THE ROLE OF FOXP3^{+} REGULATORY T-CELLS IN TRANSPLANT TOLERANCE

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I dedicate this thesis to my darling wife, Katie.

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Abbreviations

A1.RAG	A1(M).RAG1$^{-/-}$
Ag	Antigen(s)
APC	Antigen presenting cell
B6 mice	C57BL/6 mice
BHK	Baby Hamster Kidney cell line
bmDC	Bone marrow derived dendritic cells
BSA	Bovine serum albumin
CBA.RAG	CBA/Ca.RAG1$^{-/-}$
CD40L	CD40 ligand or CD154
CFSE	Carboxyflurescein diacetate succinimidyl ester
CTLA-4	Cytotoxic T lymphocyte Antigen 4
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal calf serum
Foxp3	Forkhead winged box protein 3
GM-CSF	Granulocyte macrophage colony stimulating factor
hCD2	Human CD2 protein
hCD52	Human CD52 protein
i.p.	Intraperitoneal
i.v.	Intravenous
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified dulbecco's medium
iTreg	Induced regulatory T cell (s)
KO	Gene knock out
LPS	Lipopolysaccharide (*E.coli* 0127:B8)
mAb	Monoclonal Ab(s)
MACS (Auto)	Magnetic cell sorting

Marilyn	Marilyn.RAG1$^{-/-}$
MHC	Major histocompatibility complex
nTreg	Natural Regulatory T cell(s)
PBS	Phosphate buffered saline
Pen-Strep	Penicillin-and Streptomycin antibiotics
RAG	Recombinase activating gene
RPMI	Roswell Park Memorial Institute
RT-QPCR	Real time quantitative PCR
s/n	Supernatant
TBAC	Tris-buffered ammonium chloride
TCR	T cell receptor
Teff	Effector T cell
TGF-β	Transforming growth factor-β (unless stated otherwise assume it refers TGF-β1)
Th	T helper cell
Th17	IL17 producing T helper
TIG	Therapeutic Immunology Group
TLR	Toll-like receptor(s)
Tris	Tris(hydroxymethyl)aminomethane
v/v	Volume/volume
w/v	Weight/volume

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Abstract

A major conceptual shift in immunology has been the recent discovery of regulatory T-cells (Treg), of which CD4^{+}Foxp3^{+} cells are already known to be essential to self-tolerance. Their role in transplant tolerance remains unproven due to the absence of a natural cell surface marker by which they can be manipulated in vivo. A transgenic B6.Foxp3^{hCD2} mouse was created to express an artificial GPI-anchored human CD2/CD52 surface fusion protein under the control of the Foxp3 promoter. Monoclonal antibodies directed against the human CD2 and human CD52 were used in B6.Foxp3^{hCD2} mice to isolate and ablate Foxp3^{+} Treg. CD4^{+}Foxp3^{+} cells were found to be crucial for transplant tolerance induced by non-ablative co-receptor and co-stimulatory blockade. In tolerant animals, Foxp3^{+} Treg are constantly required to suppress effector T-cells still capable of causing tissue damage. Remarkably, tolerated tissue contains T-cells capable of rejecting it, but these are prevented from doing so by therapeutically induced Foxp3^{+} Treg. Finally, induced Foxp3^{+} cells sustain tolerance by converting naive T-cells into the next generation of Foxp3^{+} cells in the periphery, providing one potential mechanism by which infectious tolerance may operate *in vivo*.

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CHAPTER 1: General Introduction

Introduction

The wealth of insight into the adaptive immune system towards the end of the 20th century was firmly founded in studies of inflammatory cells. So great was the emphasis on antigen specific effector cells that many other components, including cells involved in regulation, were at times forced to take a back seat. Inflammation may be conspicuously dramatic in a research setting, but for a co-ordinated multicellular organism, it is a damaging, energy sapping and potentially fatal process when allowed to continue without restraint. There is increasing evidence that regulatory T-cells (Treg) are integral in maintaining homeostasis by establishing the appropriate immunological set point for a given tissue. They provide a background tone of tolerance that helps determine how effector cells respond during those relatively uncommon times of infection, trauma or ischaemia.

The discovery that short-term antibody mediated blockade of T-cell signals could generate long-term tolerance has provided modern medicine with an opportunity to improve greatly the treatment of autoimmune diseases, transplant rejection and tumour immunity (Benjamin and Waldmann 1986; Qin, Cobbold et al. 1993; Waldmann and Cobbold 1998). Moreover, understanding and then re-directing the mechanisms by which the immune system self-regulates represents a conceptual shift in the way we approach disease management.

This thesis explores the crucial roles that $Foxp3^{+}CD4^{+}$ regulatory T-cells fulfil in transplant tolerance induced by co-receptor and co-stimulatory blockade.

1.1 An overview of immune tolerance

1.1.1 Central tolerance: positive and negative thymic selection of T-cells

Each developing thymocyte has a specific T-cell receptor (TCR) generated through a process of gene loci re-combination that has the potential to produce a huge variety of receptor specificities (Hedrick, Cohen et al. 1984; Hedrick, Nielsen et al. 1984; Yanagi, Yoshikai et al. 1984). Thymocytes that interact with peptide presented by the major histocompatibility complex (MHC) with moderate to high affinity are positively selected for further development in comparison to those that bind self MHC with low affinity (Zinkernagel and Doherty 1974; Zinkernagel, Callahan et al. 1978). However, so efficient is the adaptive immune system at generating receptors to potentially any antigenic determinant that TCR to self antigen can also be generated.

