weeks after transfer, and the magnitude of the increase was similar in recipients of un-depleted and CD25-depleted thymocytes. This is *prima facie* evidence for conversion of some $CD25^-$ $Foxp3^-$ cells to $Foxp3^+$ regulatory T cells. One caveat to

this interpretation is that the depleted thymocytes contained small numbers of $Foxp3^+$ $CD4^+$ T cells. However, the proportion of $Foxp3^+$ cells in non-depleted thymocytes was much higher and this was not reflected in a significantly greater increase in the proportion of $Foxp3^+$ donor-derived cells in recipients of these cells compared with recipients of CD25 depleted thymocytes. Secondly, changes in proportion of $Foxp3^+$ cells can result from apoptosis of immature thymocytes after injection. However, since the proportion of donor cells in the recipient lymph nodes remained constant between day one and two weeks following the injection, the period in which the increase in the proportion of $Foxp3^+$ cells occurred, it is unlikely that the death of large numbers of $Foxp3^-$ donor cells could account for this phenomenon. The donor thymocyte population that was transferred represents a heterogenous mixture of cells at different stages of maturity, some of which may not be amenable to conversion into regulatory T cells until a certain stage of maturity has been reached, and this may account for our failure to observe a more rapid increase in the proportion of $Foxp3^+$ T cells amongst the donor cells. However, it is ultimately not possible to determine whether the increase in the proportion of regulatory T cells was due to the maturation of regulatory T cell precursors into regulatory T cells, following a pre-programmed differentiation pathway using this approach. Overall, the results do not support rapid conversion of a large proportion of RTE to regulatory T cells soon after their entry into the periphery, but they are consistent with a more limited conversion of some RTE-like thymocytes to regulatory T cells after artificial introduction into the circulation. It is not possible to exclude the possibility that emigration of $Foxp3^+$ cells from the thymus is more rapid than emigration of conventional T cells and that this might contribute to the high proportion of Foxp3+ cells amongst RTE in the first 24 hours after intra-thymic labelling. In addition, the high proportion of regulatory T cells amongst RTE could also reflect the transient expression of Foxp3 by some RTE as they encounter cognate antigen in the periphery.

4.3.3 Recent thymic emigrants expressing the transcription factor Foxp3 bear features of anergised T cells

The level of TCR expression by both $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ thymocytes was found to be significantly lower than that of $CD25^-$ $Foxp3^-$ thymocytes (Figure 4.2.13). Furthermore, the level of TCR expression by the $Foxp3^+$ subset of RTE, observed as little as two hours after intrathymic labelling, was lower than for the $Foxp3^-$ subset. This finding suggests that lower expression of the TCR is an inherent feature of $Foxp3^+$ regulatory T cells. Interestingly, the difference in TCR expression level observed between $Foxp3^+$ regulatory T cells and their $Foxp3^-$ counterparts in cells obtained from the lymph nodes was greater the difference between $Foxp3^+$ RTE and $Foxp3^-$ RTE. This difference could be due to the continual recognition of self antigen by regulatory T cells (Fisson *et al.* 2003), leading to progressive downregulation of the TCR over time. Of further interest was the finding that the difference in TCR expression level between regulatory T cells and conventional T cells in the thymus more closely resembled that found among lymph node cells in the periphery than the difference between $Foxp3^+$ RTE and $Foxp3^-$ RTE. This raises the possibility that some of the regulatory T cells in the thymus represent T cells that have recirculated back to the thymus (Bell *et al.* 1995). This would complicate the interpretation of the origin of $Foxp3^+$ cells in the thymus and what proportion are newly generated regulatory T cells.

4.3.4 Phenotypic changes in recent thymic emigrants that express Foxp3 following their arrival in the periphery

The phenotypic maturation of $Foxp3^+$ and $Foxp3^-$ RTE was monitored in the spleen and lymph nodes using the markers Thy-1, CD45RC and CD8α. Interestingly, while the proportions of Thy-1^+ RTE declined in both the $Foxp3^+$ and $Foxp3^-$ subsets of RTE, Thy-1 was lost more rapidly by the $Foxp3^+$ subset (Figure 4.2.15). This finding suggests that the higher proportion of Thy-1 expressing on $CD25^+$ $Foxp3^+$ regulatory

T cells relative to resting and activated RTE underestimates how recently this subset has emigrated from the thymus. Previous studies in which the maturation of a cohort of RTE was examined by intrathymic injection, followed by thymectomy 24 hours later, found that Thy-1 was downregulated in approximately one week, under lymphopenic conditions which are likely to expedite this process. Because the experiments described herein utilised intact animals in order to avoid disruptions to the physiology of RTE, and the export of RTE is continuous, the phenotypic changes observed over the period following the intra-thymic injection of FITC probably underestimate the rate of maturation of RTE.

Foxp3$^+$ and Foxp3$^-$ RTE also differed in expression of CD45RC. While the proportion of Foxp3$^-$ RTE expressing CD45RC in the lymph nodes increased steadily with time after intra-thymic labelling, the proportion of CD45RC$^+$ cells in the Foxp3$^+$ subset remained relatively constant (Figure 4.2.16). There was however, a late rise in the proportion of Foxp3$^-$ RTE expressing CD45RC, although this remained lower than in the Foxp3$^-$ subset. These data suggested that both antigen experience and recent thymic origin contribute to the CD45RC$^-$ phenotype of CD25$^+$ Foxp3$^+$ regulatory T cells.

A previous report has linked expression of the CD8α homodimer with RTE. It was found that a proportion of CD4$^+$ T cells that expresses CD8α disappeared rapidly following thymectomy (Kenny *et al.* 2004). However, whether RTE express CD8α was not investigated and the relationship of the CD8α^+ cells to regulatory T cells has not been explored. Interestingly, two hours after intra-thymic injection of FITC only ~10% of the RTE in the lymph nodes expressed CD8α. However, the proportion of CD8α expressing Foxp3$^+$ RTE increased 6 fold within 48 hours, while the proportion of CD8α expressing Foxp3$^-$ RTE remained stable (Figure 4.2.17). Similar results were observed in the spleen, but the proportion of Foxp3$^+$ RTE expressing CD8α reached only 60% by 7 days after intra-thymic labelling. These findings suggest that expression of CD8α by RTE is induced in the periphery, specifically on regulatory T cells and that this may be result of encounter with cognate self antigen. The difference in expression of CD8α by Foxp3$^+$ RTE in the lymph nodes and spleen may

reflect differences in recruitment to these sites, or perhaps differences in the engagement of cells with cognate antigens.

4.3.5 Summary

In conclusion, ~ 30% of $CD25^+$ $Foxp3^+$ regulatory T cells were found to express the RTE associated marker Thy-1. This proportion was higher than observed in $CD25^-$ $Foxp3^+$ putative regulatory T cells (~15%) and in conventional resting and activated CD4+ T cells (~20%). Interestingly, ~70% of both $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells had the phenotype $CD45RC^-$, which is shared with antigen-experienced conventional T cells and with both $CD4^+$ single positive thymocytes and newly-released RTE. The CD8α homodimer was expressed by ~30% of $CD25^+$ $Foxp3^+$ regulatory T cells, while only small proportions of the other $CD4^+$ T cell subsets expressed the molecule. Approximately half of all $CD4^+$ T cells that express CD8α were found to also express Foxp3. Adult thymectomy confirmed that maintenance of numbers of Thy-1 expressing resting T cells and $CD25^+$ $Foxp3^+$ T cells is dependent on the thymus, while the numbers of Thy-1^+ $CD25^-$ $Foxp3^+$ cells were less directly affected by thymectomy. The finding that the $CD45RC^-$ subsets of resting T cells and $CD25^+$ $Foxp3^+$ regulatory T cells were reduced by only 50% by thymectomy suggests that this antigen experience subset is maintained partially by the thymus and partially by peripheral regeneration and/or greater longevity of the cells. The comparative resistance to thymectomy of the $CD25^-$ $Foxp3^+$ subset suggests that these cells are more distantly related to RTE and have greater potential for generation in the periphery. In the case of the CD8α homodimer, the minority of resting T cells expressing CD8α were affected by thymectomy, suggesting that many of these cells are not of recent thymic origin. However the large proportion of $CD8\alpha^+$ $CD25^+$ $Foxp3^+$ cells that disappeared following thymectomy, probably represent recent emigrants from the thymus. In contrast, the numbers of $CD8\alpha^+$ $CD25^-$ $Foxp3^+$ T cells were least affected by thymectomy, suggesting again that this subset has a less direct dependence on the thymus for maintenance of cell numbers. While only a

low proportion of thymocytes express Foxp3, the proportion of RTE that expresses Foxp3 increases rapidly in the first 24 hours after their release from the thymus. This increase could not be accounted for by either the differential localisation of $Foxp3^+$ and $Foxp3^-$ RTE, the proliferation of $Foxp3^+$ RTE after their arrival in the periphery, or the conversion of $Foxp3^-$ RTE into regulatory T cells. Nevertheless, evidence was obtained that some $Foxp3^-$ thymocytes (model $Foxp3^-$ RTE) may be converted to Foxp3+ regulatory cells in the periphery. Alternative explanations for the observed transient increase in the proportion of $Foxp3^+$ RTE during the early period after intra-thymic labelling are RTE that express the molecule transiently after encountering cognate antigen, or that the export of regulatory T cells from the thymus occurs more rapidly than export of their conventional counterparts. These alternatives were not explored further.

With respect to maturation of RTE following release from the thymus, $Foxp3^+$ RTE observed within two hours of intra-thymic labelling expressed lower levels of TCR compared with the $Foxp3^-$ RTE, and levels lower than thymic $CD4^+$ single positive $Foxp3^+$ T cells. These findings suggest that expression of the TCR is down-regulated by $Foxp3^+$ RTE, either around the time of release from the thymus or after the cells have entered the periphery. The latter possibility is supported by the observation that the difference in TCR expression between regulatory and conventional T cells is more pronounced when comparison is made between thymic $Foxp3^+$ cells and $Foxp3^+$ cells resident in normal lymph nodes and spleen. This suggests that RTE continue to down-regulate levels of TCR expression as they mature, possibly as a result of sequential encounters with cognate antigens. This maturation process involves also down-regulation of Thy-1, which is more rapid in the $Foxp3^+$ subset of RTE than in the conventional $Foxp3^-$ subset. Unlike the $Foxp3^-$ subset of RTE, $Foxp3^+$ RTE do not rapidly up-regulate expression of CD45RC, possibly as a result of early encounter with self antigen after emerging into the periphery. Furthermore, the proportion of $Foxp3^+$ RTE that expresses the $CD8\alpha$ homodimer increased 6 fold within the first 180 hours after intra-thymic labelling, suggesting $CD8\alpha$ is induced specifically in regulatory T cells after emigration from the thymus, possibly as a result of encounter with cognate antigens.

In conclusion, regulatory T cells have a dependence on continuing thymic function for their maintenance, have many similarities with RTE and have been shown formally to be part of the RTE population. The question of extra-thymic origin of regulatory T cells by conversion of RTE was not resolved definitively, but evidence was obtained that RTE-like thymocytes can give rise to $Foxp3^+$ cells after intravenous adoptive transfer to normal recipients. Importantly, irrespective of the site at which regulatory T cells are generated, it appears that the cells complete their maturation in the periphery. Relative to the $Foxp3^+$ subset of RTE, the $Foxp3^-$ subset down-regulates the TCR, more rapidly down-regulates Thy 1, retains expression of the $CD45RC^-$ phenotype and up-regulates expression of CD8α. These changes are consistent with encounter with cognate antigens in the periphery and are likely to be related intimately to the function of the cells in maintenance of peripheral tolerance

Chapter 5: The differentiation and turnover of regulatory T cells

5.1 Introduction

T cells in the periphery are comprised of a mixture of naïve, memory, effector (Th1, Th2 or Th17) and regulatory T cells, which have different lifespans (Sprent 1993, Vukmanovic-Stejic *et al.* 2006). T cell numbers are continually replenished through the production of new T cells by the thymus, which reach the periphery as RTE and are preferentially incorporated into the peripheral T cell pool (Vrisekoop *et al.* 2008). In young animals, the number of RTE produced by the thymus is directly proportional to the mass of productive thymic tissue, and unlike other T cell subsets, their number is not regulated by homeostatic cytokines until they have been in the peripheral circulation for approximately three weeks (Berzins *et al.* 1999). The maturation of RTE into naïve T cells is accompanied by their acquisition of CD45RC in rats (Hosseinzadeh and Goldschneider 1993), equivalent as a marker of differentiation to CD45RA in humans (Seddiki *et al.* 2006). Naïve T cells are a considerably long lived population (months in rodents and years in humans) that proliferate at a very slow rate and recirculate through the blood, where they can be recruited into lymph nodes (Mackay *et al.* 1990, Tough and Sprent 1995). However, the recruitment of naive T cells into the Peyer's patches or spleen occurs only rarely (Pabst and Binns 1989).

While naïve T cells are primarily restricted to the blood and secondary lymphoid tissues, when they are presented with foreign antigens by professional APCs they can become activated and differentiate into effector T cells that acquire the ability to migrate into peripheral tissues from the blood before draining into lymph nodes through afferent lymph (Mackay *et al.* 1990, von Andrian and Mackay 2000). These antigen experienced T cells are comprised of a mixture of different T cells, some of which proliferate at a faster rate with a lifespan of a few weeks, while some remain dormant following their generation and provide immunological memory that probably lasts for many years in humans (Tough and Sprent 1994).

The activation of naïve T cells is facilitated by professional APCs known as dendritic cells that obtain antigen in the peripheral tissues and localise to lymph nodes to generate immune responses (Croft 1994). Activated T cells rapidly localise to the B

cell areas of the lymph node to stimulate antibody production, but most of these cells die soon after their activation (Jenkins *et al.* 2001). The surviving T cells are known as effector T cells, which downregulate their expression of CD45RA (CD45RC in rats) as they differentiate towards memory T cells through the acquisition of molecules such as CD134 that provide them with survival signals and extend their longevity (Weinberg *et al.* 2004). The memory T cells that are generated through the differentiation of effector T cells include a population of "central" memory T cells that express the lymph node homing chemokine receptor CCR7 and high levels of CD62L required for binding to HEV and recruitment into secondary lymphoid tissues (Sallusto *et al.* 1999).

Central memory T cells require secondary lymphoid tissue for their maintenance, and their recirculation patterns strongly resemble those of naïve T cells (von Andrian and Mackay 2000, Obhrai *et al.* 2006). Interestingly, central memory T cells are capable of generating tissue-homing "effector" T cells upon restimulation, that express low levels of CCR7 and CD62L but high levels of LFA-1 and $\alpha 4\beta 1$ integrin molecules that facilitate binding to endothelial cells at sites of inflammation (Ricard *et al.*1998, Sallusto *et al.* 1999, Jenkins *et al.* 2001).

Effector T cells constitutively secrete proinflammatory cytokines to recruit other leukocytes to the site of infection (Sallusto *et al.* 1999). The process through which effector T cells selectively accumulate at the site of infection is not normally directed by antigen, but instead appears to involve a passive process in which they are only able to survive and proliferate in the tissues that contain same cytokine mixture that was present in the lymph nodes in which they were generated (Westermann *et al.* 2001). Although the lifespans of the central and effector subsets of memory T cells have not been quantified directly, memory T cells appear to proliferate at two distinct rates. The majority of memory T cells divide rapidly with a lifespan in the order of a few weeks, while a smaller proportion of memory T cells are long lived cells that proliferate slowly and remain quiescent in the absence of antigen and probably have a lifespan in the order of many years in humans (Tough and Sprent 1994). Thus it is likely that the central memory T cell population that recirculates through the lymph

nodes and generates effector memory T cells may comprise the long-lived component of T cell memory, while effector memory T cells have a shorter lifespan.

Under conditions of lymphopenia, naïve T cells expand in response to IL-7 in order to maintain their number through a process known as homeostatic proliferation (Boyman *et al.* 2009). In contrast, memory T cells do not appear to be subject to homeostatic regulation by IL-7, as T cells lose responsiveness to IL-7 following antigen stimulation (Webb e*t al.* 1999). Interestingly regulatory T cells, which are also believed to be antigen experienced cells, are also unresponsive to IL-7 and their numbers appear to be maintained by frequent encounter with cognate self antigens in the periphery (Fisson *et al.* 2003). Recent studies have shown that the regulatory T cells in different anatomical compartments have vastly different specificities (Cozzo *et al.* 2003, Lathrop *et al.* 2008). In fact, it appears that all subsets of T cells are maintained through low affinity contacts with self MHC molecules, which enables them to proliferate and maintain their numbers (Martin *et al.* 2006). However, regulatory T cells also require exogenous IL-2 for proliferation, which is a characteristic that they share with anergised T cells (Taams *et al.* 1999).

The inability of regulatory T cells to produce IL-2 is associated with functional defects in the signalling pathways downstream of the TCR, another characteristic shared with anergised T cells (Hickman *et al.* 2006). In addition, the transcription factor Foxp3 binds to and inhibits the transcription factor NFAT (nuclear factor of activated T cells), which is required for the transcription of IL-2 (Bettelli *et al.* 2005). The requirement of regulatory T cells for exogenously produced IL-2 in order to proliferate is a consequence of their expression of the cyclin dependent kinase inhibitor $p27_{kip}$, which arrests the cells in the G_1 phase of the cell cycle. Exogenous IL-2 enables regulatory T cells to downregulate $p27_{kip}$, express cyclin E and cyclin A, and thus proliferate (Li *et al.* 2005). Evidence for the homeostatic role of IL-2 in maintaining regulatory T cells *in vivo* has been provided by experiments using antibodies to either neutralise IL-2, or block CD25, the high affinity IL-2 receptor alpha chain CD25. The neutralisation of IL-2 with antibodies *in vivo* leads to a selective reduction in the number of regulatory T cells, culminating in the development of autoimmune disease (Setoguchi *et al.* 2005).

Blocking CD25, which is utilised by regulatory T cells for capturing IL-2, also reduces the number of regulatory T cells *in vivo* and enhances autoimmune disease (Kohm *et al.* 2006). However, treatment with some anti-CD25 antibodies appears to induce phenotypic and functional changes in both regulatory T cells and DC, complicating the interpretation of these findings (Mnasria *et al.* 2008).

In a recently published edition of the Journal of Clinical Investigation, Vukamanivic-Stejic and colleagues (2006) measured the proliferation rate and turnover of $CD45RO^+$ $CD25^{high}$ regulatory T cells in human subjects. The lifespan of $CD45RO^+$ $CD25^{high}$ cells as determined by Vukamanivic-Stejic *et al.* appears to be short, with a half-life of around 8 days. However, the narrowly defined phenotype that was used to identify regulatory T cells may limit the interpretation of this result, as it only includes a minority of the regulatory T cell population. Although this subset of T cells is enriched with regulatory T cells, it may contain other T cells. Furthermore, it only represents a minority of the regulatory T cell population. Regulatory T cells are predominantly found within the $CD45RO^+$ subset in adult humans, however many of these regulatory T cells do not express CD25 at high levels or at all (Figure 3.2.2, Sather *et al.* 2007). In addition, a functionally suppressive $CD45RA^+$ population (equivalent to $CD45RC^+$ in rats) persists throughout adult life in humans (Seddiki *et al*. 2006), and was not taken into consideration by Vukamanivic-Stejic and colleagues (2006).

In addition, under lymphopenic conditions in mice $CD25^-$ $Foxp3^+$ putative regulatory T cells have been shown to gain CD25 expression (de Lafaille *et al.* 2004), which could complicate the interpretation of measurements of $CD45RO^+$ $CD25^{high}$ cells. However, the stability of CD25 expression by regulatory T cells under normal physiological conditions has not been investigated in detail, and the relationship is between $CD25^-$ $Foxp3^+$ putative regulatory T cells and their $CD25^+$ $Foxp3^+$ counterparts it is unclear.

Thus the measurements of $CD45RO^+$ $CD25^{high}$ regulatory T cell disappearance by Vukamanivic-Stejic *et al.* may be of limited use, since the cells they have investigated

represent only a small proportion of the regulatory T cell population, and it is unclear whether changes phenotype could contribute to their apparent rate of disappearance.

In order to address this issue, techniques were developed to examine proliferation in both the $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ subsets of regulatory T cells. These include a technique to enable the simultaneous detection of CD25, intracellular Foxp3, and the Ki67 proliferation-associated antigen, In addition, a technique was developed to simultaneously detect CD25, Foxp3, and bromodeoxyuridine (BrdU) incorporated into the DNA of cells that have divided *in vivo*. These techniques were then used to measure the proliferation rates and turnover of $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ regulatory T cells in normal non-lymphopenic rats.

5.2 Results

5.2.1 Validation of the staining procedure for the simultaneous detection of the intracellular antigens Ki67 and Foxp3

In order to evaluate whether regulatory T cell subsets are actively moving through cell cycle, their expression of the Ki67 proliferation associated antigen, a molecule expressed by cells in all stages of the cell cycle except the resting (G_0) phase (Gerdes *et al.* 1983) was examined. Standard protocols for the detection of Ki67 utilise a paraformaldehyde fixation followed by treatment with a saponin permeablisation buffer (Spargo *et al.* 2001). However, the simultaneous detection of Foxp3 was not possible using this method. A new protocol utilising the Foxp3 staining set reagents supplied in the kit provided by eBiosciences (product number 72-5775) was developed to stain Foxp3 and Ki67 simultaneously. This approach involved fixing the cells with the Foxp3 staining set fixative, then staining for Ki67 by an indirect technique in a saponin buffer, before staining for Foxp3 using the Foxp3 staining set permeablisation buffer (see methods). This approach enabled the detection of Ki67 without adversely affecting the detection of Foxp3, which was not detectable using saponin staining buffers or following paraformaldehyde fixation. To validate the new

technique that enabled simultaneous Foxp3and Ki67 detection, high-density lymphocytes and larger low-density lymphocyte blasts were separated by Optiprep density gradient centrifugation and analysed for the presence of the Ki67 proliferation associated antigen (Figure 5.2.1). It was found that small, high-density lymphocytes contained a significantly smaller proportion of $Ki67^+$ cells than in the low-density fraction, which are enriched in lymphocyte blasts (p= 0.04).

5.2.2 Detecting proliferation in effector T cells and regulatory T cells

In order to provide a comparison for investigating the proliferation status of regulatory T cells, the presence of Ki67 within $CD4^+$ MHC II^+ effector T cells isolated from lymph nodes was also investigated. In rats, effector T cells can be identified by their expression of MHC II (Spargo *et al.* 2001), which is thought to be acquired by both the transfer of membrane from APCs to T cells during T cell activation, and through endogenous synthesis (Patel *et al.* 2001). Strikingly, on average 83.7% (SD=2.97) of the MHC II^+ T cells were found to contain the Ki67 proliferation associated antigen, indicating that they are in cell cycle (Figure 5.2.2). In contrast, an average of 34.6% (SD=0.92) of $Foxp3^+$ T cells were found to contain the Ki67 proliferation associated antigen.

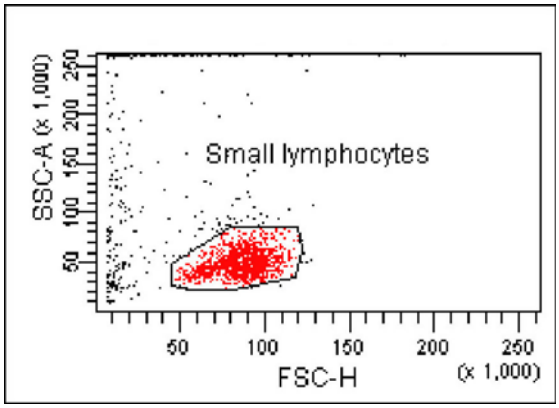
5.2.3 Analysis of proliferation in $CD4^+$ T cell subsets

In order to detect proliferation in subsets of $CD4^+$ T cells, $CD4^+$ T cells were purified by negative selection from lymph node cells and stained for CD25, Foxp3 and Ki67. A dot plot of CD25 and Foxp3 expression was gated into quadrants (Figure 5.2.3) to allow the separate analysis of resting T cells ($CD25^-$ $Foxp3^-$), activated T cells ($CD25^+$ $Foxp3^-$), regulatory T cells ($CD25^+$ $Foxp3^+$) and putative regulatory T cells ($CD25^-$ $Foxp3^+$). The proportion of $Ki67^+$ cells was higher in activated T cells than in resting T cells (Figure 5.2.4), but this difference was not significant (p=0.2). In contrast, both $CD25^+$ $Foxp3^+$ regulatory T cells, and $CD25^-$ $Foxp3^+$ putative regulatory T cells

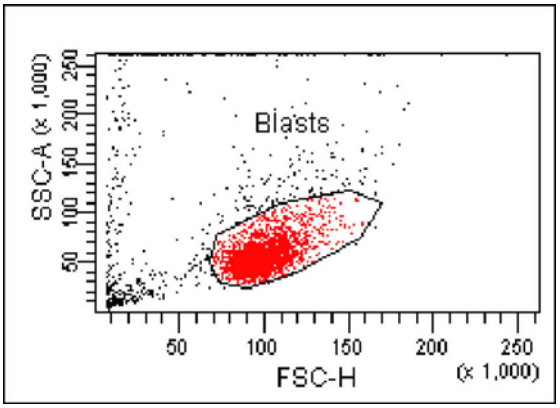
Figure 5.2.1 Validation of the optimised staining procedure for simultaneous detection of the intracellular antigens Ki67 and Foxp3

$CD4^+$ T cells purified by negative selection from pooled lymph node cells were separated using an Optiprep density gradient centrifugation (see methods) into high density and low-density lymphocytes. The two populations were then stained with antibody against the Ki67 antigen (as described in Methods 2.7.7) and examined by flow cytometry. Small, high-density lymphocytes (A) and large low-density lymphocytes (B) were gated as shown and analysed for expression of Ki67. There was a significantly higher percentage of stained cells in the large low density fraction (C) than in the small, high density fraction (D) ($p= 0.04$) as determined using a paired two-tailed student's t test.

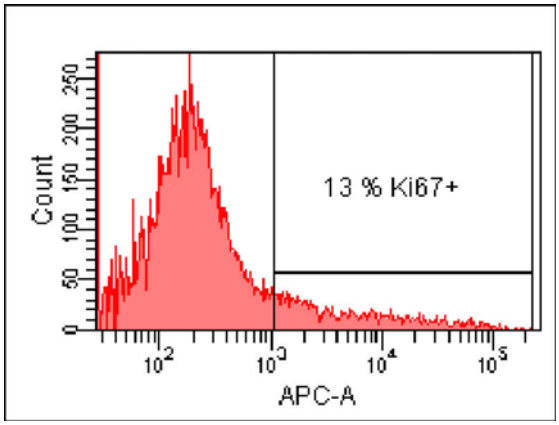
A


Small lymphocytes
SSC-A (x 1,000)
FSC-H
(x 1,000)

B


Blasts
SSC-A (x 1,000)
FSC-H
(x 1,000)

C


13 % Ki67+
Count
APC-A

D

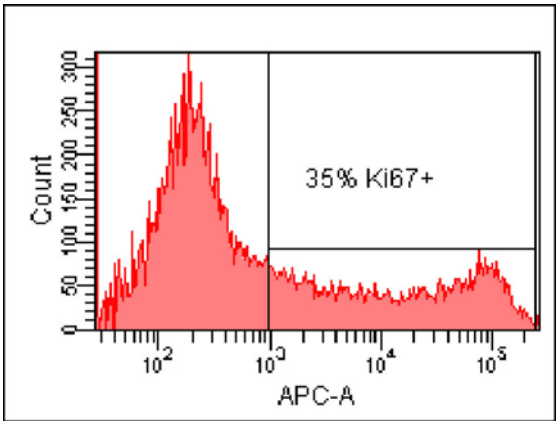
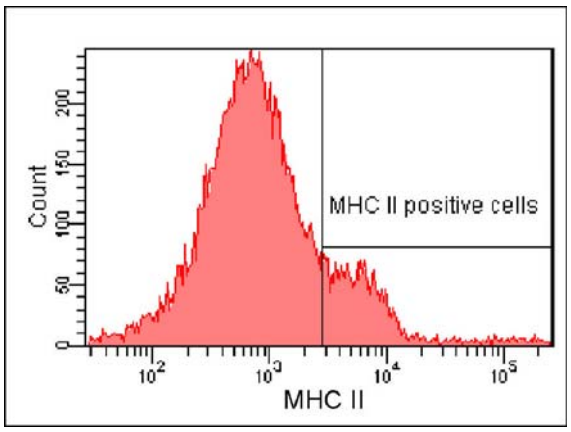

35% Ki67+
Count
APC-A

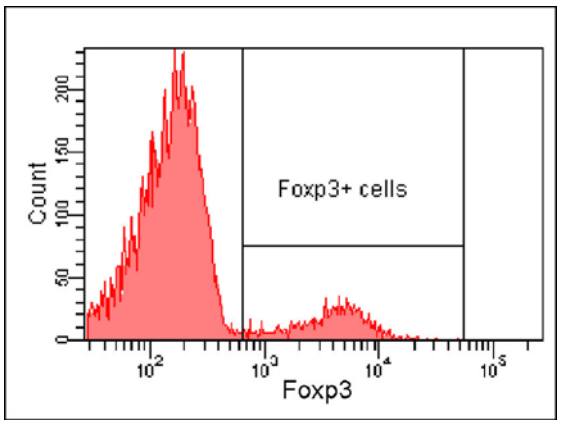
Figure 5.2.2 Expression of Ki67 by MHC II$^+$ effector T cells and Foxp3$^+$ regulatory T cells

CD4$^+$ T cells were purified from lymph node cells by immunomagnetic depletion of CD8$^+$ T cells and B cells. The cells were then stained with antibodies against Ki67, MHC II, and Foxp3. MHCII$^+$ putative effector T cells were gated (A) and found to be 82% Ki67$^+$ (C). Also, Foxp3$^+$ regulatory T cells were gated (B) and found to be 34% Ki67$^+$ (D). Representative of three experiments.

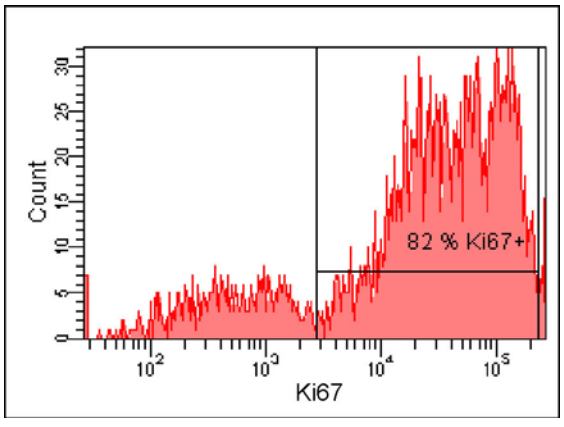
A


Count
MHC II positive cells
MHC II

B


Count
Foxp3+ cells
Foxp3

C


Count
82 % Ki67+
Ki67

D

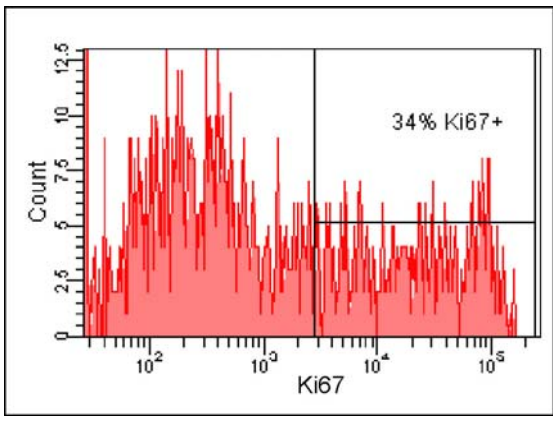
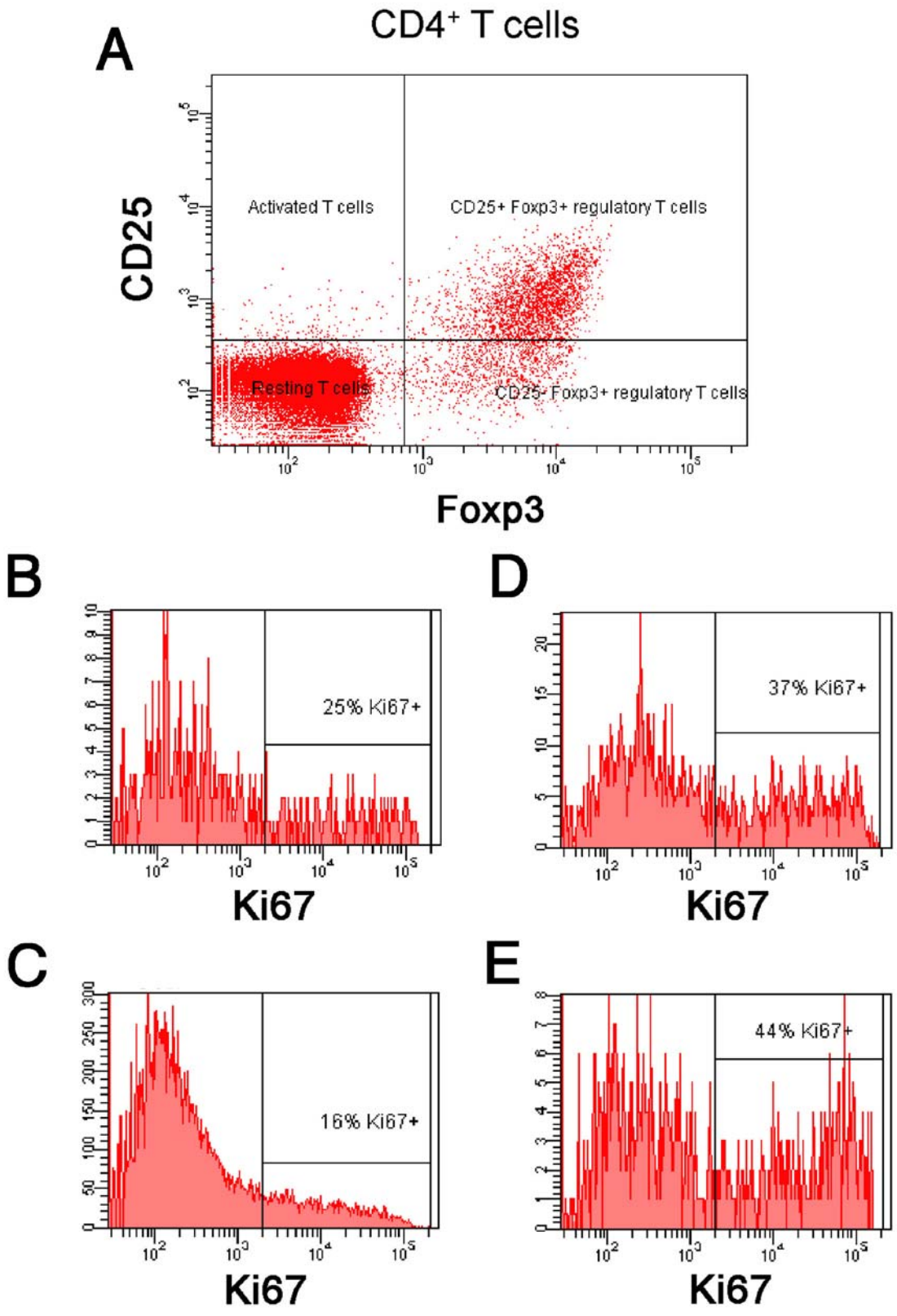

Count
34% Ki67+
Ki67

Figure 5.2.3 Simultaneous analysis of the proliferation marker Ki67 in subsets of $CD4^+$ T cells

$CD4^+$ T cells were purified from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph node cells by immunomagnetic depletion of $CD8^+$ T cells and B cells. The cells were then stained with antibodies against Ki67, CD25, and Foxp3. A dot plot obtained by simultaneous analysis of CD25 and Foxp3 expression was gated into quadrants for separate analysis of resting and activated conventional T cells, $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells (A). Expression of the proliferation associated Ki67 antigen within the activated (B), resting (C), $CD25^+$ $Foxp3^+$ regulatory T cell (D) and $CD25^-$ $Foxp3^+$ putative regulatory T cell (E) subsets is indicated as percentages. Representative of 3 experiments.


A
CD4+ T cells
CD25
Foxp3
Activated T cells
CD25+ Foxp3+ regulatory T cells
Resting T cells
CD25- Foxp3+ regulatory T cells
B
25% Ki67+
Ki67
D
37% Ki67+
Ki67
C
16% Ki67+
Ki67
E
44% Ki67+
Ki67

contained significantly greater proportions of $Ki67^+$ cells than resting T cells (p=0.04 and p=0.004 respectively).

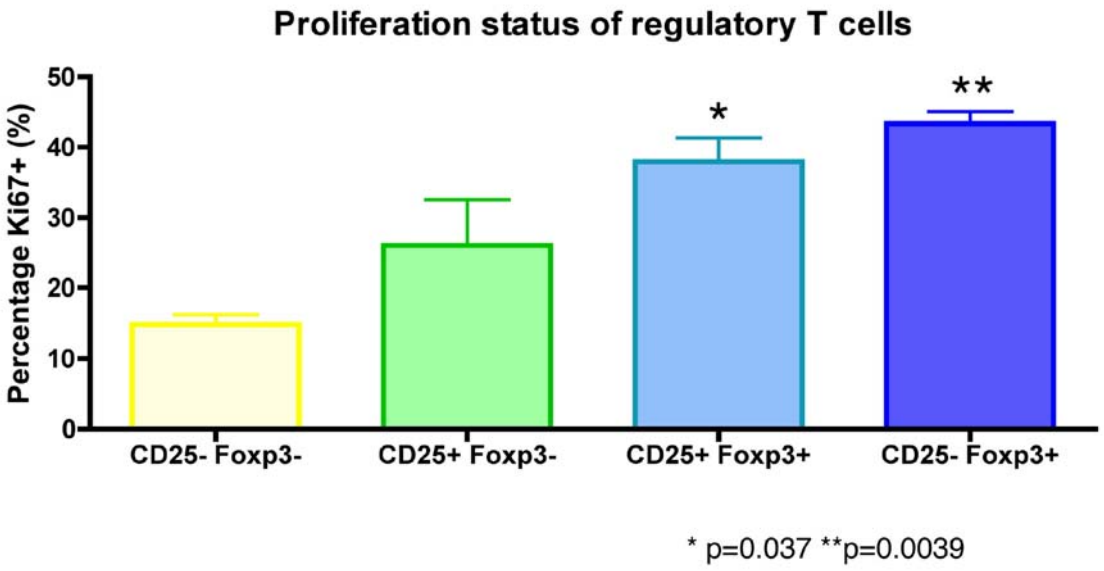
5.2.4 Measurement of the proliferation rates of peripheral blood, lymph node and thoracic duct lymph $CD4^+$ T cell subsets

The measurement of BrdU incorporation through standard techniques, involving paraformaldehyde (Tough and Sprent 1994) or ethanol fixation (Carayon and Bord 1992), with Tween-20 based staining buffers did not enable the simultaneous or subsequent detection of Foxp3. Therefore, a new technique was developed, in which cells were first stained for CD25 and Foxp3 using the Foxp3 staining set (eBiosciences), then fixed using the Foxp3 staining set reagents again before DNA digestion and staining for incorporated BrdU at room temperature. Staining for incorporated BrdU was identical to that seen using published techniques on the same sample, with no loss of Foxp3 detection. Proliferation of $CD4^+$ T cells subsets in peripheral blood, lymph nodes, and thoracic duct lymph was examined by an extended pulse of BrdU. Rats were administered with an injection of 1 mg of BrdU in saline *i.p.* every hour for 3 h. Animals were killed at 0, 1, 8, 16 and 24 h following the first injection and $CD4^+$ T cells were then isolated from blood the and lymph node samples to assess the proliferation of the resting, activated, $CD25^+$ $Foxp3^+$ regulatory T cell and $CD25^-$ $Foxp3^+$ putative regulatory T cell subsets. In the case of thoracic duct lymph, the thoracic ducts of rats were cannulated before receiving 3 mg in saline (3mg/ml) by tail vein injection. Lymph was collected in serial fractions corresponding to 0-2 h, 2-4 h, 4-6 h, 6-12 h and 12-24 h post injection.

It was found that a low proportion of resting T cells recovered from peripheral blood (mean 1.36%, SD=1.1) (Figure 5.2.5), lymph nodes (mean 1.13%, SD=0.12) (Figure 5.2.6), and thoracic duct lymph (mean 0.5%, SD=0.17) (Figure 5.2.7) had incorporated BrdU in the 24 h following administration. In contrast, activated T cells obtained from the blood (mean 6.7%, SD=0.78) and lymph nodes (mean 9.7%, SD=4.0) had incorporated more BrdU, although activated T cells taken from thoracic duct lymph had not proliferated to the same extent (mean 2.43%, SD=0.15).

Figure 5.2.4 Quantitative estimate of the proportion of regulatory T cells in the cell cycle determine by detection of Ki67

$CD4^+$ T cells were isolated from pooled cervical, coeliac, mesenteric, inguinal and popliteal lymph nodes by immunomagnetic depletion of B and $CD8^+$ T lymphocytes. The cells were then stained with antibodies against CD25, Foxp3 and Ki67 and analysed by flow cytometry. T cells subsets were gated as shown in Figure 5.2.3A and each was analysed for expression of Ki67 antigen. The proportion of $Ki67^+$ cells was higher in the activated $CD25^+$ T cell subset, than in the resting ($CD25^-$ $Foxp3^-$) cells, but this difference was not significant as determined using a paired two-tailed student's t test (p=0.07). In contrast, the proportion of $Ki67^+$ cells was significantly higher in $CD25^+$ $Foxp3^+$ regulatory T cells than in resting T cells (p=0.04). In addition, $CD25^-$ $Foxp3^+$ putative regulatory T cells also contained a significantly greater proportion of $Ki67^+$ cells compared with resting T cells (p=0.004). Data represent the means ±SD of 3 independent experiments.


Proliferation status of regulatory T cells
Percentage Ki67+ (%)
50
40
30
20
10
0
*
**
CD25- Foxp3-
CD25+ Foxp3-
CD25+ Foxp3+
CD25- Foxp3+
* p=0.037 **p=0.0039

Interestingly, while $CD25^+$ $Foxp3^+$ regulatory T cells had an equivalent proliferation rate to activated T cells in the blood (mean 6.3%, SD=2.4), they proliferated more slowly in lymph nodes (mean 3.3%, SD=0.52) and thoracic duct lymph (mean 2.6%, SD=0.7). Strikingly, the incorporation of BrdU by $CD25^-$ $Foxp3^+$ putative regulatory T cells has higher than in all other subsets in the lymph nodes (mean 8.4%, SD=1.7), lymph (mean 8.8%, SD=0.85), and blood (mean 15.2%, SD=4.61).

5.2.5 Quantitative measurement of the proliferation of conventional and regulatory $CD4^+$ T cells

In order to measure the rate of proliferation of regulatory T cells in young adult rats, the incorporation of BrdU into the DNA of dividing cells was measured over a 7-day period during of continuous BrdU administration via the drinking water. Proliferation of T cells within the cervical, mesenteric and celiac lymph nodes was measured separately, although no differences were observed between the different lymph nodes analysed. For convenience, results from the cervical lymph nodes are shown.

The incorporation of BrdU was essentially linear with time for all subsets of $CD4^+$ T cells. Resting T cells were labelled at a rate of ~0.5% per day (Figure 5.2.8). In contrast, $CD25^+$ $Foxp3^-$ activated T cells and $CD25^+$ $Foxp3^+$ regulatory T cells incorporated BrdU at the approximately equal rates of ~3% and ~2.9% per day respectively. Strikingly, the rate of incorporation of BrdU by the $CD25^-$ $Foxp3^+$ putative regulatory T cell subset was ~7.5%, such that ~50% of $CD25^-$ $Foxp3^+$ putative regulatory T cells were labelled after seven days of BrdU administration. Therefore, assuming that the size of the T cell pool remained constant (which was the case measuring T cell numbers in LN and the spleen), $CD25^+$ $Foxp3^+$ regulatory T cells and $CD25^-$ $Foxp3^+$ putative regulatory T cells have half lives of ~18 days and ~7 days respectively.

Figure 5.2.5 Recently divided cells in the $CD4^+$ subsets in peripheral blood

Normal seven week old DA CD45.2 rats received hourly intra-peritoneal injections of 1mg BrdU in saline for 3 hours. Animals were killed at 0, 1, 8, 16 and 24 h after the first injection, mononuclear cells were prepared by lymphoprep density gradient centrifugation and $CD4^+$ T cells were isolated by immunomagnetic depletion of $CD8^+$ T cells and B cells. $CD4^+$ T cells were distinguished from contaminating $CD4^+$ monocytes by staining with antibody against the $\alpha\beta$TCR. Samples were stained also with antibodies against CD25, intracellular Foxp3 and incorporated BrdU (see Methods 2.7.9). The proportion of cells that had incorporated BrdU in each T cell subset is expressed as a percentage. Data represent the means ±SD of 3 animals per time point.