The first line of defence against these potentially auto-reactive immune cells is the thymus, which deletes self-reactive T-cells with high avidity T-cell receptors (TCRs) for self antigens; so called negative selection (Kappler, Roehm et al. 1987; Rocha and von Boehmer

1991). Thymic medullary cells have since been shown to express a wider range of organ specific antigens than previously thought, allowing the elimination of a greater number of self reactive T-cells. This pathway is dependent on the expression of *autoimmune regulator (Aire)* gene and loss of function mutations cause a rare, type 1 Autoimmune Polyendocrinopathy Syndrome (Anderson, Venanzi et al. 2002; Liston, Lesage et al. 2003).

Negative selection is an imperfect process and, even in healthy people, a significant proportion of auto-reactive T-cells escape. The fact that lymphocytes isolated from normal

Immune Tolerance

Central tolerance

Potentially auto-reactive lymphocytes are eliminated in central lymphoid tissue (e.g. thymus and bone marrow)

Peripheral tolerance

I*gnorance:* Functional and physical barriers that limit the access of auto-reactive lymphocytes to self antigen

Clonal deletion: T-cells in the periphery undergo apoptosis in response to recognition of cognate antigen under certain conditions

Anergy: Lymphocytes are rendered hyporesponsive after binding to antigen in the absence of co-stimulatory signals

Regulation: A subset of lymphocytes which actively prevent the activation of self reactive lymphocytes

Box 1: Overview of immune tolerance

healthy individuals can be activated by self antigen plus potent adjuvant argue in favour of additional barriers that can prevent autoimmunity; collectively called peripheral tolerance (Liblau, Tournier-Lasserve et al. 1991; Walker and Abbas 2002; Danke, Koelle et al. 2004). Understanding the factors that are critical to the integrity of peripheral tolerance has the potential to refine organ transplant therapy, the treatment of autoimmune disease and adjuvant cancer therapies.

1.1.2 General mechanisms of peripheral tolerance

A number of peripheral mechanisms exist to prevent auto-reactive cells that have escaped negative selection from damaging self tissue. In general, these include peripheral clonal deletion, induction of a state of clonal anergy, tissue specific barriers to the infiltration of activated immune cells (immune privilege) and active regulatory cells (Box 1).

Delivery of cognate antigen at high concentration was found to result in clonal deletion of mature T-cells (Webb, Morris et al. 1990; MacDonald, Baschieri et al. 1991; Zhang, Martin et al. 1992). The cells typically proliferated for a number of cycles before undergoing apoptosis. This process was found to be depend on intact, functional apoptotic pathways, suggesting that clonal deletion may be a cell intrinsic regulatory process aimed at preventing the continued survival of over-reactive cells (Strasser, Harris et al. 1991; Bouillet, Metcalf et al. 1999). On recognising cognate antigen, some T-cells have been shown to enter an alternative state of hypo-responsiveness characterised by a reduction in the rate of

proliferation and decreased production of IL-2 (Jenkins, Chen et al. 1990; Schwartz 1990; Lechler, Chai et al. 2001; Schwartz 2003). This state of anergy can be reversed for some cells by increased ectopic expression of IL-2. Several different methods of inducing anergy have been described and include TCR recognition of antigen without the necessary co-stimulatory signals, for example; in the absence of proliferative cytokines (DeSilva, Urdahl et al. 1991), in the presence of anti-inflammatory cytokines (Groux, Bigler et al. 1996) and following blockade of co-stimulatory signals (Judge, Wu et al. 1999). In addition, recognition of modified peptides (peptide partial agonists) was also shown to generate anergic cells (Sloan-Lancaster, Evavold et al. 1993), Collectively, they imply that in the absence of inflammatory conditions which would normally favour T-cell activation, cells can be diverted into a state of anergy (Chen, Cobbold et al. 2004). The relative role of anergic cells in transplant tolerance is explored further in Chapter 6.

The interest in tissue specific immune privilege historically stemmed from early observations of delayed rejection of foreign grafts when they were placed in sites such as the eye or the hamster cheek pouch. Even before the landmark paper on foetal allograft tolerance (Billingham, Brent et al. 1953), Medawar had found that skin grafts transplanted in the anterior chamber of the eye survived longer than those transplanted onto the flank of rabbits (Medawar 1948). Several sites have been shown to exhibit what was originally described as ‘immune privilege’ (Billingham and Boswell 1953) and these include, the ventricles and striatum of the brain, the placenta, the ovaries, the testes, the hamster cheek pouch and the eye (Mellor and Munn 2008). Of these, the eye is perhaps the best studied and for the purposes of this thesis demonstrates a number of key principles of tissue immune privilege.

Firstly, exposure of ocular antigen to potentially reactive T-cells is limited. The expression of MHC I by corneal and vascular endothelial cells is low (Wang, Kaplan et al. 1987), in keeping with the finding that $CD4^+$ cells, rather than $CD8^+$ Tc cells, are the chief cell mediator of corneal rejection (Ayliffe, Alam et al. 1992; Joo, Pepose et al. 1995). The cornea also lacks antigen presenting cells (APCs) expressing MHC II presented antigen (Streilein, Toews et al. 1979). This makes it less likely that activated T-cells will pass into the anterior chamber of the eye.