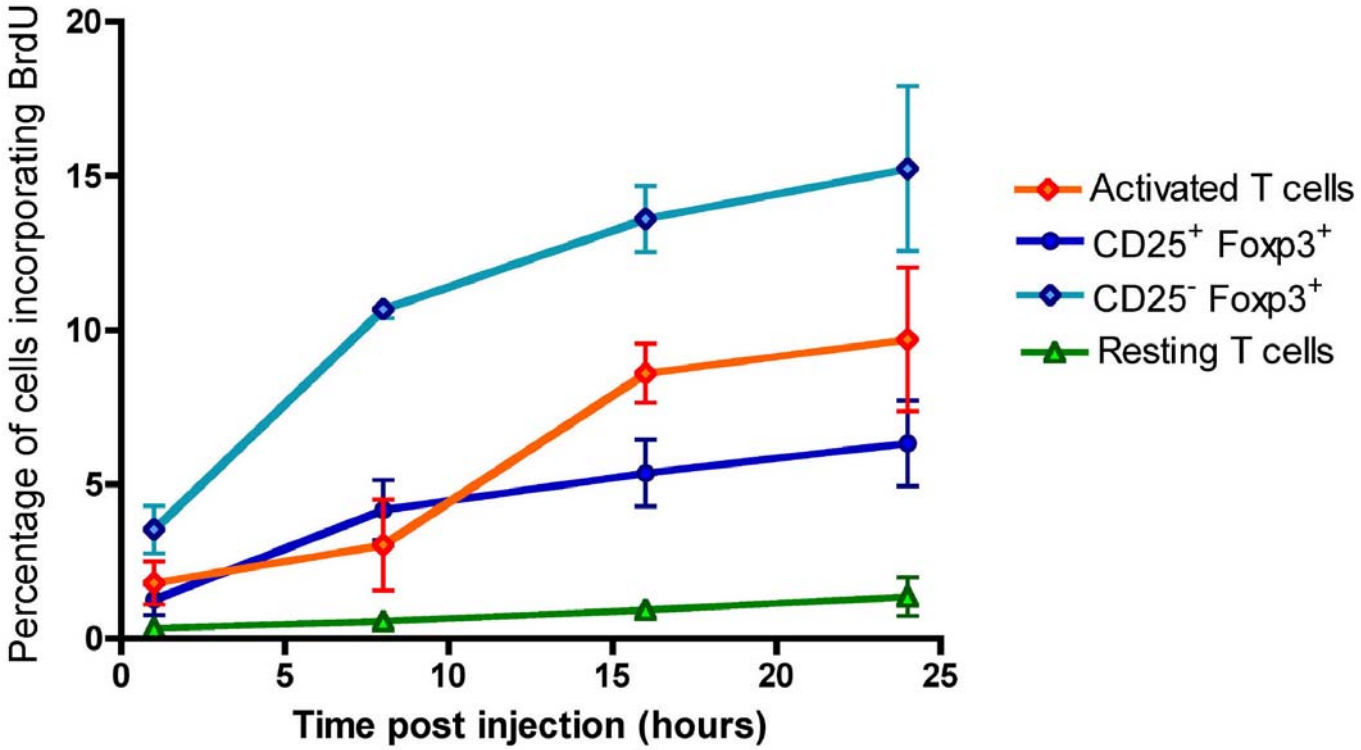

Percentage of cells incorporating BrdU
20
15
10
5
0
0
5
10
15
20
25
Time post injection (hours)
Activated T cells
CD25+ Foxp3+
CD25- Foxp3+
Resting T cells

Figure 5.2.6 Recently divided subsets of $CD4^+$ T cells in lymph nodes

Normal seven week old DA CD45.2 rats received hourly intra-peritoneal injections of 1mg BrdU in saline for 3 hours. Animals were killed at 0, 1, 8, 16 and 24 h after the first injection and cell suspensions were prepared from pooled cervical, celiac, mesenteric, inguinal and popliteal lymph nodes. $CD4^+$ T cells were purified by immunomagnetic depletion of $CD8^+$ T cells and B cells. Samples were then stained with antibodies against CD25, intracellular Foxp3 and incorporated BrdU (see Methods 2.7.9). The proportion of each subset that had incorporated BrdU is expressed as a percentage. Data represent the means ±SD of 3 animals per time point.

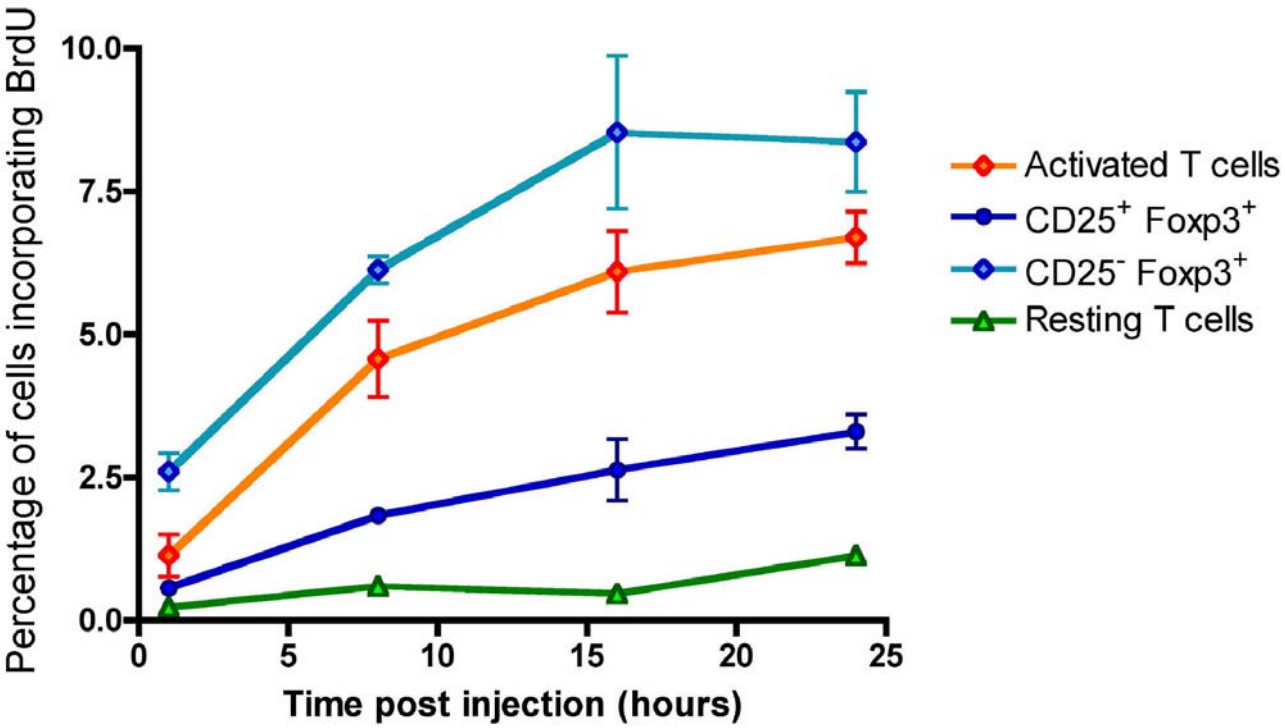
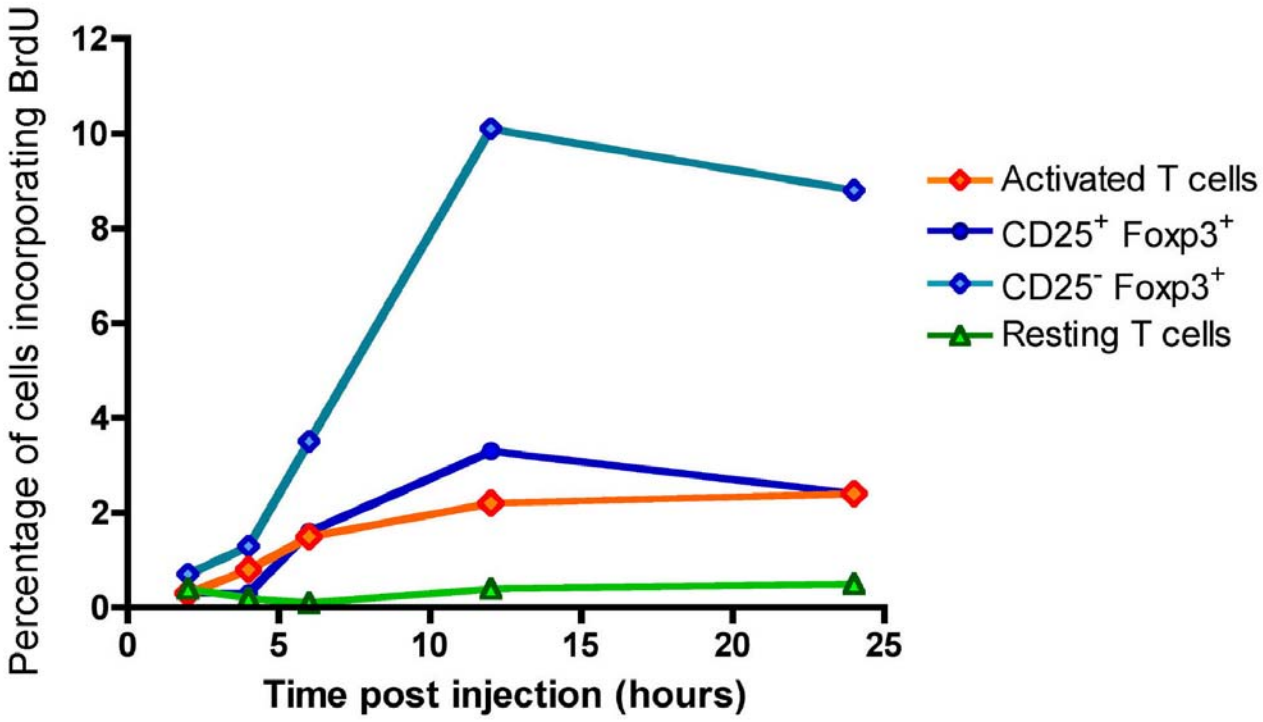

Percentage of cells incorporating BrdU
10.0
7.5
5.0
2.5
0.0
0
5
10
15
20
25
Time post injection (hours)
Activated T cells
CD25+ Foxp3+
CD25- Foxp3+
Resting T cells

Figure 5.2.7 Recently divided cells in the $CD4^+$ T cell subsets in central lymph

Central lymph was intercepted by cannulation of the thoracic duct of 7 week old DA CD45.2 rats (see Methods 2.1.9). At the time of surgery, the rats received a 1ml intravenous injection of 3mg/ml BrdU in saline. Thoracic duct lymph was collected in serial samples corresponding to 0-2 h, 2-4h, 4-6h, 6-12h and 12-24h post injection. $CD4^+$ T cells were isolated by immunomagnetic depletion of $CD8^+$ T cells and B cells, and the purified cells were then stained with antibodies against CD25, Foxp3, and BrdU (see Methods 2.7.9). The proportion of each T cell subset that had incorporated BrdU is expressed as a percentage. Data represent the means ±SD of 3 animals per time point.


Percentage of cells incorporating BrdU
12
10
8
6
4
2
0
0
5
10
15
20
25
Time post injection (hours)
Activated T cells
CD25+ Foxp3+
CD25- Foxp3+
Resting T cells

5.2.6 Turnover of sconventional and regulatory CD4$^+$ T cells

In order to verify the turnover rates of the Foxp3$^+$ T cell subsets, the rates of disappearance of BrdU labelled T cells within each subset were measured after the withdrawal of BrdU from the drinking water. As in the previous experiment, rats were provided with BrdU in their drinking water for a period of seven days. At the end of this period, the animals were returned to normal drinking water, and the proportions of BrdU labelled cells in each of the subsets was determined over a further 14 days. After an early decline in the first three days after BrdU withdrawal, the proportion of labelled CD25$^-$ Foxp3$^+$ putative regulatory T cells remained essentially constant for the remainder of the observation period (Figure 5.2.9). In contrast, the proportions of labelled cells in other subsets declined progressively with time. This observation seemed to contradict the previously observed rapid proliferation rate in the CD25$^-$ Foxp3$^+$ subset. It was therefore hypothesised that this apparent discrepancy may be accounted for by the conversion of CD25$^+$ Foxp3$^+$ regulatory T cells to the CD25$^-$ Foxp3$^+$ phenotype. This possibility was investigated directly by the isolation of CD25$^+$ Foxp3$^+$ regulatory T cells and adoptive transfer into normal CD45-congenic recipients.

5.2.7 Isolation of CD4$^+$ CD25^{bright} cells by FACS

In order to isolate CD25$^+$ Foxp3$^+$ T cells, use was made of the higher expression of CD25 by regulatory T cells compared with activated T cells (see Figure 3.2.2). Genetically marked CD4$^+$ CD25^{bright} T cells were isolated daily under sterile conditions from pooled DA CD45.2 lymph node cells using high speed fluorescence activated cell sorting (FACS) (Figure 5.2.10). Each daily preparation contained at least 1 x 10^6 cells of which >99% had the CD25^{bright} phenotype and >98% expressed Foxp3. The purified cells were transferred by daily intravenous injection into a CD45 congenic host for 5 days, allowing transfer of a 5 x 10^6 cohort of genetically marked CD25$^+$ Foxp3$^+$ regulatory T cells per host. The phenotype of the adoptively transferred cells was then assessed weekly by sampling arterial blood.

Figure 5.2.8 The proliferation rate of regulatory T cell subsets assessed by continuos labelling with BrdU

Rats were given 0.8 mg/ml BrdU in their drinking water for 7 days continuously and groups of three were killed at intervals to obtain cervical lymph nodes. $CD4^+$ T cells were isolated by immunomagnetic depletion of $CD8^+$ T cells and B cells, and the purified cells were then stained with antibodies against CD25, Foxp3 and BrdU (see Methods 2.7.9). The cells were analysed by flow cytometry for expression of CD25 and Foxp3 and for BrdU incorporated into the DNA during the period of labelling. The proportion of each subset that had incorporated BrdU is expressed as a percentage. Data represent the mean ±SD of 3 animals per time point.

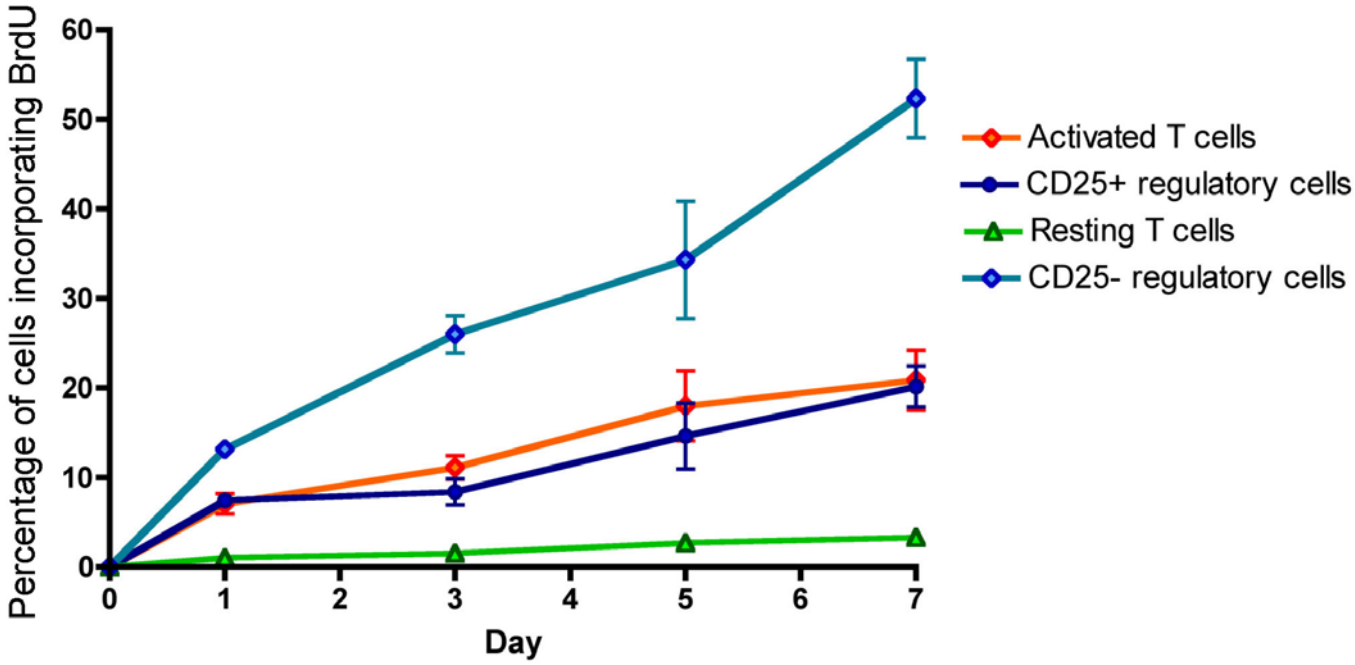

Percentage of cells incorporating BrdU
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
Day
Activated T cells
CD25+ regulatory cells
Resting T cells
CD25- regulatory cells

Figure 5.2.9 Turnover of incorporated BrdU label by subsets of regulatory T cells

Ras before (Figure 5.2.8) rats were given BrdU (0.8mg/ml) in their drinking water as before for seven days. They were then returned to normal drinking water and groups of three were killed at intervals to obtain cervical lymph nodes. $CD4^+$ T cells were isolated by immunomagnetic depletion of $CD8^+$ T cells and B cells, and the purified cells were then stained with antibodies against CD25, Foxp3 and BrdU (see Methods 2.7.9). The cells were analysed by flow cytometry for expression of CD25 and Foxp3 and for BrdU incorporated into the DNA during the period of labelling. The proportion of each subset that had incorporated BrdU is expressed as a percentage. Data shown represent the mean ±SD of 3 animals per time point.

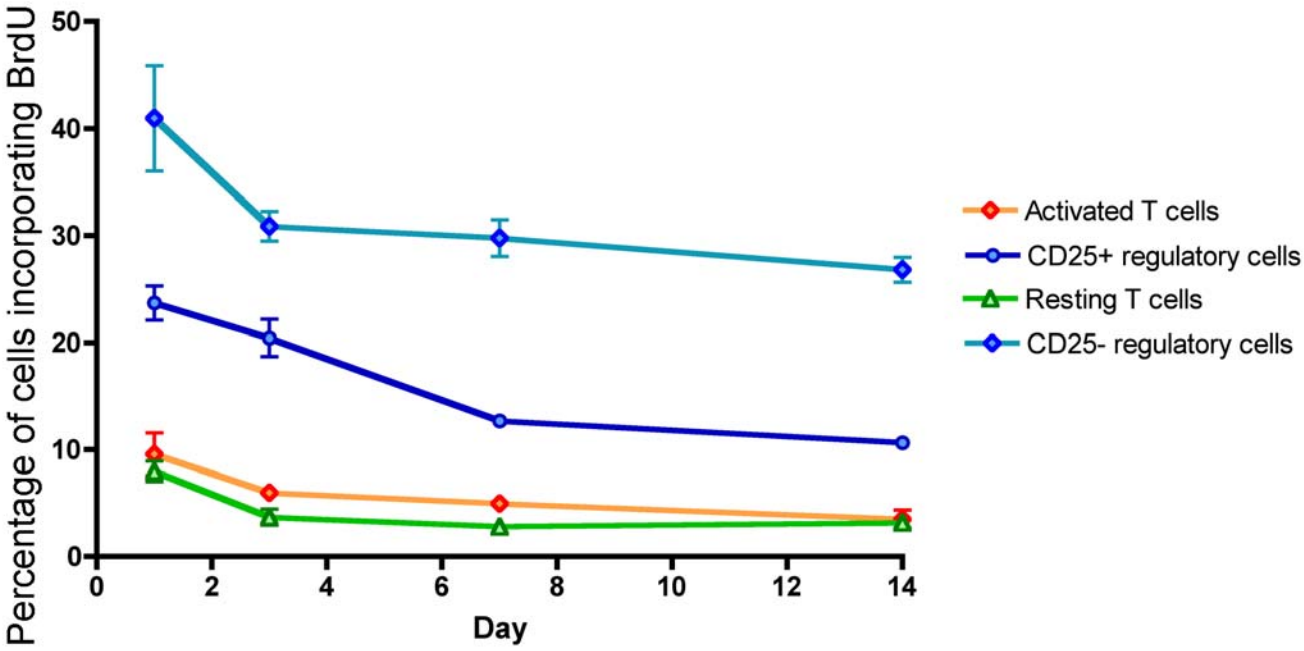
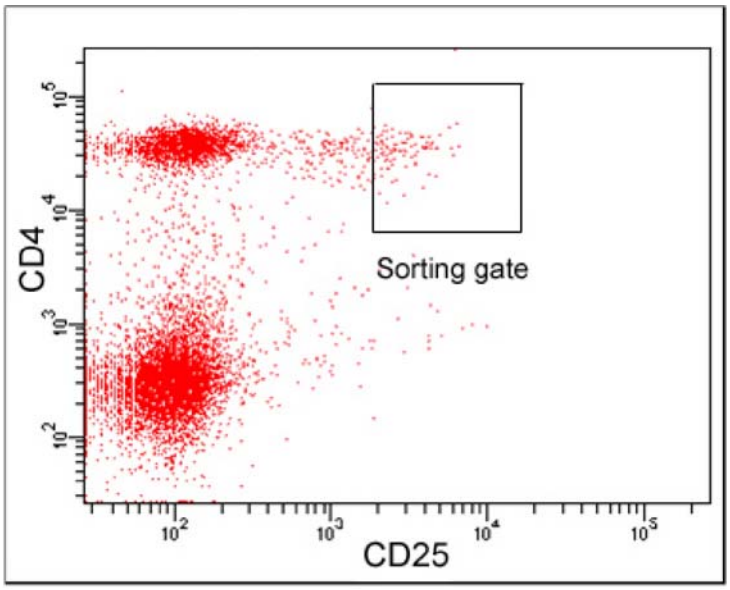

Percentage of cells incorporating BrdU
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Day
Activated T cells
CD25+ regulatory cells
Resting T cells
CD25- regulatory cells

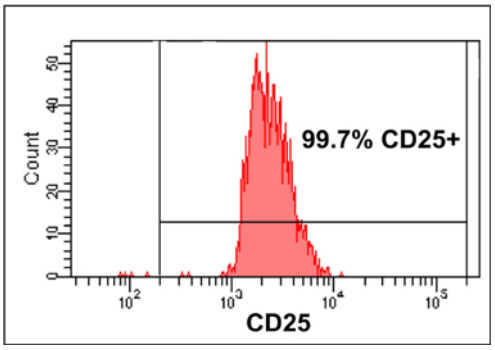
Figure 5.2.10 Preparation of $CD4^+$ $CD25^{bright}$ regulatory T cells for adoptive transfer

Lymph node cells were prepared from pooled cervical, mesenteric, celiac, popliteal and inguinal lymph nodes of DA CD45.2 donors and stained with antibodies against CD4 and CD25. The FACS was used to purify $CD4^+CD25^{bright}$ cells, gated as shown to include the 50% of cells with the highest fluorescence for CD25 (A). Sorted cells routinely contained >99% $CD25^+$ cells (B) and >98% expressed Foxp3 (C). Representative data from 20 separations of $CD4^+$ $CD25^{bright}$ T cells by FACS.

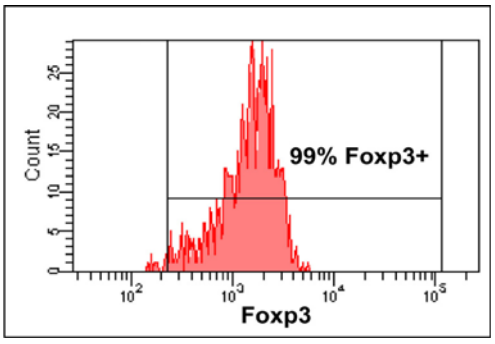
A


CD4
CD25
Sorting gate

B


Count
CD25
99.7% CD25+

C


Count
Foxp3
99% Foxp3+

5.2.8 Loss of CD25$^+$ Foxp3$^+$ regulatory T cells

In order to monitor the phenotype of adoptively transferred CD25$^+$ Foxp3$^+$ cells, weekly arterial blood samples were taken (1ml) from the tails of the recipients, using a heparin containing syringe. Blood mononuclear cells were isolated by lymphoprep density gradient centrifugation and stained for the $\alpha\beta$TCR, CD45.2 and CD25. Flow cytometric analysis revealed that the proportion of donor-derived T cells that continued to express CD25 decreased in a linear fashion following adoptive transfer (Figure 5.2.11). By 25 days after the transfer, less than 20% of donor cells were CD25$^+$ while they had retained Foxp3 expression.

5.2.9 Isolation of *in vivo* matured CD25$^-$ Foxp3$^+$ T cells

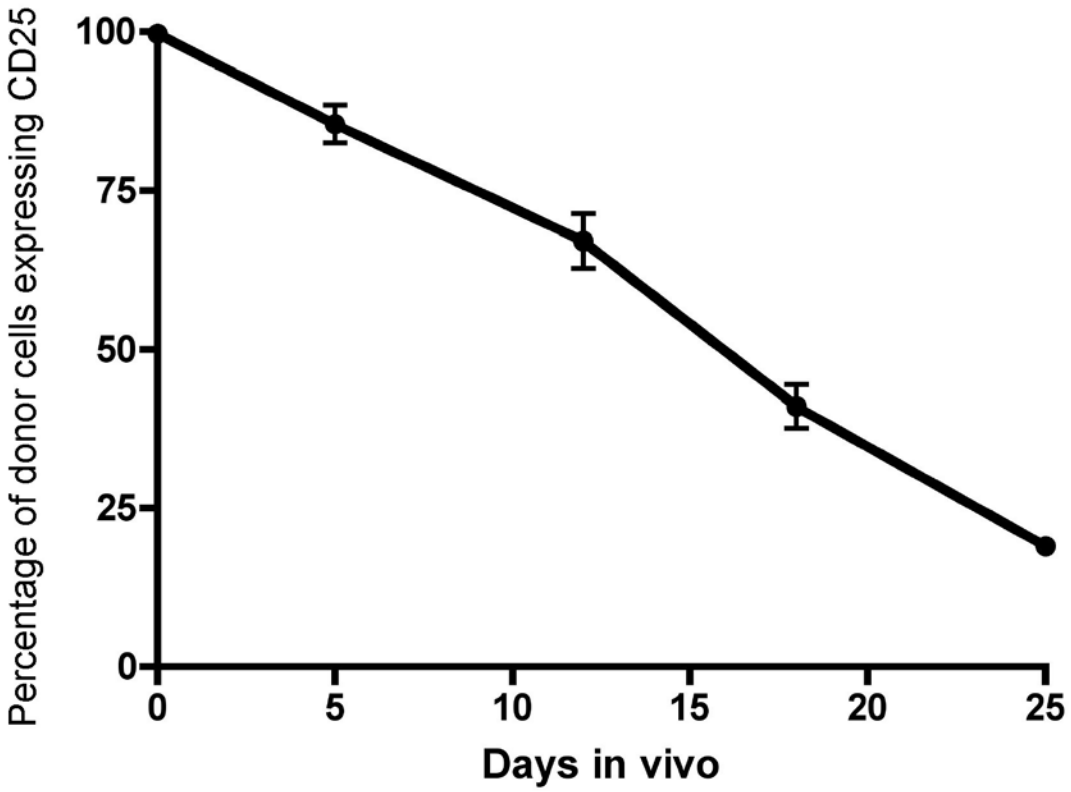
The lack of a specific cell surface marker has prevented the direct purification of CD25$^-$ Foxp3$^+$ putative regulatory T cells for functional investigations. However, the phenotypic transition of adoptively transferred CD25$^+$ Foxp3$^+$ regulatory T cells into CD25$^-$ Foxp3$^+$ putative regulatory T cells provides an opportunity for their isolation through the use of their CD45.2 genetic marker. In order to re-isolate *in vivo* matured CD25$^-$ Foxp3$^+$ T cells, pooled cervical, mesenteric, celiac, popliteal and inguinal lymph nodes were collected from recipients at 25 days after transfer, at which time ~80% of the donor cells had the CD25- Foxp3+ phenotype (Figure 5.2.11A).

Cells prepared from the lymph nodes were stained with antibodies against the $\alpha\beta$TCR, CD45.2 and CD25, to enable the isolation of donor CD45.2$^+$ CD25$^-$ T cells by FACS (Figure 5.2.12). Interestingly, while only ~20% of donor cells in the blood continued to express CD25 at 25 days following adoptive transfer, ~35% of the donor-derived cells obtained from the lymph nodes still expressed the molecule. Importantly, all of the donor cells isolated from lymph nodes after 25 days had retained their expression of Foxp3. This procedure was performed on 3 recipients of CD25+ Foxp3+ cells, and the recovery of CD25$^-$ Foxp3$^+$ donor cells was approximately 2-4 x 10^4 cells per recipient.

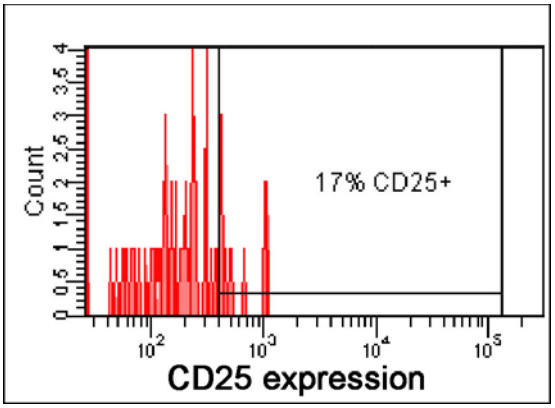
Figure 5.2.11 Loss of CD25 expression by adoptively transferred $CD4^+$ $CD25^{bright}$ regulatory T cells

$CD4^+$ $CD25^{bright}$ cells prepared by FACS from lymph node cells of DA CD45.2 donors (see Figure 5.2.10) were transferred to DA CD45.1 recipients by intravenous injection. A total of 5×10^6 cells was transferred by daily injections over five days. The day of the last injection serves as day 0. Arterial blood samples (~1ml) were obtained at regular intervals and. PBMNC were isolated by lymphoprep density gradient centrifugation. Samples of PBMNC were stained with antibodies against the $\alpha\beta$TCR, the CD45.2 donor marker and CD25 and analysed by flow cytometry. The proportion of donor cells expressing CD25 decreased in a linear fashion with time following adoptive transfer (A) (Data represent the means ±SD of 4 experiments). By 25 days after the transfer, less than 20% of the donor derived cells in the blood of recipients expressed CD25 (B), but essentially all expressed Foxp3 (C). Representative of 4 experiments.

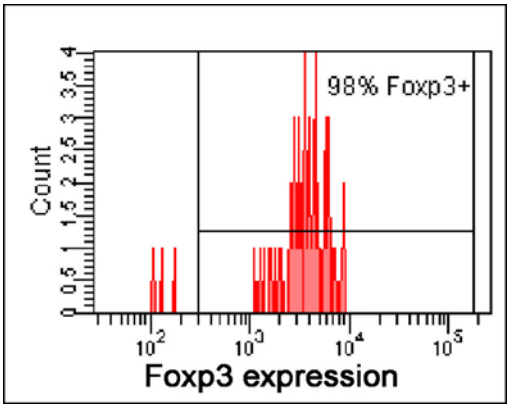
A


Percentage of donor cells expressing CD25
100
75
50
25
0
0
5
10
15
20
25
Days in vivo

B


Count
17% CD25+
CD25 expression

C


Count
98% Foxp3+
Foxp3 expression

5.2.10 Functional analyses of *in vivo* matured CD25$^-$ Foxp3$^+$ putative regulatory T cells

The regulatory activity of purified *in vivo* matured CD25$^-$ Foxp3$^+$ T cells was assessed by their ability to inhibit an allogeneic mixed lymphocyte reaction (MLR). First, an assay for measuring regulatory T cell suppressive activity was established using purified CD25$^+$ Foxp3$^+$ regulatory T cells. FACS purified CD25^{bright} Foxp3$^+$ regulatory T cells were prepared from the lymph nodes of DA CD45.2 rats and added (1 x 10^3, 5 x 10^3 or 1 x 10^4 cells per well) to co-cultures containing 1 x 10^3 syngeneic APC and 1 x 10^4 allogeneic T cells from hooded PVG/c rats. The most potent inhibitory effect was seen when 1 x 10^4 regulatory T cells were used (Figure 5.2.13), and these conditions were used to evaluate the regulatory activity of CD25$^-$ Foxp3$^+$ T cells.

To generate CD25$^-$ Foxp3$^+$ T cells, CD4$^+$CD25^{bright} cells were prepared from CD45.2 donors (5 x 10^6 total) and transferred to congenic DA CD45.1 recipients in daily doses of ~1 x 10^6 cells. Twenty five days later, CD25$^-$ Foxp3$^+$ cells were reisolated from the lymph nodes of the recipients by FACS (see above) and assayed for regulatory function in the allogeneic MLR (1 x 10^4 cells per well). Freshly isolated CD25^{bright} Foxp3$^+$ T cells from normal DA CD45.2 rats were used in parallel MLRs as a positive control (1 x 10^4 cells per well). *In vivo* matured CD25$^-$ Foxp3$^+$ T cells and CD25^{bright} Foxp3$^+$ T cells were equipotent in suppressing the proliferation of T cells (Figure 5.2.14), despite their loss of CD25 expression *in vivo*. This procedure was performed on three recipients of CD25^{bright} Foxp3$^+$ T cells and consistently demonstrated the potent activity of CD25$^-$ Foxp3$^+$ T cells in suppressing T cell proliferation.

5.3 Discussion

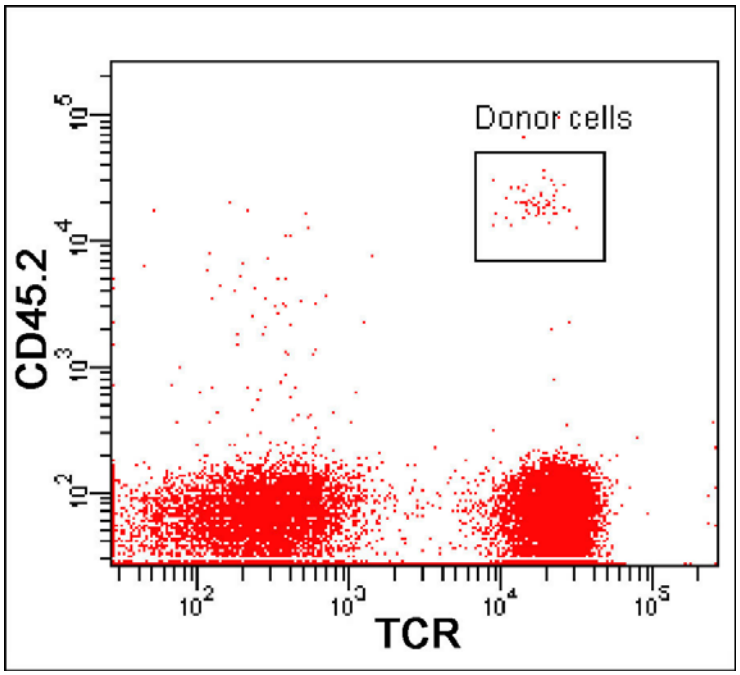
5.3.1 The proliferative status of conventional and regulatory T cells

The proliferation associated Ki67 antigen is expressed in all phases of the cell cycle except the resting (G_0) phase (Gerdes *et al.* 1983). Although Ki67 appears to be

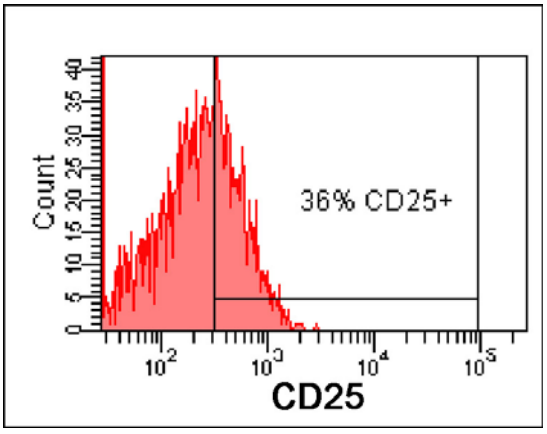
Figure 5.2.12 Isolation of *in vivo* matured regulatory T cells

$CD4^+CD25^{bright}$ cells from DA CD45.2 donors (5×10^6 total), prepared as described in Figure 5.2.10 were transferred to a DA CD45.1 recipient by daily intravenous doses of ~1×10^6 cells. Lymph node cells from pooled cervical, mesenteric, celiac, popliteal and inguinal lymph nodes were obtained at 25 days after transfer. Lymphocytes were prepared from the pooled lymph nodes and stained with antibodies against the $\alpha\beta$TCR, CD45.2 and CD25. In preparation for sorting donor-derived T cells were identified as shown (A). At this time, approximately 35% of the donor-derived T cells expressed CD25 (B), and essentially all expressed Foxp3 (C). Yields of flow sorted $CD25^-$ $Foxp3^+$ *in vivo* matured cells ranged from 2-4 x 10^4 cells per animal. Representative of four independent experiments.

A


Donor cells
CD45.2
TCR

B


36% CD25+
Count
CD25

C

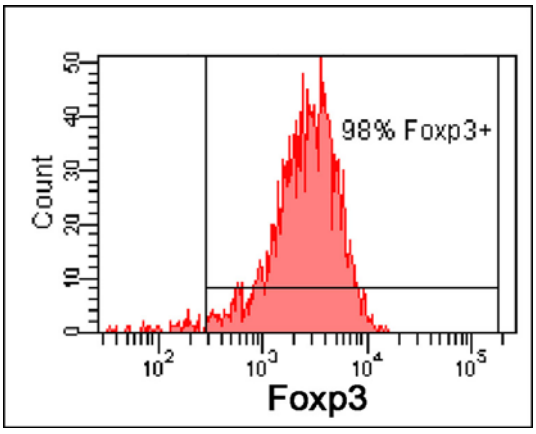

98% Foxp3+
Count
Foxp3

Figure 5.2.13 Optimisation of mixed lymphocyte reaction to test the activity of regulatory T cells

To establish an in vitro system for assessing the suppressive activity of regulatory T cells, they were included in allogeneic MLR at several ratios with respect to responder cells. Antigen presenting cells were enriched from suspensions of DA CD45.2 rat spleen cells by immunomagnetic depletion of B and T cells. Before use, they were treated with mitomycin C to prevent proliferation of contaminating T cells. The resulting preparation was included in the MLR at 1 x 10^3 cells per well. Allogeneic responder T cells ($CD4^+$ $CD25^-$ T cells) were prepared from suspensions of PVG/c lymph node cells by immunomagnetic depletion of B cells, $CD8^+$ T cells and $CD25^+$ T cells. Responder cells were used at 1 x 10^4 cells per well. $CD25^{bright}$ $Foxp3^+$ regulatory T cells were isolated by FACS from suspensions of normal DA CD45.2 lymph node cells that had been stained with antibodies against CD4 and CD25 (see Figure 5.2.10). Regulatory T cells were added to the MLR at either 1 x 10^3, 5 x 10^3 or 1 x 10^4 cells per well (responder:regulatory ratios of 10:1, 2:1 and 1:1 respectively). Cells were co-cultured for 4 days in round-bottomed 96 well plates, and then pulsed with [^{3}H] TdR (1 µCi per well) for 18 h before harvesting. Control wells contained APC alone, responder cells alone or regulatory cells alone. Data represent the [3H] TdR incorporation (counts per minute) per well (mean ±SD, n=5 replicate wells).

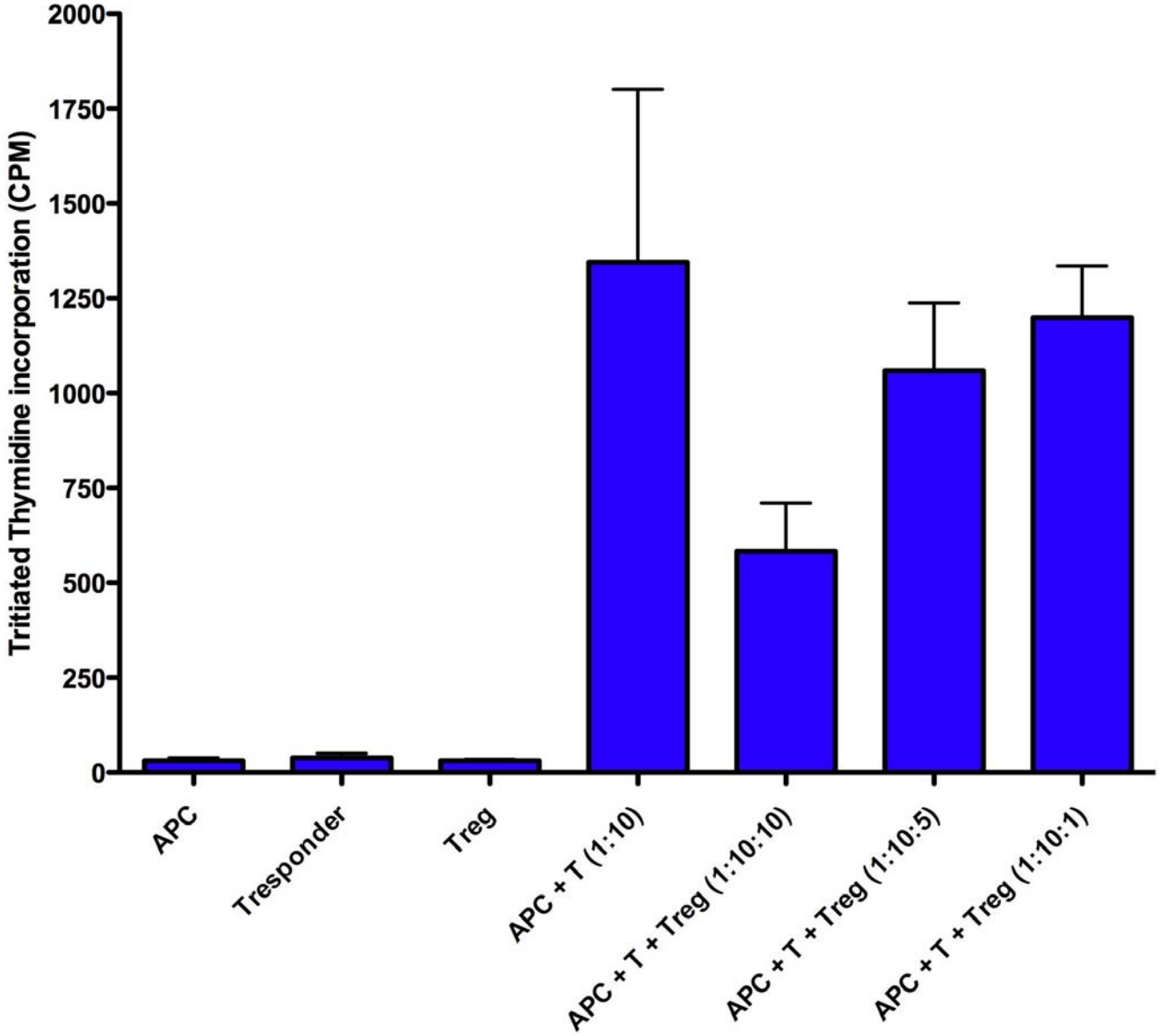
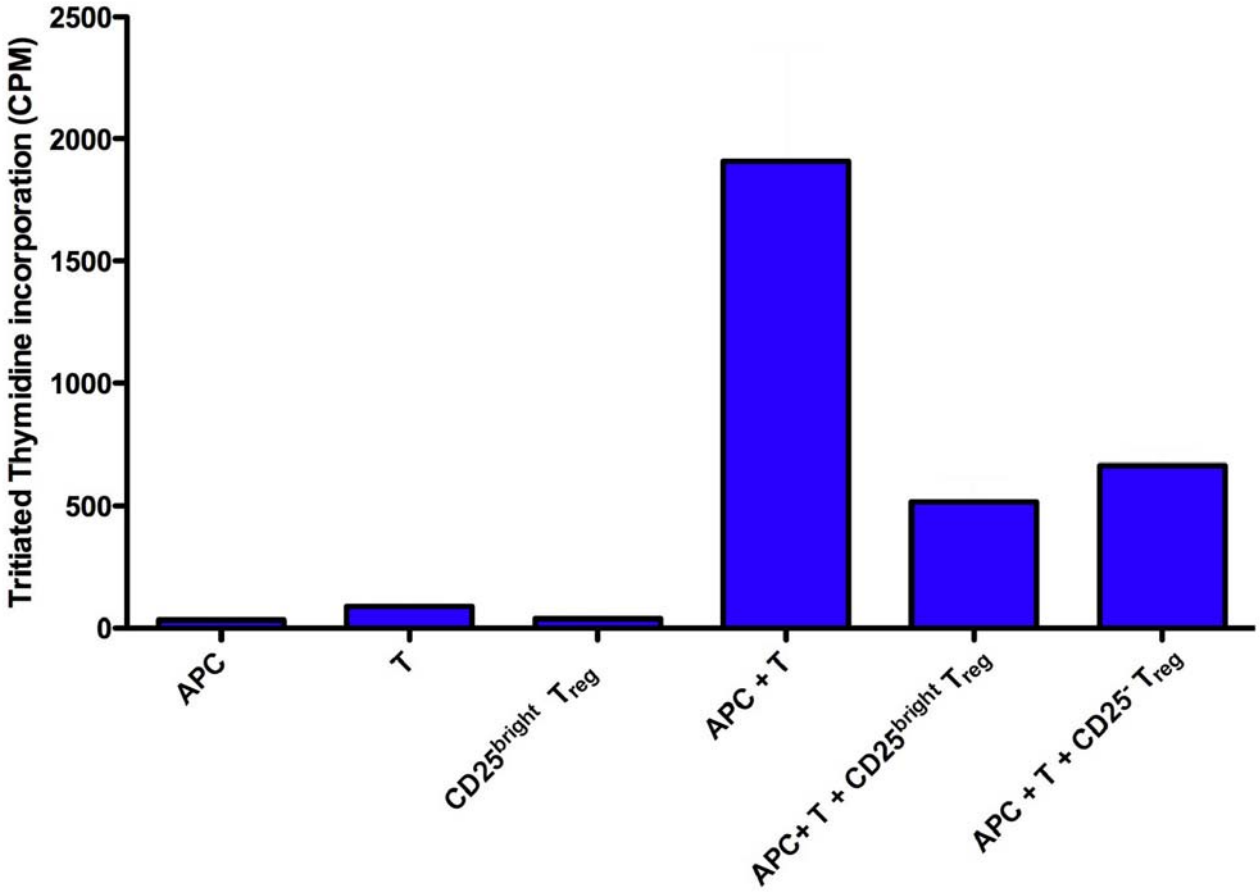

Tritiated Thymidine incorporation (CPM)
2000
1750
1500
1250
1000
750
500
250
0
APC
Tresponder
Treg
APC + T (1:10)
APC + T + Treg (1:10:10)
APC + T + Treg (1:10:5)
APC + T + Treg (1:10:1)

Figure 5.2.14 Analysis of the regulatory function of *in vivo* matured CD25^{-} Foxp3^{+} T cells

CD4^{+}CD25^{bright} cells from DA CD45.2 donors (5 x 10^6 total) were sorted (see Figure 5.2.10) and transferred to a DA CD45.1 recipient in daily doses of ~1 x 10^6 cells. The day of the last transfer was deemed to be "day 0". At day 25 after transfer, the recipient was killed and a cell suspension was prepared from the pooled cervical, mesenteric, celiac, popliteal and inguinal lymph nodes and CD25^{-} Foxp3^{+} cells (*in vivo* matured regulatory T cells) were prepared by FACS (see Figure 5.2.12). Antigen presenting cells were enriched from suspensions of DA CD45.2 spleen cells by immunomagnetic depletion of B and T cells and then treated with mitomycin C (see Figure 5.2.13). Cells were included in the MLR as follows - mitomycin-treated APC, 1 x 10^3 cells per well; responder cells 1x 10^4 cells per well; *in vivo* matured CD25^{-} Foxp3^{+} cells, 1x 10^4 cells per well. In order to provide a comparison, freshly isolated CD25^{bright} Foxp3^{+} T cells from normal DA CD45.2 rats were used in parallel MLRs (1 x 10^4 cells per well). Cells were co-cultured for 4 days in round-bottomed 96 well plates, and then pulsed with [^{3}H] TdR (1 µCi per well) for 18 h before harvesting. Control wells contained APC alone, responder cells alone or regulatory cells alone. Data shown are counts per minute (CPM) per well (means of two replicate wells). Representative of three experiments.


Tritiated Thymidine incorporation (CPM)
2500
2000
1500
1000
500
0
APC
T
CD25bright Treg
APC + T
APC+ T + CD25bright Treg
APC + T + CD25- Treg

required for cell proliferation (Duchrow *et al.* 1995), it remains detectable in cells that have been arrested in cell cycle due to treatment with chemical inhibitors (Van Oijen *et al*.1998), and it has been proposed that it may identify chromosomal scaffolding used for the reorganisation of DNA during replication (Verheijen *et al.* 1989). The antigen has been shown to be a useful marker for proliferating cells within malignant neoplasia, correlating with patient survival in cases of multiple myeloma, prostate cancer, and breast carcinoma (Schlozen and Gerdes 2000).However, some studies of breast cancer have failed to show a correlation between Ki67 expression and patient survival (Heatley 2002). Staining of Ki67 antigen has been used also to distinguish between benign and malignant lymphocytes on the basis of proliferative activity and it is of value as a diagnostic tool to aid in the identification of lymphoproliferative disorders (Bryant *et al*. 2006).

In order to assess the proliferation of regulatory T cells, a protocol enabling the simultaneous detection of the intracellular antigens Foxp3 and Ki67 was developed. The MHC II^+ subset of $CD4^+$ T cells (Figure 5.2.2) had the highest proportion of $Ki67^+$ cells (~80%), a figure similar to that found by Spargo *et al.* (2006) in $CD4^+$ MHC II^+ T cells infiltrating the synovium of rats with adjuvant-induced arthritis. This proportion was higher (Figure 5.2.3) than in “activated” conventional T cells defined by the phenotype $CD25^+$ $Foxp3^-$ (~25%). Because MHC II is expressed by activated T cells in rats (Spargo *et al.* 2001), and as shown by Mabarrack *et al.* (2008) not all MHC II^+ $CD4^+$ $Foxp3^-$ T cells express CD25, this finding suggests that the $MHCII^+$ $CD25^-$ subset is enriched in cells that are in cell cycle. This interpretation explains the relatively high proportion of $Ki67^+$ T cells found in the “resting” $CD25^-$ $Foxp3^-$ subset (Figure 5.2.3) and significant level of labelling by BrdU observed in these cells (Figure 5.2.5). The MHC II^+ $CD25^-$ subset would be contained within this so-called resting population.

Approximately 30-40% of the $Foxp3^+$ cells were found to express the Ki67 antigen (Figure 5.2.2), indicating that there is robust proliferation in this subset *in vivo*, presumably sustained by the paracrine action of IL-2 released from activated T cells. This finding is consistent with findings by others of relatively high turnover of

regulatory T cells (Vukmanovic-Stejic *et al.* 2006). Interestingly, a consistent finding was that the $CD25^-$ $Foxp3^+$ subset (putative regulatory T cells) contained a higher proportion of $Ki67^+$ cells than the $CD25^+$ $Foxp3^+$ subset (regulatory T cells), although the proportions in cell cycle were higher in both than in the resting population of conventional T cells (Figure 5.2.4). It was important, therefore, to compare the steady state rates of proliferation of regulatory and conventional T cells *in vivo*, using a quantitative approach.

5.3.2 Quantitative measurements of regulatory T cell proliferation *in vivo*

Conventional $CD4^+$ T cells are derived from RTE, which mature into naive T cells in the periphery. Following encounter with antigen in the presence of sufficient costimulation, naïve T cells become activated, proliferate rapidly and differentiate into a mixture of short lived effector T cells and long-lived memory T cells (Sprent 1993). However, a component of the memory T cell pool is thought to be short lived and rapidly proliferating, and these cells may represent effector memory T cells that can localise to sites of infection (Jenkins *et al.* 2001). An approach to studying the turnover of T cell subsets is to observe the rate of labelling of the cells by continuously administered BrdU (Tough and Sprent 1995). Naïve $CD4^+$ T cells, which comprise the majority of peripheral $CD4^+$ T cells, were found to be a relatively quiescent population that remains in interphase for long periods of time and has a half life of ~3 months in mice (Tough and Sprent 1994). Consistent with these findings, a pulse of BrdU labelled only a small proportion of the $CD25^-$ $Foxp3^-$ cells from blood, lymph nodes and thoracic duct lymph, but a higher proportion of the activated $CD25^+$ $Foxp3^-$ subset (Figures 5.2.5 - 5.2.7).

As predicted from the proportion of $Ki67^+$ cells, $CD25^+$ $Foxp3^+$ regulatory T cells contained a higher proportion of labelled cells than the resting T cell subset. However, the proportion of labelled cells in this subset was lower than in activated conventional $CD4^+$ T cells in the blood and lymph node compartments. Thus, although a higher

proportion of CD25$^+$ Foxp3$^+$ T cells were in cell cycle relative to activated T cells (Figure 5.2.4), fewer were in S phase of the cell cycle during the pulse of BrdU (Figures 5.2.5 - 5.2.7). One explanation of this observation is that the proliferation of CD25$^+$ Foxp3$^+$ cells may be limited *in vivo* by extrinsic factors, such as exogenously produced IL-2 (Li *et al.* 2005). Strikingly, the proportion of labelled cells was highest in the CD25$^-$ Foxp3$^+$ putative regulatory T cell subset. This observation highlights a paradoxical difference between the two Foxp3$^+$ subsets. While approximately equal proportions of CD25$^-$ Foxp3$^+$and CD25$^+$ Foxp3$^+$ cells expressed the Ki67 antigen and thus were in cell cycle (Figure 5.2.4), the proportion incorporating a pulse of BrdU was approximately three times higher in the CD25$^-$ Foxp3$^+$ subset than in the CD25$^+$ Foxp3$^+$ subset. In order to determine whether this difference is due to faster proliferation within the CD25$^-$ Foxp3$^+$ subset, the proliferation of CD4$^+$ T cell subsets was measured over a seven day period.

If the size of a population of cells is constant, and the rates of proliferation and turnover are at equilibrium, then the time taken for 50% of the population to incorporate a label such as BrdU will be equal to the time taken for the same proportion of cells to turnover (i.e. the half life of the cell population). To investigate proliferation rates and turnover of the CD4$^+$ T cell subsets, BrdU was provided to rats continuously in the drinking water and incorporation was assessed in cells obtained from the cervical, mesenteric and celiac lymph nodes. Using this approach, the approximate half-life of the CD25$^-$ Foxp3$^-$ resting T cells was estimated to be ~3 months (Figure 5.2.8). While this figure is consistent with the reported turnover rate of naïve T cells (Tough and Sprent 1994), it may be an under-estimate due to the presence of some MHC II$^+$ activated cells in the CD25$^-$ Foxp3$^-$ subset (see above). Interestingly, the CD25$^+$ Foxp3$^+$ regulatory T cells had an estimated half life of ~18 days, similar to that of the activated CD25$^+$ Foxp3$^-$ subset (~17 days). This rate of turnover is equivalent to that of memory T cells (Tough and Sprent 1994), where it is thought that numbers are maintained by recurring antigenic stimulation (Sprent et al.1993), a situation similar to that proposed for the maintenance of regulatory T cells (Fisson *et al.* 2003). The results are at variance with the conclusions of a study by Vukmanovic-Stejic *et al.* (2006) on regulatory T cells in humans. In that study, the

half-life of regulatory T cells, identified as $CD4^+$ $CD45RO^+$ $CD25^{hi}$ cells, was reported to be ~8 days. The apparent turnover of the $CD25^-$ $Foxp3^+$ putative regulatory T cell subset, based on the rate of labelling by continuous administration of BrdU, was estimated to be ~7 days. However, when BrdU was withdrawn, the proportion of labelled $CD25^-$ $Foxp3^+$ cells remained relatively constant, despite progressive falls in the proportions of labelled calls in the $CD25^+$ $Foxp3^-$ and $CD25^+$ $Foxp3^+$ subsets.

This raised the possibility that the paradoxically rapid rate of BrdU labelling but slow decay of labelled cells after BrdU withdrawal in the $CD25^-$ $Foxp3^+$ subset may be due to labelled cells from another $CD4^+$ T cell subset differentiating into $CD25^-$ $Foxp3^+$cells, essentially resupplying this population with labelled cells. The most likely candidate for this precursor population is the $CD25^+$ $Foxp3^+$ subset, and if these cells naturally lose their expression of CD25, it may account for the shorter estimate of half-life of regulatory T cells reported by Vukmanovic-Stejic *et al*. (2006) in humans. The use of CD25 as a marker to define regulatory T cells in that scenario would lead to the misinterpretation that loss of the molecule is a reflection of cell death. If the interpretation that $CD25^+$ $Foxp3^+$ convert into $CD25^-$ $Foxp3^+$ cells is correct, the overall half-life of regulatory T cells might be closer to ~24 days (~17 days as $CD25^+$ $Foxp3^+$ cells then ~7 days as $CD25^+$ $Foxp3^+$ cells). Direct evidence was sought for this conversion.

5.3.3 Phenotypic maturation of $CD25^-$ $Foxp3^+$ T cells into $CD25^+$ $Foxp3^+$ regulatory T cells

The observation that the proportion of $CD25^-$ $Foxp3^+$ T cells labelled with BrdU remained stable for a paradoxically long time despite an apparent rapid rate of proliferation and calculated short half life (Figure 5.2.9) suggested that the cells were being replenished from another population source. The most likely source was the $CD25^+$ $Foxp3^+$ subset, which progressively lost the BrdU label after the withdrawal of BrdU from the drinking water. The conversion of these cells into $CD25^-$ $Foxp3^+$ T cells

through the loss of CD25 expression could effectively "top up" the $CD25^-$ $Foxp3^+$ T cell pool and resupply it with labelled cells. This hypothesis was supported by the results obtained from the adoptive transfer of purified $CD25^+$ $Foxp3^+$ regulatory T cells transferred into congenic hosts. The donor cells steadily lost their expression CD25, while retaining their expression of Foxp3 (Figure 5.2.11). This observation contrasts with the findings reported by de Lafaille *et al.* (2004), which indicated that $CD25^-$ $Foxp3^+$ cells gain CD25 expression to become $CD25^+$ $Foxp3^+$ T cells under conditions of lymphopenia. This difference may be due to the effect of lymphopenia on the homeostasis of regulatory T cells, which was disrupted under lymphopenic conditions (Figure 4.2.8). The finding that $CD25^+$ $Foxp3^+$ regulatory T cells give rise to $CD25^-$ $Foxp3^+$ putative regulatory T cells supports the interpretation of the kinetic studies (Sections 5.2.5 and 5.2.6) and also the observation that the $CD25^+$ $Foxp3^+$ subset has a closer relationship to RTE than the $CD25^-$ $Foxp3^+$ subset (Figure 4.2.1).

Interestingly, the proportion of donor cells that had down-regulated CD25 was approximately two-fold in the blood (Figure 5.2.11) than in the lymph nodes (Figure 5.2.12). This observation is consistent with the circulating cells lying downstream of those in the lymph nodes, suggesting that a generative population of $CD25^+$ $Foxp3^+$ T cells in lymph nodes produces $CD25^-$ $Foxp3^+$ T cells for export to the circulation, probably via efferent lymph (Figure 5.2.7). The latter may represent a regulatory effector population *en route* to the peripheral tissues, where they are enriched (Section 3.2.8).

5.3.4 $CD25^-$ $Foxp3^+$ T cells have regulatory function

In vivo maturation of genetically marked $CD25^+$ $Foxp3^+$ T cells after adoptive transfer made it possible to purify viable $CD25^-$ $Foxp3^+$ T cells from host lymph nodes. These cells were used to determine whether the loss of CD25 is accompanied by changes in regulatory potency. $CD25^-$ $Foxp3^+$ T cells of donor origin were retrieved from lymph nodes 25 days after adoptive transfer using the FACS (Figure 5.2.12) and examined functionally for their ability to suppress an allogeneic MLR. $CD25^-$ $Foxp3^+$ T cells were

found to be equally potent as $CD25^+$ $Foxp3^+$ regulatory T cells isolated from a normal donor in their ability to suppress the MLR. Thus, maturation of $CD25^+$ $Foxp3^+$ T cells to $CD25^-$ $Foxp3^+$ T cells is accompanied by a retention of regulatory T cell activity and henceforth, these cells will be referred to simply as a subset of regulatory T cells. This finding has important implications as cells with this phenotype are enriched within the lamina propria (Figure 3.2.18) and in inflamed synovium (Figure 3.2.19). The cells may, therefore, comprise an "effector" regulatory T cell population that localises to sites of inflammation, regulating local immune responses. Furthermore, the fact that these cells are enriched within the subpopulation of T cells that is recruited into the normal thymus suggests that they may play a role modulating thymic function. Thus generation of tissue homing "effector" $CD25^-$ $Foxp3^+$ regulatory T cells may be a key role of the more sessile the $CD25^+$ $Foxp3^+$ regulatory T cell population. This $CD25^+$ subset may recirculate through secondary lymphoid tissue, where they could be regarded as the equivalent of "central" memory regulatory T cells, and by analogy their engagement with cognate antigens could produce "effector regulatory T cells".

5.3.5 Summary

In summary, a method was developed for the simultaneous detection of the Ki67 proliferation associated antigen and Foxp3, and this was utilised to assess the proportion of regulatory and conventional $CD4^+$ T cell subsets in cell cycle. This revealed that only a small proportion of $CD25^-$ $Foxp3^-$ resting T cells expressed Ki67 antigen, while most of the activated conventional T cells defined by expression of MHC II (and a smaller proportion of activated T cells defined by their expression of CD25) were in cell cycle. Interestingly, the proportions of both $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ regulatory T cells in cell cycle were higher than in the $CD25^+$ subset of conventional activated T cells.

In order to measure the proliferation of regulatory T cells using a more direct, quantitative approach, a technique was developed to enable the detection of BrdU label incorporated into the DNA of cells that had divided. Using a period of continuous BrdU administration, it was found that $CD25^-$ $Foxp3^-$ resting T cells incorporated the

BrdU label at a slow rate, with an approximate half-life of 3 months, consistent with the low proportion of these cells in cell cycle. However, $CD25^+$ $Foxp3^+$ regulatory T cells incorporated the BrdU label at a much faster rate, with an approximate half life of 18 days, which was comparable to that of $CD25^+$ activated T cells (~17 days). Strikingly, the rate of incorporation of BrdU into the $CD25^-$ $Foxp3^+$ T cell subset was much more rapid, giving these cells a half-life of ~7 days. The loss of the BrdU label from each subset was progressive, with the exception of the $CD25^-$ $Foxp3^+$ T cell population, where a high proportion of the cells paradoxically retained their BrdU label for a prolonged period of time following the cessation of BrdU administration.

It was therefore hypothesised that the paradoxically slow disappearance rate of the BrdU label from the $CD25^-$ $Foxp3^+$ T cell population may be due to the conversion of $CD25^+$ $Foxp3^+$ regulatory T cells into $CD25^-$ $Foxp3^+$ T cells. This hypothesis was supported by findings from an adoptive transfer system in which $CD4^+$ $CD25^{bright}$ ($Foxp3^+$) T cells were transferred into a normal congenic host, where they downregulated CD25 over 25 days while retaining their expression of Foxp3. The ability of the generated $CD25^-$ $Foxp3^+$cells to suppress an allogeneic MLR was confirmed, indicating that they do indeed constitute a subset of regulatory T cells. This rapidly proliferating regulatory T cell population is enriched in the lamina propria and inflamed synovium, and may represent a population of tissue homing “effector” regulatory T cells that regulate local immune responses in peripheral tissues.

Chapter 6: The suppressive mechanism of regulatory T cells

6.1 Introduction

Regulatory T cells suppress the proliferation and effector functions of other T cells through mechanisms that remain unclear, despite extensive investigations. It was originally believed that regulation might be achieved by preferential capture of IL-2 by the high affinity IL-2 receptor α chain (CD25), which is expressed at high levels by some regulatory T cells (Sakaguchi *et al.* 1995). Alternatively, it was proposed that suppression might be mediated by secretion of humoral inhibitory factors such as IL-10 and/or TGF-β, or by signals that lead to the generation of cytotoxic tryptophan catabolites such as kynurenine (Miyara and Sakaguchi 2007). However, later studies revealed a requirement for regulatory T cells to interact physically with antigen presenting cells, indicating that soluble factors do not play an essential role in the suppressive mechanism of regulatory T cells (Takahashi *et al.* 1998). In agreement with this finding, it has been shown that regulatory T cells require stimulation through their TCR to trigger their suppressive mechanism, but once activated, the mechanism is completely antigen non-specific (Thornton and Shevach 2000). There are currently several alternative theories about how regulatory T cells might inhibit the proliferation and effector functions of other T cells, some of which involve direct interactions between the respective T cells.

Regulatory T cells express the inhibitory costimulatory molecule CTLA-4, which can bind to the costimulatory molecules CD80 and CD86 expressed by mature APC. However, since T cells can express CD80 and CD86 following stimulation (Sabzevari *et al.* 2001), it has been proposed that a direct interaction between CTLA-4 expressed by regulatory T cells and CD80 or CD86 expressed by stimulated T cells can deliver an inhibitory signal that curtails T cell activation (Miyara and Sakaguchi 2007). While this mode of action is supported by studies showing that the engagement of CD80 or CD86 on effector T cells by regulatory T cells expressing CTLA-4 can prevent autoimmune disease (Paust *et al.* 2004), other studies have shown that T cells that do not express CD80 or CD86 remain highly susceptible to regulation by regulatory T cells *in vitro*. These findings indicate that a CTLA-4-mediated mechanism is not a

necessary part of the suppressive mechanism associated with regulatory T cells (Takahashi *et al.* 2000, May *et al.* 2007).

Another proposed mechanism of regulatory T cell suppression involves the action of lymphocyte activation gene 3 (LAG-3), a molecule that binds MHC class II molecules and is upregulated on the surface of regulatory T cells following TCR stimulation (Huang *et al.* 2004). LAG-3 displayed by regulatory T cells is suggested to inhibit the activation and proliferation of effector T cells by binding to MHC II molecules displayed by the APC to the effector T cells (Miyara and Sakaguchi 2007). However, this mode of action seems improbable when one considers the size of leukocytes compared with the molecules that they express on their plasma membranes. Molecules on the surface of regulatory T cells would be unable to access the immunological synapses that form between the APC and the effector T cell. Although LAG-3 is certainly able to deliver signals that lead to the inhibition of T cell proliferation, the absence of overt autoimmune disease in LAG-3 deficient mice suggests that the molecule is unlikely to play a major role in the suppressive mechanisms of regulatory T cells (Huang *et al.* 2004).

Regulatory T cells are known to express membrane-bound TGF-β when stimulated in the presence of IL-2, and it is thought this may utilised *in vivo* to control intestinal inflammation (Nakamura *et al.* 2004). Although some studies have shown that the regulation of colitogenic T cells is inhibited by neutralisation of TGF-β, this phenomenon did not appear to be associated with effects on membrane bound TGF-β expressed by the regulatory T cells (Kullberg *et al.* 2005). Indeed, rather than being involved with the suppressive mechanism of regulatory T cells, TGF-β appears to play a role in the expansion or differentiation of regulatory T cells (Huber *et al.* 2004, Kullberg *et al.* 2005).

Thus mechanisms that involve the direct delivery of inhibitory signals from regulatory T cells to effector T cells do not appear to be necessary components of the suppressive mechanism associated with regulatory T cells. However, new research

suggests that regulatory T cells may have actions that are similar to those of anergised T cells, inhibiting T cell proliferation by conditioning APC in a manner that reduces their stimulatory capacity. T cells are anergised by encounter with cognate antigen in the absence of additional costimulatory signals. A consequence of this state is that when the anergised cells interact with APC, they are unable to up-regulate CD154 and provide stimulation through CD40 to protect APC from apoptosis (Bowen *et al.* 1995, Koppi *et al.* 1997). Failure to up-regulate CD154 is thought to be due to ubiquitination of CD154 by gene associated with anergy in lymphocytes (GRAIL), which targets the molecule for degradation by the proteosome (Lineberry *et al.* 2008). Anergised T cells thus effectively condition DC to arrest their maturation and inhibit their expression of costimulatory molecules, indirectly depriving T cells of stimulation (Frasca *et al.* 2003). The considerable similarities between regulatory T cells and anergic T cells (see Chapter 3) raises the possibility that the cells share common mechanisms of suppression, which involve conditioning APC to reduce their capacity to activate other T cells.

Supporting this concept, regulatory T cells have been shown to swarm around DCs *in vivo*, out-competing other T cells for space on the DC surface and limiting their access to stimulatory signals (Tang and Krummel 2006, Hugues *et al.* 2006). Regulatory T cells appear not to form stable associations with effector T cells during active suppression, and instead inhibit stable interactions between effector T cells and DC (Tang *et al.* 2005, Tadokoro *et al.* 2006). Interestingly, like anergised T cells, regulatory T cells have been consistently shown to inhibit the maturation of DC and their expression of the costimulatory molecules CD80 and CD86 by the cells (Cederbom *et al*. 2000, Houot *et al.* 2006, Veldhoen *et al.* 2006, Onishi *et al.* 2008). Furthermore, there is evidence that the ligation of CD154 to CD40 expressed on the surface of immature DC prevents regulatory T cells from being able to exert their suppressive activities, while promoting the capacity of immature DC to mature and stimulate other T cells (Serra *et al.* 2003). These observations suggest strongly that the interaction of both regulatory T cells and anergised T cells with DC fails to provide survival signals in the form of CD154 abrogating their ability upregulate CD80 and CD86 and to activate T cells is abrogated (Frasca *et al.* 2003).

Experiments were undertaken to determine whether the effects of regulatory T cells on the proliferation and effector functions of other T cells is mediated by conditioning of APC. The functional consequences of treating lymph node DCs with syngeneic regulatory T cells were tested in a modified MLR system. After depletion of the regulatory T cells, the conditioned APC were added to allogeneic responder T cells and stimulation was compared to cultures in which regulatory T cells were present for the duration of the MLR. The objective of this protocol was to allow the effects of regulatory T cells to be separated spatially and temporally from any effects that they might have directly on the responder population.

6.2 Results

6.2.1 Isolation of lymph node dendritic cells

In order to obtain a pure population of professional APC, lymph node cells were prepared by enzymatic digestion of lymph nodes and DC were first enriched by Percoll density gradient centrifugation. B and T cell blasts were then removed by immunomagnetic depletion, resulting in a preparation of cells that contained <1% T cells (see Figure 6.2.3C). In order to test the stimulatory activity of the purified cells, they were cocultured with CD25-depleted allogeneic responder T cells in a one way mixed lymphocyte reaction (MLR). The purified lymph node DC were found to be almost 3-fold more potent as stimulator cells in a one way MLR than spleen APC prepared by immunomagnetic depletion of B and T lymphocytes from a suspension of splenocytes (Figure 6.2.1). To examine the dose dependence of APC in the MLR, purified lymph node DC were titrated from 10^3 to 10^5 per well into a one way MLR containing 2 x 10^5 allogeneic responder T cells per well. The lymph node DC stimulated responder T cell proliferation in a dose-dependent manner across the range of concentrations tested (Figure 6.2.2).

Figure 6.2.1: Lymph node DC are potent antigen presenting cells

Lymph node dendritic cells were isolated from pooled mesenteric, coeliac, inguinal, popliteal, axcillary, and cervical lymph nodes, which were homogenised and digested with 7.8 U/ml collagenase type I and 3.4 U/ml DNAse I. Lymph node DC were separated from lymphocytes using a Percoll step-gradient and contaminating T cells removed by labelling cells with R73 (anti-TCR) and immunomagnetically depleting them. Splenic APC were prepared by labelling a suspension of spleen cells with OX33 (anti-CD45RA), MARK-1 (anti-rat Ig κ chain) and R73 (anti-TCR), and immunomagentically depleting B and T cells. APC were used at 1 x 10^3 cells per well and responder T cells, which consisted of allogeneic negatively selected $CD4^+$ $CD25^-$ T cells from Pvg/c lymph nodes were used at 2 x 10^5 cells per well. Cells were cocultured for 4 days, then pulsed with 1 μCi tritiated thymidine per well and harvested 18 h later. Representative of two experiments.

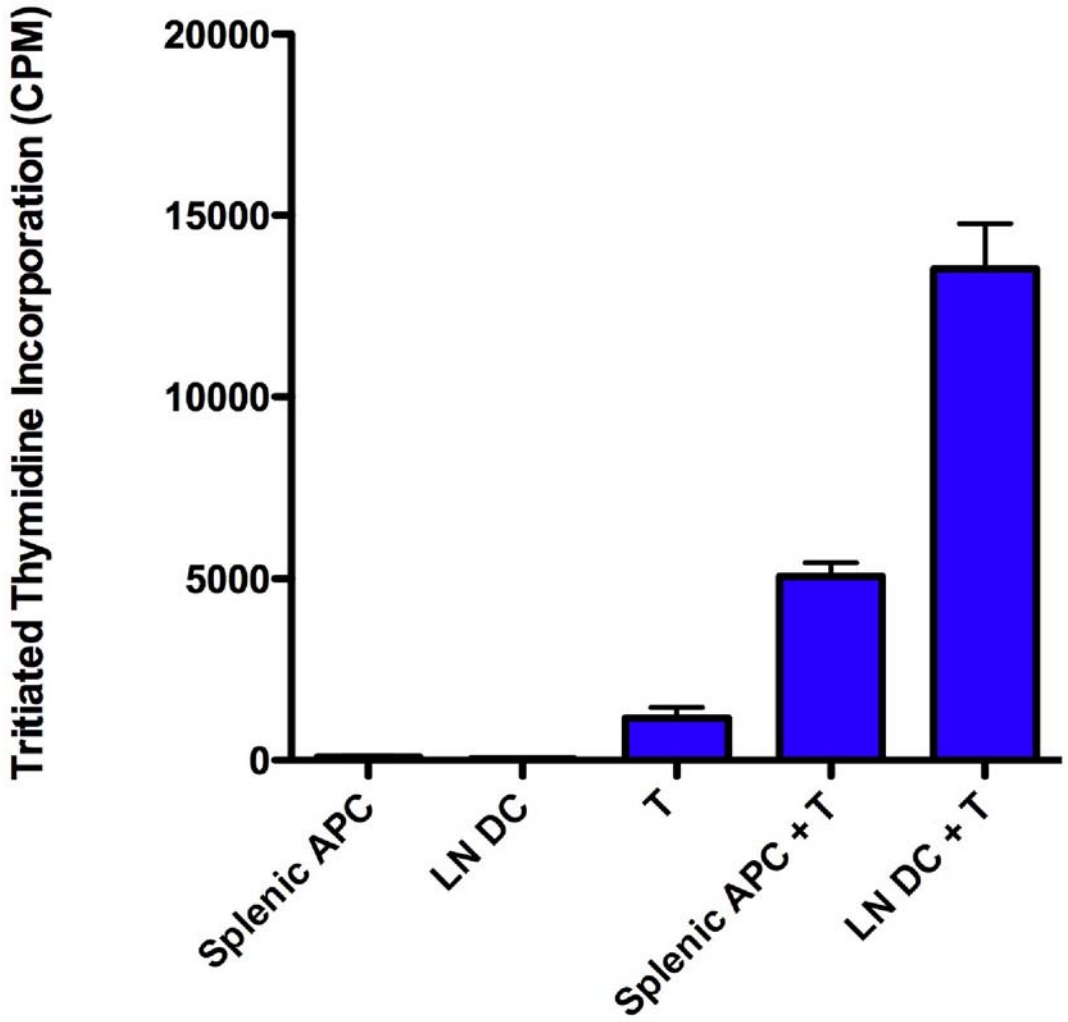
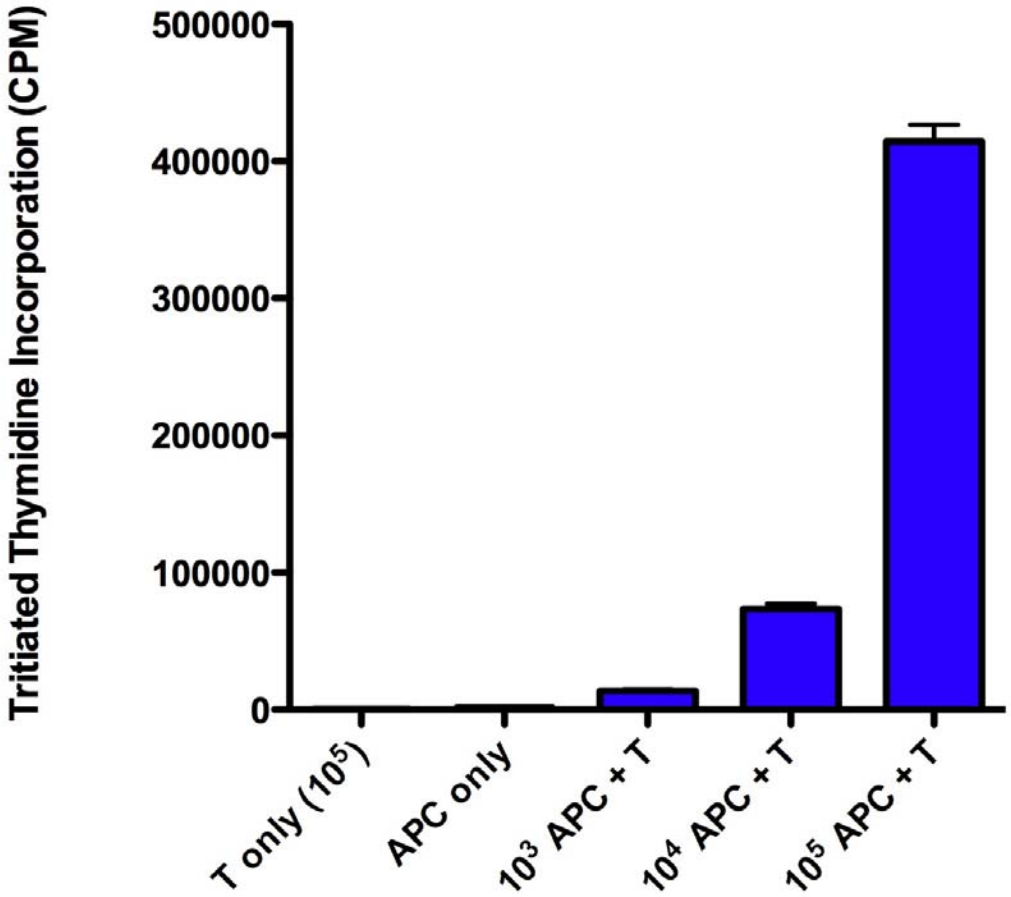

Tritiated Thymidine Incorporation (CPM)
20000
15000
10000
5000
0
Splenic APC
LN DC
T
Splenic APC + T
LN DC + T

Figure 6.2.2: Lymph node DC stimulate T responder cells in a dosage dependent manner

Lymph node DC were isolated from lymph nodes by mechanical disruption and enzymatic digestion followed by Percoll density centrifugation and immunomagnetic T cell depletion, and used to stimulate the proliferation of allogeneic $CD4^+$ $CD25^-$ responder T cells. Lymph node DC were included into cultures of 2 x 10^5 allogeneic responder T cells per well at 10^3, 10^4, or 10^5 cells per well, and incubated for 4 days before being pulsed with 1 μCi tritiated thymidine per well and harvested 18 h later. Representative of two experiments.


Tritiated Thymidine Incorporation (CPM)
500000
400000
300000
200000
100000
0
T only (10^5)
APC only
10^3 APC + T
10^4 APC + T
10^5 APC + T

6.2.2 Multi-stage in vitro assay to investigate the suppressive mechanism of regulatory T cells

A multi-stage mixed lymphocyte reaction was designed in order to investigate whether inhibition of T cell proliferation by regulatory T cells involves conditioning of APC to reduce their ability to stimulate responder T cells, or whether the regulatory T cells act directly on responder T cells. In two sets of replicate cultures that were incubated in parallel, lymph node DC (5 x 10^4 per well) were either conditioned by incubation with 2 x 10^5 FACS purified syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells per well, or they were left untreated. After 48 hours, one of the replicate cultures was taken and cells in each well resuspended in EDTA to release the regulatory T cells from the DC, and subjected to an immunomagnetic T cell depletion, to remove the regulatory T cells from the coculture. The untreated DC culture in the same replicate was subjected to the same treatment to ensure that any differences observed were not due to the differential handling of DC. The conditioned and untreated DC (5 x 10^4 per well) were then cocultured with 2 x 10^5 allogeneic $CD4^+$ $CD25^-$ responder T cells, which were also added to each well of the parallel replicates that were not depleted of T cells.

The MLRs were allowed to proceed for four days, at which time the cultures were pulsed with 1 μCi tritiated thymidine and harvested 18h later. Thus the assay design allows evaluation of whether regulatory T cells must make contact with responder T cells in order to inhibit their proliferation, or whether regulation is independent of responder T cells and results from the conditioning of APC. An essential caveat to interpretation of this experiment is that there must be effective depletion of T cells from the conditioned DC prior to the MLR. Purified lymph node DC contained <1% T cells (Figure 6.2.3C), while wells co-cultured with regulatory T cells contained ~70% T cells (Figure 6.2.3D). After the 48 hour co-culture, cells were treated with EDTA and subjected to immunomagnetic depletion using mAb R73, resulting in <1% contamination with T cells (Figure 6.2.3E), a figure no greater than in untreated DC that received a second round of depletion (Figure 6.2.3F)

There was vigorous incorporation of tritiated thymidine by responder cells in positive control wells that received untreated allogeneic DCs, with no significant incorporation in negative control wells that contained purified preparations of either DC alone, responder T cells alone or regulatory T cells alone. Expected inhibition of responder cell proliferation was observed in the cultures that contained regulatory T cells throughout the MLR (Figure 6.2.3A). Importantly, equal inhibition of responder T cell proliferation ($p=0.93$ using a paired two tailed student's t test) was observed in the cultures that contained purified conditioned DC (Figure 6.2.3B). These findings indicate that no direct contact between regulatory T cells and responder T cells is required for the inhibition of the MLR. Thus, in this model of inhibition of a primary immune response by naïve $CD4^+$ T cells, contact between regulatory T cells and APCs is sufficient to demonstrate the suppressive effect of the regulatory T cells. It was concluded that regulatory T cells can condition APC in a manner that reduces their ability to stimulate the proliferation of allo-reactive naive T cells (Figure 6.2.3).

To examine whether the conditioning effect of regulatory T cells on APC is specific to the $CD25^+$ $Foxp3^+$ subset, or could be substituted by co-culture with conventional $CD4^+$ T cells, lymph node DC (5×10^4 per well) were incubated with either 2×10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells, 2×10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, the cultures were labelled with mAb R73 and depleted of T cells using immuno-magnetic beads. Replicate aliquots of 5×10^4 DC from each preparation were removed and incubated with allogeneic responder T cells for 4 days prior to pulsing with 1 μCi tritiated thymidine for 18h. As shown in Figure 6.2.4, DC conditioned with $CD25^-$ $CD4^+$ T cells supported similar levels of responder cell proliferation to DC that were untreated. In contrast, the level of responder cell proliferation was significantly lower ($p=0.0005$) in cultures containing DC that had been conditioned by regulatory T cells.

Figure 6.2.3: Determination of the effect of co-culture with regulatory T cells on the ability of DC to stimulate T cell proliferation

In two parallel cultures, lymph node DCs (5 x 10^4 per well) were incubated with 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells per well (T_{reg}), or were left untreated. In the control MLR (A), allogeneic $CD4^+$ $CD25^-$ responder T cells were added after 48h, while in the test MLR (B), the syngeneic regulatory T cells were depleted after 48h prior to the addition of responder T cells. The MLRs were allowed to proceed for 4 days following the addition of allogeneic responder T cells, then pulsed with 1 μCi tritiated thymidine and harvested 18h later. The depletion of regulatory T cells from the culture of DC and regulatory T cells, and the mock depletion of T cells from the DC culture was confirmed by flow cytometry. Prior to T cell depletion, the culture of lymph node DC alone contained <1% T cells (C), and the coculture of regulatory T cells contained ~75% T cells (D), and following the immunomagnetic depletion of T cells both cultures contained <1% T cells (E and F). Representative of 4 experiments.

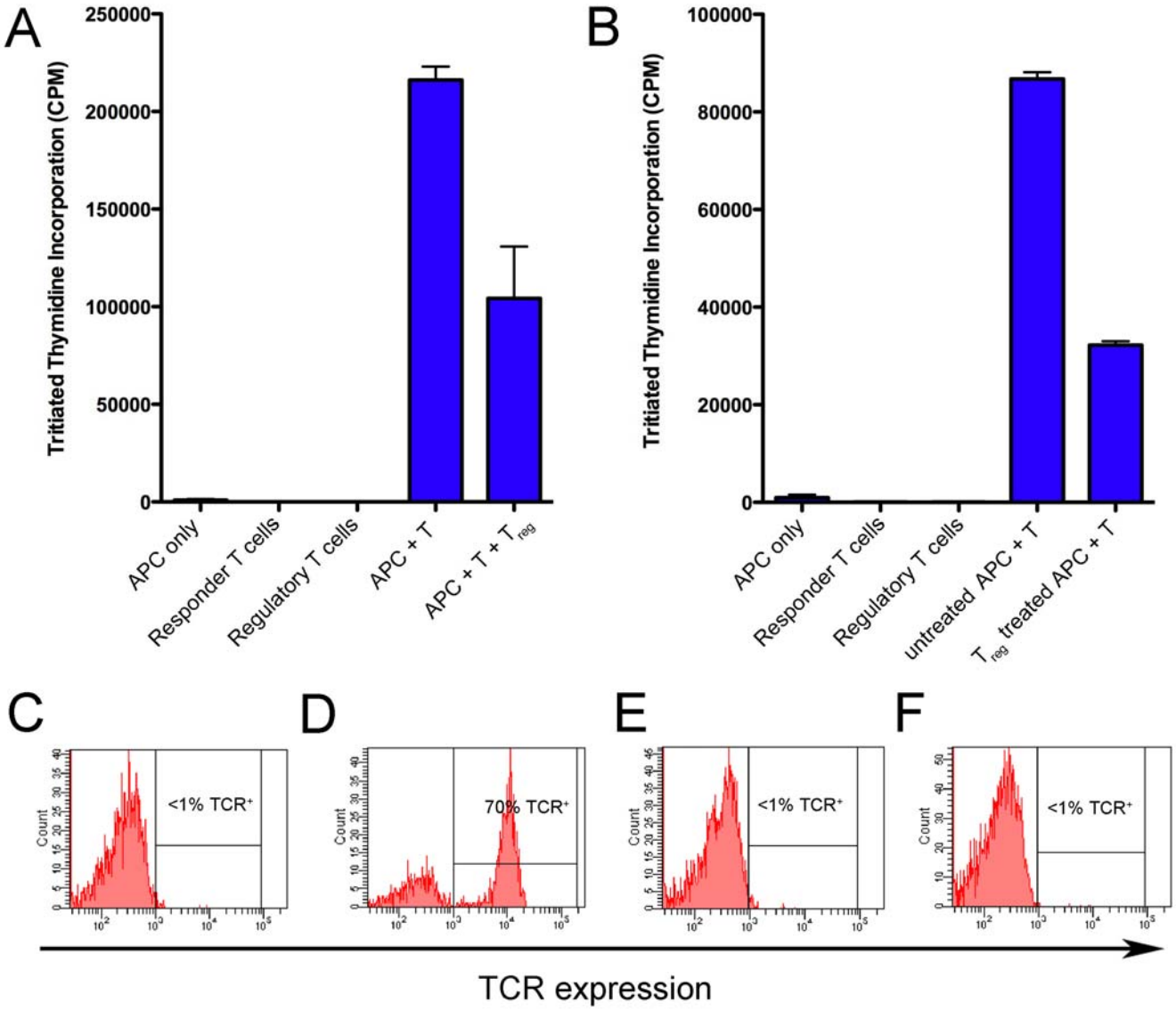
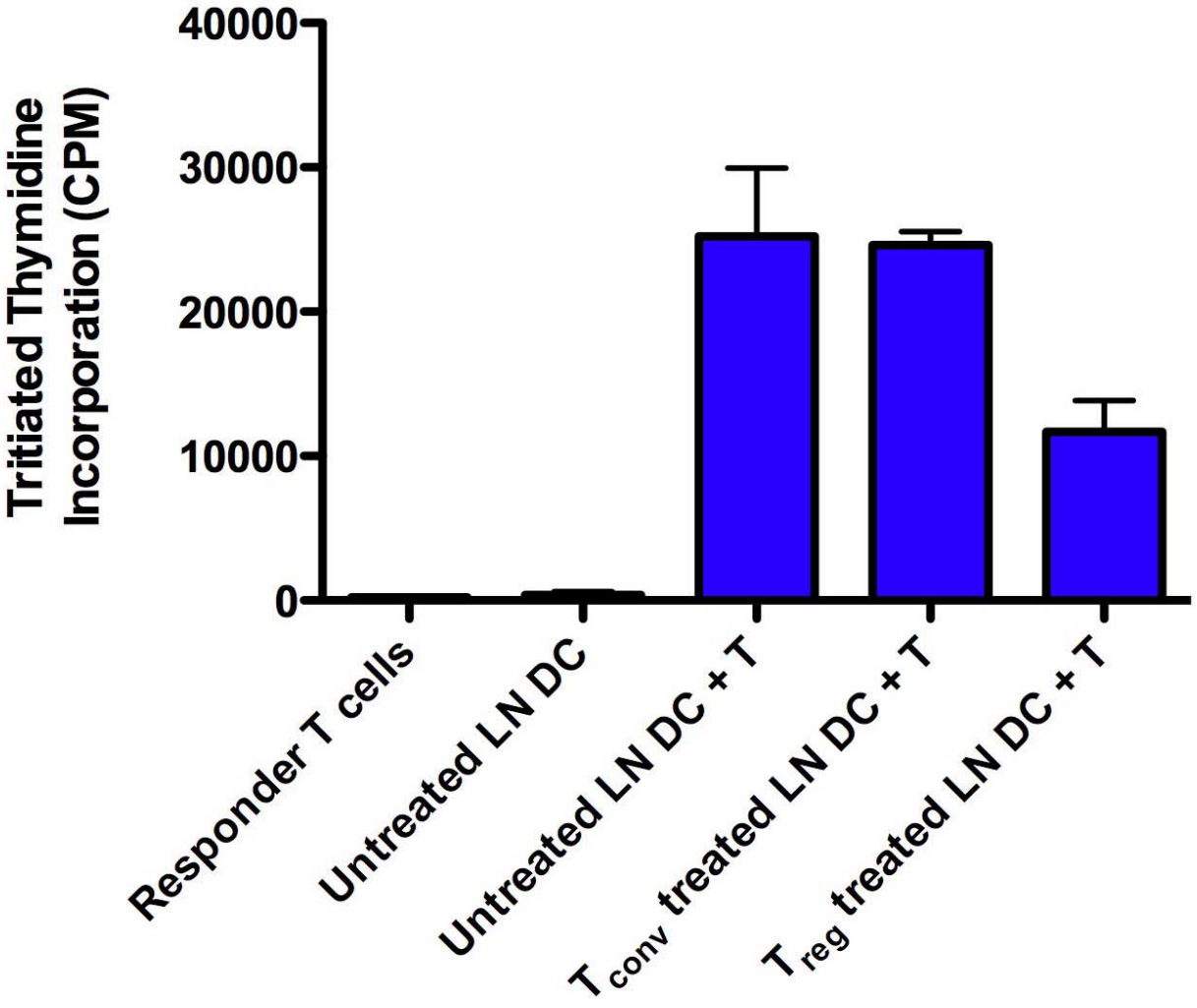

A
Tritiated Thymidine Incorporation (CPM)
250000
200000
150000
100000
50000
0
APC only
Responder T cells
Regulatory T cells
APC + T
APC + T + T reg
B
Tritiated Thymidine Incorporation (CPM)
100000
80000
60000
40000
20000
0
APC only
Responder T cells
Regulatory T cells
untreated APC + T
T reg treated APC + T
C
<1% TCR+
D
70% TCR+
E
<1% TCR+
F
<1% TCR+
Count
TCR expression

Figure 6.2.4: Functional analysis of regulatory T cell conditioned DC prepared for microarray differential gene expression analysis

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. To evaluate their stimulatory capacity, DCs from each group (5 x 10^4) were incubated with allogeneic responder T cells for 4 days, then pulsed with 1 μCi tritiated thymidine and harvested 18h later. Representative of 4 experiments.