Factors within the anterior chamber have been shown to inhibit antigen driven T-cell proliferation and the release of inflammatory cytokines, so providing an additional functional barrier against any activated cells that do succeed in entering (Taylor, Streilein et al. 1992; Taylor, Streilein et al. 1994). Surprisingly, ocular tolerance of alloantigen injected into the anterior chamber can spread beyond the local environment to influence systemic tolerance, so called anterior chamber associated immune deviation (ACAID) proposed to involve a TGF-β dependent camero-splenic immune axis (reviewed in (Streilein 2003)).

1.1.3 Harnessing active mechanisms of peripheral tolerance

Pioneering work in the 1980's, in which a short course of non depleting anti-CD4 antibodies was shown to generate long term tolerance to foreign proteins, led to the development of multiple non depleting antibody regimens that could induce tolerance to minor and major histocompatibility mismatched graft tissue (Benjamin, Cobbold et al. 1986;

Gutstein, Seaman et al. 1986; Qin, Cobbold et al. 1987). Much of what is known about therapeutically induced tolerance has come from these types of transplantation studies, partly because they offer the practical advantage of control over a number of important factors. These include the timing of tolerance induction and subsequent intervention, the type and site of tissue, the antigenic burden, and the type of immune cells under investigation. At the heart of these studies is the now well established triad of induced transplant tolerance;

-**Dominance**; in that once tolerance to foreign tissue has been established using blocking but not ablative anti-CD4 or anti-CD8 mAb, it cannot be broken by the addition of potentially reactive T-cells (Scully, Qin et al. 1994).

-**Infectious tolerance**; when CBA mice were made tolerant to B10.BR skin using non depleting mAb blockade of CD4 and CD8, the resulting tolerance was resistant to injection of up to 50 million splenocytes from litter mates. Moreover, since the original CBA mice were transgenic and expressed a human CD2 reporter on the surface of all their T-cells, ablative anti-human CD2 mAb was used to deplete the original tolerant T-cells, leaving behind only those injected T-cells from wild type littermates. Despite the loss of the original tolerant cells (found to be specifically the $CD4^+$ subset), graft tolerance was maintained by the new population. This demonstrated that tolerance can be passed on from one $CD4^+$ cell population to another – hence infectious (Qin, Cobbold et al. 1993).

-**Linked suppression**; in which an immune system (**A**) previously tolerised to one set of minor mismatched antigens (**B**) could accept third party minor antigens (**C**) if these were co-expressed in the second tissue graft (eg skin from F1 **B**x**C** progeny) but not if two skin grafts (one from **B** and one from **C**) were placed side by side in the same graft bed (Davies, Leong et al. 1996; Wise, Bemelman et al. 1998).

These findings collectively suggested that an immune system could become tolerant to foreign antigen so long as it encountered that antigen in the absence of activating signals. Thereafter, that state of tolerance resisted challenges from potentially reactive cells and was self-perpetuating in that subsequent generations of cells could themselves be induced to take up the mantle of preserving tolerance. Finally, linked suppression demonstrated that antigens needed to be presented either by the same antigen presenting cell (APC) or at least, in very close proximity within a tissue microenvironment.

1.2 Identifying regulatory T-cells; the discovery of Foxp3

In spite of these breakthroughs, the inability to verify which was the critical regulatory cell subset limited the understanding and exploitation of those mechanisms underlying therapeutic tolerance. This may also have been one of the barriers to an early and wider acceptance of regulatory (Treg) cells. CD25 (the surface bound IL-2 receptor alpha chain) was initially thought to be the best marker of Treg (Kuniyasu, Takahashi et al. 2000) and

$CD25^{+}CD4^{+}$ cells were subsequently found to enhance foreign graft survival on adoptive transfer (Jiang, Tsang et al. 2006; Feng, Wood et al. 2008; Xia, Shah et al. 2009). However, CD25 can also be found on the surface of recently activated $CD4^{+}$ lymphocytes regardless of whether these cells behave with a regulatory phenotype and conversely, CD25- cells are also able to mediate transplant tolerance (Graca, Thompson et al. 2002). Treg cells may also express CD62L, GITR, CTLA-4 and CD45, but again, neither one is a specific marker (Sakaguchi, Sakaguchi et al. 1995; Herbelin, Gombert et al. 1998; Read 2006). In addition, the emergence of many other $CD4^{+}$ cell subsets described as having a regulatory phenotype has added to the confusion. These include TGFβ secreting Th3 cells (Weiner 2001), IL-10 secreting Tr1 cells (Levings, Sangregorio et al. 2002), $Foxp3^{+}$ T-cells (Bennett, Christie et al. 2001; Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003) and most recently $Foxp3^{neg}$ iT(R)35 cells (Collison, Chaturvedi et al. 2010),

An important breakthrough occurred in 2001 when Brunkow et al. discovered what subsequent studies have shown to be the best marker of Treg cells to date - a new member of the forkhead winged-helix family of transcription regulators called Foxp3 (Brunkow 2001). The gene was found in naturally occurring X-linked mutant scurfy mice characterised by lymphocyte infiltration into liver (hepatomegaly), spleen (progressive thrombocytopaenia and anaemia), gut (malabsorption secondary to chronic diarrhoea), and lymph nodes. So aggressive is the uncontrolled immunopathology that these typically small and scaly mice rarely live more than four weeks (Godfrey, Wilkinson et al. 1991). In the same year, a collaborative group found a mutation in the human ortholog of Foxp3 in patients with IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome (Bennett,

Christie et al. 2001). IPEX is a rare and fatal disorder, presenting typically in male patients within the first 6 months of age with a clinical triad of watery diarrhoea, eczematous dermatitis, and endocrinopathy; most commonly insulin-dependent diabetes mellitus and/or thyroiditis (Gambineri, Torgerson et al. 2003). Gastrointestinal histology reveals a gross inflammatory infiltrate, villous atrophy and mucosal erosion. As with scurfy mice, the skin is often involved, commonly leading to eczema but patients can also develop exfoliative dermatitis and erythroderma. Treatment is primarily with immunosuppressants until a well matched bone marrow donor can be found (Bennett and Ochs 2001).

1.3 Foxp3^{+} Regulatory T- cells

Two years following the identification of Foxp3, two groups independently published concurrent findings that Foxp3 was essential to T regulatory cell development and function in vivo (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003). Although there were a number of differences in their approach, in general they both demonstrated that Foxp3 gene expression is greatest in CD4^{+} cells, that the ability to suppress T effector cell function in vitro can be conferred by transfection with the *foxp3* gene, and finally that transfected Foxp3^{+} CD4^{+} lymphocytes were able to suppress cell mediated autoimmunity when injected into *foxp3*$^{-/-}$ *scurfy* mice. A third group compared the original *foxp3*$^{-/-}$ *scurfy* mice with transgenic Foxp3 mice expressing increased levels of the gene (Khattri, Cox et al. 2003). Again, Foxp3 expression was associated with a supressive phenotype in vitro. In vivo, the Foxp3 transgenic mice were crossed with (Cytotoxic T lymphocyte Antigen 4) CTLA-4$^{-/-}$ mice and survived for at

least 80 days compared to the usual CTLA-4 $^{-/-}$ strain which succumbed to fatal autoimmunity within a month. Fontenot et al. later followed up their initial work by showing that Foxp3 expression is responsible for suppressive activity of CD4^{+} cells, irrespective of whether these cells are CD25^{+} or not (Fontenot, Rasmussen et al. 2005).

The discovery that Foxp3 is neither specific to regulatory cells nor the immune system as a whole questioned whether *foxp3-/- scurfy* mice were a faithful representation of an immune system without Foxp3 Treg (Zuo, Wang et al. 2007; Chang, Zheng et al. 2008). Similarly, it was possible that Foxp3 expression was most important during early immune development and that its subsequent regulatory roles in later life were redundant. These concerns were answered by the finding that targeted ablation of Foxp3^{+} cells in previously healthy adult mice resulted in a severe immunopathology similar to that found in scurfy foxp3$^{-/-}$ mice (Kim, Rasmussen et al. 2007; Kim, Lahl et al. 2009). It seemed that Foxp3^{+} Treg were therefore responsible for the maintenance of self tolerance throughout life.

Foxp3^{+} Treg have subsequently been promoted above all others as essential for graft tolerance by largely descriptive data, typically involving the enhancement of graft survival by adoptive transfer of Foxp3^{+} rich, but impure, CD25^{+}CD4^{+} populations (Feng, Wood et al. 2008; Xia, Shah et al. 2009), and the induction of Foxp3^{+} Treg as a result of tolerogenic protocols (Cobbold, Castejon et al. 2004; Battaglia, Stabilini et al. 2006; Turnquist, Raimondi et al. 2007). The reliance on descriptive data is principally because Foxp3 is an intracellular transcription factor and analysis of its expression involved permeabilisation of the cells of

interest. Demonstrating definitive evidence of a genuine operational role in transplant tolerance therefore requires a reporter protein by which specifically Foxp3$^+$ Treg can be manipulated in vivo.

This has been achieved in a murine model known as DEREG, (DEpletion of REGulatory T-cells) in which all Foxp3$^+$ cells co-express the high affinity receptor for diphtheria toxin. In vivo depletion of Foxp3$^+$ cells by diphtheria toxin resulted in severe immunopathology, even in previously healthy adult mice. The resulting phenotype was so dramatic that the authors concluded that Foxp3$^+$ Treg were the master regulator and without them there is no regulation of any importance (Kim, Rasmussen et al. 2007). Although one group has used this model to demonstrate a role for Foxp3$^+$ Treg in certain strains that can naturally accept MHC mismatched renal grafts without immunosuppression (Miyajima, Chase et al. 2011), it has limitations for investigating induced, long term, graft tolerance. This is mainly because treated mice suffer from global immune dysregulation and very few survive longer than three weeks.

The model is also highly dependent on the dosing of the diphtheria toxin (DT), which is composed of two subunits. Subunit A catalyses the transfer of ADP-ribose from NAD to the polypeptide chain elongation factor 2, so halting cell protein synthesis and leading to cell apoptosis. It is such an efficient enzyme that even one molecule can result in cell death (Yamaizumi, Mekada et al. 1978). The limiting step of DT cell ablation is therefore the interaction of subunit B with the diphtheria toxin receptor (DTR). Mice express the low affinity

receptor for DT, but unlike simians and humans, they lack the high affinity DTR. This is the basis for the DTR ablation strategy originally demonstrated in a transgenic murine hepatitis model in which only liver cells artificially expressed the high affinity DTR (Saito, Iwawaki et al. 2001). Although the dose of DT that could result in non specific cell death was not originally published, the investigators recommended a maximum one off dose of 50μg/kg. Kim et al. needed to use repeated doses of 50μg/kg every 48 hours for optimal Treg cell ablation. This is equivalent to 6μg per 25 gram C57BL/6 mouse per week, far more than the regimen to ablate B and T-cells published in the original Nature Methods paper (Buch, Heppner et al. 2005). The most 'stringent' course in the Methods' article consisted of 100ng three times daily for 7 days; a total load of 2.1μg per animal per week. Bearing in mind that all murine cells express the low affinity receptor for DT, it is possible that these higher doses of DT are sufficient to cause non specific collateral cell death. Foxp3 mRNA has also been found in other cells, particularly epithelial cells, again raising concern that the toxin may be contributing to systemic inflammation by killing bystander cells (Chen, Chen et al. 2008; Kim, Lahl et al. 2009).