Tritiated Thymidine Incorporation (CPM)
40000
30000
20000
10000
0
Responder T cells
Untreated LN DC
Untreated LN DC + T
T conv treated LN DC + T
T reg treated LN DC + T

6.2.3 Identification of genes in dendritic cells altered by regulatory T cells, using differential gene expression microarray analysis

Untreated purified lymph node DC, and conditioned DC that had been re-purified after incubation with regulatory T cells, were prepared as described above. As also described above (Figure 6.2.4), an additional control group was added, in which DC were "conditioned" by incubation with syngeneic conventional $CD4^+$ $CD25^-$ T cells. As discussed in 6.2.2, only the DC conditioned with regulatory T cells exhibited reduced capacity to stimulate allogeneic responder T cells (Figure 6.2.4). RNA was purified from approximately 2×10^5 purified DC from each of the treatment groups. Three replicates were generated to enable the microarray analysis of differential gene expression. Following conditioning with T cells, re-purified DC were homogenised in TRIzol and RNA was extracted using an RNeasy kit, according to the manufacturer's instructions (Qaigen). RNA samples were supplied to the Adelaide Microarray Centre, where it was confirmed to be of suitable quality using an RNA Bioanalyser (Agilent). Total RNA was then reverse transcribed and coupled to Cy dye and hybridised to GeneChip Gene 1.0 ST Microarray chips (Affymetrix) prior to scanning as described in Materials and Methods (Section 2.12). The Partek Genomics Suite 6.3 software package was used to analyse the data. Raw Affymetrix intensity measurements of all probe sets were background corrected, normalised and summarised into gene expression level measurements using the robust multiarray average (RMA) method, which corrects for cDNA loading (Kim *et al.* 2007). A one-way ANOVA was performed to identify differentially expressed genes, which were then ranked in order of ascending p values. It was found that the expression of many molecules had changed in DCs conditioned with regulatory T cells compared with both DCs treated with conventional $CD4^+$ $CD25^-$ T cells (Table 6.1) and untreated DCs (not shown).

The genes identified as being differentially expressed between DCs treated with conventional T cells and DCs treated with regulatory T cells were also differentially expressed between untreated DCs and DCs treated with regulatory T cells. Importantly, it was found molecules known to be associated with T cells were not found among the differentially expressed genes, indicating that the results obtained

are unlikely to be artefacts of T cell contamination. Interestingly, the majority of genes expressed differentially by DCs treated with regulatory T cells compared to the other groups were genes that were upregulated, including a considerable number that appear to encode putative serine/threonine kinases.

One of the genes with up-regulated transcripts in DC conditioned with regulatory T cells (Entrez ID 293591) encodes a CD163-like molecule (Figure 6.2.5). Although the function of this molecule is not known, it may be similar to that of CD163, which is a scavenger receptor expressed by monocytes and macrophages that captures haemoglobin molecules complexed to haptoglobin (Abraham and Drummond 2006). Binding of haemoglobin-haptoglobin complexes by CD163 on macrophages is known to induce the secretion of IL-10, which aids in the resolution of inflammation resulting from haemolysis (Philippidis *et al.* 2003). If stimulation through the CD163-like molecule were to have a similar effect on the release of anti-inflammatory cytokines by DC, this might point to its relevance following up-regulation by regulatory T cells. In order to assess the level of similarity between CD163 and the CD163-like molecule, a protein-protein Basic Local Alignment Search Tool (BLAST) alignment was conduced using the National Centre for Biotechnology Information (NCBI) website. This revealed 38% amino acid identity between the two peptides, and similarity between 53% of the amino acid residues (Figure 6.2.6). Furthermore, the CD163-like molecule contained 8 cysteine rich scavenger receptor domains, one less than CD163 itself, suggesting that it may have a very similar structure to CD163, and possibly some similarities in function.

Transcripts of genes encoding 11 novel protein kinases were upregulated in DC that had been treated with regulatory T cells. These included three kinases with putative four point-1, ezrin, radixin and moesin (FERM) domains (Figure 6.2.8A-C),and eight putative serine/threonine kinases (Figure 6.2.9 and 6.2.10). As these putative kinases have not yet been formally named, they will be referred to by their Entrez identification (ID) numbers. The FERM domain is believed to mediate the localisation of signalling proteins to the interior side of the plasma membrane, and it is typically found in proteins that mediate the attachment of the cytoskeleton to the

Table 6.1 Differentially expressed genes in DCs treated with regulatory T cells

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. The reisolated DCs were then homogenised in Trizol and their RNA was purified using a RNeasy kit (Qiagen). RNA samples were then analysed using GeneChip Gene 1.0 ST Microarray chips (Affymetrix) at the Adelaide Microarray Centre to identify differentially expressed genes. Differential expression of genes is reported as the expression in DCs treated with regulatory T cells relative to DCs treated with conventional T cells.

Entrez ID	Gene Title	Fold Change	Regulation in APC + T_{reg}	P.Value	Type
293591	CD163 molecule-like 1 /// similar to scavenger receptor cysteine-rich type 1 protein M160 precursor	2.36	up	0.000079	Scavenger receptor
171062	natural killer cell group 7 sequence	3.96	down	0.002479	granule/vesicle associated
365140	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	2.40	up	0.002847	Putative Kinase
502285	similar to putative pheromone receptor (Go-VN4) (predicted)	1.54	up	0.003327	Olfactory associated
502271	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	3.01	up	0.004518	Putative Kinase
116651	E2F transcription factor 5	1.98	down	0.004671	Cell cycle
679206	similar to FERM domain containing 1	2.57	up	0.005586	Putative FERM Kinase
308209	similar to putative protein kinase (predicted) /// similar to FERM domain containing 1	2.67	up	0.005730	Putative FERM Kinase
365139	similar to serine/threonine kinase /// similar to FERM domain containing 1	2.82	up	0.005987	Putative Kinase
502273	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	3.24	up	0.006003	Putative Kinase
685793	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	2.72	up	0.006495	Putative Kinase
298461	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	3.10	up	0.006603	Putative Kinase
685793	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	3.06	up	0.006751	Putative Kinase
365132	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	2.70	up	0.006962	Putative Kinase
365132	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	2.96	up	0.007203	Putative Kinase
502273	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	2.70	up	0.007782	Putative Kinase
498544	hypothetical protein LOC498544 (Kinase)	1.52	up	0.009158	Putative Kinase
685793	similar to serine/threonine kinase /// similar to putative protein kinase (predicted)	2.54	up	0.009737	Putative Kinase
689299	similar to FERM domain containing 1	2.24	up	0.010116	Putative FERM Kinase
246097	Fas (TNF receptor superfamily, member 6)	1.58	down	0.013679	Inhibitory molecule
293599	olfactory receptor 300 (predicted)	1.43	up	0.014007	Olfactory associated
171519	calcitonin-related polypeptide, beta	1.70	down	0.014253	Inhibitory molecule
499342	CD274 antigen	1.68	down	0.015034	Inhibitory molecule
293491	similar to yippee-like 3 (predicted)	1.40	up	0.016259	Inhibitory molecule
365149	similar to putative protein kinase (predicted) /// similar to KP78b CG17216-PA	2.25	up	0.016791	Putative Kinase
361604	synaptotagmin-like 2 (predicted)	1.71	up	0.017974	granule/vesicle associated
494535	trace-amine-associated receptor 7h/e/b/g /// trace amine-associated receptor 7C ///	1.52	up	0.018880	Olfactory associated
361617	centaurin, delta 2	1.45	up	0.019675	signal transducer
405039	olfactory receptor 310 (predicted)	1.39	up	0.019855	Olfactory associated
680891	similar to Splicing factor 3B subunit 5 (SF3b5) (Pre-mRNA splicing factor SF3b 10 kDa subunit)	1.50	down	0.020402	mRNA splicing factor
688574	similar to DNA-directed RNA polymerase II largest subunit (RPB1)	2.93	up	0.020643	RNA polymerase
292615	putative zinc finger protein /// similar to Ca^{2+}-sensing receptor/// similar to putative pheromone receptor (Go-VN5)	1.49	up	0.020833	Olfactory associated

Figure 6.2.5 Expression of CD163-like molecule by DC conditioned by regulatory T cells

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. The reisolated DCs were then homogenised in Trizol and their RNA was purified using a RNeasy kit (Qiagen). RNA samples were then analysed using GeneChip Gene 1.0 ST Microarray chips (Affymetrix) at the Adelaide Microarray Centre to identify differentially expressed genes. Transcripts of the CD163-like molecule (Entrez ID 293591) were found to be expressed at more than two-fold higher levels in DC treated with regulatory T cells (APC + T_{reg}) compared to untreated DC (APC) or DC treated with conventional T cells (APC + T).

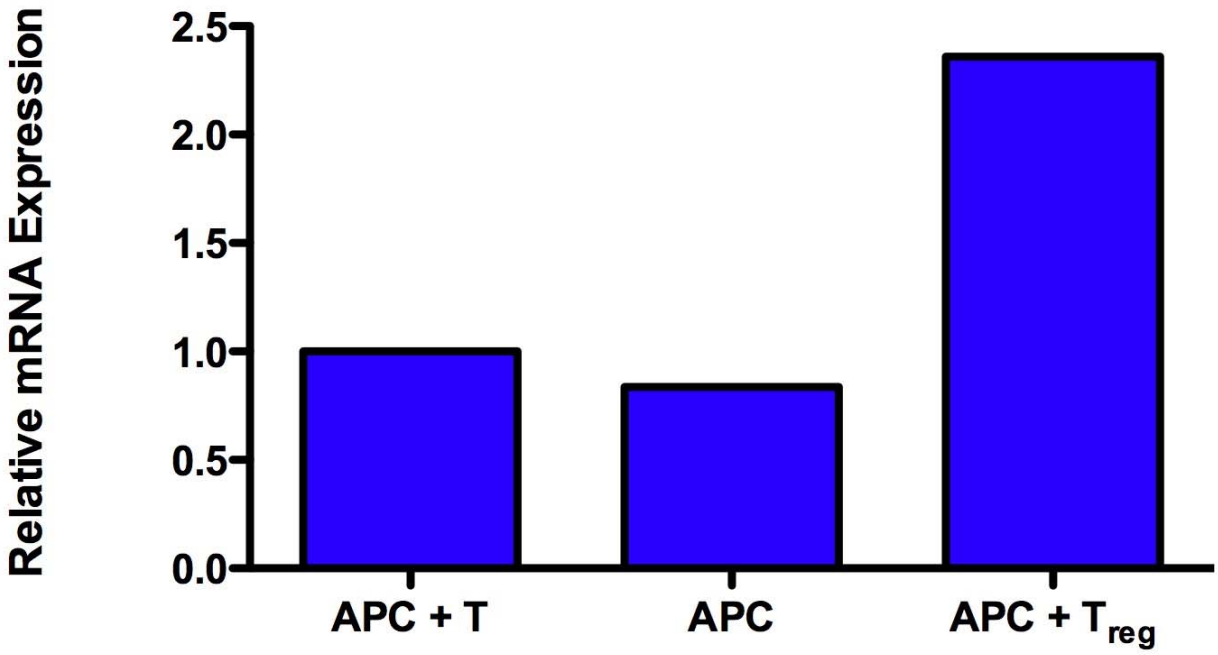
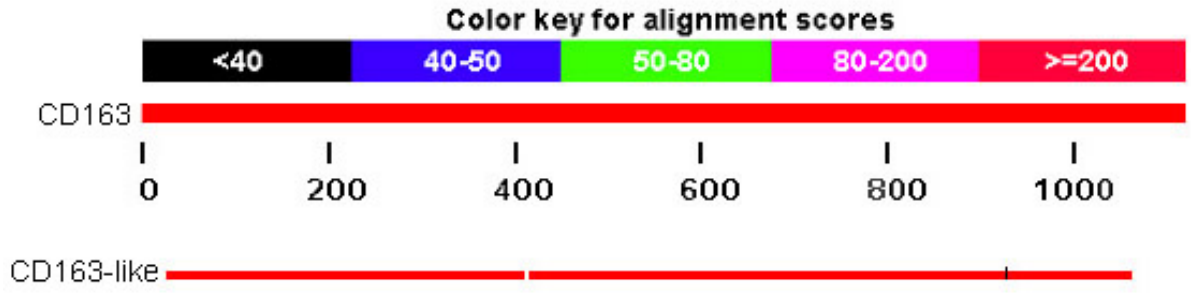

Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
APC + T
APC
APC + Treg

Figure 6.2.6 Peptide sequence comparison of the CD163-like molecule with CD163

The peptide sequence of the CD163-like molecule (Entrez ID 293591) was aligned with CD163 itself using a protein-protein BLAST alignment (NCBI), revealing that 38% of the amino acid residues were identical, and 53% of residues were similar, such that a high alignment score was achieved (A). The peptide sequences were also analysed for conserved domains using a specialised BLAST tool (NCBI), revealing the presence of 8 cysteine rich scavenger receptor domains in the CD163 like molecule (B), compared with 9 cysteine rich scavenger receptor domains in CD163 itself (C).

A


Color key for alignment scores
<40
40-50
50-80
80-200
>=200
CD163
0
200
400
600
800
1000
CD163-like

B


Query seq.
Superfamilies
1
125
250
375
500
625
750
875
995
SRCR superfamil
SRCR superfam
SRCR superfami

C

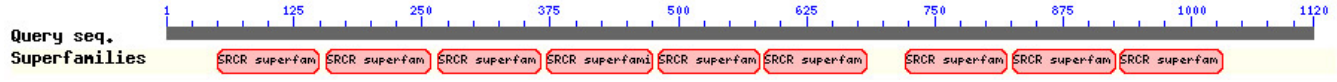

Query seq.
Superfamilies
1
125
250
375
500
625
750
875
1000
1120
SRCR superfam
SRCR superfami

Figure 6.2.7 The expression of natural killer group 7 sequence by DC conditioned with regulatory T cells.

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. The reisolated DCs were then homogenised in Trizol and their RNA was purified using a RNeasy kit (Qiagen). RNA samples were then analysed using GeneChip Gene 1.0 ST Microarray chips (Affymetrix) at the Adelaide Microarray Centre to identify differentially expressed genes. Transcripts encoding the natural killer group 7 sequence molecule (Entrez ID 171062) were expressed at much lower levels in DC treated with regulatory T cells (APC + T_{reg}) compared to untreated DC (APC) or DC treated with conventional T cells (APC + T).

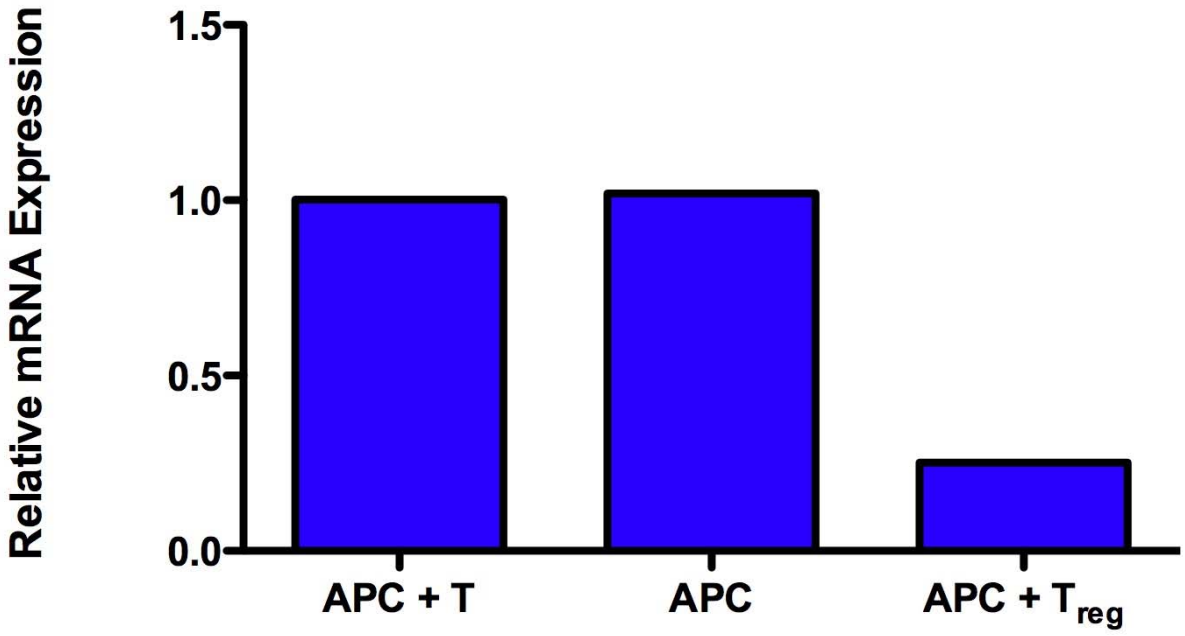
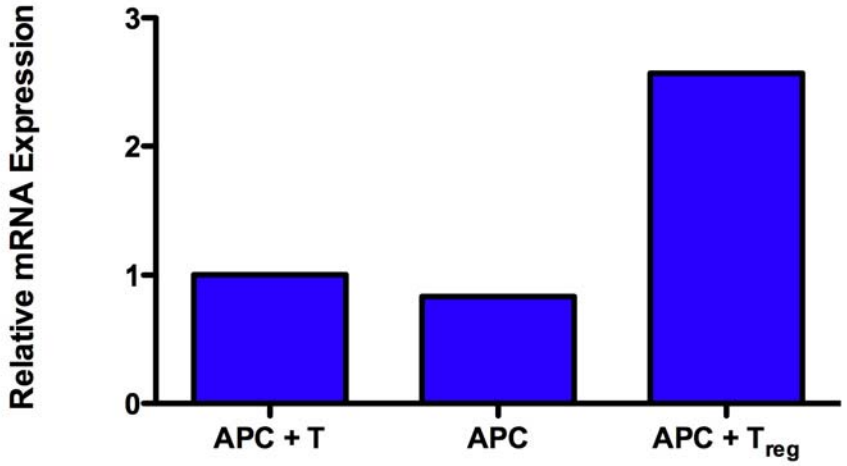

Relative mRNA Expression
1.5
1.0
0.5
0.0
APC + T
APC
APC + Treg

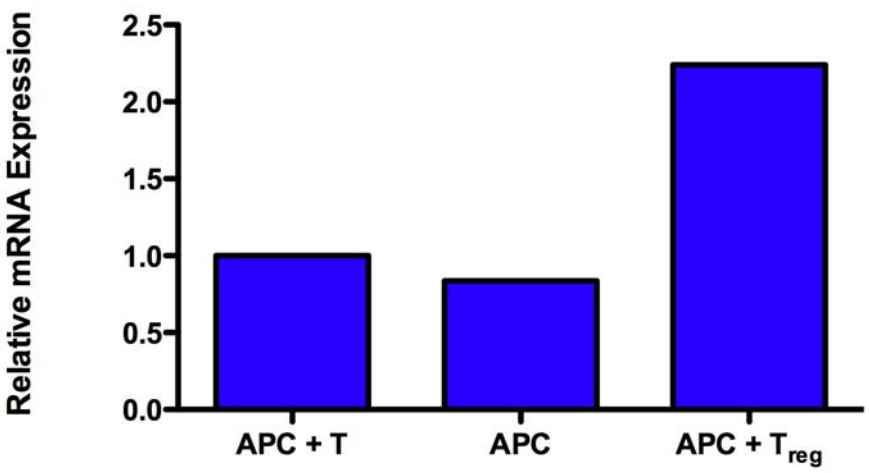
Figure 6.2.8 The expression of putative FERM-domain containing kinases by DC conditioned with regulatory T cells.

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. The reisolated DCs were then homogenised in Trizol and their RNA was purified using a RNeasy kit (Qiagen). RNA samples were then analysed using GeneChip Gene 1.0 ST Microarray chips (Affymetrix) at the Adelaide Microarray Centre to identify differentially expressed genes. Transcripts encoding the putative four point-1, ezrin, radixin and moesin (FERM) domain containing kinases 679206 (A), 689299 (B), and 308209 (C) were expressed at 2-3 fold higher levels in DC treated with regulatory T cells (APC + T_{reg}) compared to untreated DC (APC) or DC treated with conventional T cells (APC + T).

A


Relative mRNA Expression
3
2
1
0
APC + T
APC
APC + Treg

B


Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
APC + T
APC
APC + Treg

C

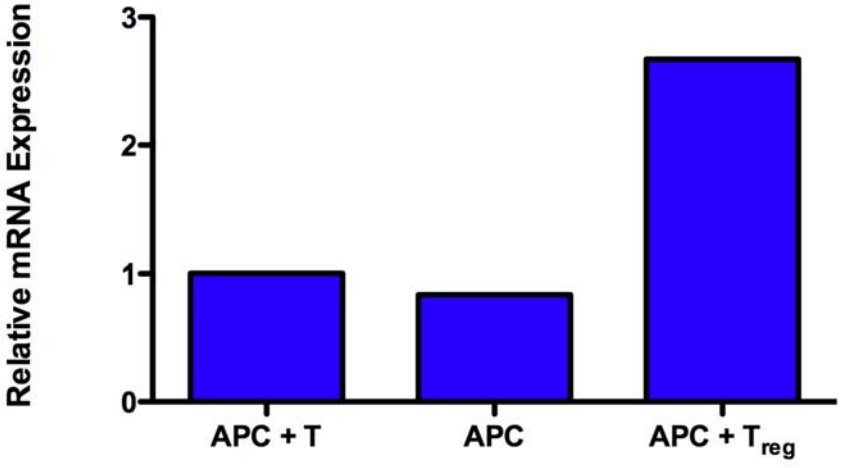

Relative mRNA Expression
3
2
1
0
APC + T
APC
APC + Treg

Figure 6.2.9 The expression of putative serine/threonine kinases by DC conditioned with regulatory T cells.

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. The reisolated DCs were then homogenised in Trizol and their RNA was purified using a RNeasy kit (Qiagen). RNA samples were then analysed using GeneChip Gene 1.0 ST Microarray chips (Affymetrix) at the Adelaide Microarray Centre to identify differentially expressed genes. Transcripts encoding the putative serine/threonine kinases 298461 (A), 365132 (B), 365139 (C), and 365140 (D) were expressed at 2-3 fold higher levels in DC treated with regulatory T cells (APC + T_{reg}) compared to untreated DC (APC) or DC treated with conventional T cells (APC + T).

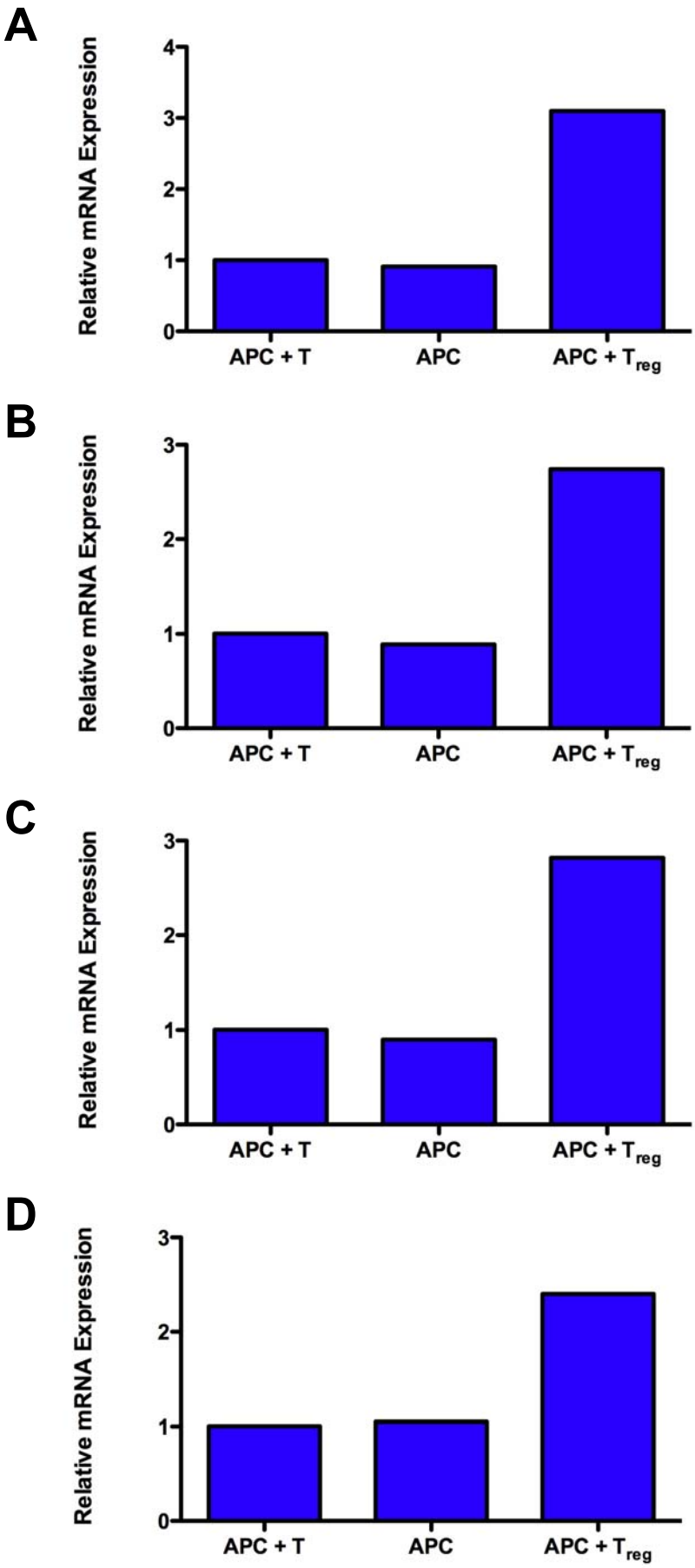
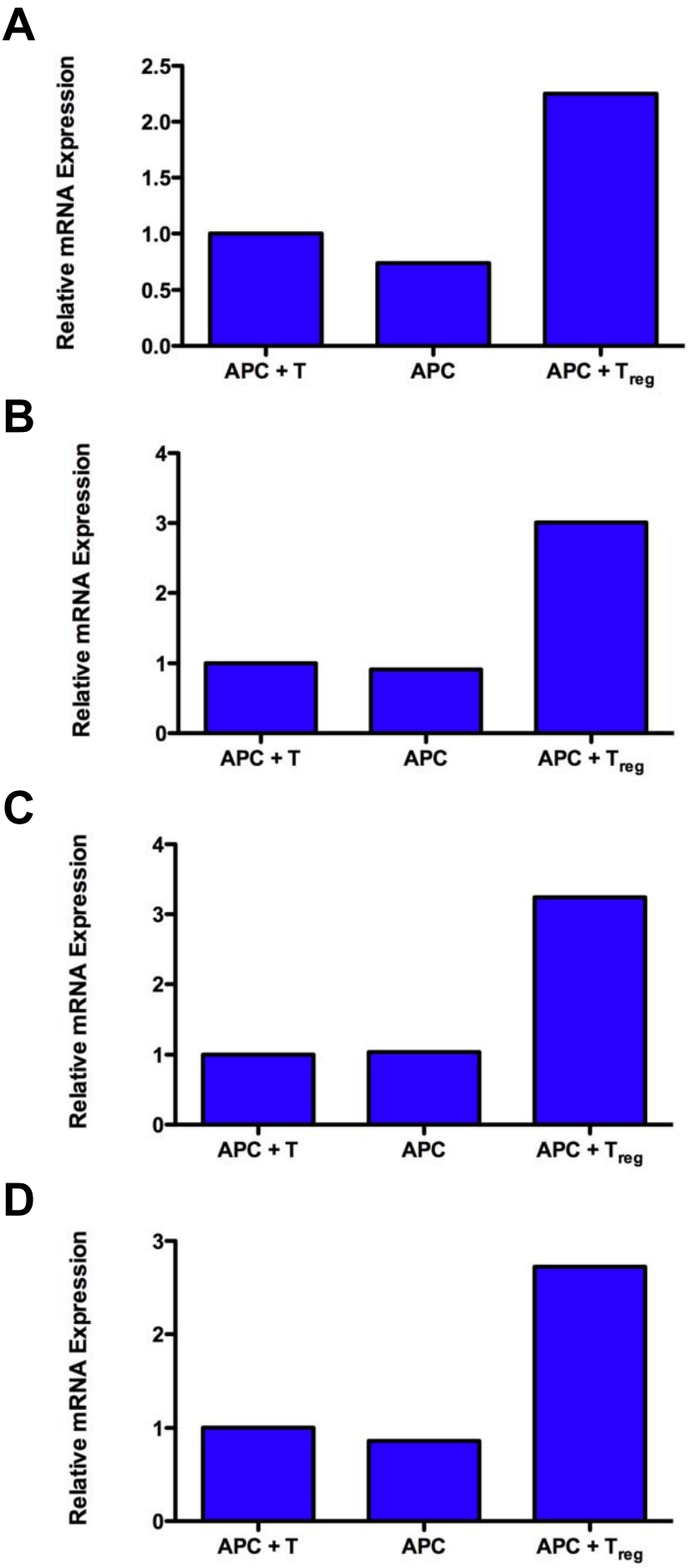

A
Relative mRNA Expression
4
3
2
1
0
APC + T
APC
APC + Treg
B
Relative mRNA Expression
3
2
1
0
APC + T
APC
APC + Treg
C
Relative mRNA Expression
3
2
1
0
APC + T
APC
APC + Treg
D
Relative mRNA Expression
3
2
1
0
APC + T
APC
APC + Treg

Figure 6.2.10 The expression of putative serine/threonine kinases by DC conditioned with regulatory T cells.

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. The reisolated DCs were then homogenised in Trizol and their RNA was purified using a RNeasy kit (Qiagen). RNA samples were then analysed using GeneChip Gene 1.0 ST Microarray chips (Affymetrix) at the Adelaide Microarray Centre to identify differentially expressed genes. Transcripts encoding the putative serine/threonine kinases 365149 (A), 502271 (B), and 502273 (C) and 685793 (D) were expressed at 2-3 fold higher levels in DC treated with regulatory T cells (APC + T_{reg}) compared to untreated DCs (APC) or DC treated with conventional T cells (APC + T).


A
Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
APC + T
APC
APC + Treg
B
Relative mRNA Expression
4
3
2
1
0
APC + T
APC
APC + Treg
C
Relative mRNA Expression
4
3
2
1
0
APC + T
APC
APC + Treg
D
Relative mRNA Expression
3
2
1
0
APC + T
APC
APC + Treg

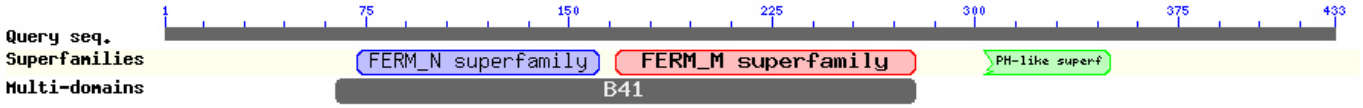
plasma membrane (Bompard *et al.* 2003). To assess whether FERM and kinase domains are present within these putative kinases, mRNA sequences were translated into protein sequences using the Expert Protein Analysis System (ExPASy) server of the Swiss Institute of Bioinformatics. The protein sequences were then analysed for the presence of conserved domains using the National Centre for Biotechnology Information (NCBI) specialised basic local alignment search tool (BLAST) for conserved protein domains. The putative FERM-domain containing kinase 679206 was predicted to contained the FERM domain (Figure 6.2.11), plus a contain a Pleckstrin-like homology domain. This was of interest because this motif is another that is thought to be involved in targeting proteins to the plasma membrane (Knight and Falke 2009). In contrast, the two other putative FERM-domain containing kinases (689299 and 308209) were found not to contain the FERM domain and were instead predicted to be serine/threonine kinases. All of the 8 putative serine/threonine kinases (298461, 365132, 365139, 365140, 365149, 502271, 502273 and 685793) plus the two identified above (689299 and 308209) were predicted to contain ATP-binding pockets, substrate binding pockets, activation and catalytic loops characteristic of serine/threonine kinases. Thus transcription of a totalof10 genes that encode serine/threonine kinases were up-regulated in the regulatory T cell-conditioned DC (Figure 6.2.12). These molecules are of particular interest because they might be components of a novel signal transduction pathway(s).

Other transcripts of unknown significance for the function of DC were up-regulated. These include transcripts from genes associated with olfaction, including two putative pheromone receptors (Entrez IDs 502285, 292615), olfactory receptor 300 (Entrez ID 293599), olfactory receptor 310 (Entrez ID 405039) and trace-amine-associated receptor 7h (Entrez ID 494535). The expression of these molecules is usually restricted to cells of the nervous system (Olender *et al.* 2008), and their increased expression in APC treated with regulatory T cells was unexpected. In addition, a transcript encoding a protein similar to a subunit of the DNA-directed RNA polymerase (Entrez ID 688574) was strongly upregulated. Several other transcripts were only slightly upregulated in DC treated with regulatory T cells, including centaurin delta 2 (Entrez ID 361617), synaptotogmin-like 2 (Entrez ID 361604),

Figure 6.2.11 Identification of conserved protein domains in putative FERM domain containing kinases expressed at higher levels in DC following treatment with regulatory T cells

The putative FERM domain containing kinases 679206 (A), 689299 (B), and 308209 (C) were analysed for conserved protein domains using a specialised BLAST tool provided by the NCBI. This analysis revealed that only the putative FERM domain containing kinase 679206 is predicted to contain a FERM domain. This molecule is also predicted to contain a Pleckstrin-like homology domain (PH-like superf), which is typically involved in the localisation of a protein to a specific cellular compartment through the binding of proteins or inositol phosphates.

A


1
75
150
225
300
375
433
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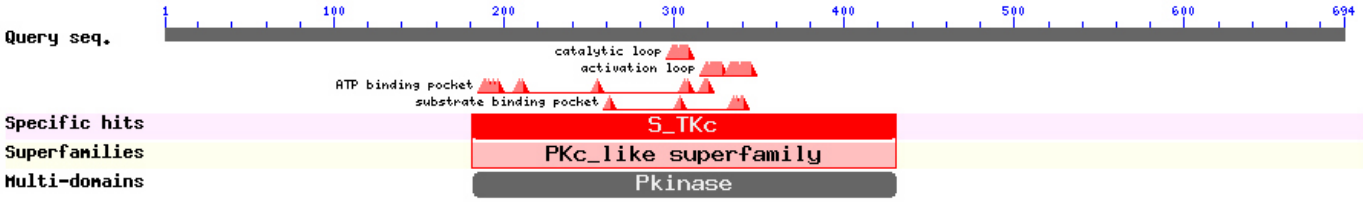
Superfamilies
Multi-domains
FERM_N superfamily
FERM_M superfamily
PH-like superf
B41

B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400
500
600
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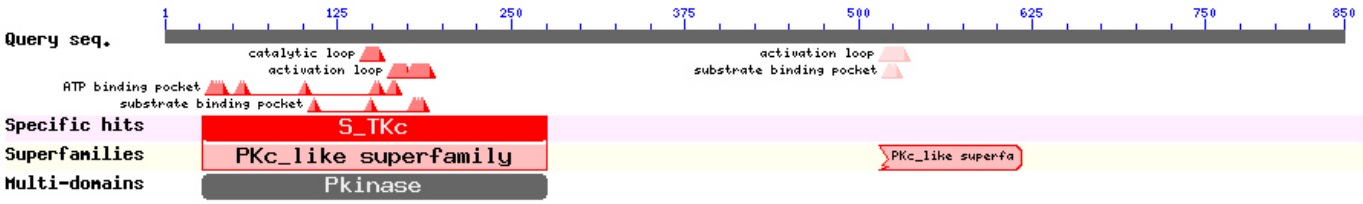
activation loop
ATP binding pocket
substrate binding pocket
Specific hits
Superfamilies
Multi-domains
S_TKc
PKc_like superfamily
Pkinase

C

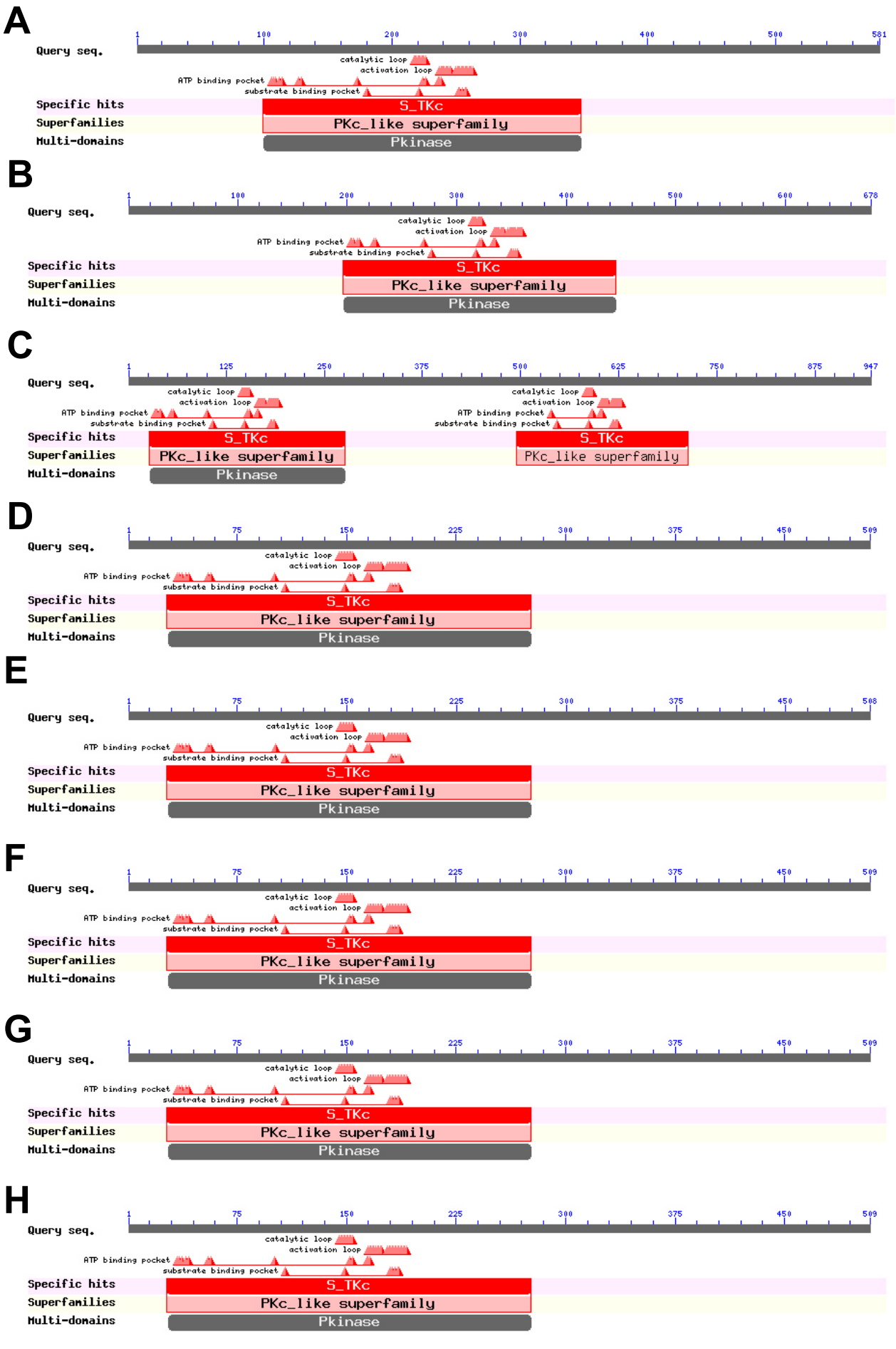

1
125
250
375
500
625
750
850
Query seq.
catalytic loop
activation loop
ATP binding pocket
substrate binding pocket
activation loop
substrate binding pocket
Specific hits
Superfamilies
Multi-domains
S_TKc
PKc_like superfamily
Pkinase
PKc_like superfa

contain a Pleckstrin-like homology domain, which is also involved in targeting proteins to the plasma membrane (Knight and Falke 2009). In contrast, the two other putative FERM-domain containing kinases were predicted not to contain the FERM domain and were instead predicted to be serine/threonine kinases. It was found that all of the 8 putative serine/threonine kinases were predicted to contain ATP-binding pockets, substrate binding pockets, activation and catalytic loops characteristic of serine/threonine kinases, giving a total of 10 upregulated serine/threonine kinases (Figure 6.2.12). It was found that the expression of E2F transcription factor 5 was reduced approximately 2-fold following the treatment of DCs with regulatory T cells (Figure 6.2.13). The E2F family of transcription factors are associated with cell cycle progression and the regulation of apoptosis (Itoh *et al.* 1995), and it is believed that E2F transcription factor 5 forms a complex with retinoblastoma gene family member p130 that acts to inhibit entry into the cell cycle (Chen *et al.* 2008). Interestingly, E2F transcription factor 5 can profoundly increase the size of cells in the absence of the transcription factor DP1, and when it is co-expressed with DP1 it can mediate cell cycle entry (Dirks *et al.* 1998). These apparently contradictory functions are thought to result from the ability of E2F transcription factor 5 to make cell cycle progression dependent on the activation of particular signal transduction cascades (DeGregori *et al.* 2006). The highly context-dependent nature of E2F transcription factor 5 activity complicates the interpretation of the finding that it is expressed at lower levels in DCs treated with regulatory T cells, which would be best investigated further using a molecular approach.