In this thesis, I have adopted an alternative transgenic Foxp3^{+} reporter model to investigate the exclusive roles of Foxp3^{+} Treg in therapeutically induced transplant tolerance - B6.Foxp3^{hCD2} mice. The model was developed in collaboration with Dr. Shohei Hori in Japan, and contains a sequence coding for a GPi-linked human CD2_CD52 fusion protein in the 3'UTR of the X-linked foxp3 gene (B6.Foxp3^{hCD2}) such that all Foxp3 expressing cells co-express the fusion protein on their cell surface (Komatsu, Mariotti-Ferrandiz et al. 2009). A series of anti-human CD2 and CD52 monoclonal antibodies were used to identify and isolate

pure populations of live Foxp3$^+$ cells based on their surface expression of the reporter protein. Ablative anti-human CD52 and CD2 monoclonal antibodies could also be used in vivo to deplete specifically Foxp3$^+$ Treg in a toxin free manner (Chapter 3). It is therefore possible to examine the role of Foxp3$^+$ Treg in tolerance to non-self antigen by exploiting transplant tolerance experiments induced by short term co-receptor and co-stimulatory blockade (Chapter 4).

1.4 Natural versus induced Foxp3$^+$ Treg

Natural Foxp3$^+$ nTreg refer to those first described as developing in the thymus shortly after the CD4$^+$CD8$^+$ double positive stage by a process involving high avidity binding of TCR to antigen presented on MHC class II (Apostolou, Sarukhan et al. 2002; Fontenot, Rasmussen et al. 2005; Larkin, Rankin et al. 2008). As a population, thymic derived nTreg have a limited TCR repertoire that is skewed toward self antigen (Jordan, Boesteanu et al. 2001; Apostolou, Sarukhan et al. 2002; Larkin, Rankin et al. 2008). When nTreg that had undergone forced deletion of *foxp3* were introduced into lymphopaenic hosts they expanded to dominate peripheral T-cell numbers and contributed to lymphocytic invasion of inflamed tissues (Williams and Rudensky 2007). This demonstrated the ability of nTreg to bind self antigen as well as their inflammatory potential in the absence of Foxp3. It was initially unclear how centrally produced nTreg with a TCR repertoire that recognised predominantly self antigen could be the cells responsible for peripherally induced transplant tolerance. In addition, TGF-β signalling was at first shown to be unnecessary for thymic development of

nTreg, whereas it was essential for the induction of transplant tolerance by co-receptor blockade (Cobbold, Castejon et al. 2004; Daley, Ma et al. 2007). As discussed in Chapter 5, the initial assumptions that the development of nTreg does not require TGF-β or cannot respond to induction of tolerance in the periphery have since been challenged ((Liu, Zhang et al. 2008) and (Watanabe, Wang et al. 2005; Proietto, van Dommelen et al. 2008) respectively). Nonetheless, the important role of TGF-β in converting naive $CD4^+$ cells into $Foxp3^+$ cells with a regulatory phenotype suggested, at the least, that an alternative pathway for generating $Foxp3^+$ Treg existed, and perhaps even a functionally separate group of peripherally induced 'iTreg' (Chen et al., 2003, Cobbold et al., 2004a, Nishimura et al., 2004).

The demonstration that $Foxp3^+$ cells could themselves convert naive $CD4^+$ cells into new $Foxp3^+$ iTreg in vitro (Andersson, Tran et al. 2008) seemed to provide a natural mechanism for infectious tolerance (Qin, Cobbold et al. 1993). However, it has proven much harder to demonstrate this process in vivo (Chapter 5), as has the relative regulatory capacity of iTreg over nTreg. This is principally because it has been almost impossible to reliably distinguish between nTreg and iTreg in conventional mice, let alone assess their respective contributions. TCR transgenic mice on a $RAG^{-/-}$ background have provided one way of examining the role of iTreg in the absence of nTreg since these mice have a population of monoclonal $CD4^+$ lymphocytes and no thymic derived nTreg. Any detectable $Foxp3^+$ cells will therefore be newly induced iTreg in response to tolerogenic conditions, as has been described following the induction of peripheral tolerance to a peptide (Kretschmer, Apostolou et al. 2005) or a skin graft (Cobbold, Castejon et al. 2004). A TCR transgenic mouse incorporating the Foxp3-hCD2_hCD52 reporter system (Marilyn.$Foxp3^{hCD2}$) is used in

Chapter 5 to explore whether iTreg associated with transplant tolerance actually perform an essential regulatory role. The ability of iTreg to recruit new Foxp3^{+} cells from naive cells in the periphery is also investigated to determine whether iTreg provide one potential mechanism by which the phenomenon of infectious tolerance might operate in vivo.