Interestingly, it was found that the expression of Fas antigen by DCs was reduced following treatment with regulatory T cells (Figure 6.2.14). The Fas antigen, a member of the tumour necrosis factor superfamily is known to induce the death of T cells that have recently been activated and as such it serves to attenuate T-cell mediated immune responses (Nagata and Golstein 1995). However, the mechanism of regulatory T cell suppression is known not to act through the killing of other T cells, although regulatory T cells are capable of killing other cells in certain situations (Miyara and Sakaguchi 2007, Grossman *et al.* 2004). Therefore it is unlikely that Fas

Figure 6.2.12 Identification of conserved protein domains in putative serine/threonine kinases expressed at higher levels in DC following treatment with regulatory T cells

The putative serine/threonine kinases 298461 (A), 365132 (B), 365139 (C), 365140 (D), 365149 (E), 502271 (F), and 502273 (G) and 685793 (H) were analysed for conserved protein domains using a specialised BLAST tool provided by the NCBI. All of the putative serine/threonine kinases were predicted to contain the ATP-binding pockets, substrate binding pockets, activation and catalytic loops characteristic of serine/threonine kinases.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Query seq.
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200
300
400
500
581
catalytic loop
activation loop
ATP binding pocket
substrate binding pocket
Specific hits
S_TKc
Superfamilies
PKc_like superfamily
Multi-domains
Pkinase
B
Query seq.
1
100
200
300
400
500
600
678
catalytic loop
activation loop
ATP binding pocket
substrate binding pocket
Specific hits
S_TKc
Superfamilies
PKc_like superfamily
Multi-domains
Pkinase
C
Query seq.
1
125
250
375
500
625
750
875
947
catalytic loop
activation loop
ATP binding pocket
substrate binding pocket
Specific hits
S_TKc
Superfamilies
PKc_like superfamily
Multi-domains
Pkinase
D
Query seq.
1
75
150
225
300
375
450
509
catalytic loop
activation loop
ATP binding pocket
substrate binding pocket
Specific hits
S_TKc
Superfamilies
PKc_like superfamily
Multi-domains
Pkinase
E
Query seq.
1
75
150
225
300
375
450
508
catalytic loop
activation loop
ATP binding pocket
substrate binding pocket
Specific hits
S_TKc
Superfamilies
PKc_like superfamily
Multi-domains
Pkinase
F
Query seq.
1
75
150
225
300
375
450
509
catalytic loop
activation loop
ATP binding pocket
substrate binding pocket
Specific hits
S_TKc
Superfamilies
PKc_like superfamily
Multi-domains
Pkinase
G
Query seq.
1
75
150
225
300
375
450
509
catalytic loop
activation loop
ATP binding pocket
substrate binding pocket
Specific hits
S_TKc
Superfamilies
PKc_like superfamily
Multi-domains
Pkinase
H
Query seq.
1
75
150
225
300
375
450
509
catalytic loop
activation loop
ATP binding pocket
substrate binding pocket
Specific hits
S_TKc
Superfamilies
PKc_like superfamily
Multi-domains
Pkinase

similar to yippee-like 3 (Entrez ID 293491), and hypothetical protein LOC498544 (Entrez ID 498544).

Transcripts from a smaller number of genes were found to be down-regulated in DC conditioned with regulatory T cells. One that was strikingly down-regulated (Figure 6.2.7) encodes the natural killer cell group 7 molecule (Entrez ID 171062). This molecule is a cytotoxic granule protein that is associated with the cytotoxic effector function of natural killer cells and cytotoxic $CD8^+$ T cells (Medley et al. 1996). It has also been identified as a molecule expressed by regulatory T cells (Sugimoto et al. 2006) and thus could arise from contaminating regulatory T cells. However, because it was expressed at lower levels in cultures of DC that had been incubated with purified regulatory T cells, it is very unlikely that the transcripts originated from residual regulatory cells.
Expression of transcripts encoding the E2F transcription factor 5 was reduced approximately 2-fold following the treatment of DCs with regulatory T cells (Figure 6.2.13). The E2F family of transcription factors are associated with cell cycle progression and the regulation of apoptosis (Itoh *et al.* 1995), and it is believed that a complex containing E2F transcription factor 5 and retinoblastoma gene family member p130 acts to inhibit entry into the cell cycle (Chen *et al.* 2008). Interestingly, E2F transcription factor 5 can increase the size of cells profoundly in the absence of the transcription factor DP1, and when it is co-expressed with DP1 it can mediate entry into the cell cycle (Dirks *et al.* 1998). These apparently contradictory functions are thought to result from the ability of E2F transcription factor 5 to make cell cycle progression dependent on the activation of particular signal transduction cascades (DeGregori *et al.* 2006). The highly context-dependent nature of E2F transcription factor 5 activity complicates the interpretation of its modified expression in DC after the treatment with regulatory T cells.

The gene encoding Fas antigen is another that appears to be transcribed at lower levels after DC are exposed to regulatory T cells (Figure 6.2.14). Ligation of the Fas antigen, a member of the tumour necrosis factor superfamily, is known to induce the death of T cells that have been activated recently. The molecule provides one of the

Figure 6.2.13 Expression of E2F transcription factor 5 by DC following treatment with regulatory T cells

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. The reisolated DCs were then homogenised in Trizol and their RNA was purified using a RNeasy kit (Qiagen). RNA samples were then analysed using GeneChip Gene 1.0 ST Microarray chips (Affymetrix) at the Adelaide Microarray Centre to identify differentially expressed genes. Transcripts encoding the E2F transcription factor 5 were found to be expressed at approximately 2-fold lower levels in DC treated with regulatory T cells (APC + T_{reg}) compared to untreated DC (APC) or DC treated with conventional T cells (APC + T).

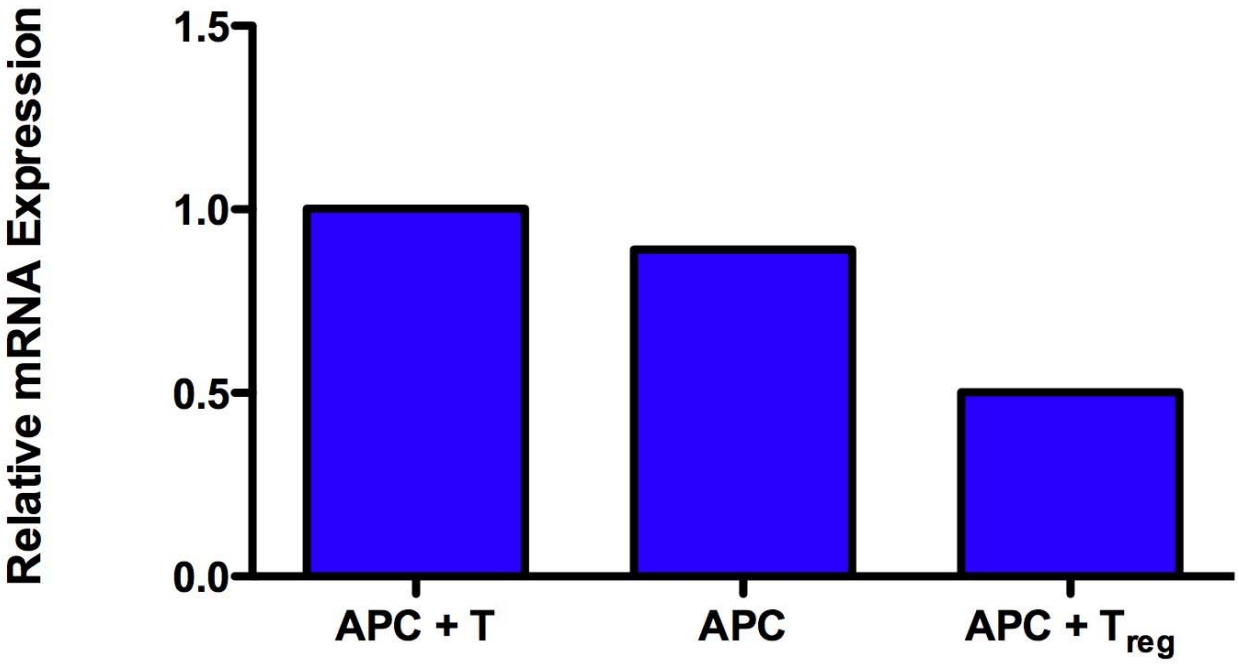
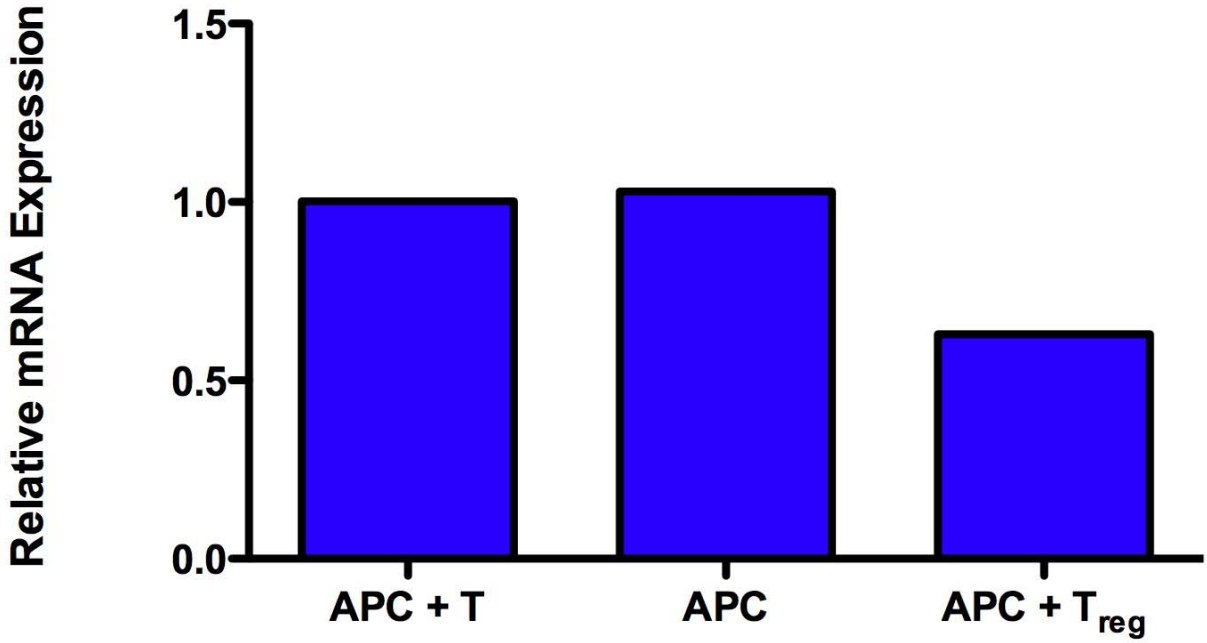

Relative mRNA Expression
1.5
1.0
0.5
0.0
APC + T
APC
APC + Treg

Figure 6.2.14 Expression of the Fas antigen by DC following treatment with regulatory T cells

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. The reisolated DCs were then homogenised in Trizol and their RNA was purified using a RNeasy kit (Qiagen). RNA samples were then analysed using GeneChip Gene 1.0 ST Microarray chips (Affymetrix) at the Adelaide Microarray Centre to identify differentially expressed genes. Transcripts encoding the Fas antigen were found to be expressed at reduced levels in DC treated with regulatory T cells (APC + T_{reg}) compared to untreated DC (APC) or DC treated with conventional T cells (APC + T).

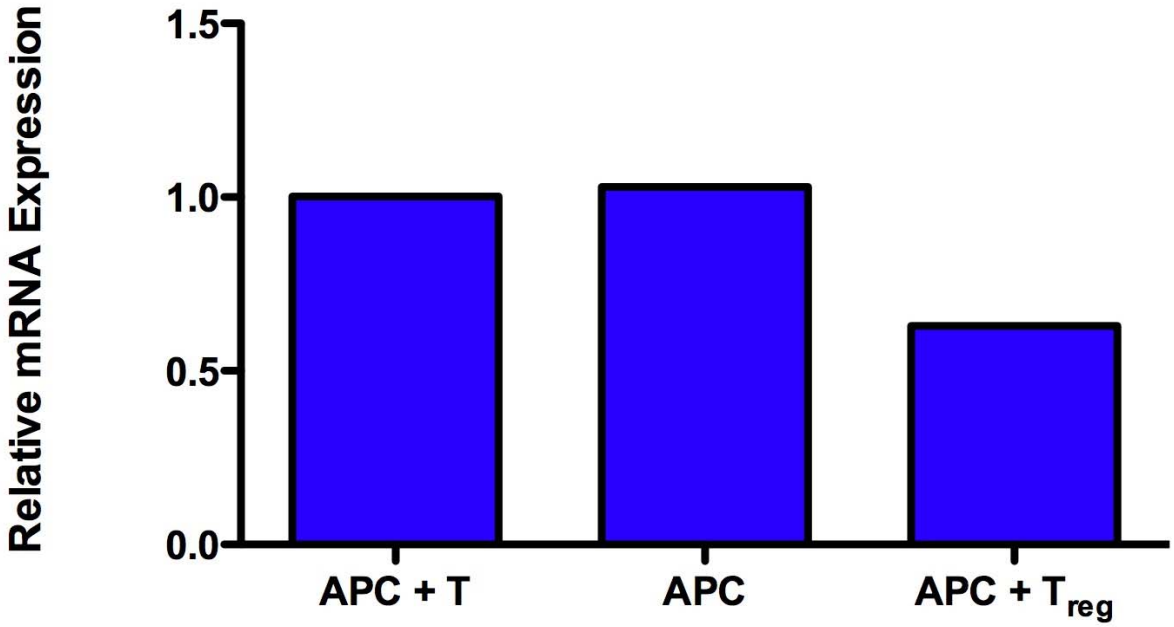

Relative mRNA Expression
1.5
1.0
0.5
0.0
APC + T
APC
APC + Treg

mechanisms involved in attenuation of T-cell mediated immune responses (Nagata and Golstein 1995). Although regulatory T cells appear to be capable of killing other cells in certain situations (Grossman *et al.* 2004), killing of conventional T cells has not been observed during suppression mediated by regulatory T cells (Miyara and Sakaguchi 2007). This makes it less likely that Fas antigen plays a role in the mechanism of regulatory T cell suppression and its downregulation by DCs treated with regulatory T cells may be incidental. In any case, the lower expression of Fas antigen on DC treated with regulatory T cells suggests it does not play a role in the suppressive mechanism associated with regulatory T cells.
Down-regulation of transcripts encoding programmed death ligand 1 (CD274), a member of the immunoglobulin superfamily of costimulatory molecules, after treatment of DC with regulatory T cells (Figure 6.2.15) was of some interest. T cell activation can be inhibited when programmed death 1 expressed on the surface of T cells binds to programmed death ligand 1 expressed by DC. This effect is observed when co-stimulation through CD28 is relatively weak, but it is not sufficiently potent to restrain T cell activation when the level of co-stimulation through CD28 is higher (Freeman *et al.* 2000). Therefore, although programmed death ligand 1 has a mechanism of action compatible with the known features of regulatory T cell suppression, it is unlikely to be involved in the mechanism by which regulatory T cells mediate suppression because transcription levels were lower (rather than higher) in DC following treatment with regulatory T cells.

Several other transcripts were slightly downregulated in DC following treatment with regulatory T cells, including calcitonin-related polypeptide (Entrez ID 171519), which is a neuropeptide that has been shown to partially inhibit IL-2 production and T cell proliferation (Xing *et al.* 2000). The lower expression of this molecule on DC treated with regulatory T cells would be expected to permit greater T cell proliferation and IL-2 production, and is thus unlikely to participate in the suppressive mechanism associated with regulatory T cells. However, the reduced expression of transcripts encoding splicing factor 3b (Entrez ID 608891), suggests that alternative RNA splicing could be a mechanism that contributes to the reduced capacity of DC treated with regulatory T cells to stimulate T cell proliferation. Although splicing factor 3b has

Figure 6.2.15 Expression of Programmed Death Ligand 1 by DC following treatment with regulatory T cells

Lymph node DCs (5 x 10^4 per well) were incubated with either 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^{bright}$ regulatory T cells (T_{reg}), 2 x 10^5 flow sorted syngeneic $CD4^+$ $CD25^-$ conventional T cells, or were left untreated. After 48h, cultures of syngeneic regulatory T cells and DCs, syngeneic conventional T cells and DCs, and DCs cultured alone were depleted of T cells by labelling them with R73 (anti-TCR) and incubating them with immunomagnetic beads. The reisolated DCs were then homogenised in Trizol and their RNA was purified using a RNeasy kit (Qiagen). RNA samples were then analysed using GeneChip Gene 1.0 ST Microarray chips (Affymetrix) at the Adelaide Microarray Centre to identify differentially expressed genes. Transcripts encoding programmed death ligand 1 (CD274) were expressed at reduced levels in DC treated with regulatory T cells (APC + T_{reg}) compared to untreated DC (APC) or DC treated with conventional T cells (APC + T).


Relative mRNA Expression
1.5
1.0
0.5
0.0
APC + T
APC
APC + Treg

previously been shown to be downregulated in mononuclear cells following measles virus infection (Zilliox *et al.* 2007), its effects on immune function remain unexplored.

Discussion

6.3.1 The mechanism of regulatory T cell suppression

Although the mechanism(s) by which regulatory T cells mediate suppression has been the subject of many investigations, it remains a subject of considerable controversy. While many groups have shown that soluble factors are unlikely to account for the suppressive activity associated with regulatory T cells (Thorton and Shevach 1998, Takahashi *et al.* 1998, Piccirillo *et al.* 2002), it is still claimed that TGF-β plays a direct role in the mechanism of regulatory T cell suppression (Nakamura *et al.* 2004). However, the effects of TGF-β may be indirect, by promoting the expansion of natural regulatory T cells or adaptive regulatory T cells (Huber *et al.* 2004),or by inhibiting the maturation of APC and their antigen presenting functions. For instance, TGF-β treated APCs have been shown to anergise T cells(Chen *et al.* 2003), and as discussed earlier, this process may have an intimate association with the differentiation of regulatory T cells. Since the suppressive activity of regulatory T cells is unaffected by the either neutralisation of TGF-β with antibodies or by the use of responder T cells that are unable to respond to the cytokine, it is unlikely that TGF-β plays a direct role in the suppressive mechanism associated with regulatory T cells (Miyara and Sakaguchi *et al.* 2007). It does appear clear, however, that the mechanism of regulatory T cell suppression involves a cell-contact dependent mechanism, and it has been argued that this involves the negative costimulatory molecule CTLA-4.

CTLA-4 transduces inhibitory signals after ligation with either CD80 or CD86, which are vital costimulatory molecules typically expressed by mature APC. However, these molecules can also be expressed by activated T cells, raising the possibility that

inhibitory signals could be transduced from regulatory T cells bearing CTLA-4 to activated T cells (Miyara and Sakaguchi 2007). However, T cells not expressing CD80 or CD86 remain highly susceptible to regulation by regulatory T cells *in vitro*, making this scenario unlikely (Takahashi *et al.* 2000, May *et al.* 2007).

Another hypothesis involves the direct inhibition of conventional T cell activation by regulatory T cells bearing LAG-3 (Huang *et al.* 2004). It has been suggested that LAG-3 on the surface of regulatory T cells can inhibit the proliferation of conventional T cells by binding MHC II molecules displayed by APC and thus interferes with antigen presentation and activation (Miyara and Sakaguchi 2007). However, it is unlikely that molecules on the surface of regulatory T cells would be able to access the immunological synapses as they form between APCs and effector T cells due to the vastly greater size of the cells compared with the molecules that protrude from their surfaces.

An alternative mechanism that requires cell-cell-contact involves the delivery of signals to DC that inhibit their capacity to activate other T cells. Indeed, new imaging techniques that are capable of observing leukocyte interactions *in vivo* have recently produced evidence that supports this hypothesis.

Presentation of antigen to T cells by DCs occurs in secondary lymphoid tissue, where intravital microscopy has revealed that T cells migrate in a random and autonomous manner along the stromal cell network (Miller *et al.* 2003). The network of stromal cells that provides a scaffold in the T cell area of the lymph node is composed mainly of fibroblastic reticular cells. These cell are rich in adhesion molecules that are continuously contacted by pseudopodial projections arising from the T cell surface. This network of fibroblastic reticular cells delimits the extent of the T cell area, and because two-photon microscopy shows that T cell movement is restricted to the surface of this fibrous network, it may serve to facilitate the compartmentalisation of T cells (Bajenoff *et al.* 2006). DC in the lymph node appear to form networks that surround the B cell follicles and extend into the T cell areas. They are particularly dense in the border zone between the T cell areas and the B cell follicles, where T

cell dependent immune responses are generated. These networks have been shown to be highly dynamic, with rapidly moving mature DC infiltrating the lymph node gradually slowing to a crawl with many of the crawling DC either joining or leaving groups of sessile DCs that continuously probe motile T cells (Lindquist *et al.* 2004). In the event of antigenic challenge, the interactions between DCs and T cells are stabilised. T cells cluster and enlarge, and then they begin to swarm before dividing and rapidly dispersing (Miller *et al.* 2002, Stoll *et al.* 2002). The phase of stabilised interactions between T cells and DC correlates with the induction of markers of T cell activation, and is likely to represent the phase in which activation occurs (Mempel *et al.* 2004). Regulatory T cells inhibit stable interactions between other T cells and DCs, and this appears to be the result of persistent interactions between regulatory T cells and DCs (Tang *et al.* 2006). Importantly, no stable association of regulatory T cells with responder T cells has been observed during active suppression *in vivo* (Tang *et al.* 2006, Tadokoro *et al.* 2006).

These observations strongly suggest that regulatory T cells may act by interfering with the activation of other T cells, and that this probably occurs through signals delivered to DC. Indeed, there is ample evidence that regulatory T cells condition DCs to inhibit their maturation and antigen presenting function (Cederbom *et al*. 2000, Misra *et al.* 2004, Houot *et al.* 2006, Veldhoen *et al.* 2006, Onishi *et al.* 2008). Therefore, in order to assess the contribution of this mechanism to the suppressive activity associated with regulatory T cells, we have designed a multi-stage *in vitro* system that enables the functional effects of regulatory T cells on DCs to be evaluated. We determined that the conditioning of DCs by regulatory T cells was sufficient to inhibit T cell proliferation, and as equivalent suppression occurs in the absence and the presence of regulatory T cell-conventional T cell interactions, this suggests that T-T interactions do not appreciably contribute to the mechanism of regulatory T cell suppression (Figure 6.2.3).

6.3.2 Putative molecular targets of regulation

The conditioning effect observed when regulatory T cells were incubated with freshly isolated DC prompted an investigation of parallel molecular changes in the cells. DC conditioned with $CD25^+$ regulatory T cells were purified and compared with DC that had been conditioned under identical conditions with $CD25^-$ conventional T cells, and also with DC that had been incubated under the same conditions in the absence of T cell. Differential gene expression in these populations was analysed by comparing relative levels of transcripts using GeneChip Gene 1.0 ST Microarray chips (Affymetrix). These chips provide total coverage of the 27,342 known rat genes through the use of approximately 26 probes spread across the full length of each gene. This coverage was made possible by the completion of the sequencing of the rat genome, which was completed in 2004 and is currently being further refined (Gibbs *et al.* 2004, Twigger *et al.* 2008). The changes in gene expression revealed through this microarray analysis require confirmation by RT-PCR in future studies. The changes in gene expression identified in this study do not provide any indication of mRNA transcription and turnover rates, which can change and affect the expression of the encoded proteins. Furthermore, the rates of protein translation and turnover are not measurable using this approach, such that the gene expression changes are only indirectly related to potential changes in protein expression. Microarray analysis of differential gene expression is also unable to detect changes at the post-transcriptional level such as protein phosphorylation, dephosphorylation, ubiquitination, cleavage or other modifications. In future studies, these changes could be investigated using a proteomics approach.

Microarray analysis of differential gene expresssion identified several significant differences between DC that had been conditioned by contact with regulatory T cells and DC that either received no contact with T cells in vitro, or were incubated with conventional T cells (containing a small proportion of $CD25^-$ $Foxp3^+$ cells). Genes with up-regulated expression could encode proteins that are involved in the mechanism of suppression by regulatory T cells, while genes with down-regulated expression may

normally direct the function of DC towards activation of T cells. Strikingly, 10 genes that were expressed at 2-3 fold higher levels in DC treated with regulatory T cells encode predicted serine/threonine kinases (Table 6.1). All 10 are predicted to contain the ATP-binding pockets, substrate binding pockets, activation loops and catalytic loops characteristic of serine/threonine kinases (Figure 6.2.12). The increased expression of these molecules suggests that they may be part of a novel signal transduction pathway that is established in DC following interactions with regulatory T cells and that this pathway is linked to the reduced capacity of the cells to activate naive T cells.

In addition, a putative signalling protein (Entrez ID 679206) predicted to contain both a FERM domain and a Pleckstrin-like domain was found to be upregulated in DCs treated with regulatory T cells. The FERM domain is common among signalling proteins, where it appears to ensure their localisation to the interior side of the plasma membrane and also serves to help anchor the cytoskeleton to the plasma membrane (Bompard *et al.* 2003). Similarly, the Pleckstrin-like domain is thought to function as an anchor that binds proteins to the rare membrane lipid phosphatidylinositol (3,4,5)-trisphosphate (Knight and Falke 2009). Therefore, it is likely that the FERM-domain containing protein 679206 is a signalling protein located at the plasma membrane, and that it may be involved in the modification of DCs by regulatory T cells.

Another gene with up-regulated transcription in DC treated with regulatory T cells encodes a CD163-like molecule (Entrez ID 293591) (Figure 6.2.5). Although this CD163-like molecule has not yet been characterised, it bears considerable similarity with CD163, and may therefore share some functional characteristics with CD163 (Figure 6.2.6). CD163 is expressed by monocytes and macrophages and it mediates the capture of haemoglobin-haptoglobin complexes that form following the lysis of erythrocytes (Moestrup and Moller 2004). Haemoglobin can cause oxidative damage to tissues and in the kidney, and it is cleared by monocytes and macrophages that metabolise the haemoglobin into free iron ions using the heme oxygenase enzyme (Abraham and Drummond 2006). Interestingly, the metabolism of haemoglobin releases carbon monoxide, which has anti-inflammatory and cytoprotective

properties, and triggers the release of IL-10 by macrophages, which further serves to attenuate inflammation resulting from the oxidative effects of haemoglobin and promote wound healing (Philippidis *et al.* 2004). The expression of the CD163-like molecule by DCs following treatment with regulatory T cells may be indicative of a transition to an “alternative activation” state similar to that described for macrophages in which DCs lose their capacity to activate T cells and may assume a more phagocytic and anti-inflammatory role, scavenging molecules such as haemoglobin that are processed into anti-inflammatory metabolites and permitting the resolution of inflammation (Moestrup and Moller 2004).

One of the transcripts that was less abundant (approx. two-fold less) following the treatment of DC with regulatory T cells was that encoding E2F transcription factor 5 (Figure 6.2.13). The E2F family of transcription factors are associated with the regulation of cell proliferation and of the process of apoptosis (Itoh *et al.* 1995). EF2 transcription factor 5 is thought to function by making entry into the cell cycle dependent on the activation of particular signal transduction cascades (DeGregori *et al.* 2006). The transition of immature DC towards mature DC is a terminal differentiation process, which is accompanied by the loss of the ability of DC to divide (Wong *et al.* 2004). Therefore, changes in the expression of the E2F transciption factor 5 may participate in the inhibition of DC maturation by regulatory T cells that has previously been described (Houot *et al.* 2006)

Transcripts encoding the cytotoxic natural killer group 7 sequence protein was found to be reduced in DCs treated with regulatory T cells, (Figure 6.2.7). This protein is expressed by natural killer (NK) cells, cytotoxic T cells and regulatory T cells (Medley *et al.* 1995, Sugimoto *et al.* 2004), where it is associated with cytotoxic granules. The preparation of lymph node DCs may have contained traces of contaminating natural killer cells that were affected by their co culture with regulatory T cells (Munz *et al.* 2008). Alternatively, the natural killer group 7 sequence protein may have been expressed on DC, which are known to express other NK cell markers in the rat,such as NK cell receptor protein 1, which may contribute to the ability of DC to kill cells that are sensitive to NK cell cytotoxic activity (Josien *et al.* 1997, Trinite *et al.* 2000,

Brissette-Storkus *et al.* 2002). Since the mechanism of regulatory T cell suppression does not involve the killing of conventional T cells, (Miyara and Sakaguchi 2007), the potential reduction of DC cytotoxic activity through decreased levels of natural killer group 7 sequence protein transcripts is unlikely to be involved in the mechanism of regulatory T cell suppression. The finding that DCs treated with regulatory T cells have a lower expression of natural killer group 7 sequence protein also suggests that the T cell depletion stage of the assay was very successful.

Transcripts encoding the Fas antigen were reduced following treatment of DC with regulatory T cells (Figure 6.2.14). Although Fas antigen is capable of attenuating T cell proliferation and survival following activation, this is due to the induction of T cell apoptosis, which is not a feature of the mechanism of regulatory T cell suppression (Miyara and Sakaguchi 2006). This makes the finding that Fas antigen is expressed at lower levels following the treatment of DC with regulatory T cells less likely to be relevant to the mechanism of regulatory T cell suppression. Similarly, transcripts encoding programmed death ligand-1 were also expressed at lower levels by DC following treatment with regulatory T cells (Figure 6.2.15), and again, this has doubtful relevance to the mechanism of regulatory T cell suppression. Nevertheless, the findings could have relevance as manifestations of functional changes in the cells, away from activation of T cells towards a new role in mediating suppressive effects.

The absence of costimulatory molecules from the list of differentially expressed transcripts obtained through microarray analysis was unexpected. Others have shown that the downregulation of the most potent costimulatory molecules CD80 and CD86 on the surface of DCs following exposure to regulatory T cells is relatively mild (Cederbom *et al*. 2000, Houot *et al.* 2006). The subtle changes in CD80 and CD86 expression previously reported may be too small to be detected using microarray analysis.

6.3.3 Summary

In summary, the work described in his chapter shows that regulatory T cells can inhibit activation of naïve T cells by conditioning DC. This conditioning effect, which is similar to that observed using anergised T cells, reduces the capacity of the DC to activate T cells. The reduction in the stimulatory capacity of DC that occurs in the presence of regulatory T cells may be associated with their mechanism of suppression. It is also consistent with the results of recent *in vivo* imaging analyses, which indicated that regulatory T cells can inhibit interactions between APC and other T cells, depriving those cells of signals that they require for activation (Tang *et al.* 2006, Tadokoro *et al.* 2006).

Transcription of several genes was increased after conditioning DCs with regulatory T cells. These molecules are thus candidates for functional roles in the mechanism of regulatory T cells suppression. They included 10 novel predicted serine/threonine kinases, suggesting that they may be part of a novel signal transduction pathway. Furthermore, transcripts encoding an additional putative signalling molecule, which is predicted to contain both a FERM domain and a Pleckstin-like domain, were up-regulated following incubation of DC with regulatory T cells.

The expression of the E2F transcription factor 5, which is involved in regulating cell cycle progression was reduced in DCs following treatment with regulatory T cells. As mature DC are non-replicating cells, the loss of this transcription factor may be associated with the subversion of DC maturation by regulatory T cells (Wong *et al.* 2004, Houot *et al.* 2006). In addition, the results suggest that this treatment also induced increased expression of a CD163-like molecule. If this molecule has functions similar to CD163, it might have a scavenger role and suggest that interaction with regulatory T cells has induced transition towards a phagocytic and/or anti-inflammatory function.

The functions of the genes that have been identified by microarray analysis can now be the subject of further investigation. After confirmation of gene expression changes

using RT-PCR, the function of differentially expressed genes could be investigated using small interfering RNA to inhibit gene expression. Alternatively, DC could be transfected with expression constructs encoding the molecules of interest, to determine whether any of them affect the ability of DC to activate T cells. A proteomics-based approach to investigating the effect of regulatory T cells on the proteins expressed by DC may reveal additional molecular changes to DC that are not detectable using microarray technology.

Chapter 7: The effect of aging on the thymic output of regulatory T cells

7.1 Introduction

The thymus is the major site of T cell differentiation, and by generating a continuos supply of new T cells, it is now thought to contribute to the maintenance of the T cell pool throughout life. In humans the thymus begins involuting soon after birth, resulting in a gradual but steady decrease in the numbers of new T cells that are produced. However, at the rate of declining T cell production observed, it is estimated that the average thymus does not cease functioning until after approximately 120 years of age (Bodey *et al.* 1997). The process of thymic involution in humans involves extensive structural changes. There is gradual replacement of the parenchyma of the cortex and medulla with connective and adipose tissues, resulting in productive areas of lymphopoiesis becoming isolated to remaining small areas of parenchymal tissue (Gruver *et al.* 2007). Destruction of thymic epithelial cells occurs during the process of involution, and this may account for reduction in the number of new T cells that enter the peripheral pool with aging humans. Nevertheless, the remaining thymic tissue remnants continue to produce new T cells (Cunningham *et al.* 2001, Hale *et al.* 2006). Although surgical removal of the thymus in patients that have undergone heart surgery does not appear to lead to clinically significant immuno-deficiency, it does result in permanent reduction in the total numbers of circulating $CD4^+$ and $CD8^+$ T cells, and a compensatory increase in the number of neutrophils (Eysteinsdottir *et al.* 2004, Halnon *et al.* 2005).). During aging in rodents, the perivascular spaces of the thymus are not invaded by adipose and connective tissues and instead, the thymus merely decreases in size (Hale *et al.* 2006, Gruver *et al.* 2007).

It has been proposed that the involution of the thymus that occurs with aging reflects a genetically preprogrammed mechanism within the thymus itself. This hypothesis is based on the results of thymic transplantation experiments, where thymuses from young animals have been found to involute at a steady rate, whether transplanted into aged matched young recipients or much older animals (Bodey *et al.* 1997). However, more recent research has shown that aged thymuses become structurally and functionally rejuvenated when transplanted into young recipients,

suggesting that factors external to the thymus also affect thymic involution (Nobori *et al.* 2006). While it is currently unclear what factors may be responsible for aged-related thymic involution, several soluble mediators of thymic involution including IL-6, leukaemia inhibitory factor (LIF), and oncostatin M have been identified (Gruver and Sempowski 2008).

Involution of the thymus is known to proceed more rapidly in males compared to females, and this has been attributed to the greater effect of male sex hormones in promoting involution, as compared with female sex hormones (Aspinall and Andrew 2001). Supporting this idea, it is known that castration in mice retards thymic involution, while among sexually intact mice, thymic involution occurs more rapidly in breeding animals compared to animals that have not ever reproduced (Henderson 1904, Heng *et al.* 2005). In addition, the withdrawal of sex steroids has been shown to regenerate the aged thymus and enhance the number of peripheral T cells in mice (Dudakov *et al.* 2009)

Cellular mediators such as antigen experienced T cells that re-enter the thymus (Westermann *et al.* 1996, Spargo *et al.* 1996) where they could potentially affect thymic involution. At the cellular level, thymic involution appears to be mediated in part through cellular interactions between T cells displaying Fas ligand and thymic epithelial cells that express Fas. This may lead to the apoptosis of epithelial cells, because age related thymic involution does not occur in the absence of the Fas pathway (Yajima *et al.* 2004).

Fas ligand is expressed predominantly by activated Th1 cells (Nagata and Golstein 1995, Dzialo-Hatton *et al.* 2001), some of which are recruited into the thymus soon after activation (Hardy *et al.* 2001). *In vivo* activation of T cells through the injection of anti-CD3 antibody induces the apoptosis of thymic epithelial cells in a Fas-dependent manner, indicating a potential cellular process that contributes to age-related thymic involution (Yajima *et al.* 2004). In addition, thymic involution can be expedited by Jagged-1 expressed on the surface of T cells, which also induces apoptosis in thymic epithelial cells (Beverly *et al.* 2006). Jagged-1 expression is typically associated with

Th2 effector T cells and not Th1 effector T cells. However, it remains possible that Th1 T cells may kill thymic epithelial cells through the display of other Notch family members, in addition to Fas-mediated killing (Dzialo-Hatton *et al.* 2001).

In rats, the involution of the thymus commences at around the week 12 of life, at which stage sexual maturity has been reached (Brelinska *et al.* 2008). Therefore, in order to study the involution of the rat thymus, and its implications for the maintenance of the regulatory T cell pool, we have chosen to investigate thymus size, morphology and phenotype, and enumerate regulatory T cells and RTE in animals 8 weeks of age, before involution has begun, and following the commencement of thymic involution at 24, 52 and 76 weeks of age. Thymuses were examined for weight, histological morphology and cell-surface markers. Numbers of regulatory T cells were estimated in the thymus and numbers of both regulatory T cells and Thy1.2^{+} RTE were enumerated in the peripheral lymphoid tissues.

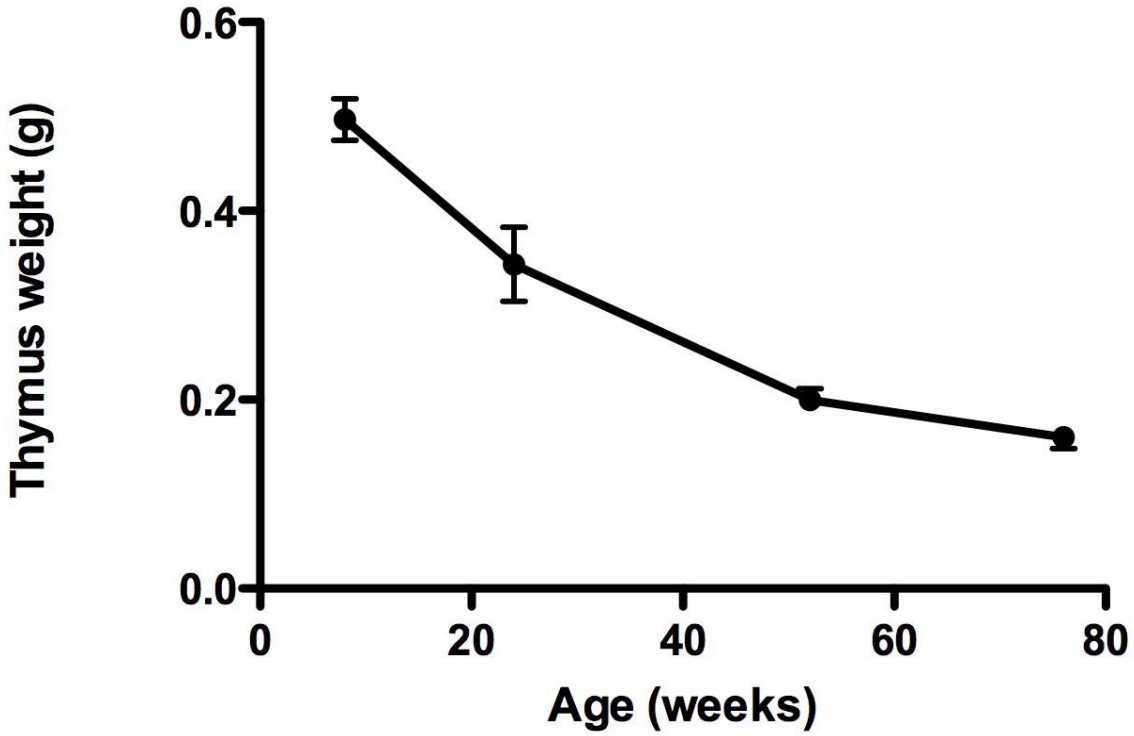
7.2 Results

7.2.1 Involution of the thymus with aging.

In order to document the changes in thymus weight and thymocyte numbers with age, rats aged 8, 24, 48 and 76 weeks were killed and the were removed for weighing, followed by gentle homogenisation to release the thymocytes. Thymus weight diminished with time (Figure 7.2.1), and this correlated with the subjective appearance of the organ. By 76 weeks of age, the average weight of the thymus had decreased by approximately 65%. Visual inspection indicated that peri-thymic fatty tissue (which was not included during weighing) was absent in 8 week old animals. However, there was a subjective increase in peri-thymic fat as rats aged, in contrast to the decline in thymus size. Since it has been reported that in humans the perivascular spaces are slowly replaced with fatty tissue during aging (Gruver *et al.* 2007), the thymuses were examined microscopically. There was no obvious increase in fat within the thymic parenchyma or between the lobules as the rats aged (Figure

Figure 7.2.1 Thymic mass decreases with aging

Thymuses from female RT7b rats aged 8, 24, 52 and 76 weeks were extracted, dissected free of fatty tissue and weighed. Four animals were used per time point.


Thymus weight (g)
0.6
0.4
0.2
0.0
0
20
40
60
80
Age (weeks)

7.2.2A-D), but numerous proteinaceous cysts were present within the medulla in thymuses from rats that were older than 76 weeks (Figure 7.2.2E).

Thymic involution, as evidenced by reduction in cell content of the thymus, was observed beyond 24 weeks of age. By 76 weeks of age, numbers of thymocytes had declined by approximately two thirds (Figure 7.2.3A), thus mirroring the decline in thymus weight (Figure 7.2.1). This decline was mirrored also by thinning of the cortex relative to the medulla (Figures 7.2.2C,D compared with Figures 7.2.2.A, B). In contrast to total thymocyte numbers, the absolute number of $Foxp3^+$ thymocytes remained relatively constant across the complete age range (Figure 7.2.3B).

In order to visualise the location of the $Foxp3^+$ thymocytes, frozen sections were prepared from the thymuses of 8 week old and 76 week old rats. When these were stained with anti-Foxp3 antibody (see Methods) the $Foxp3^+$ regulatory T cells were confined almost entirely to the medullary region in thymuses from rats in both age groups (Figures 7.2.4 and 7.2.5). Furthermore, as expected, staining of Foxp3 had a nuclear localisation. In agreement with the finding that the number of $Foxp3^+$ thymocytes remained stable as the total number of thymocytes decreased, the density of $Foxp3^+$ cells was higher in the medulla of thymuses from the older animals.

7.2.2 Contraction of the peripheral T cell pool with aging

In order to determine whether the age related thymic involution is accompanied by a reduction in the number of T cells in the peripheral pool, numbers of $CD4^+$ and $CD8^+$ T cells were estimated in the lymph nodes and spleens of animals aged 8, 24, 52 and 76 weeks. Numbers of $CD4^+$ T cells declined in a linear fashion by approximately two thirds between 8 weeks and 76 weeks of age (Figure 7.2.6A), while the numbers of $CD8^+$ T cells also declined but at an initially slower rate (Figure 7.2.6B).

Figure 7.2.2 Age-related changes in thymic architecture that occur with aging

Thymuses from rats aged 8, 24, 52 and 76 weeks were cut into 7μm sections, then stained with haematoxylin and eosin to visualise their cellular structure. Thymuses from rats aged 8 weeks (A) were found to have the largest cortical regions (C), deep within which medullary (M) regions were present. Thymuses from rats aged 24 weeks (B) had a slightly less organised structure, and by 52 weeks of age (C) the cortical regions surrounding the medulla had thinned considerably. At 76 weeks of age (D) the cellular structure of the thymus still resembled that of younger animals, despite the appearance of proteinaceous cysts (E). Images were taken at 10X magnification.