1.5 Antigen specific Foxp3^{+} iTreg

Given that all the CD4^{+} T-cells in TCR transgenic mice are monoclonal for a particular peptide, the antigen specificity of iTreg can also be explored using the Marilyn.Foxp3^{hCD2} model previously described. From the outset it seemed possible that Treg cell function might be antigen specific since the generation of Foxp3^{+} cells, whether nTreg or iTreg, requires TCR-Ag recognition. But this does not mean that Treg necessarily perform their regulatory function in an antigen specific manner, nor that the outcome of Treg activity is restricted to the antigen that triggers them. For example, the autoimmune gastritis (AIG) mouse is a model of immune regulation in which the H^{+}/K^{+} ATPase gastric pump is a major T-cell target. If a H^{+}/K^{+} knock out mouse was created, one might expect that a regulatory cell population from this mouse would lack Treg that could recognise the target protein and so would not be able to prevent AIG on adoptive cell transfer. In reality this is not the case, and Treg cells from H^{+}/K^{+} knockout mice are still able to prevent the development of AIG in WT athymic mice infused with WT CD25– effector T-cells (Zwar, Read et al. 2006). The two major limitations to studies of this kind are that the outcome is measured in terms of antigen specific effector activity, not

regulatory activity and the possibility that antigen specific Treg can perform some of their regulatory function in a non-specific way, for example through the release of anti-inflammatory cytokines such as IL-10 (Asseman, Mauze et al. 1999) or TGF-β (Fahlen, Read et al. 2005) is not excluded. This would be consistent with antigen-specific T-helper cells that, once activated, can influence responses to bystander antigens.

While specificity to a single defined antigen may not be an essential characteristic of functional Treg cells, there is evidence that it is at least beneficial. In vitro generated iTreg from transgenic mice expressing a single TCR for a particular pancreatic islet antigen displayed enhanced suppressive properties in vitro compared to a general population of polyclonal nTreg (Tang, Henriksen et al. 2004; Tarbell, Yamazaki et al. 2004). Infusion of these iTreg into NOD (non obese diabetic) mice achieved similar suppression of autoimmunity as twenty times as many nTreg cells (Tarbell, Yamazaki et al. 2004).

Lastly, the relative stability of Foxp3 expression in induced iTreg compared to nTreg is one factor that may determine a functional difference. Much of the ongoing research in this area has followed in vitro generated iTreg cultured in the presence of APCs, cognate antigen and TGF-β. The addition of other cytokines can divert cell differentiation, for example adding IL-6 promotes Th-17 cells (Harrington, Hatton et al. 2005; Park, Li et al. 2005). When transferred into in vivo models, a large proportion of cells down-regulate their foxp3 expression (Kretschmer, Apostolou et al. 2005; Koenecke, Czeloth et al. 2009) and some have been shown to revert back to a T-helper cell phenotype (Zhou, Bailey-Bucktrout et al.

2009). This has led some authors to question whether a subset of iTreg exist with sufficient plasticity to become helper or regulatory cells depending on the environment in which they find themselves (Komatsu, Mariotti-Ferrandiz et al. 2009). Alternatively, cells that transiently express Foxp3 may represent one phase of peripheral development leading to a fully differentiated, stable, Foxp3^{+} Treg. Others define 'true' Foxp3^{+} Treg by set of epigenetic changes previously shown to result in sustained expression of Foxp3 (Polansky, Kretschmer et al. 2008), which means that any cells transiently expressing Foxp3 are, by that definition, excluded from the debate (Zheng, Josefowicz et al. 2010).

TCR transgenic mice are, by definition, so different from conventional mice that it is still not clear how important antigen specific iTreg are in the context of a co-ordinated polyclonal immune regulation. The aim of Chapter 6 was to investigate whether tolerance induction resulted in peripherally induced iTreg in an otherwise conventional immune system lacking Foxp3^{+} nTreg. Given that depletion of Foxp3^{+} nTreg typically results in fatal immunopathology (Kim, Rasmussen et al. 2007; Lahl, Loddenkemper et al. 2007), much of the initial effort was directed at developing a protocol that did not lead to the early death of mice.

1.6 The physiology of Foxp3+ Treg

Understanding those factors that induce cells to express Foxp3 and behave as regulatory cells, although mainly in vitro, is an important start to understanding the role Treg cells play in the coordination of immune regulation.

1.6.1 TGF-β

Transforming growth factor beta (TGF-β) to date is the most reliable instigator of Foxp3+ cells. Of the three TGF-β members, TGF-β1 is the principal type expressed in the immune system and binds to tetrameric cell surface receptors initiating kinase activity (Derynck and Zhang 2003). After TGF-β1 deficient mice were shown to develop T-cell dependent fatal immunopathology that could not be rescued under germ free conditions, it was thought that TGF-β was essential in establishing T-cell dependent immune tolerance (Shull, Ormsby et al. 1992; Letterio, Geiser et al. 1996; Boivin, Ormsby et al. 1997).

Fantini et al. demonstrated that when purified splenic CD4+ cells from FVB ('Friend leukemia virus B') mice were cultured for 5 days in the presence of TGF-β rich medium (1ng/ml) and stimulated with anti-CD3 plus anti-CD28 they expressed both the Foxp3 mRNA and protein (Fantini, Becker et al. 2004). Whereas, those cells cultured without TGF-β failed to express Foxp3. A similar result was obtained for human CD4+ lymphocytes cultured with TGF-β, that produced more TGF-β themselves and showed negligible production of

inflammatory cytokines such as IL-4 and IFN-γ. Interestingly, they also produced less IL-10 than control cells cultured without TGF-β. The role of IL-10 in immune regulation is discussed in more detail below. That same month, a second group submitted similar results using purified $CD4^{+}CD25$- splenic cells from C57BL/6 mice cultured with antigen presenting cells (APCs), anti-CD3 and TGF-β for seven days (Chen, Jin et al. 2003). They found that $CD4^{+}CD25^{-}$ cells could be induced by TGF-β to upregulate Foxp3, display anergy in vitro, and suppress $CD4^{+}$ cell proliferation in vivo.