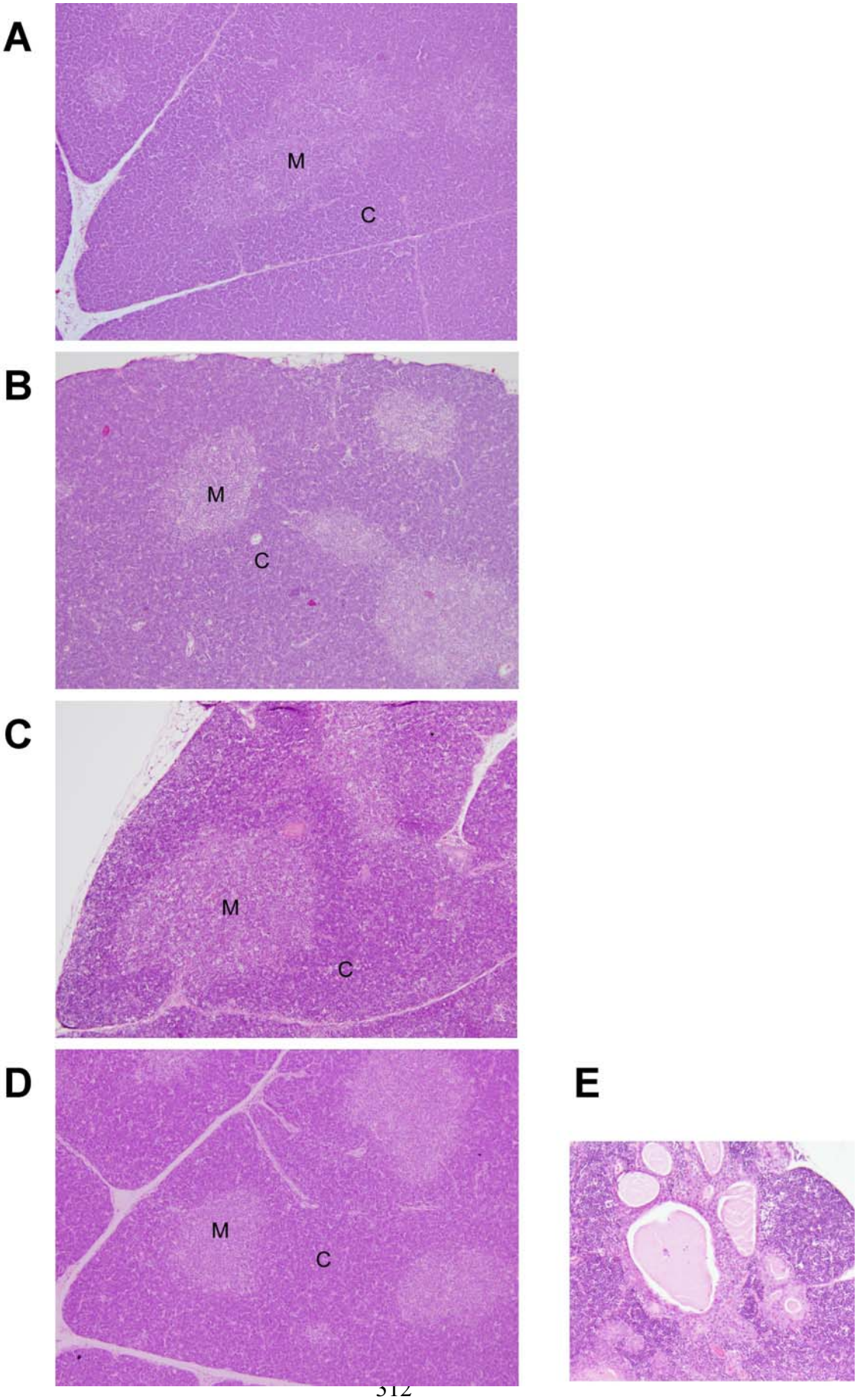
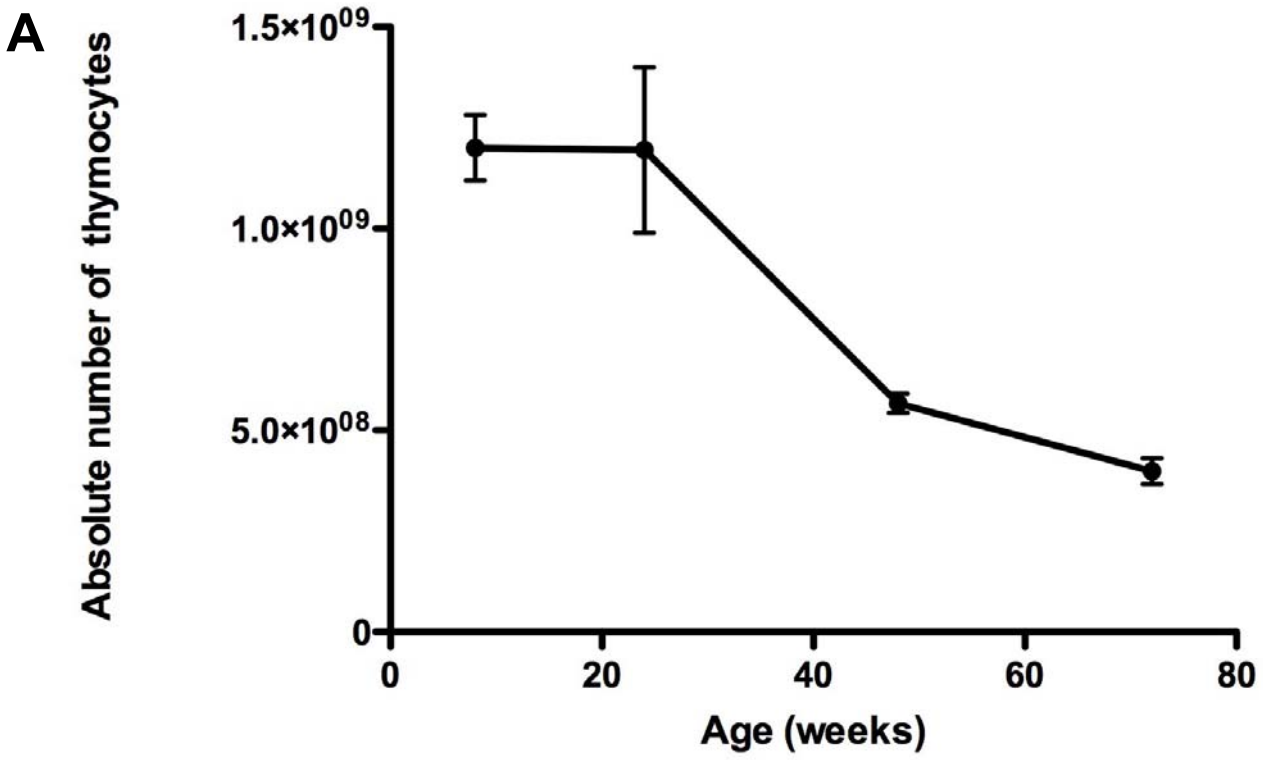

A
M
C
B
M
C
C
M
C
D
M
C
E

Figure 7.2.3 The number of thymocytes decreases with thymic involution, while the number of Foxp3$^+$ thymic regulatory T cells remains stable

Thymuses from rats aged 8, 24, 52 and 76 weeks were homogenised and thymocytes enumerated using a haemocytometer (A). In order to enumerate regulatory T cells, a sample of 1 x 10^6 thymocytes was stained for Foxp3, and the proportion of Foxp3$^+$ cells in the sample then multiplied by the total number of thymocytes to determine the absolute number of Foxp3$^+$ thymic regulatory T cells (B). 4 animals were used per time point.


A
Absolute number of thymocytes
1.5×10^09
1.0×10^09
5.0×10^08
0
0
20
40
60
80
Age (weeks)

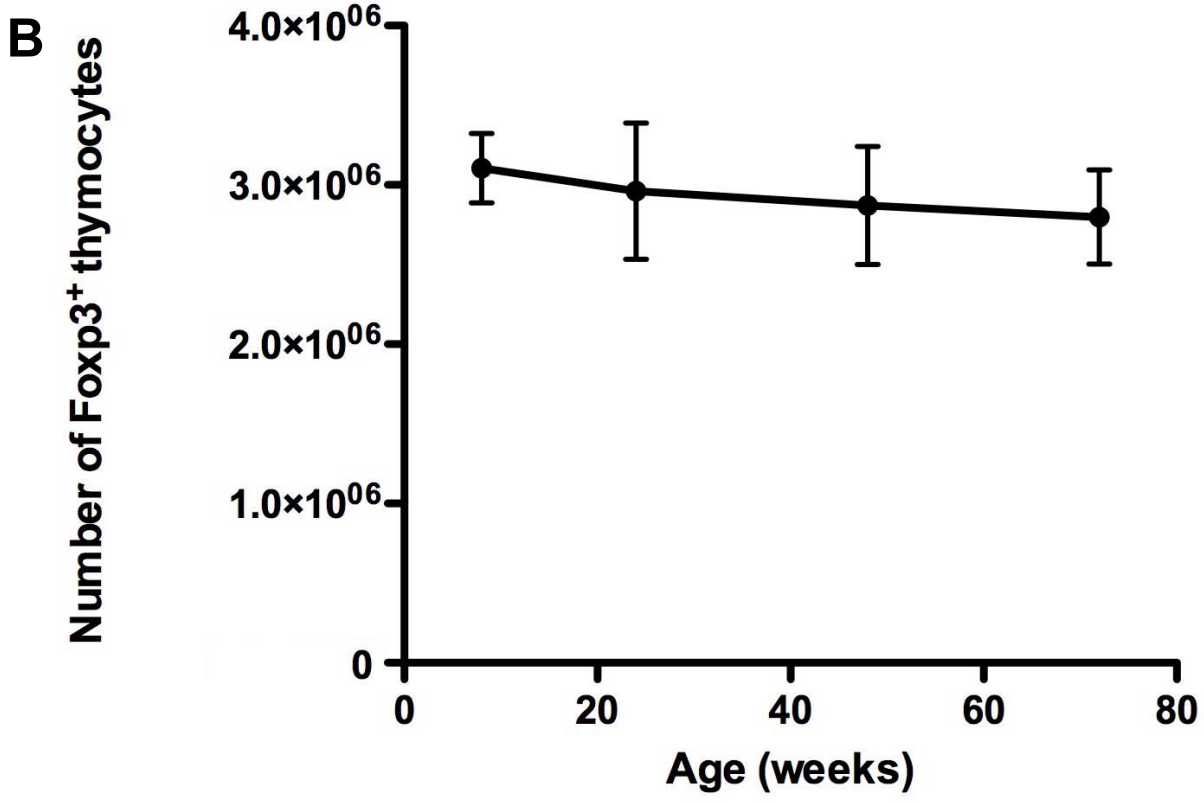

B
Number of Foxp3+ thymocytes
4.0×10^06
3.0×10^06
2.0×10^06
1.0×10^06
0
0
20
40
60
80
Age (weeks)

Figure 7.2.4 Immunohistochemical identification of Foxp3$^+$ T cells within the thymus of young adult rats

7μm sections of thymic tissue from 8 week old female RT7b rats were stained for Foxp3 (A), or with an isotype control (B). Foxp3$^+$ thymocytes were found to be confined to the medulla (M) with only the occasional Foxp3$^+$ thymocyte appearing in the surrounding cortex (C). Images were taken at 40X magnification.

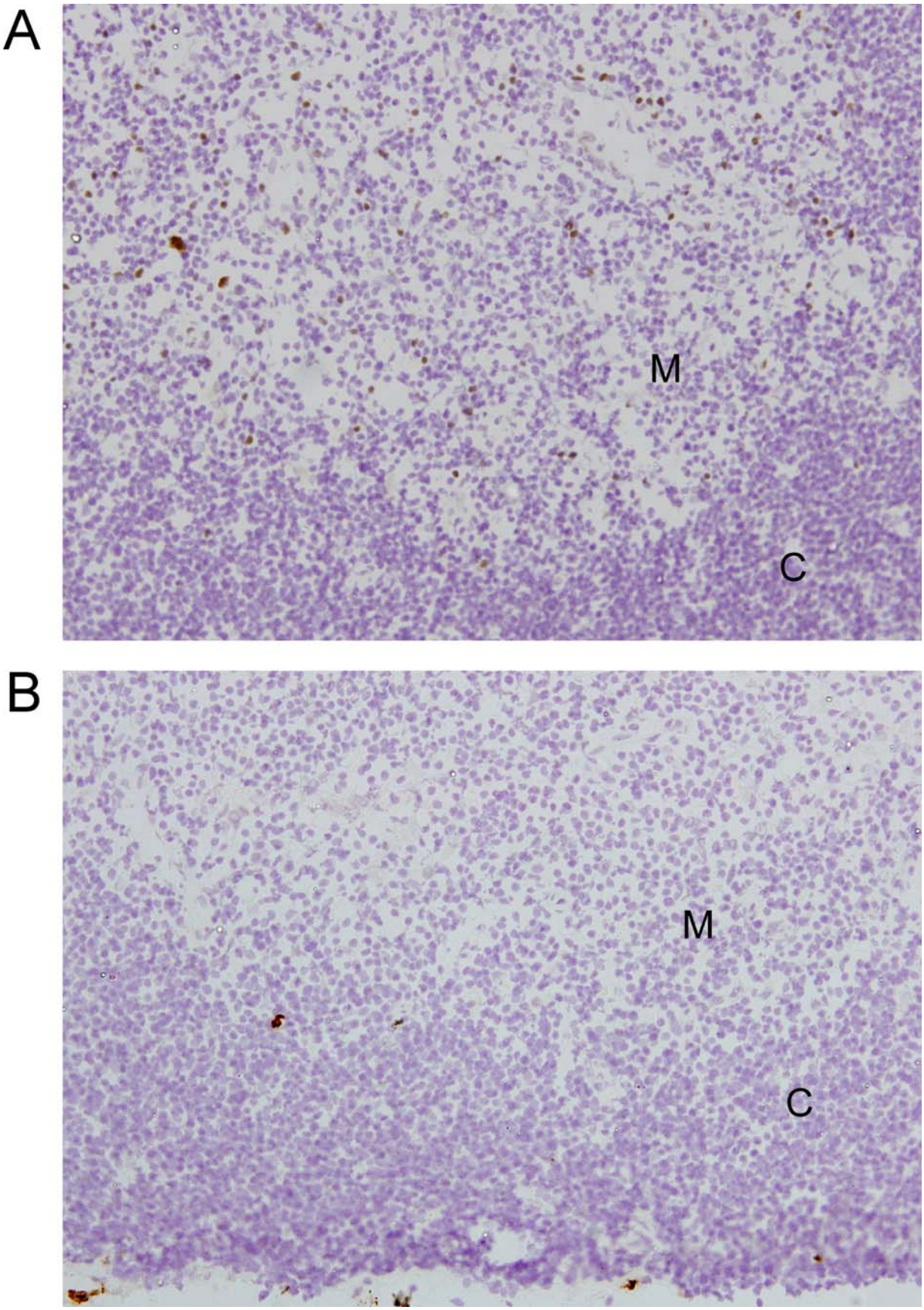

A
M
C
B
M
C

Figure 7.2.5 Immunohistochemical identification of Foxp3^{+} T cells within the thymus of aged adult rats

7μm sections of thymic tissue from 76 week old female RT7b rats were stained for Foxp3 (A), or with an isotype control (B). Foxp3^{+} thymocytes were found to be confined to the medulla (M) with only the occasional Foxp3^{+} thymocyte appearing in the surrounding cortex (C).

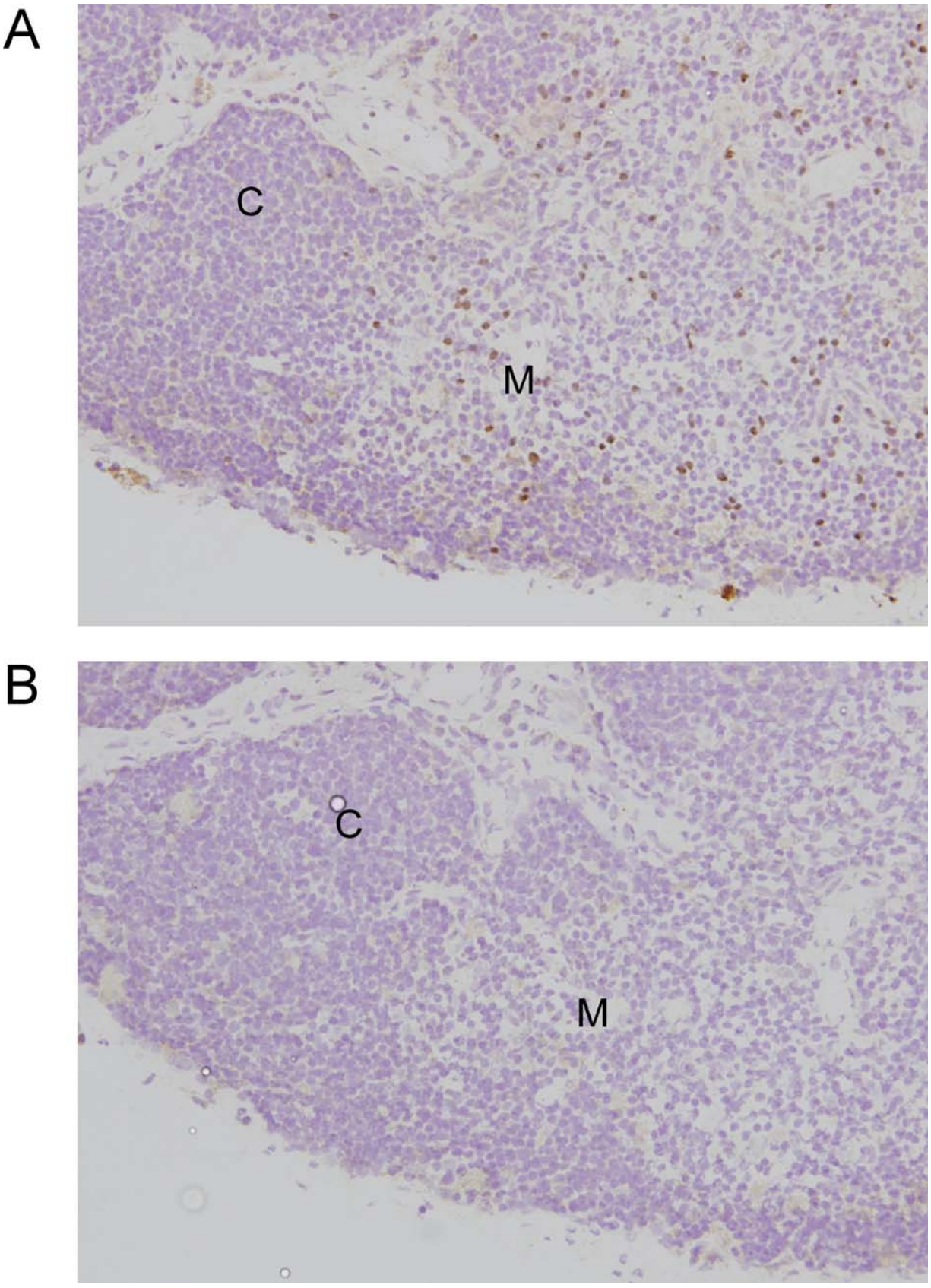
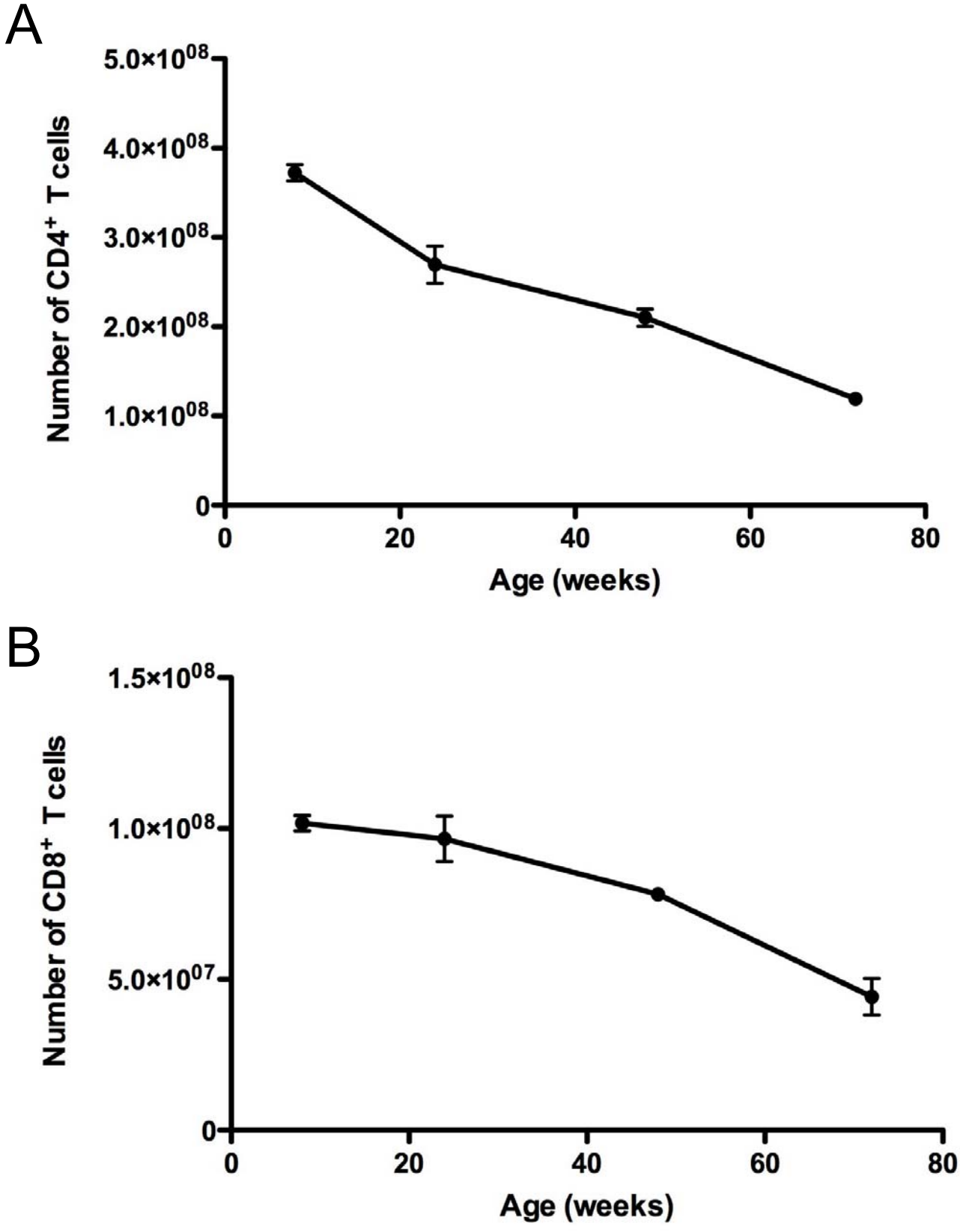

A
C
M
B
C
M

Figure 7.2.6 The absolute number of both $CD4^+$ and $CD8^+$ T cells in the lymph nodes and spleen decline with aging

Lymph nodes and spleens from rats aged 8, 24, 52 and 76 weeks were homogenised separately, enumerated using a haemocytometer and samples were then taken and stained for $CD4^+$ T cells and $CD8^+$ T cells. The total number of $CD4^+$ and $CD8^+$ T cells residing in the lymph nodes and spleen for each animal was determined arithmetically following the enumeration of each T cell subset in each separate tissue sample. Three animals were used per time point.


A
5.0×10^08
4.0×10^08
3.0×10^08
2.0×10^08
1.0×10^08
0
Number of CD4+ T cells
0
20
40
60
80
Age (weeks)
B
1.5×10^08
1.0×10^08
5.0×10^07
0
Number of CD8+ T cells
0
20
40
60
80
Age (weeks)

In order to investigate how thymic involution correlates with the organisation of secondary lymphoid tissues, lymph nodes and spleens from two month old animals were compared histologically with their counterparts from animals aged 12 months and 18 months. In lymph nodes, it was found that over time the thymus-dependent paracortical region, the major area containing T cells, decreased steadily in both size and cellularity (Figure 7.2.7A-C). As a consequence, the medulla appeared to occupy a greater proportion of the cross-sectional area of the lymph nodes (not shown). Follicles containing B cells retained approximately the same size and cellularity with age. Similar observations were made in all of the lymph nodes that were examined (mesenteric, axilliary and celiac). Lymph nodes from rats aged more than 24 weeks contained yellowish deposits in the paracortex and medulla which are probably composed of oxidised fats and known to collect in macrophages with age (Boaro *et al.* 1998). Deposits of lipofuchsin were particularly prominent in the celiac lymph nodes, which drain the liver.

Within the spleen, areas of white pulp were smaller and less connected in older rats than in those that were two months old (Figure 7.2.8). The peri-arteriolar lymphatic sheaths (PALS, thymus dependent areas) were smaller in 76 week old rats (Figure 7.2.8B) compared with 8 week old rats (Figure 7.2.8A) and their cellularity was reduced markedly. The adjacent follicles were of similar size in the two age groups, although due to the smaller area of PALS, they occupied a larger proportion of the cross-sectional area of the white pulp. For the same reason, the marginal zone appeared more prominent in spleens from 76 week old rats. A greater proportion of the splenic cross-sectional area was red pulp, and it contained large deposits of lipofuchsin, presumably within macrophages.

To relate the histological findings in lymph nodes to changes in specific populations of lymphocytes, serial frozen sections were prepared from lymph nodes of young (8 week old) and aged (76 week old) rats. These were stained with mAbs OX33 (B cells), W3/25 ($CD4^+$ T cells), OX39 (activated and regulatory T cells), and FJK-16s ($Foxp3^+$ regulatory T cells). The pattern of CD4 staining in lymph nodes from young rats was as expected (Barclay 1981), with $CD4^+$ T cells localised mainly in the

Figure 7.2.7 Gradual loss of lymphoid tissue containing T cells from lymph nodes with aging

Lymph nodes from rats aged 8, 24, 52 and 76 weeks of age were cut into 7um sections and stained with eosin and haematoxylin. It was found that 2 month old rats (A) had the most paracortical tissue (P), within which T cells reside. B cell containing follicles (F) were present on the outer edges of the lymph nodes, and some medullary regions (M) were present. The lymph nodes of rats aged 52 weeks (B) had more medulla, and there appeared to be a loss of T cells surrounding the follicles, both on the outer edge of the lymph node surface, and from the paracortex. It was found that by 76 weeks of age (C), marked atrophy of the paracortex had occurred, such that B cell follicles appeared more isolated from the remaining lymphoid tissue.

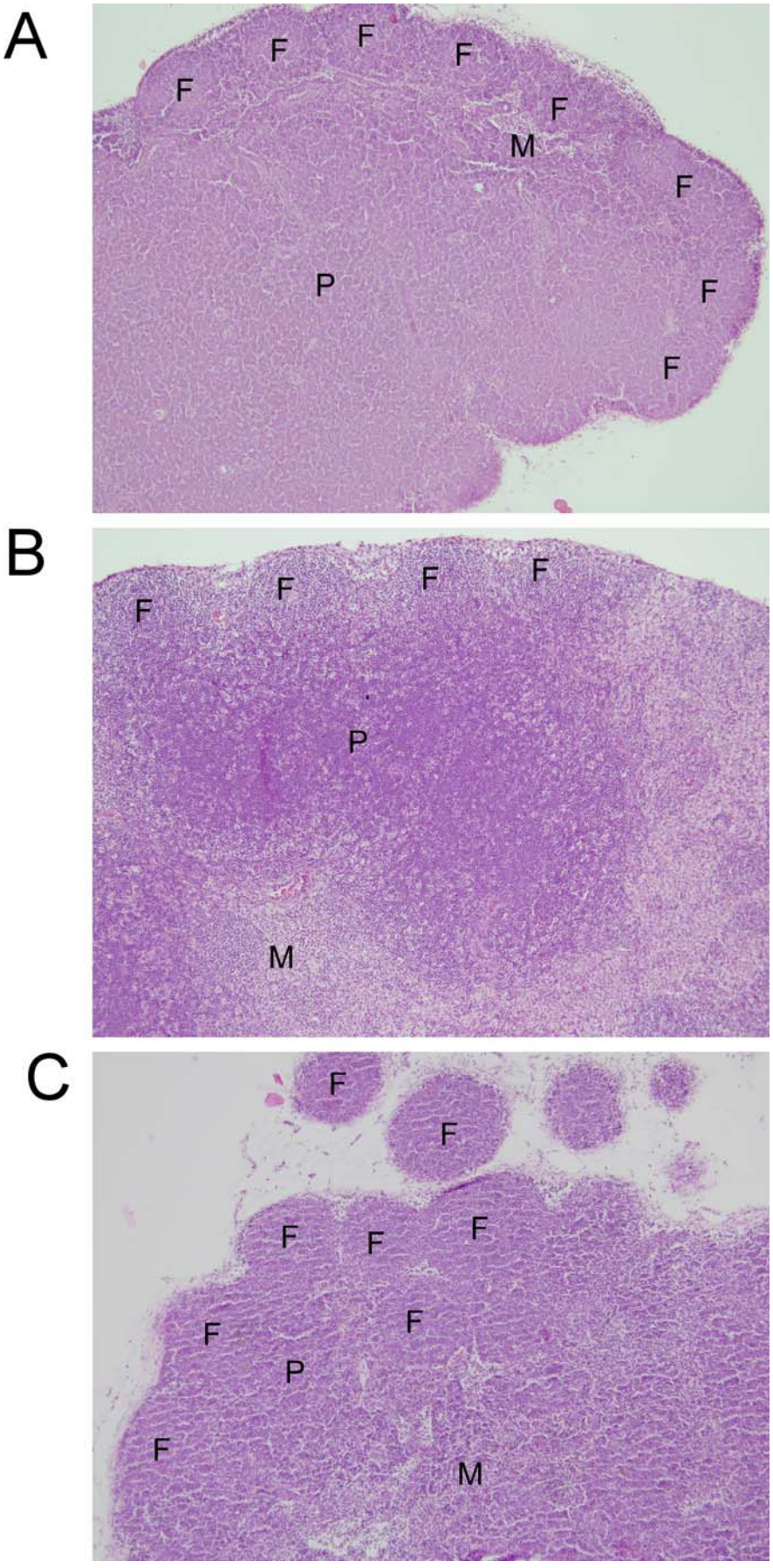
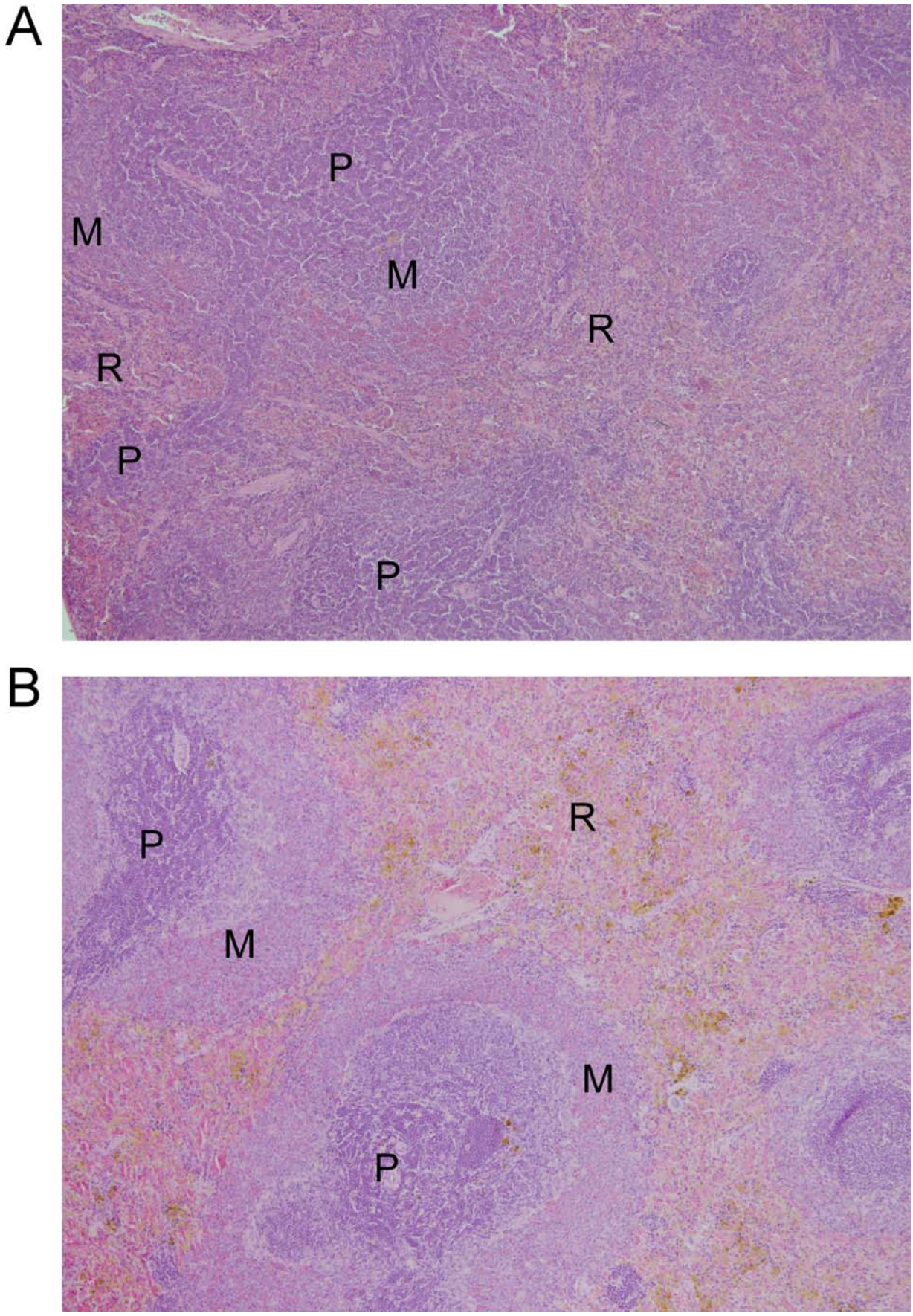

A
F
F
F
F
F
M
F
P
F
F
B
F
F
F
F
P
M
C
F
F
F
F
F
F
P
F
M

Figure 7.2.8 Gradual loss of white pulp containing T cells from the spleen with aging

Spleens from rats aged 8 weeks (A) were cut into sections 7μm thick and stained with eosin and haematoxylin. Periarteriolar lymphoid sheath (P) tissue containing T cells was surrounded by a layer of Marginal zone (M) tissue, comprised of B cells and macrophages. The red pulp (R) surrounds these white pulp areas, which are often interconnected by bridges of tissue. In animals aged 76 weeks (B), a marked contraction of the periarteriolar lymphoid sheath had occurred, with a visible loss of cellularity, especially in certain regions.


A
P
M
M
R
R
P
P
B
R
P
M
M
P

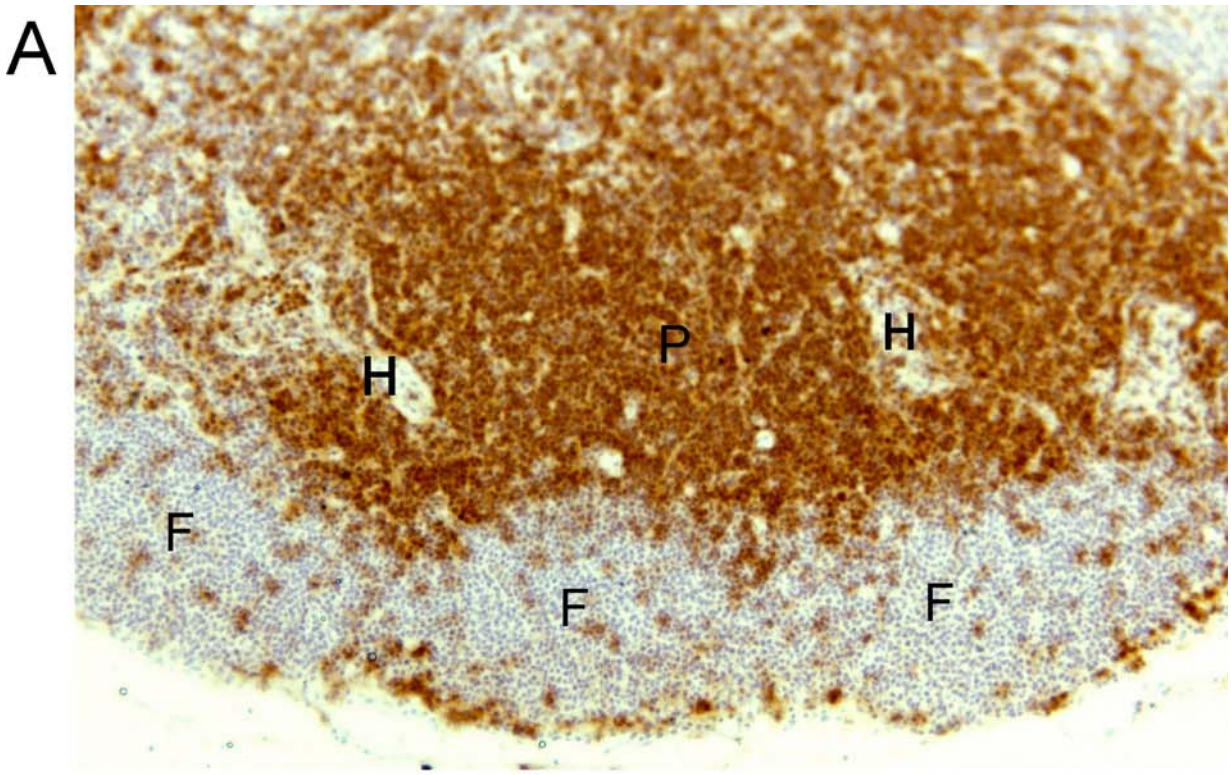
paracortex, with small numbers of $CD4^+$ T cells within the follicles and forming a peri-follicular rim (Figure 7.2.9). High endothelial venules (HEV) were conspicuous within the paracortex. Also as expected, B cells were localised mainly to the follicles (Figure 7.2.10). Having defined the structural compartments within the lymph nodes, serial sections were examined to detect the distribution of lymphocytes that express markers that identify regulatory T cells. The distribution of $CD25^+$ cells, which are predominantly regulatory T cells but also include some activated T cells, was similar to that of $CD4^+$ T cells (Figure 7.2.11A). They were mainly found in the paracortex, with small numbers in the peri-follicular area, and occasional cells located within the follicles. $Foxp3^+$ cells shared a similar distribution with $CD25^+$ T cells, being predominantly located within the paracortex (Figure 7.2.12A). It is noteworthy that occasional $Foxp3^+$ cells were detected within HEV, suggesting that these cells are part of the pool of lymphocytes that recirculates through lymph nodes.

In lymph nodes from aged rats (76 weeks of age) the location of $CD4^+$ T cells was very similar to that found in lymph nodes from their younger counterparts (Figure 7.2.13A). However, the relative cross-sectional area occupied by paracortex was smaller, as observed by histological examination (see Figure 7.2.7). Interestingly, there appeared to be fewer HEV in the paracortex of the lymph nodes from older animals, and those that were present had a different morphology. The endothelial cells had a more flattened appearance, so that the HEV were noticeably thinner. The three unstained follicular areas in the cortex (Figure 7.2.13A) contained B cells (Figure 7.2.14A), although the plane of the latter section passed through only the peripheries of the follicles.

In order to determine the location of regulatory T cells within the lymph nodes of aged rats, sections were stained for CD25 (Figure 7.2.15). $CD25^+$ T cells were localised to the paracortex, with some cells present also in the peri-follicular areas. The distribution of $Foxp3^+$ regulatory T cells correlated closely with the distribution of $CD25^+$ cells (Figure 7.2.16). Compared with the lymph nodes from younger rats, the density of regulatory T cells appeared to be higher in those from aged rats.

Figure 7.2.9 Immunohistochemical identification of $CD4^+$ T cells within the lymph node tissues of young adult rats

Lymph node sections 7μm thick were prepared from 8 week old female RT7b rats, and stained for CD4 (A), or with an isotype control (B). It was found that $CD4^+$ T cells were mainly localised to the paracortex (P), which contained high endothelial venules (H) and while some cells lined the outer edge of B cell follicles (F), very few $CD4^+$ T cells were present in the follicles. These images were photographed at 20X magnification.


A
H
P
H
F
F
F

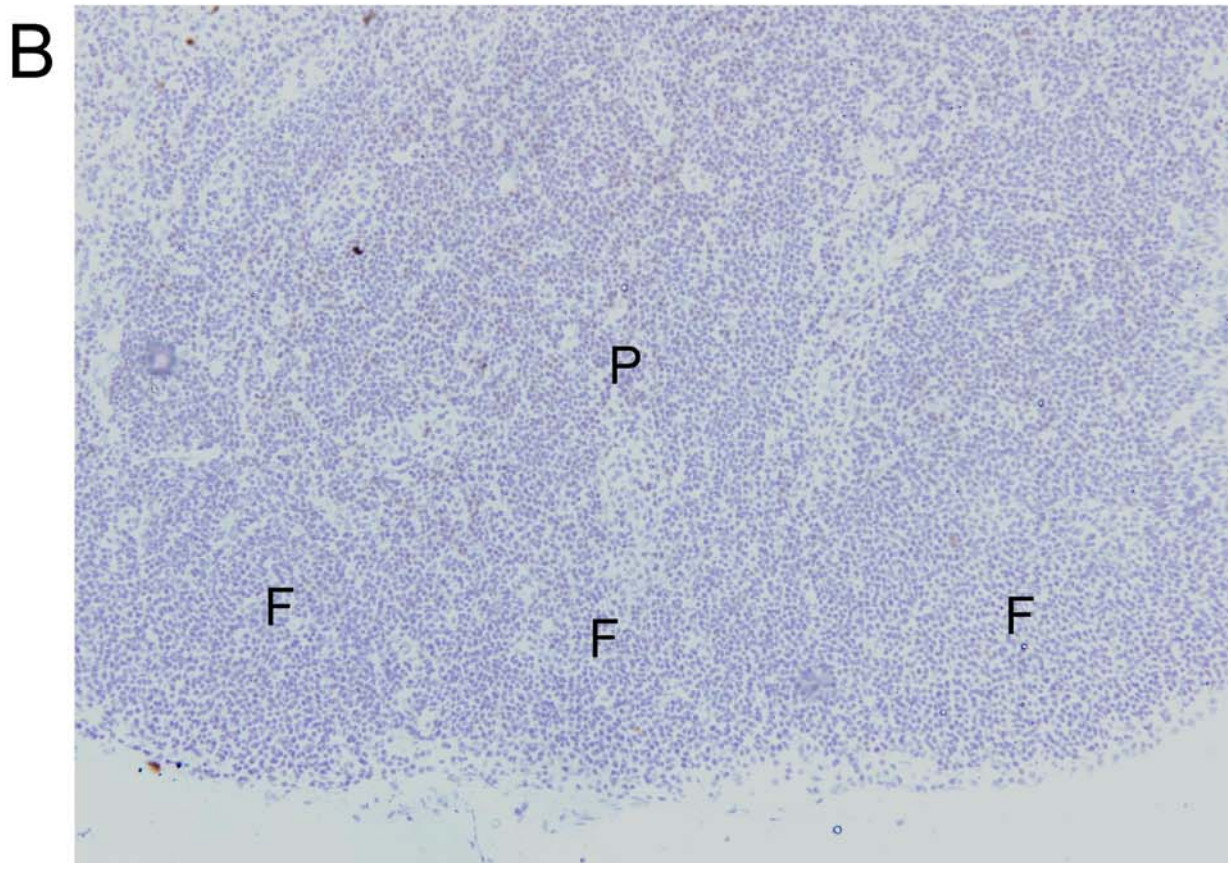

B
P
F
F
F

Figure 7.2.10 Immunohistochemical detection of B cells within the lymph node tissues of young adult rats

Lymph node sections 7μm thick were prepared from 8 week old female RT7b rats, and stained with OX33, which recognises an isoform of CD45 specifically expressed by B cells. It was found that B cells were predominantly located within the B cell follicles (F), and they were present in equal proportions to T cells in the sparsely populated medulla (M). Only the occasional B cell was present within the paracortex (P). This image was photographed at 20X magnification.

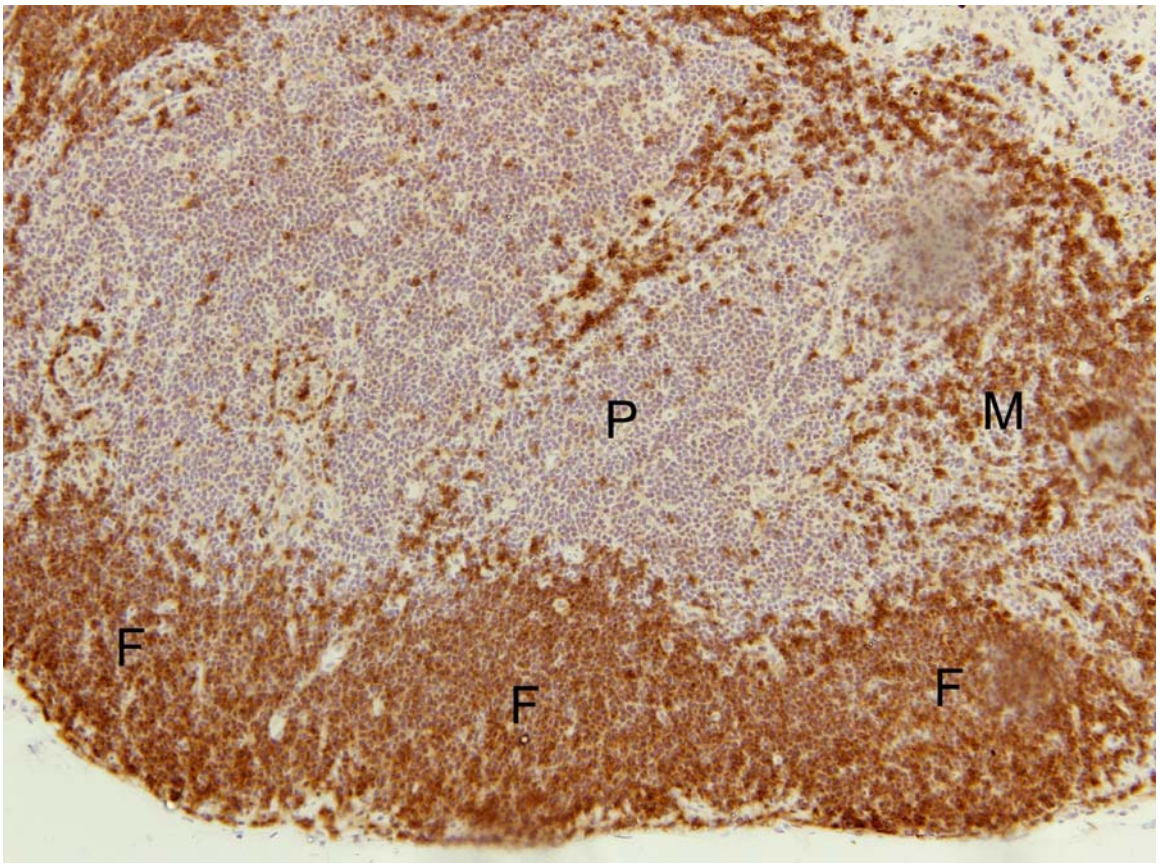

P
M
F
F
F

Figure 7.2.11 Immunohistochemical detection of $CD25^+$ T cells within the lymph node tissues of young adult rats

Sections of lymph node 7μm in thickness from 8 week old rats were stained for CD25, a cell surface marker expressed by regulatory T cells but also activated T cells. It was found that the pattern of staining observed closely resembled that of $CD4^+$ T cells, with the bulk of $CD25^+$ cells located in the paracortex (P), a small population surrounding the B cell follicles (B), and the occasional cell inside the B cell follicles. This image was photographed at 20X magnification.