TGF-β signalling involves activation of 'mothers against decapentaplegic homologue' (SMAD) proteins, particularly SMAD2 and SMAD3. In addition, there is a highly conserved non-coding region in the FOXP3 locus that is TGF-β sensitive and has binding sites for SMAD as well as NFAT. In this way, TGF-β is thought to augment the induced expression of Foxp3 on TCR activation via cAMP dependent activation of cAMP-responsive-element-binding protein (CREB) and activating transcription factor (ATF) (Tone, Furuuchi et al. 2008)

Systemic anti-TGF-β mAb treatment had already been shown to abrogate the tolerance induced by non depleting anti-CD4 blockade in female mice with a single TCR recognising the male peptide (Cobbold, Castejon et al. 2004). To investigate a crucial role for TGF-β in iTreg dependent transplant tolerance, a dominant negative TGF-β receptor (dnTgfbr2) A1.RAG-/- mouse was created (Daley, Ma et al. 2007). Using an A1.RAG-/- mouse helped control for the increased reactivity of B cells previously seen in TGF-β receptor gene

knockout mice (Cazac and Roes 2000), although the higher proportion of NK cells remained without control (Laouar, Sutterwala et al. 2005). Female mice then received male skin grafts with a short course of anti-CD4 mAb. Whereas long term graft survival was achieved in 'wild type' mice receiving anti-CD4 mAb, it failed in the dnTgfbr2 strain.

The acceptance of diverse genome wide minor transplantation antigens was also shown to be TGF-β dependent in this model (Daley, Ma et al. 2007). In terms of the critical timing of TGF-β signalling to induced tolerance, dnTgfbr2 conventional mice rejected B10.BR skin grafts after 40 days if anti-TGF-β mAb was administered for three weeks starting the day before transplantation. This occurred despite graft transplantation under the cover of normally tolerising co-receptor and co-stimulatory blockade. TGF-β is therefore required at the time of transplantation to prevent graft rejection and induce long term tolerance.

It is not clear how important TGF-β is to the maintenance of graft acceptance since T-cells from dnTgfbr2 mice failed to expand when transferred into lymphocyte depleted hosts. This meant that they could not reliably be used in models that assess the ability of tolerant cells to prevent a new population from rejecting a graft. Similarly, an alternative approach using anti-TGF-β mAb treatment is limited by the indeterminate efficacy of the antibody in vivo (Daley, Ma et al. 2007). Fantini et al found that antibody blockade of TGF-β signalling from iTreg to T-effector cells only impaired the anti-proliferative effect of iTreg by ~25% (Fantini, Becker et al. 2004).

Although TGF-β may be required for Treg induction in vitro, it is still unclear whether it is essential for Treg action in vivo thereafter.

1.6.2 CTLA-4

Cytotoxic lymphocyte antigen 4 (CTLA-4 aka CD152) is a cell surface expressed member of the immunoglobulin super family. While other T-cells only express CTLA-4 on activation, Treg cells constitutively express it (Takahashi, Tagami et al. 2000). Furthermore, CTLA-4 knockout mice develop fatal autoimmune multiorgan inflammation (Waterhouse, Penninger et al. 1995) and evidence of colitis, as well as tumour immunity, can be elicited by antibody blockade of CTLA-4 (Leach, Krummel et al. 1996; Read, Malmstrom et al. 2000). CTLA-4 therefore appeared to be integral to immune tolerance.

Given that CTLA-4 is also expressed on activated effector T-cells causing their down regulation on binding with APC expressing CD80 or CD86, the extent to which CTLA-4 exerts its inhibitory effects via $Foxp3^+$ Treg cells alone was unclear. To help answer this question, BALB/c mice expressing Cre under the control of the Foxp3 promoter were crossed with mice expressing a floxed form of the CTLA-4 gene. In this way, CTLA-4 expression was unchanged in all other cell types except in the Treg population that was CTLA-4-/- in >98% of cells (Wing, Onishi et al. 2008). Compared to complete CTLA-4 knockout mice that became moribund within three weeks, those mice that were only CTLA-4 negative in terms of Treg cells alone

remained apparently healthy for seven weeks and only latterly developed a systemic immunopathology characterised by myocarditis, gastritis, lymphadenopathy, splenomegaly and gross oedema. While the absence of CTLA-4 was not found to impair nTreg cell development in the thymus, it did impair the suppressive capacity of Treg in vitro and in vivo (Wing, Onishi et al. 2008). One pathway of CTLA-4 dependent regulation may operate by stripping co-stimulatory molecules off dendritic cells, a mechanism that can be enhanced by (Programmed Death 1) PD-1 signalling (Kaufmann and Walker 2009; Schmidt, Wang et al. 2009).

The Foxp3 Treg dependent CTLA-4 knock out model may not have been perfect (control Cre Foxp3 mice had fewer Foxp3 Treg cells than wild type mice and abnormally high levels of antiparietal antibodies) but it succeeded in demonstrating a Treg cell specific role for CTLA-4 and highlighted it as a potential therapeutic target to influence Treg cell function (Chapter 7).