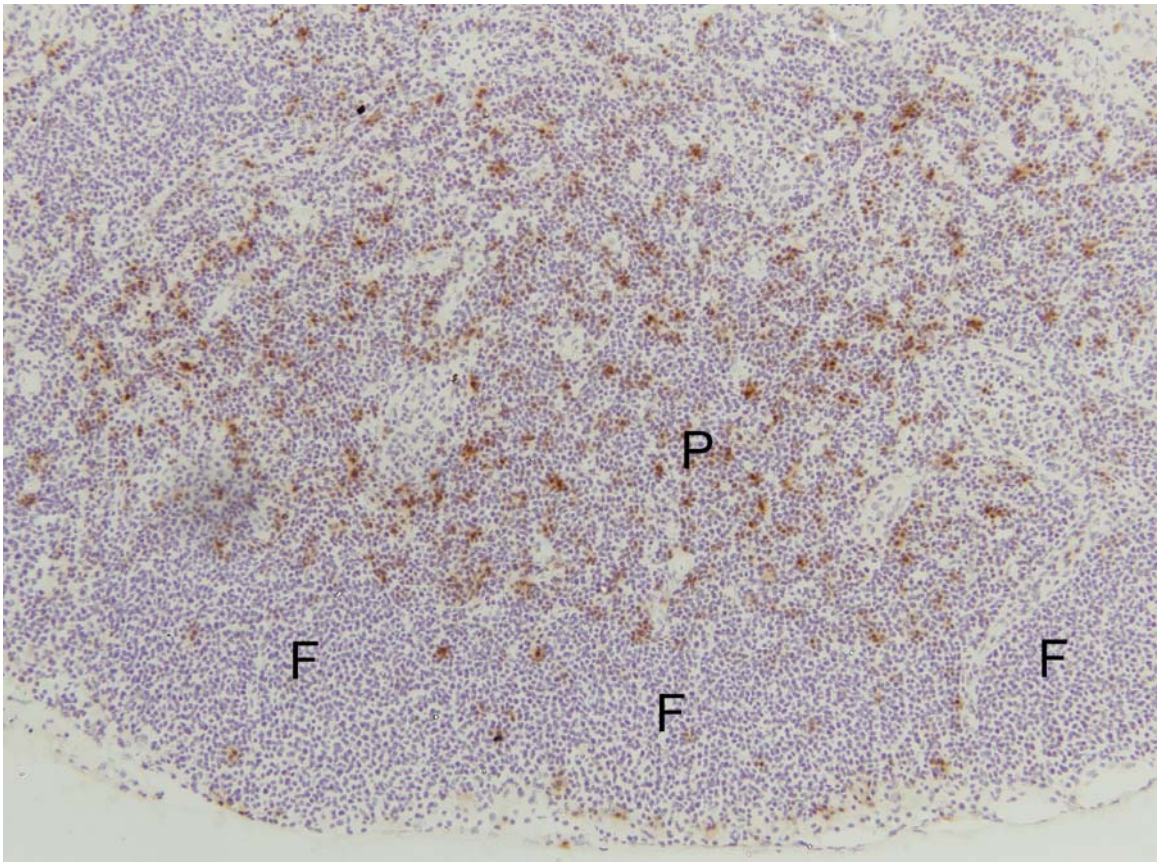

P
F
F
F

Figure 7.2.12 Immunohistochemical identification of Foxp3^{+} T cells within the lymph node tissues of young adult rats

Foxp3^{+} T cells were identified in 7μm thick sections of lymph nodes taken from 8 week old rats (A). The paracortex (P) contained the most Foxp3+ T cells, which were also present in some B cell follicles (F) and on the outer surface of the follicles (B). The image in (A) was photographed at 20X magnification, and the image in (B) was photographed at 40X magnification.

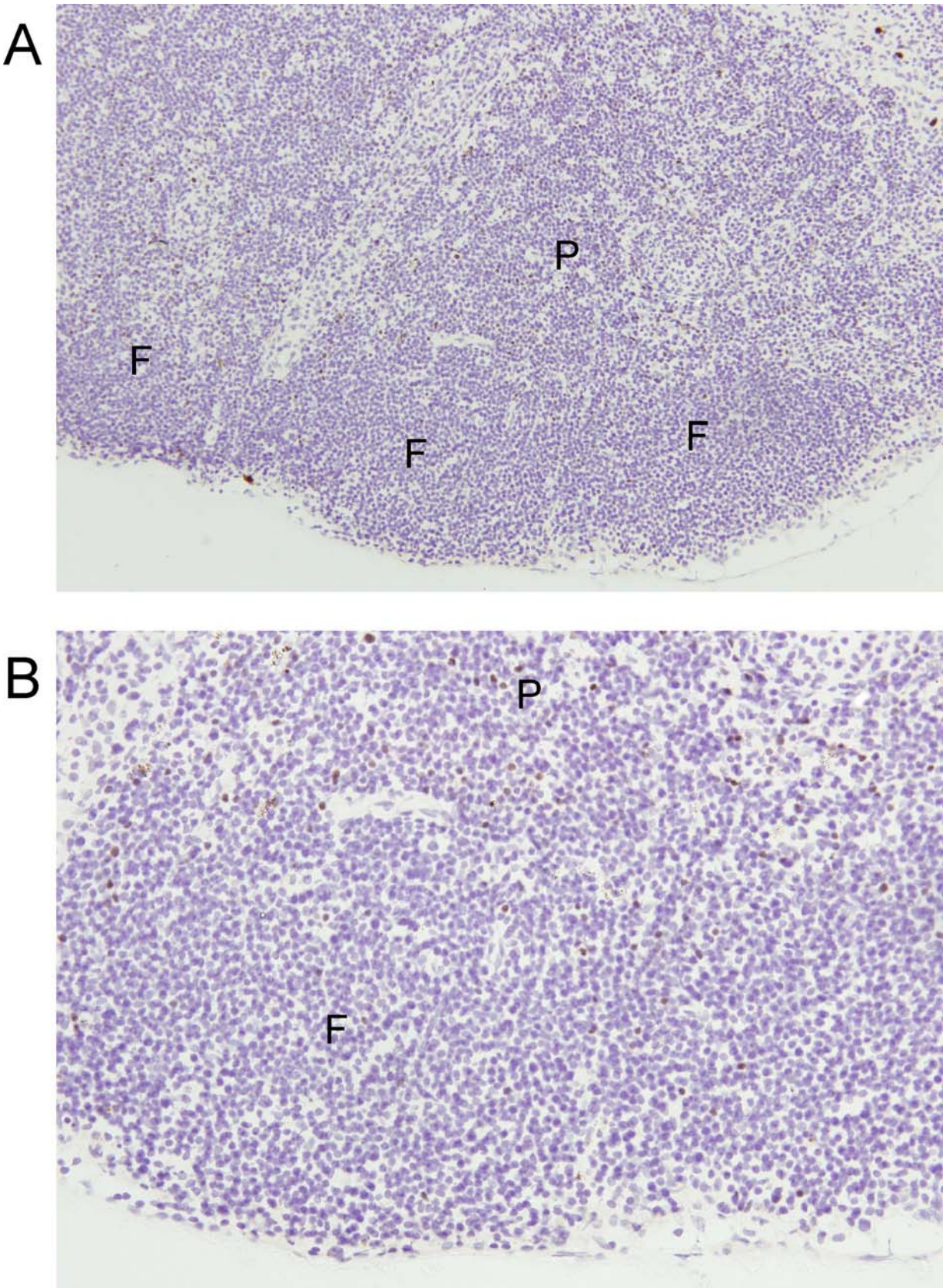

A
P
F
F
F
B
P
F

Figure 7.2.13 Immunohistochemical characterisation of $CD4^+$ T cells within the lymph node tissues of aged adult rats

Lymph node sections 7μm thick were prepared from 76 week old female RT7b rats, and stained for CD4, which was detected by a secondary antibody conjugated to HRP. It was found that $CD4^+$ T cells were mainly localised to the paracortex (P), which was smaller than in younger animals, contained smaller and less frequent HEVs (H) and formed a less discrete boundary with the B cell follicle areas (F). This images was photographed at 20X magnification.

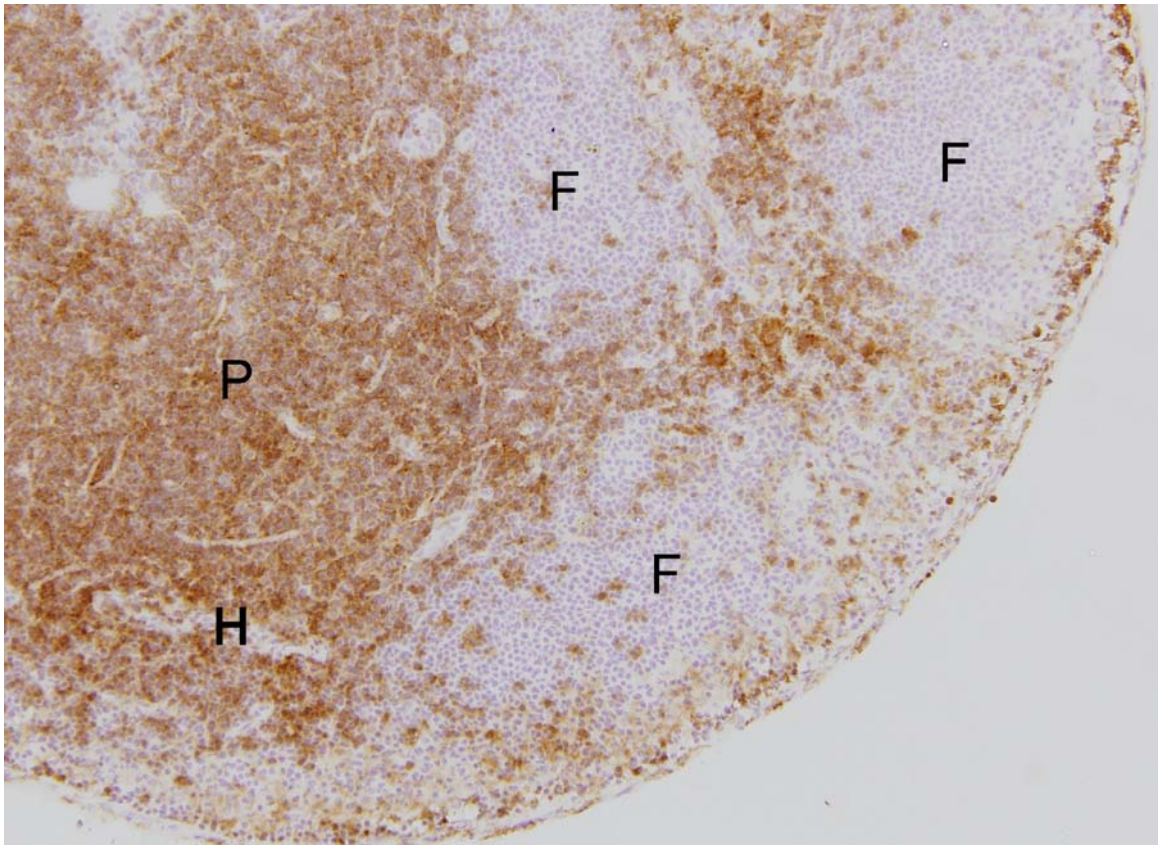

F
F
P
F
H

Figure 7.2.14 Immunohistochemical characterisation of B cells within the lymph node tissues of aged rats

Lymph node sections 7μm thick were prepared from 76 week old female RT7b rats, and then stained with OX33, which recognises an isoform of CD45 specifically expressed by B cells. B cells were predominantly located within the B cell follicles (F), which were very disorganised at their boundary. Several B cells were scattered throughout the paracortex (P). A knife mark is present on this section. This image was photographed at 20X magnification.

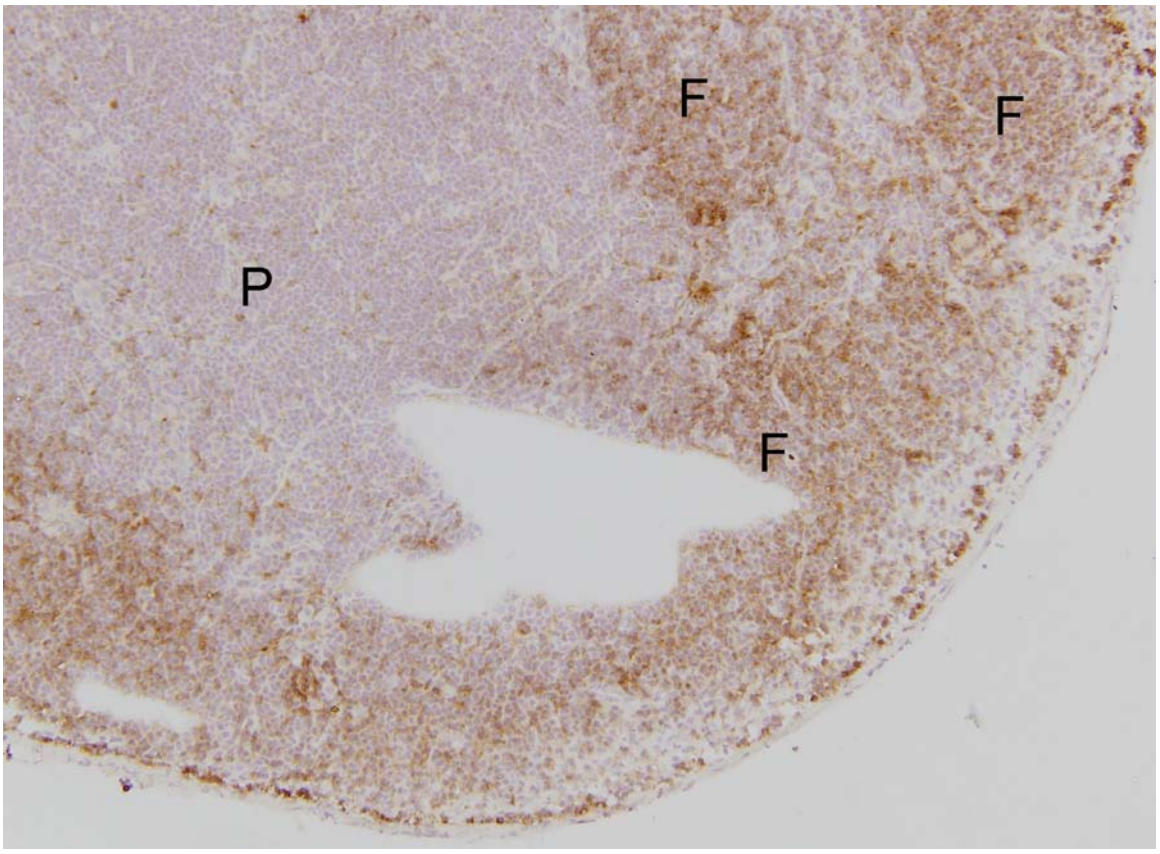

F
F
P
F

Figure 7.2.15 Immunohistochemical identification of $CD25^+$ T cells within the lymph node tissues of aged rats

Sections of lymph node 7μm in thickness taken form animals 76 weeks of age were stained for CD25, which identifies mostly regulatory T cells but also some activated T cells. It was found that the pattern of staining observed closely resembled that of $CD4^+$ T cells, with the bulk of $CD25^+$ cells located in the paracortex (P), a small population surrounding the B cell follicles (B), and the occasional cell inside the B cell follicles. This image was photographed at 20X magnification.

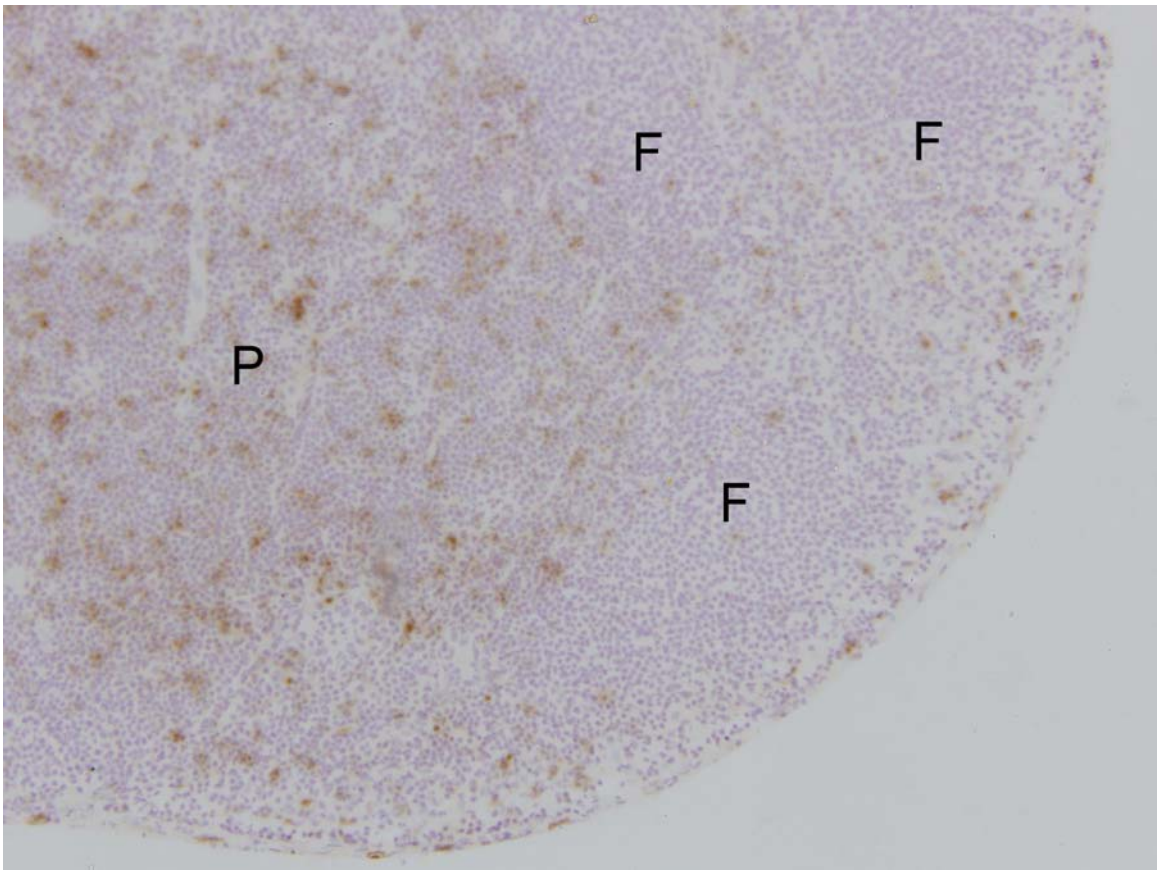

F
F
P
F

Figure 7.2.16 Immunohistochemical identification of Foxp3$^+$ T cells within the lymph node tissues of aged rats

In order to detect Foxp3$^+$ T cells, 7μm thick sections of lymph nodes taken from 76 week old rats were stained for Foxp3 (A) or an isotype control (B). The paracortex (P) contained the most Foxp3$^+$ T cells, which were present in higher concentrations compared to younger animals. In addition, regulatory T cells were also present in some B cell follicles (F) and on the outer surface of the follicles. These images were photographed at 20X magnification.

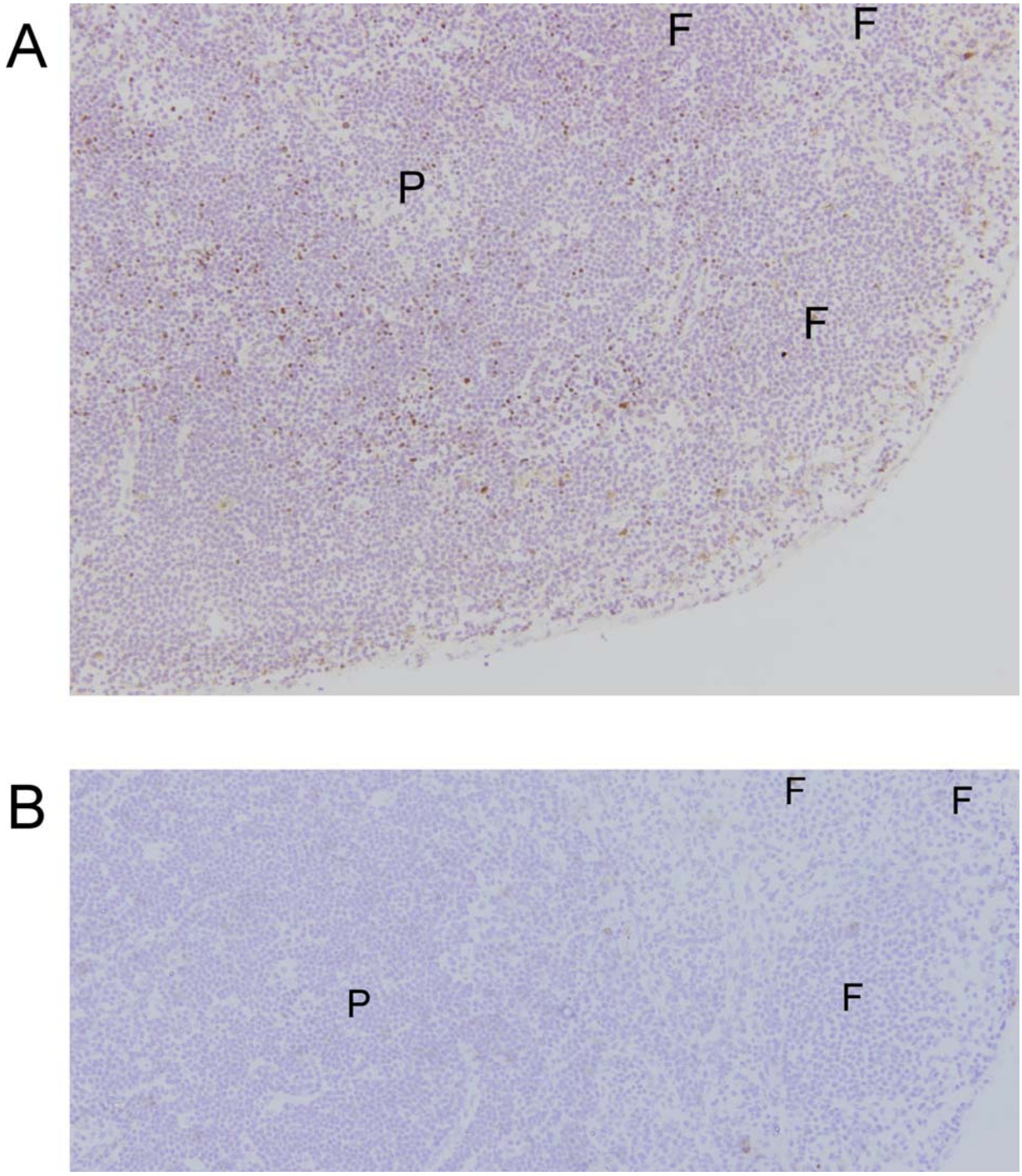

A
F
F
P
F
B
F
F
P
F

Figure 7.2.17 The number and frequency of recent thymic emigrants declines with aging

In order to enumerate recent thymic emigrants, samples of LN and spleen cells were stained for CD4, and the remaining cells labelled with OX33, MARK-1 and 341 and then incubated with goat anti-mouse beads to negatively select $CD4^+$ T cells. Negatively selected $CD4^+$ T cells were stained with antibodies against Thy1 to identify $Thy1^+$ $CD4^+$recent thymic emigrants and the total number of these cells residing in the lymph nodes and spleen for each animal was determined following their enumeration in each separate tissue sample (A). The proportion of $Thy1^+$ cells amongst $CD4^+$ T cells is indicated as a percentage of the total $CD4^+$ T cell population (B). Three animals were used per time point.

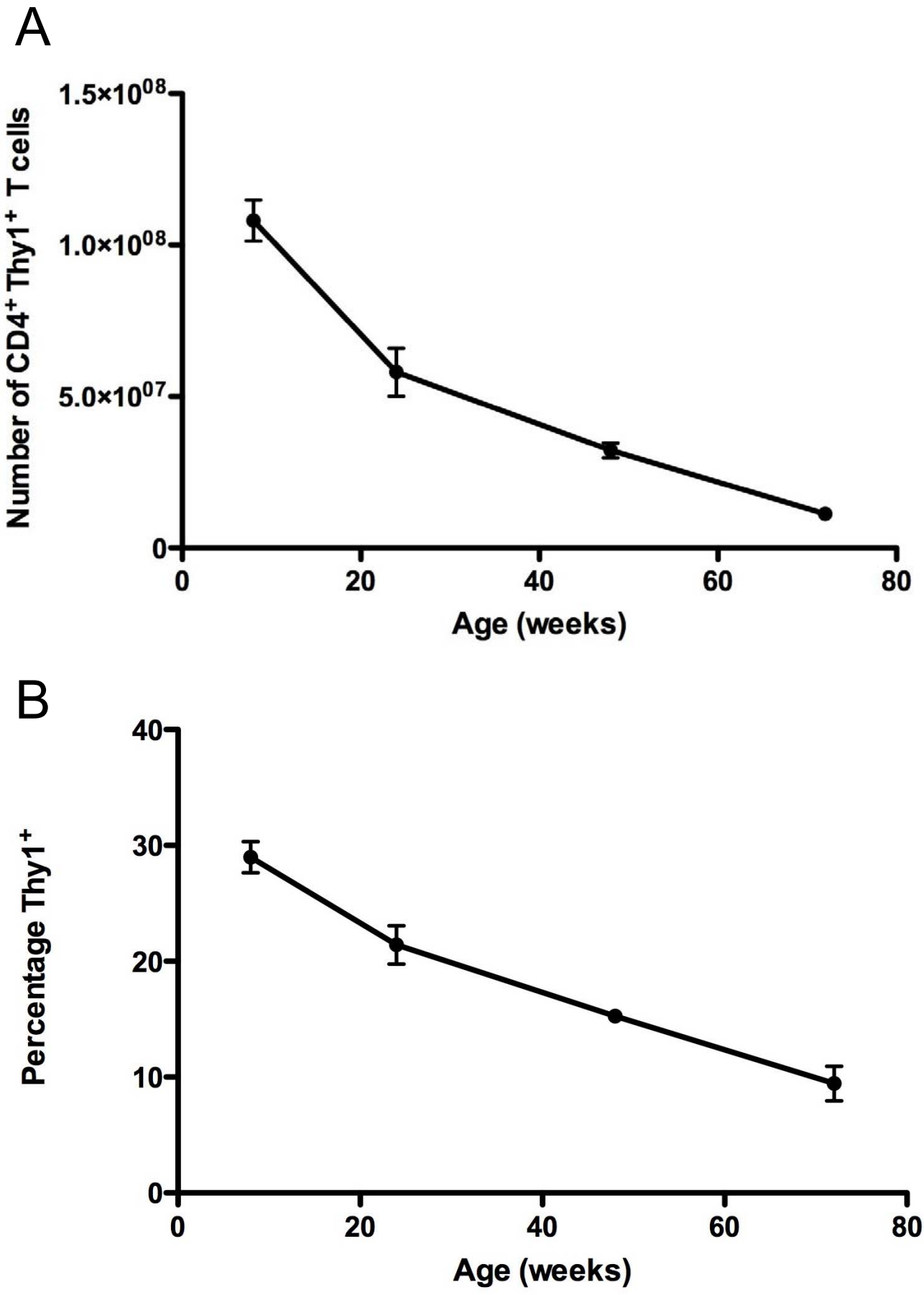

A
Number of CD4+ Thy1+ T cells
1.5×10^08
1.0×10^08
5.0×10^07
0
0
20
40
60
80
Age (weeks)
B
Percentage Thy1+
40
30
20
10
0
0
20
40
60
80
Age (weeks)

Figure 7.2.18 The number of CD8α^+ Foxp3$^+$ T cells in the lymph nodes and spleen decreases with aging

In order to enumerate regulatory T cells expressing the CD8α homodimer, samples of LN and spleen cells were stained for CD4, and the remaining cells labelled with OX33, MARK-1 and 341 and then incubated with goat anti-mouse beads to negatively select CD4$^+$ T cells. Negatively selected CD4$^+$ T cells were stained with antibodies against CD8α, CD25 and Foxp3 to enable the enumeration of regulatory T cells expressing the CD8α homodimer. Three animals were used per time point.

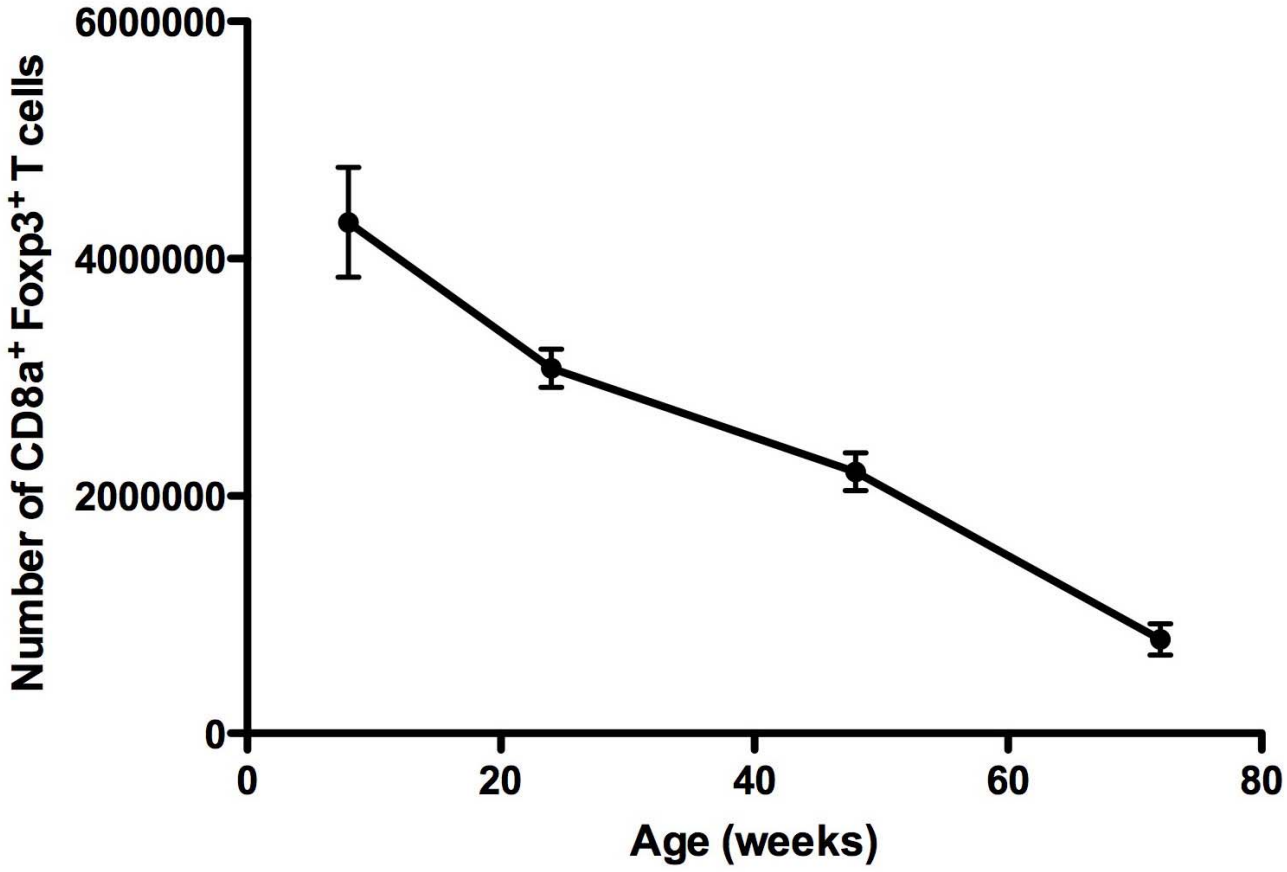
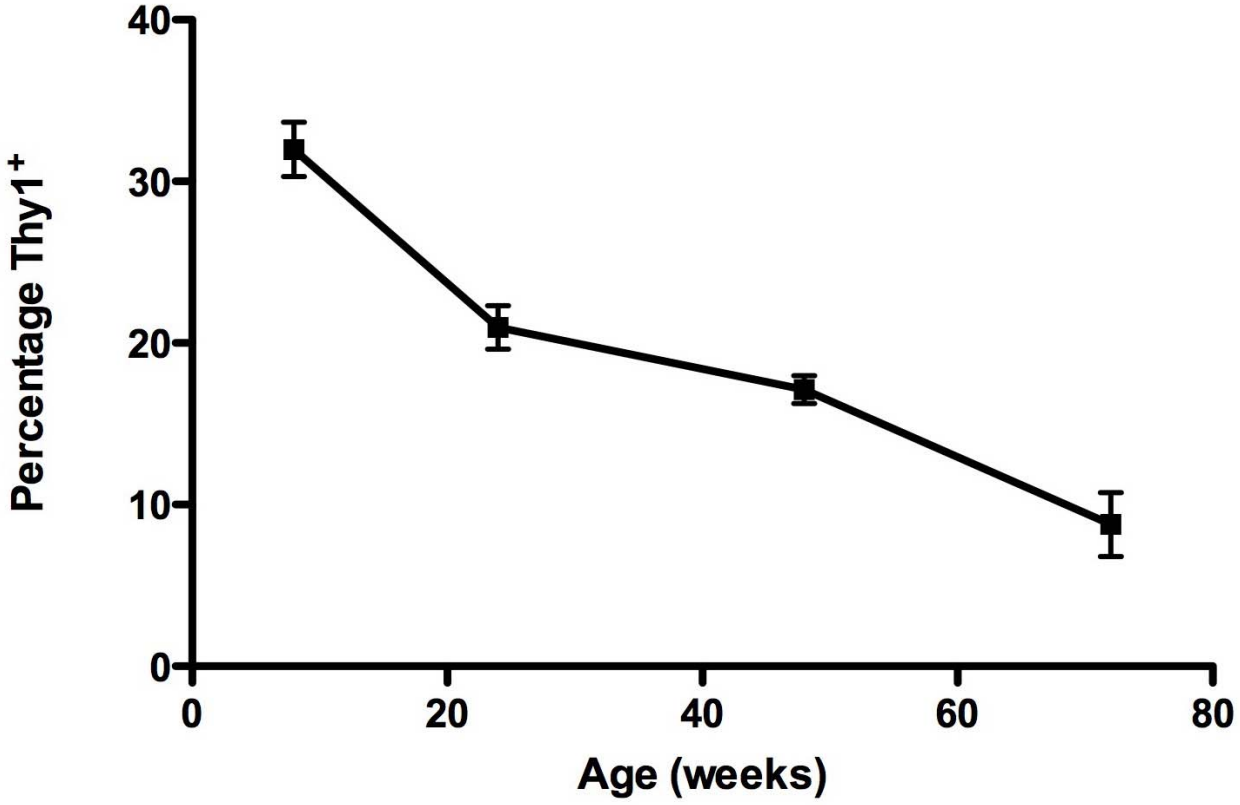

6000000
4000000
2000000
0
Number of CD8a+ Foxp3+ T cells
0
20
40
60
80
Age (weeks)

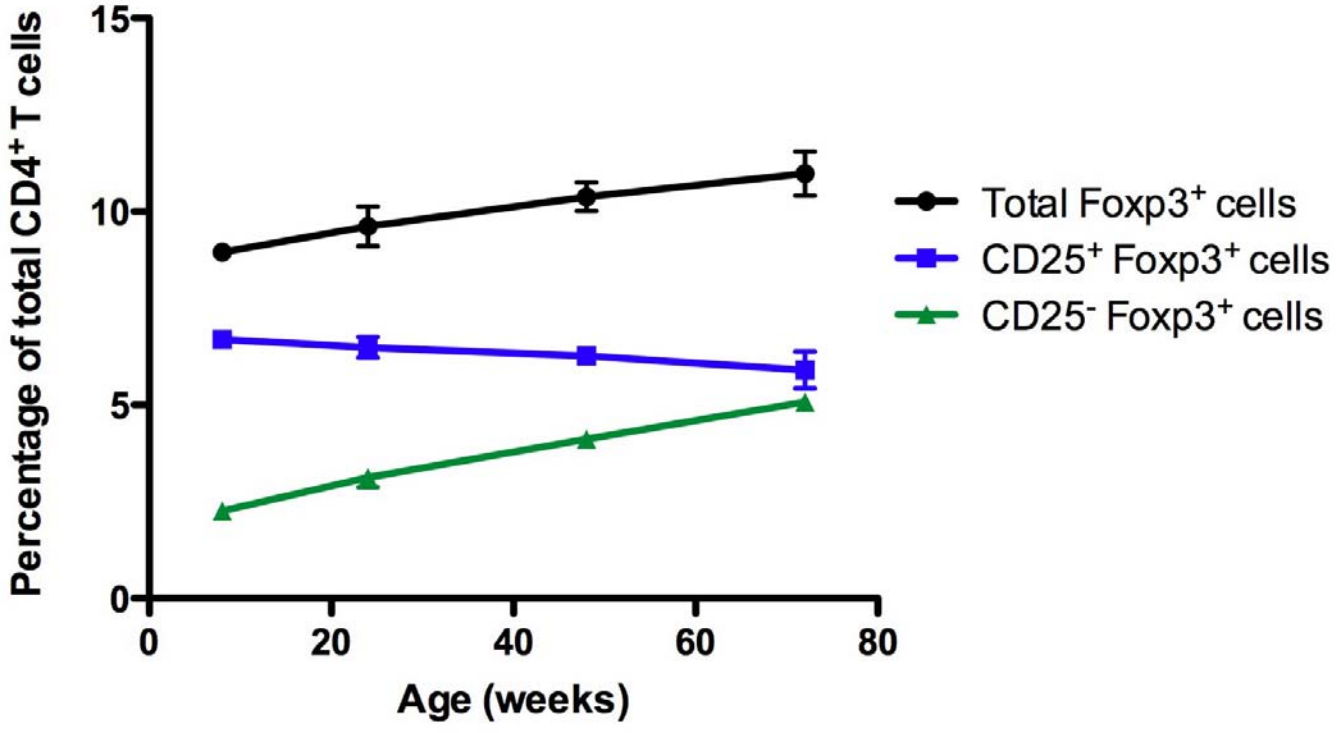
Figure 7.2.19 The proportion of $CD25^-$ $Foxp3^+$ regulatory T cells increases with aging

Lymphocytes from spleen and lymph node samples taken from rats aged 8, 24, 52 and 76 weeks were stained for CD4 to enumerate $CD4^+$ T cells, and separately labelled with labelled with OX33, MARK-1 and 341 and then incubated with goat anti-mouse beads to negatively select $CD4^+$ T cells. Negatively selected $CD4^+$ T cells were stained with antibodies against CD25, Foxp3 and Thy1 to enable the enumeration of RTE and regulatory T cell subsets. The number of $CD25^+$ $Foxp3^+$ regulatory T cells expressing Thy1 was determined for rats aged 8 to 76 weeks (A). The proportion of $CD4^+$ T cells expressing Foxp3 and in the $CD25^-$ $Foxp3^+$ and $CD25^+$ $Foxp3^+$ regulatory T cell subsets is reported as a percentage (B).

A


Percentage Thy1+
40
30
20
10
0
0
20
40
60
80
Age (weeks)

B


Percentage of total CD4+ T cells
15
10
5
0
0
20
40
60
80
Age (weeks)
Total Foxp3+ cells
CD25+ Foxp3+ cells
CD25- Foxp3+ cells

7.2.3 Reduction in thymic output with aging

The absolute number of $CD4^+$ RTE in the spleen and pooled LN of animals was determined by flow cytometry, using cell suspensions prepared from animals aged 8, 24, 52 and 76 weeks. The numbers of RTE reported are the sum of those determined in the spleen and in the pooled LN. There were 10-fold fewer $CD4^+$ RTE in the suspensions prepared from rats aged 76 weeks compared with those aged 8 weeks (Figure 7.2.17A). This decline was more marked than the decline in numbers of $CD4^+$ T cells, with the result that there was an approximate 3-fold reduction in the frequency of $Thy1^+$ cells amongst $CD4^+$ T cells (Figure 7.2.17B). $CD8\alpha^+$ $Foxp3^+$ cells declined more than 5-fold in number over the same period of time (Figure 7.2.18).

In order to determine how the decline in the thymic output of RTE affects the composition of the regulatory T cell pool, estimates were made of the proportions of $CD25^+$ $Foxp3^+$ regulatory T cells that expressed Thy1 at various ages. The percentage of $CD25^+$ regulatory cells that expressed Thy1 declined with aging (Figure 7.2.19A), in a manner similar to that observed in conventional $CD4^+$ T cells (Figure 7.2.17B. The proportion of total $CD4^+$ T cells that expressed Foxp3 (Figure 7.2.19B) increased steadily, from ~9% at 8 weeks of age to ~11% at 76 weeks of age ($p=0.05$).

Over the same period, the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells declined marginally over this period, but this decrease was not significant ($p=0.12$). Thus the increase in total $Foxp3^+$ cells was due to a steady increase in the proportion of $CD25^-$ $Foxp3^+$ cells ($p=0.007$). Numbers of $CD25^-$ $Foxp3^+$ and $CD25^+$ $Foxp3^+$ cells were approximately equal by 76 weeks of age.

7.3 Discussion

7.3.1 Involution of the rat thymus with age

The involution of the thymus is thought to result from a combination of factors, including the disorganisation of epithelial cell networks, the induction of apoptosis in thymocytes by activated T cells displaying death-inducing ligands, that enter the thymus from the periphery, and the cumulative effects of soluble factors such as cytokines and hormones that promote thymic atrophy. In humans, the perivascular spaces surrounding blood vessels are expanded by replacement of thymic tissue with adipose tissue, which gradually leads to the isolation of productive thymic tissues over time (Gruver *et al.* 2007). However, despite the progressive loss of thymic mass during ageing in rats (Figure 7.2.1), the basic architecture of the thymus was maintained (Figure 7.2.2). There was an increase in peri-thymic fat but no replacement of thymic tissue by adipose tissue in rats. Although it is widely accepted that age-related thymic involution occurs in humans, the variation in thymic size within the population is considerable. To illustrate this point, in one study it was found that wet thymus weight ranged from 2 to 54 grams, while the two subjects with the largest thymuses were one month old, and 77 years old (Kendall *et al.* 1980). Thus, it is striking that we found such little variation in thymic weights amongst animals of the same age, and a steady decline in thymic weight with age. The enormous variation in thymus size found within the human population is thought to be partly the result of stress induced thymic involution, which can be triggered by malnutrition, pregnancy, emotional distress or infection (Gruver and Sempowski 2008), and is partly due to the greater effect of steroid sex hormones on thymic involution in males compared with females. Single sex, the inbred nature of the rats and the controlled environment in which they were raised probably accounts for the small variation in thymus size observed within each age cohort.

The structural changes we observed in the thymus with aging included a trend toward increasing disorganisation in the medulla, with the appearance of regions containing proteinaceous cysts (Figure 7.2.2). The cysts we observed were primarily located within the medulla, and probably represent developmental remnants of the thymopharyngeal duct left over from the formation of the thymus (Farr *et al.* 2002, Pearse 2006).

Changes in the amount and character of the thymic epithelium may also affect its ability to provide survival and proliferation signals to thymocyte precursors recruited from the bone marrow, and thus affect the productivity of the thymus (Jenkinson *et al.* 2005). In particular, the volume of cortical thymus appeared to be affected most. As this is the main proliferative compartment in the thymus (Petrie 2002), it is not clear whether this reflects simply loss of thymocytes, or loss of cortical epithelium required to maintain thymocyte development. In either case, it might be expected to lead to a decline in export of new T cells to the periphery

The productivity of thymic tissue was adversely affected by aging, as the wet thymic mass (excluding peri-thymic adipose tissue) had decreased approximately 2.5 fold by 76 weeks of age while the numbers of $Thy1^+$ RTE in the periphery had decreased 10-fold. These results are in contrast to reports that in sheep, the productivity of thymic tissue is unaffected by aging (Cunningham *et al.* 2001). Although the 3-4 year old aged sheep (equivalent to 40-50 human years of age) that were used in those experiments had smaller thymus sizes than those found in lambs, there is no evidence that their peripheral lymphocyte pool was yet declining in size. Thus the finding that the productivity of thymic tissue remains constant with aging (Cunningham *et al.* 2001) may not be applicable to animals of a more advanced age.

7.3.2 The effect of age on the thymic output of regulatory T cells

The process of aging was accompanied by a decline in the proportion of RTE among $CD4^+$ T cells, indicating reduced thymic output (Figure 7.2.17). In addition, the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells (which we have shown are associated with RTE, see section 4.2) amongst $CD4^+$ T cells also declined with aging (Figure 7.2.19). Interestingly, although the absolute number of thymocytes decreased with aging, the number of $Foxp3^+$ regulatory T cells found in the thymus was not affected (Figure 7.2.3). The $Foxp3^+$ regulatory T cells we identified were predominantly located in the medulla, where the most developed T cells are situated, including those that have returned to the thymus from the periphery (Westermann *et al.* 1996). The higher concentration of $Foxp3^+$ cells in the thymuses of aged rats (Figure 7.2.5) is an

interesting finding that may reflect a shift towards the production of regulatory T cells in old age, in order to fortify the aging immune system against the onset of autoimmune disease. We have previously determined that the lifespan of regulatory T cells is short compared to conventional T cells, with the half life of the $CD25^+$ $Foxp3^+$ subset ~18 days, and the half life of the $CD25^-$ $Foxp3^+$ subset ~7 days (Figure 5.2.8). The continuous production of regulatory T cells by the thymus may therefore be required to replace regulatory T cells that have expired, in order to maintain immune homeostasis.

However, while the continued production of regulatory T cells by the thymus may be required to avert autoimmunity, it is unlikely that the higher frequency of regulatory T cells detected in the thymus of aged animals is the result of a shift towards the production of regulatory T cells. It was observed that the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells (which are associated with RTE, see section 4.2) amongst $CD4^+$ T cells in the periphery actually declined with aging (Figure 7.2.19).

Rather than representing a shift towards the production of regulatory T cells by the thymus with aging, the higher frequency of regulatory T cells observed in the thymus as animals become older may be the result of the recirculation of regulatory T cells from the periphery back into a diminishing volume of thymic tissue. The preferential recirculation of regulatory T cells back to the thymus was earlier demonstrated in Chapter 3 (Figures 3.2.18 and 3.2.20), thus lending credibility to such a scenario. Although it is not clear whether the recirculation of regulatory T cells to the thymus is incidental, or if they are mediating a specific biological role, it would be expected that their immunosuppressive properties would have biological consequences in the thymus. In a manner analogous to activated T cells, regulatory T cells that recirculate back to the thymus may display their inhibitory ligands to thymic epithelial cells, to induce their apoptosis and contribute to the process of thymic involution (Yajima *et al.* 2004, Beverly *et al.* 2006).

In contrast, the proportion of $CD25^-$ $Foxp3^+$ regulatory T cells amongst $CD4^+$ T cells in the lymph nodes and spleen increased with aging. Thus, as the export of RTE wanes

with age, regulatory T cells may be maintained by conversion of cells from the $CD25^+$ $Foxp3^+$ regulatory T cell population to the tissue homing $CD25^-$ $Foxp3^+$ regulatory T cell population.

7.3.4 Summary

The involution of the rat thymus that occurs with aging is accompanied by the loss in the absolute number of thymocytes and a decrease in both the size and mass of thymic tissue. While in humans thymic tissue is slowly replaced by adipose tissue in the perivascular spaces, this study indicated that the thymic structure of the rat largely survives intact into old age, as fat is deposited on its exterior. However, the productivity of the thymic tissue (excluding peri-thymic fat) declined with aging. The number of $Foxp3^+$ cells in the thymus was unaffected by aging, despite reductions in the total number of thymocytes (Figure 7.2.3). Although the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells among $CD4^+$ T cells declined with aging as thymic output waned, the proportion of $CD25^-$ $Foxp3^+$ regulatory T cells among $CD4^+$ T cells increased with aging, potentially providing a mechanism to compensate for the declining thymic output of regulatory T cells.

Chapter 8: General Discussion

8.1 The recent thymic origin and differentiation of regulatory T cells

Regulatory T cells are believed to differentiate within the thymus through their selection by self peptides. (Bensinger *et al.* 2001, Apostolou *et al.* 2002, Fontenot and Rudensky 2004, Caton *et al.* 2004, Larkin *et al.* 2007). The thymic selection of regulatory T cells has been examined using transgenic mice that express a model "self" peptide from a foreign organism constitutively. Although these mice contain normal proportions of regulatory T cells, when they are crossed with mice that contain a transgenic TCR that also recognises the model peptide, a high proportion of the TCR transgenic thymocytes and peripheral T cells are regulatory T cells (Jordan *et al.* 2001). The cells that mediate the thymic selection of regulatory T cells are thymic cortical epithelial cells, which also mediate the positive selection of thymocytes (Bensinger *et al.* 2001). It has been proposed that regulatory T cells represent the most self reactive T cells that avoid negative selection and that they have a high avidity for self antigens (Schwartz *et al.* 2005). However, the thymic selection of regulatory T cells has been more thoroughly examined using a transgenic mouse model that enables the expression of the model "self" peptide to be modulated to achieve different levels of expression, which has led to a different interpretation of these findings.

While the proportion of $CD25^+$ immunoregulatory thymocytes bearing a transgenic TCR that recognises the model self peptide does increase with increasing expression of the model self peptide, it appears that this is an artefact of the negative selection of $CD4^+$ $CD25^-$ thymocytes (van Santen *et al.* 2004). In fact, the number of $CD25^+$ thymocytes is only slightly affected by the extensive negative selection of $CD4^+$ $CD25^-$ thymocytes that occurs with the expression of the model peptide. While the number of $CD25^+$ thymocytes bearing a transgenic TCR marginally increases when the model self peptide is expressed at high levels, this is accompanied by the same increase in the number of $CD25^+$ thymocytes not bearing the transgenic TCR, and is due to the non-specific effects of homeostatic proliferation induced by lymphopenia (Kawahata

et al. 2002, van Santen *et al.* 2004). Thus, the expression of cognate antigen has no specific effect on the numbers of regulatory T cells that appear in the thymus, and the "thymic selection" of regulatory T cells that has been described is an artefact of the differential sensitivity of regulatory T cells and conventional T cells to negative selection. In fact, it appears that $CD25^+$ thymocytes are approximately 3-fold less sensitive to negative selection compared to their $CD4^+$ $CD25^-$ thymocyte counterparts (van Santen *et al.* 2004). This finding suggests that regulatory T cells are unlikely to recognise antigen with high avidity, since high avidity interactions with self peptide MHC complexes are known to lead to negative selection (Sprent and Webb 1995). Indeed, we have shown that regulatory T cells have a low expression of CD5 (Figure 3.2.7), a molecule upregulated during the maturation of T cells in proportion to the avidity of the TCR-MHC interaction (Azzam *et al.* 1998), which supports the idea that regulatory T cells do not recognise self with high avidity. Furthermore, recent work has shown that regulatory T cells are not strongly autoreactive and in fact recognise non-self antigens with high avidity (Pacholczyk *et al.* 2007). Indeed, the mechanism of regulatory T cell suppression can be triggered by peptides that are only weakly cross-reactive with the TCRs of regulatory T cells (Larkin et al. 2007), implicating low avidity recognition of self antigen by regulatory T cells. It may be that regulatory T cells recognise highly prevalent self-antigens with low avidity, and are rendered anergic through the serial engagement of their TCR by these antigens. Supporting this idea, the differentiation of regulatory T cells appears to be dependent on the expression of MHC II molecules, suggesting that TCR-MHC interactions may play a role in the induction of Foxp3 (Fontenot *et al.* 2005).

We hypothesised that regulatory T cells may require continual replenishment from the thymus due to their pro-apoptotic nature (Taams *et al.* 2001) and thus be closely associated with RTE. The rat is an ideal model for the study of RTE due to the specific expression of Thy-1 (CD90) by this population, which is downregulated as RTE mature in the periphery (Douglas 1972). In contrast, the expression of Thy-1 occurs on all T cells in mice, while in humans it is only found on a minority of thymocytes, confounding its use in the investigation of RTE (Kroczek *et al.* 1986). When we examined the expression of Thy-1 by regulatory T cells with that of resting

$CD4^+$ T cells, we found that a higher proportion of $CD25^+$ $Foxp3^+$ regulatory T cells expressed Thy-1 than resting T cells (Section 4.2.1), and furthermore, the negative peak of Thy-1 expression on $CD25^+$ $Foxp3^+$ regulatory T cells had a higher MFI than resting $CD4^+$ T cells, suggesting that unlike resting T cells they do not have time to fully downregulate Thy-1 before they expire. Thy-1 expressing regulatory T cells and resting T cells were confirmed to be RTE as they rapidly disappeared following thymectomy (Section 4.2.2). Interestingly, a lower proportion of $CD25^-$ $Foxp3^+$ regulatory T cells expressed Thy1 compared with their $CD25^+$ counterparts, which we have shown is likely due to the unidirectional differentiation of $CD25^+$ $Foxp3^+$ regulatory T cells to a $CD25^-$ $Foxp3^+$ regulatory T cell phenotype (Section 5.2).

In addition, a large proportion of $CD25^+$ $Foxp3^+$ regulatory T cells expressed the CD8α homodimer, which is known to be expressed by some RTE but also by other T cells following activation (Kenny *et al.* 2004). Strikingly, we found that in fact CD8α^- RTE are found exclusively within the $CD25^+$ $Foxp3^+$ regulatory T cell population, and that the remainder of CD8α^- cells are mainly located within the resting $CD4^+$ T cell pool and are not RTE (Section 4.2.2). Furthermore, and contrary to our expectations it was found that CD8α is exclusively upregulated by $Foxp3^+$ RTE only after they have entered the periphery, possibly as a result of contact with self antigens (Section 4.2.3). The finding that regulatory T cells express the CD8α homodimer is also interesting considering that the now discredited suppressor T cells were described as expressing the CD8α chain (Lyt 2) along with the CD8β chain (Lyt 3), which we have found is not expressed by regulatory T cells (Ishii *et al.* 1981).

The expression of the CD45RC isoform, which is absent on both RTE and memory T cells did not occur on the majority of both $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ T cells, while most resting T cells were of a $CD45RC^+$ naïve T cell phenotype. Following thymectomy, it was found that approximately half of the $CD25^+$ $Foxp3^+$ regulatory T cells that were $CD45RC^-$ disappeared, suggesting that half of this population is comprised of RTE, and the remainder may be antigen experienced cells (Section 4.2.2). However, none of the $CD25^-$ $Foxp3^+$ regulatory T cells appeared to be RTE, probably because they are older than their $CD25^+$ $Foxp3^+$precursors. It was found

that unlike other RTE, which upregulate CD45RC as they differentiate towards naïve T cells in the periphery, the upregulation of CD45RC was inhibited in Foxp3$^+$ RTE, possibly due to antigen recognition.

The close association of CD25$^+$ Foxp3$^+$regulatory T cells with RTE was further emphasised by the high proportion of regulatory T cells among RTE soon after their entry into the periphery as determined using an intrathymic labelling approach to identify RTE (Section 4.2.3). It was hypothesised that this effect may be due to the rapid proliferation of regulatory T cells, so this possibility was tested by administering a series of BrdU injections over the first 24 h following intrathymic labelling. Interestingly, proliferation was found to be insufficient to account for the increases in the proportion of Foxp3$^+$ cells in the first 24 h of RTE entry into the periphery. Therefore, the potential for Foxp3$^-$ RTE to convert into RTE was assessed by adoptively transferring either whole unfractionated thymocytes, or thymocytes depleted of Foxp3$^+$ cells into congenic hosts, and measuring the proportion of Foxp3$^+$ donor cells that could be recovered. It was found that while the proportion of donor cells remained stable for several weeks, an increase in the proportion of donor cells that expressed Foxp3 was observed in recipients of both whole unfractionated thymocytes and thymocytes depleted of regulatory T cells. Furthermore, a similar rate of increase was observed between the two groups, indicating that the depletion of regulatory T cells from thymocyte cell suspensions prior to adoptive transfer was not affecting the increase in the proportion of regulatory T cells. Thus it appears that the conversion of some Foxp3$^-$ RTE into regulatory T cells may account in part for the close association of RTE with regulatory T cells. The interpretation of these findings is limited however by the heterogenous nature of donor thymocytes used, which may be at differing stages of maturity and thus converting into regulatory T cell more asynchronously than the cohort of RTE we studied using intrathymic labelling. This limitation may be reflected in the considerable difference in timing and possibly the magnitude observed between the increases in the proportion of Foxp3$^+$ cells among RTE (within 24 h), and the increases in the proportion of Foxp3$^+$ cells among donor thymocytes (which occurred between 2 and 14 days following adoptive transfer). Due to these limitations, it is not possible to determine the extent to which the conversion

of Foxp3$^-$ RTE into regulatory T cells can account for the high proportion of Foxp3$^+$ cells amongst RTE. The high proportion of regulatory T cells among RTE may also reflect either the transient expression of Foxp3 by some RTE as they encounter cognate antigen in the periphery, or the genuine conversion of a large proportion of RTE into regulatory T cells upon antigen contact that soon perish by apoptosis.

8.2 Regulatory T cells bear markers of failed activation, but proliferate vigorously *in vivo*.

Regulatory T cells bear many phenotypic and functional characteristics associated with anergic T cells, which are T cells that have recognised antigen in the absence of costimulatory signals and have become refractory to activation.
Regulatory T cells contain artefacts typical of failed activation including impaired protein kinase C activity and defects in Ras, MEK and ERK signal transduction pathways downstream of the TCR (Li *et al.* 2005, Hickman *et al.* 2006). Indeed, the molecular basis of their dependency on IL-2 has the same molecular basis as for anergised T cells, their expression of the cyclin dependent kinase inhibitor $p27_{kip}$, which arrests them the G_1 phase of the cell cycle (Kubsch *et al.* 2003). Exogenous IL-2 enables regulatory T cells to downregulate $p27_{kip}$, express cyclin E and cyclin A, and proliferate (Li *et al.* 2005). Furthermore, by increasing the stimulation of signal transduction pathways downstream of the TCR, the anergic state of regulatory T cells can be broken, resulting in their production of IL-2 and proliferation (Hickman *et al.* 2006). Regulatory T cells share the expression of many distinctive cell surface molecules including CD25, CTLA-4, TGF-β receptor 1 and IL-10 with anergised T cells (Taams *et al.* 1998, Lechner *et al.* 2001, Knoechel *et al.* 2006). Interestingly, anergised T can inhibit the proliferation and effector functions of other T cells, which they achieve by reducing the capacity of APCs to deliver stimulatory signals (Marelli-Berg *et al.* 1997, Kubsch *et al.* 2003, Frasca *et al.* 2003). However, the mechanism of regulatory T cell suppression remains unclear. In order to investigate the hypothesis that regulatory T cells may represent T cells that have been anergised *in vivo*, the phenotype of regulatory T cells was investigated using flow cytometry to determine if

they bear phenotypic features of failed activation. Indeed, when we assessed the expression of the TCR and its co-receptor CD4 by regulatory T cells, we found that they were expressed at significantly lower levels compared with resting T cells (Section 3.2), which is a characteristic feature of anergised T cells (Jordan *et al.* 2000). The ligation of the TCR induces its degradation in the absence of T cell activation through a process that involves the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ (D'Oro *et al.* 1997, von Essen *et al.* 2004). However, activated T cells are able to recycle internalised TCR complexes and return them to the cell surface following the phosphorylation of the CD3γ chain and subsequent activation of protein kinase C (Salio *et al.* 1997, Dietrich *et al.* 1998). The loss of TCR expression and its coreceptor CD4 from the surface of anergised T cells (Taams *et al.* 1999) is likely to be the result of the degradation of the entire TCR signalling complex, which is not separated into its individual components as it is internalised and enters the lysosomal degradation pathway (von Essen *et al.* 2004). Therefore, since regulatory T cells bear distinctive features of anergised T cells, we decided to investigate their proliferation and turnover *in vivo*, before extending these studies further by determining if regulatory T cells share their mechanism of suppression with anergised T cells.

In order to determine the proliferation characteristics and lifespan of regulatory T cells, their expression of the Ki67 proliferation-associated antigen, which is expressed by cells in all phases of the cell cycle except the resting G_o phase (Gerdes *et al*.1983), was analysed. We developed a technique enabling the simultaneous detection of surface CD25, intracellular Foxp3 and the intracellular Ki67 antigen for subsequent flow cytometry analysis. Using this technique, we determined that approximately a third of both $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ regulatory T cells were in cell cycle in the lymph nodes, a smaller proportion compared to effector T cells, but a significantly greater proportion compared with resting T cells (Section 5.2.1). It has been reported that the half-life of regulatory T cells of the phenotype $CD45RO^+$ $CD25^{high}$ is approximately 8 days in humans (Vukmanovic-Stejic *et al.* 2006). However, these results were obtained using narrowly defined phenotype that only includes a minority of the regulatory T cell population and may not therefore be representative. Regulatory T cells are predominantly $CD45RO^+$ in adult humans,

although a functionally suppressive $CD45RA^+$ population (equivalent to $CD45RC^+$ in rats) persists throughout adult life (Seddiki *et al*. 2006). In addition, while $CD25^{high}$ cells are enriched for regulatory T cells, some regulatory T cells do not express CD25 at high levels or at all (Figure 3.2.2, Sather *et al.* 2007).

Therefore, we developed a technique enabling the simultaneous detection of cell surface CD25, intracellular Foxp3 and BrdU incorporated into the DNA of dividing cells. Using this approach, the $CD25^+$ $Foxp3^+$ regulatory T cell subset was found to have an approximate half life of ~18 days, similar to that of activated T cells (~17 days) and much shorter than that of resting T cells (~3 months) (Section 5.2.5). Furthermore, $CD25^+$ $Foxp3^+$ regulatory T cells were shown to differentiate into $CD25^-$ $Foxp3^+$ regulatory T cells, which were rapidly dividing with a half life of ~7 days. Thus, our findings indicate that the overall half-life of regulatory T cells is closer to ~25 days, contradicting the results obtained by Vukmanovic-Stejic *et al.* (2006), that suggest a half life of approximately 8 days.

8.3 The differentiation of regulatory T cells and their trafficking *in vivo*

Regulatory T cells emerge into the periphery from the thymus amongst $Foxp3^-$RTE, some of which appear to also convert into regulatory T cells. Interestingly, while the majority of regulatory T cells in the thymus and amongst RTE express CD25, it has been reported that $CD25^-$ $Foxp3^+$ cells can differentiate into $CD25^+$ $Foxp3^+$ regulatory T cells *in vivo* (de Lafaille *et al.* 2004), apparently contradicting our findings that $CD25^+$ $Foxp3^+$ regulatory T cells are more closely associated with RTE compared to their $CD25^-$ counterparts (Section 4.2).

The conversion of polyclonal $CD4^+$ $CD25^-$ T cells into $CD4^+$ $CD25^+$ regulatory T cells appears to only occur under certain conditions. A proportion of $CD4^+$ $CD25^-$ T cells readily convert into regulatory T cells when transferred into newborn wild type mice,

but this is not observed when the cells are transferred into adult wild type recipients (de Lafaille *et al.* 2004). However, when the adult recipients are transgenic animals that express a monoclonal transgenic TCR, conversion is observed, and when the adult recipients are lymphopenic animals, the conversion of $CD4^+$ $CD25^-$ T cells into $CD4^+$ $CD25^+$ regulatory T cells occurs even more readily (de Lafaille *et al.* 2004). Thus, it appears that the conversion of $CD4^+$ $CD25^-$ T cells into $CD4^+$ $CD25^+$ regulatory T cells only occurs under lymphopenic conditions, and is associated with homeostatic expansion.

In order to determine the normal direction of regulatory T cells differentiation under physiological conditions, we adoptively transferred genetically marked, pure $CD4^+$ $CD25^{bright}$ T cells expressing Foxp3 into a normal, lymphocyte replete congenic host. It was hypothesised that we would observe a uni-directional differentiation of these cells into $CD25^-$ $Foxp3^+$ cells on the basis of several observations. We have shown that the majority of regulatory T cells in the thymus express CD25 (Section 3.2), and that $CD25^+$ $Foxp3^+$ regulatory T cells are closely associated with RTE, while $CD25^-$ $Foxp3^+$ regulatory T cells are not (Section 4.2). Furthermore, although $CD25^-$ $Foxp3^+$ T cells appear to proliferate rapidly, they do not lose their incorporated BrdU label at a rapid rate, suggesting that another cell population continually replenishes them (Section 5.2). Indeed, following adoptive transfer we observed that $CD4^+$ $CD25^{bright}$ T cells steadily lost CD25 expression in a linear fashion, such that by 25 days following adoptive transfer, less than 20% of the donor cells still expressed CD25, and the majority of donor cells were of the $CD25^-$ $Foxp3^+$ phenotype. Interestingly, the blood contained a higher proportion of donor cells of the $CD25^-$ $Foxp3^+$phenotype, suggesting that they may egress from the lymph nodes, which is also indicated by their lower expression of CD62L and higher expression of LFA-1 compared with their $CD25^+$ precursors (Section 3.2).

In order to determine the destinations to which recirculating $CD25^-$ $Foxp3^+$regulatory T cells recirculate to, we obtained recirculating lymphocytes by intercepting them as they drained through the thoracic duct towards the bloodstream. After adoptive

transfer of unfractionated recirculating lymphocytes into a normal congenic host, it was found that $CD25^+$ $Foxp3^+$ regulatory T cells mainly localised to secondary lymphoid tissue, consistent with their high expression of CD62L. In contrast, donor $CD25^-$ $Foxp3^+$ regulatory T cells had selectively migrated into the lamina propria, where host $CD25^-$ $Foxp3^+$ regulatory T cells were also enriched (Section 3.2). This selective migration occurred despite their low expression of CD103, which binds to E-cadherin exclusively expressed in the gut epithelium, and the much higher expression of CD103 by their $CD25^+$ counterparts, which were present in the lamina propria in much smaller numbers. Therefore, we hypothesised that $CD25^-$ $Foxp3^+$ regulatory T cells represent a tissue homing subset of regulatory T cells, which may localise to sites of inflammation to regulate the immune response. In order to test this hypothesis, we adoptively transferred recirculating lymphocytes from normal animals into animals that were experiencing adoptively transferred arthritis. However, although the donor regulatory T cells migrating into the lamina propria were still mostly of the $CD25^-$ $Foxp3^+$ phenotype, the majority of donor regulatory T cells infiltrating the SRT expressed CD25. This finding may either reflect the genuine recruitment of $CD25^+$ $Foxp3^+$ regulatory T cells, which are relatively sessile, into the SRT, or it may be due to the re-expression of CD25 by infiltrating $CD25^-$ $Foxp3^+$ regulatory T cell subset, which is known to occur in the presence of antigen stimulated T cells (de Lafaille *et al.* 2004). Interestingly, $CD25^-$ $Foxp3^+$ regulatory T cells were consistently found to be enriched within the population of cells that can recirculate back to the thymus, where they comprise up to 50% of the donor cells that return to the thymus. Thus, $CD25^-$ $Foxp3^+$ regulatory T cells may also play a role in modulating thymic function.

8.4 The suppressive mechanism of regulatory T cells

It was originally proposed that the suppressive activity associated with regulatory T cells might be associated with their expression of CD25, through the sequestration of IL-2 produced by activated T cells (Thornton and Shevach 1998). Alternatively, has been proposed that regulatory T cells might inhibit other T cell proliferation through

the secretion of humoral inhibitory factors such as IL-10 and TGF-beta (Nakamura *et al.* 2004). However, *in vitro* assays have revealed a requirement for regulatory T cells to physically interact with antigen presenting cells for inhibition to be observed, indicating that soluble factors do not play a role in the mechanism of regulatory T cell suppression (Takahashi *et al.* 1998). This finding has led many groups to speculate that membrane-bound TGF-beta on the surface of regulatory T cells may act to inhibit the proliferation of other T cells *in vivo* (Nakamura *et al.* 2004). Although some studies have shown that the effector functions of colitogenic T cells are inhibited in the presence of TGF-beta, this phenomenon is not associated with membrane bound TGF-beta (Kullberg *et al.* 2005).

Regulatory T cells constitutively express the inhibitory costimulatory molecule CTLA-4, which can bind to CD80 and CD86 costimulatory molecules typically found on the surface of APCs. However, since T cells can also acquire CD80 and CD86 following stimulation (Sabzevari *et al.* 2001), the direct interaction of CTLA-4 expressed by regulatory T cells with CD80 or CD86 expressed by other T cells may deliver an inhibitory signal that can curtail T cell activation (Miyara and Sakaguchi 2007). Alternatively, the ligation of CTLA-4 may induce the production of the enzyme indolamine 2,3-dioxygenase which catalyses the conversion of the amino acid tryptophan into kynurenine and other cytotoxic metabolites that might kill proliferating T cells (Fallarino *et al.* 2006). While some research has indicated that the engagement of CD80 or CD86 on effector T cells by regulatory T cells expressing CTLA-4 can prevent autoimmune disease (Paust *et al.* 2004), other studies have shown that T cells not expressing CD80 or CD86 remain highly susceptible to regulation by regulatory T cells *in vitro*, suggesting that CTLA-4 does not participate in the suppressive mechanism of regulatory T cells (Takahashi *et al.* 2000, May *et al.* 2007).

Alternatively, the mechanism of regulatory T cell suppression may involve lymphocyte activation gene 3 (LAG-3), a molecule that is upregulated on the surface of regulatory T cells following TCR stimulation and binds MHC class II molecules (Huang *et al.* 2004). LAG-3 displayed by regulatory T cells has been proposed to inhibit the

proliferation of effector T cells by binding MHC II molecules presented by APCs to the effector T cells to interfere with their activation (Miyara and Sakaguchi 2007). However, it is unlikely that molecules on the surface of regulatory T cells would be able to access the synapse between APCs and other T cells, due to the much greater size of leukocytes compared to the molecules they express on their plasma membrane. Alternatively, LAG-3 may deliver signals to APCs that reduce their ability to activate T cells. However, its role in the suppression of T cell proliferation by regulatory T cells appears to be dispensable as LAG-3 deficient animals do not spontaneously develop autoimmune disease (Huang *et al.* 2004). Thus, despite intensive investigations, the mechanism of regulatory T cell suppression has remained poorly defined. In order to address this issue, we have adopted an alternative strategy based on the extensive similarities between regulatory T cells and anergic T cells. Although it is continually asserted in the literature that regulatory T cells and anergised T cells are different types of T cells (Kuniyasu *et al.* 2000, Knoechel *et al.* 2006), there is no evidence to support this. We therefore investigated the possibility that regulatory T cells may possess a similar mechanism of suppression to that which has been described for anergised T cells (Marelli-Berg *et al.* 1997, Kubsch *et al.* 2003, Frasca *et al.* 2003).

Anergised T cells are T cells that have become refractory to activation following their encounter with antigen in the absence of costimulatory signals. Due to the defects in the signal transduction machinery downstream of the TCR, anergised T cells are unable to provide survival signals such as CD154 to APCs upon antigen presentation (Bowen *et al.* 1995, Koppi *et al.* 1997). As a consequence of this, the maturation and expression of costimulatory molecules by APCs is impaired, and their capacity to stimulate other T cells is lost (Frasca *et al.* 2003). Interestingly, regulatory T cells have been shown to inhibit the maturation of DC and their expression of the costimulatory molecules CD80 and CD86 (Cederbom *et al*. 2000, Houot *et al.* 2006, Veldhoen *et al.* 2006, Onishi *et al.* 2008). Furthermore, there is evidence that the ligation of CD154 to CD40 expressed on the surface of immature DC prevents regulatory T cells from being able to exert their suppressive activities (Serra *et al.* 2003).

Therefore, a multi-stage *in vitro* assay was designed to determine if the mechanism of regulatory T cell suppression involves their modification of DC to reduce their capacity to stimulate other T cells. This system involved treating DC either with regulatory T cells, conventional T cells, or leaving them untreated, before reisolating them and testing their ability to activate allogeneic T cells. It was found that there was little difference in the suppression observed in cultures in which DC were treated with regulatory T cells prior to the addition of responder T cells, compared with the suppression observed in cultures in which regulatory T cells remained in the culture for the duration of the experiment, and found little indication of direct regulatory T cell-responder T cell inhibitory mechanisms (Section 6.2). This finding is supported by information obtained using new imaging techniques that are capable of observing leukocyte interactions *in vivo.*

Strikingly, no stable association of regulatory T cells with responder T cells has been observed during active suppression *in vivo,* which is characterised by persistent interactions between regulatory T cells and DC that inhibit stable interactions between responder T cells and DC (Tang *et al.* 2005, Tadokoro *et al.* 2006). These observations strongly suggest that regulatory T cells may act by interfering with the activation of other T cells, and that this probably occurs through signals delivered to DC. In order to identify these signals, we isolated regulatory T cell conditioned DC, and compared them with DC that were conditioned with $CD25^-$ conventional T cells and also untreated DC by differential gene expression analysis using a microarray to identify putative molecular targets of regulation.

Strikingly, 10 predicted serine/threonine kinases were expressed at 2-3 fold higher levels in DC treated with regulatory T cells compared to untreated DC and DC treated with conventional T cells (Section 6.2). The increased expression of these serine/threonine kinases following the treatment of DC with regulatory T cells suggests that a novel signal transduction pathway may be established in DC that may limit their ability to activate other T cells. Furthermore, an additional a putative signalling protein (Entrez ID 679206) predicted to contain both a FERM domain and a Pleckstrin-like domain was found to be upregulated in DC treated with regulatory T

cells. The presence of these domains suggests that the protein is a signalling protein located at the plasma membrane, and its induced expression following the treatment of DC with regulatory T cells suggests that it may be involved in the functional modification of DC by regulatory T cells.

Interestingly, it was found that the expression of E2F transcription factor 5 was reduced approximately 2-fold following the treatment of DC with regulatory T cells (Section 6.2). This transcription factor is associated with the regulation of cell cycle progression, which it achieves by making entry into the cell cycle dependent on the activation of particular signal transduction cascades (DeGregori *et al.* 2006). Although the highly context-dependent nature of E2F transcription factor 5 activity limits the interpretation of its downregulation following the treatment of DC with regulatory T cells, it may be important for capacity of DC to stimulate T cells and should be examined in future functional analyses.

The expression of a novel CD163-like molecule (Entrez ID 293591) was increased more than two fold in DC treated with regulatory T cells compared to untreated DC or DC treated with conventional T cells. It was found that this molecule had a very similar composition to CD163, which mediates the capture of haemoglobin-haptoglobin complexes that form following the lysis of haemoglobin-rich erythrocytes to limit the oxidation of tissues by haemoglobin (Moestrup and Moller 2004). The metabolism of haemoglobin following its capture by CD163 releases anti-inflammatory metabolites and induces IL-10 secretion by the monocytes and macrophages that express CD163 (Philippidis *et al.* 2004). Therefore, the expression of the CD163-like molecule by DC following treatment with regulatory T cells may reflect a transition towards a more phagocytic and anti-inflammatory lifestyle.

The expression of the Fas antigen was reduced following the treatment of DC with regulatory T cells (Section 6.2). Although Fas antigen is capable of attenuating T cell proliferation and survival, this is due to the induction of T cell apoptosis following activation, which is not a feature of the mechanism of regulatory T cell suppression (Miyara and Sakaguchi 2006). The finding that Fas antigen is expressed at lower

levels following the treatment of DC with regulatory T cells is unlikely to have any relevance to the mechanism of regulatory T cell suppression. It was also found that programmed death ligand-1 was expressed at lower levels by DC following treatment with regulatory T cells. While programmed death ligand-1 is capable of inhibiting weak costimuatory signals, its potency is insufficient to account for the inhibition of T cell proliferation associated with regulatory T cells. Furthermore, its lower expression on DC exposed to regulatory T cells makes the likelihood of its relevance to the mechanism of regulatory T cell suppression remote.

8.5 The effect of ageing on the thymic output of regulatory T cells and implications for the pathogenesis of autoimmune disease

The process of ageing is accompanied by a reduction in the rate of T cell production by the thymus, eventually culminating in the contraction in the size of the T cell pool. The decline in thymic productivity in humans occurs due to extensive structural changes that are associated with the gradual replacement of the thymus cortex and medulla with connective and adipose tissues that invade from the perivascular spaces, such that areas of productive thymic tissue become isolated over time (Gruver *et al.* 2007). In contrast, we observed that in rodents thymic involution is only associated with mild increase in disorganisation, and a pronounced reduction in the size of the thymus, with the accumulation of adipose tissues occurring outside the thymus itself (Section 7.2).

Thymic involution was originally thought to result from a genetically preprogrammed mechanism, as thymi from young animals involute at a steady rate whether transplanted into aged matched young recipients or much older animals (Bodey *et al.* 1997). However, when aged thymi are transplanted into young recipients they become structurally and functionally rejuvenated, suggesting that factors external to the thymus induce thymic involution (Nobori *et al.* 2006). Certainly the character of T cells in the periphery changes with ageing, which is associated with the accumulation of $CD25^{-}$ $Foxp3^{+}$ T cells and a declining ability of T cells to proliferate (Nishioka *et al.* 2006). Thus the more immunosuppressive nature of T cells in older animals, some of

which may re-enter the thymus may be one factor that promotes thymic involution with ageing.

T cells are known to cause significant thymic involution with ageing through their display of the Fas ligand to thymic epithelial cells, which induces their apoptosis and so reduces the productivity of thymic tissue. In the absence of Fas pathway, age related thymic involution does not occur (Yajima *et al.* 2004). Fas ligand is predominantly expressed by activated T cells (Nagata and Golstein 1995), a proportion of which recirculate back to the thymus soon after activation and induce apoptosis in thymic epithelial cells (Hardy *et al.* 2001, Yajima *et al.* 2004). In addition, thymic involution can be expedited by Jagged-1 expressed on the surface of T cells, which also induces apoptosis in thymic epithelial cells (Beverly *et al.* 2006). The recirculation of regulatory T cells back to the thymus may also contribute to thymic involution, as it is known that they are capable of killing autologous T cells, monocytes and DC through perforin pathway (Grossmann *et al.* 2004). Interestingly, the population of T cells that recirculate back to the thymus is enriched for $CD25^-$ $Foxp3^+$ regulatory T cells, which may interact with thymic epithelial cells to affect thymic involution (Section 3.2). In addition to cellular mediators of thymic involution, several soluble mediators of thymic involution have been identified, including IL-6, leukaemia inhibitory factor, and oncostatin M (Gruver and Sempowski 2008).

Interestingly, although thymic involution was accompanied by a reduction in the absolute number of thymocytes, the number of $Foxp3^+$ regulatory T cells found in the thymus was not affected (Section 7.2). These $Foxp3^+$ regulatory T cells were predominantly located in the medulla, which contains newly forming T cells at an advanced stage of maturity, and T cells that have recirculated back to the thymus (Westermann *et al.* 1996). A clearly higher concentration of $Foxp3^+$ cells in the thymic tissue of aged rats was observed compared with their younger counterparts, although this may not reflect a shift towards the production of regulatory T cells in old age, since there was no increase in the proportion of Thy-1^+ RTE that expressed Foxp3 in the periphery. Furthermore, the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells amongst $CD4^+$ T cells in the periphery was found to decline with ageing, suggesting

that an increased influx of regulatory T cells from the thymus does not occur in older animals. Alternatively, the high concentration of regulatory T cells in the thymuses of aged rats may reflect the increased re-entry of T cells from the periphery (Hale *et al.* 2006), which we have shown are enriched for $CD25^-$ $Foxp3^+$ regulatory T cells (Section 3.2).

The process of ageing was found to be accompanied with a decline in the proportion of RTE among $CD4^+$ T cells, and a decline in the proportion of $CD25^+$ $Foxp3^+$ regulatory T cells, which are associated with RTE. However, the proportion of $CD25^-$ $Foxp3^+$ regulatory T cells, which differentiate from $CD25^+$ $Foxp3^+$ regulatory T cells increases steadily with ageing, resulting in an increase in the proportion of $Foxp3^+$ cells amongst $CD4^+$ T cells in the periphery. This process may reflect a mechanism to compensate for the reduction in thymic output that occurs with ageing. Regulatory T cells may be maintained in aged rats through the conversion of cells from the $CD25^+$ $Foxp3^+$ regulatory T cell population to the tissue homing $CD25^-$ $Foxp3^+$ regulatory T cell population.

8.6 Future Prospects

The close association of $CD25^+$ $Foxp3^+$ regulatory T cells with RTE that we characterised was demonstrated by their expression of markers associated with RTE that disappeared following thymectomy, and by the high proportion of regulatory T cells amongst RTE. Although our investigations into the expression of Foxp3 by RTE indicated that some conversion of $Foxp3^-$ RTE into regulatory T cells is likely, it was not possible to accurately determine to what extent these cells contribute to the total population of regulatory T cells. The use of thymocytes in place of RTE limited our interpretation of these experiments due to the heterogenous maturation state of the thymocytes, which confounded comparisons between them and the more homogenous cohort of RTE due to the delayed and asynchronous kinetics of conversion following the adoptive transfer of thymocytes. In order to overcome these difficulties, it may be possible to use a transgenic model in which RTE express one

fluorescent tag (Boursalian *et al.* 2004) and regulatory T cells express another (Wan and Flavell 2005). In such a situation, it would be feasible to isolate Foxp3$^-$ RTE in large enough numbers by flow sorting to be able to observe their phenotype after adoptive transfer into a wild-type host, to determine what proportion of them may convert into regulatory T cells.

We determined that CD25$^+$ Foxp3$^+$ regulatory T cells generate rapidly dividing CD25$^-$ Foxp3$^+$ regulatory T cells which migrate into the peripheral tissues. Although the profile of adhesion molecules expressed by these two subsets correlated well with their behaviour *in vivo,* it is likely that their expression of chemokine receptors may provide further useful information. While the progressive loss of CD25 expression during the maturation of regulatory T cells provided a useful phenotypic difference that could be used to identify CD25$^-$ Foxp3$^+$ tissue-homing regulatory T cells under normal conditions, we found that the regulatory T cells that infiltrated inflamed tissue had the phenotype CD25$^+$ Foxp3$^+$. Thus it was not clear whether this observation reflects the re-acquisition of CD25 expression by CD25$^-$ Foxp3$^+$ tissue-homing regulatory T cells upon their arrival in the inflamed tissue, or the genuine recruitment of CD25$^+$ Foxp3$^+$ regulatory T cells, which we have shown are relatively sessile. Thus, the expression of chemokine receptors by regulatory T cells subsets may enable a more reliable method of identifying subsets of tissue homing regulatory T cells that may avoid the problem of the potential re-expression of CD25 by CD25$^-$ Foxp3$^+$ regulatory T cells (de Lafaille *et al.* 2004).

In order to determine whether the appearance of CD25$^+$ Foxp3$^+$ regulatory T cells in inflamed tissues is the result of the re-expression of CD25 by infiltrating CD25$^-$ Foxp3$^+$ regulatory T cells, a transgenic system could be used in which regulatory T cells can be identified through their expression of a fluorescent marker under the control of the endogenous Foxp3 promoter (Wan and Flavell 2005). Using this system, the isolation of CD25$^-$ Foxp3$^+$ cells by flow sorting provide considerably larger yields than the *in vivo* maturation system we used to study these cells. Thus, it would be feasible to transfer these purified cells into a congenic host affected by adoptively

transferred arthritis and determine if they indeed re-acquire CD25 expression following their entry into the inflamed SRT.

The considerably more rapid proliferation of both $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ regulatory T cells compared with other $CD4^+$ T cells subsets is a useful finding that may enable their neutralisation with the use of reagents such as cyclophosphamide, which may be beneficial to patients suffering from cancer. Indeed, regular low doses of cyclophosphamide in cancer patients that were administered to decrease tumour angiogenesis have been shown to selectively reduce the numbers of circulating regulatory T cells and release both natural killer cells and cytotoxic T cells from suppression to enable the killing of tumour cells (Ghiringhelli *et al.* 2006).

The accumulation of regulatory T cells that we observed with ageing was an unexpected finding, as we had determined that $CD25^+$ $Foxp3^+$ regulatory T cells were closely associated with RTE, and we observed an age related decline in the productivity of the thymus. However, the accumulation of regulatory T cells that we observed was due to an increase in the proportion of $CD25^-$ $Foxp3^+$ regulatory T cells, which are not closely associated with RTE. We did not find any evidence of spontaneous autoimmune disease in older animals in our histological analyses of susceptible organs, and so it appears that the conversion of $CD25^+$ $Foxp3^+$ regulatory T cells into $CD25^-$ $Foxp3^+$ regulatory T cells may compensate for the slowing thymic output of regulatory T cells that occurs with ageing. However, the continued thymic output of some regulatory T cells in aged rats may not occur at a sufficient rate in humans, which become more susceptible to autoimmune disease with ageing. Therefore, future research involving human subjects would be valuable, and it may be possible to develop novel strategies for combating autoimmune disease, through the administration of cytokines such as IL-7, Keratinocyte growth factor, thymic stromal lymphopoetin, human growth hormone or leptin, which stimulate thymopoesis and may enable the replenishment of regulatory T cells and lead to the suppression of autoimmune disease (Gruver *et al.* 2007).

The mechanism of regulatory T cell suppression has been persistently pursued by researchers in the hope of identifying its molecular basis, which may enable the generation of new drugs capable of emulating their action (Game *et al.* 2008). Cellular based therapies involving the use of regulatory T cells are being developed that involve the rapid *in vitro* expansion of regulatory T cells obtained from patients that can then be returned to them to treat autoimmune disease (Tang *et al.* 2004), but this approach is limited by the need to make separate preparations of cells for each patient, which is time consuming and labour intensive. Alternatively, regulatory T cells can be expanded *in vivo* thorough the administration of a CD28 superagonist antibody, which would obviate the need for cellular based therapies (Lin and Hunig 2003). However, serious adverse effects in human subjects receiving anti-CD28 antibodies have been reported, which involved a “cytokine storm” similar to that observed in toxic shock syndrome, and the viability of this kind of approach is questionable (Suntharalingam *et al.* 2006). In order to investigate the mechanism of regulatory T cells suppression, a multi-stage *in vitro* assay was developed to determine if they act in a similar manner to anergised T cells, by conditioning APCs. We found that regulatory T cells do condition APCs to reduce their ability to activate other T cells, and identified putative molecular targets of regulation by differential gene expression microarray analysis.

The expression of 10 putative serine/threonine kinases was found to be higher in DC following their interaction with regulatory T cells. These novel kinases were predicted to contain the ATP-binding pockets, substrate binding pockets, activation and catalytic loops that are characteristic of serine/threonine kinases using a conserved domain finding BLAST provided by the NCBI server. In addition, another putative signalling molecule containing domains that suggest it is targeted to the apical side of the plasma membrane was upregulated. These 11 novel signalling molecules may form a signal transduction pathway that is involved in limiting the ability of APCs to activate T cells following interactions with regulatory T cells. Furthermore, the E2F transcription factor 5, which is thought to regulate cell cycle progression by making it dependent on specific signal transduction pathways was downregulated by DC following treatment with regulatory T cells. Although the highly context-dependent

nature of E2F transcription factor 5 activity complicates the interpretation of this finding, it is possible that the transcription factor may be required for the ability of DC to activate T cells and is targeted for downregulation by regulatory T cells. We also observed that the expression of a CD163-like molecule was induced following the treatment of DC with regulatory T cells, which may reflect a transition towards a more phagocytic anti-inflammatory role.

In order to validate these putative molecular targets of regulatory T cell suppression, further experiments that assess the functional contribution of each identified molecule to the mechanism of regulatory T cells suppression should be undertaken. These experiments may involve the use of small interfering RNA (siRNA) to inhibit the expression of molecules of interest by APCs, which could be then tested *in vitro* using a mixed lymphocyte reaction in order to assess the functional outcome.

8.7 Conclusions

In conclusion, we have shown that regulatory T cells bear characteristic features of failed activation, and may represent T cells that have been anergised *in vivo*. We have demonstrated that $CD25^+$ $Foxp3^+$ regulatory T cells are closely associated with RTE and they differentiate into $CD25^-$ $Foxp3^+$ regulatory T cells in the periphery while retaining their suppressive activity. The turnover of regulatory T cells was found to be rapid, and they are continually replaced by the thymus, which is consistent with the idea they may represent T cells that have been anergised through the recognition of self antigen. The thymic output of regulatory T cells continues into old age, where the conversion of $CD25^+$ $Foxp3^+$ regulatory T cells into $CD25^-$ $Foxp3^+$ T cells appears to compensate for the declining thymic output of $CD25^+$ $Foxp3^+$ regulatory T cells, in order to maintain the regulatory T cell pool. Using an *in vitro* assay we assessed whether regulatory T cells share their mechanism of suppression with that of anergised T cells, which reduce the ability of DCs to activate other T cells. It was found that regulatory T cells do condition DCs to reduce their stimulatory capacity,

and we identified several novel molecules as putative targets of regulatory T cell suppression in DCs by differential gene expression microarray analysis. These putative molecular targets of regulation represent prime targets for rational drug design, in order to generate compounds that emulate the biological activity of regulatory T cells for the treatment of autoimmune disease. Such an approach may overcome the problems encountered by *in vivo* regulatory T cell expansion strategies, and the current limitations of cellular replacement therapies for the treatment of autoimmune disease.

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