1.6.3 Cytokines

Prior to the discovery of Foxp3, $CD4^+$ Treg cells were characterised by their high expression of CD25; the alpha receptor for interleukin 2 (IL-2)(Kuniyasu, Takahashi et al. 2000). The complete IL-2 receptor is composed of alpha, beta and gamma chains. Of these, the beta chain has the longest intracellular tail and is able to bind and activate JAK 1. This facilitates autophosphorylation recruiting adaptor proteins such as SHC that in turn activate

MAP and PI3 kinases (Johnston, Bacon et al. 1996). STAT 5 is also recruited and on dimerisation translocates to the nucleus. In Treg cells, despite not having an effect on other downstream signalling pathways such as PI3K, IL-2 signalling has been shown to upregulate STAT 5, and binding sites for STAT5 have been found on the Foxp3 gene in both mice and humans (Burchill, Yang et al. 2007; Yao, Kanno et al. 2007). In the presence of TGF-β, IL-2 results in the increased survival of $CD4^{+}CD25^{+}Foxp3^{+}$ murine and human cells but many lose their anergic and suppressive properties (Fontenot, Gavin et al. 2003). Thus, IL-2 may be helpful in Treg cell induction without being necessary for suppression.

If the CD25 cell surface protein is the non signalling alpha chain of the IL-2 receptor, how does IL-2 influence Treg cells? It is likely that IL-2 signaling persists through other completely formed trimeric IL-2 receptors. Indeed, there is evidence for significant redundancy both in terms of the signal and the receptor; IL-4, Il-7, IL-9 and IL-15 can all bind to the IL-2 receptor, it takes a double negative mutation of the gamma chain (not just the alpha or beta chain) to result in a complete lack of Foxp3 T reg cells, and chimeras of the IL-2 receptor beta chain plus an IL-7 receptor alpha chain are able to re-establish IL-2 dependent Treg signaling (Burchill, Yang et al. 2007; Zheng, Sharma et al. 2007). One school of thought is that the CD25/alpha IL-2 receptor mops up IL-2 within a given tissue microenvironment and thereby starves effector T-cells of sufficient IL-2 levels for prolonged survival (Thornton and Shevach 1998; Pandiyan, Zheng et al. 2007).

The local tissue is increasingly emerging as an important micro-environment, in which APCs could present antigen under certain 'tolerogenic conditions' and induce Foxp3$^+$ Treg. It is not clear which are the key factors that determine those tolerogenic conditions and whether they are harnessed in vivo by specifically Foxp3$^+$ Treg to recruit new converts. For example, IL-10 is the important regulatory axis required to prevent immunopathology of gastro-intestinal mucosa, regardless of whether Foxp3$^+$ Treg secrete it (Asseman, Mauze et al. 1999; Uhlig, Coombes et al. 2006). One of the most important factors is clearly TGF-β (Cobbold, Castejon et al. 2004; Li, Wan et al. 2006; Coombes, Siddiqui et al. 2007; Daley, Ma et al. 2007) and its abundance during tissue healing may be one way of generating iTreg while effector activity is inhibited by co-receptor blockade. Once the graft tissue is well healed, Treg remain perhaps to determine the degree of immune regulation specific to that tissue (Chapter 5). Foxp3$^+$ Treg might also exploit a number of well conserved pathways, that limit the exhaustive effects of inflammation, to protect the tissue from infiltrating effector cells. These could include the induction of APC metabolic enzymes such as IDO (Indoleamine 2,3-dioxygenase) that depletes local amino acids (Cobbold, Adams et al. 2009) or membrane bound CD73 converting inflammatory adenosine into anti-inflammatory AMP (Dr. F. Regateiro, DPhil Thesis 2009) (Mandapathil, Hilldorfer et al. ; Kobie, Shah et al. 2006). In this way, Foxp3$^+$ Treg may be one important factor in determining a state of relative immune privilege in accepted graft tissue.

It remains to be seen whether the induction of non-self tolerance by co-receptor blockade exploits Foxp3 dependent mechanisms whose natural role is to maintain (and at

times return) normal homeostasis to tissues that are continually vulnerable to stressors such as infection, ischaemia and trauma.

1.7 Aims of the thesis

- To investigate the exclusive roles of Foxp3$^+$ Treg in transplant tolerance induced by short term immune blockade

- To explore where and when Foxp3$^+$ Treg act to ensure therapeutically-induced tolerance to non-self

- To demonstrate a functional role for induced Foxp3$^+$ iTreg generated through the induction of transplant tolerance

- To investigate whether iTreg are required for tolerance in conventional immune systems

CHAPTER 2: Materials and Methods

2.1 Reagents, solutions and tissue culture media

All solutions were prepared in endotoxin-free water produced by the milliQ-Advantage system (Millipore). Unless otherwise stated, all reagents were obtained from Sigma.

2.1.1 Phosphate-buffer saline (PBS) x 1

1 x PBS was prepared by diluting 1/10 a commercial stock of 10 x PBS (Lonza) with milliQ prepared water (mQ). When necessary, the final solution was filtered through a 0.2μm filter.

2.1.2 Tissue culture reagents

R10 medium was prepared under sterile conditions using the following:

RPMI-1640 medium (Lonza)
10% v/v Foetal Calf Serum (FCS - Gibco)
50 ug/ml Penicillin+Streptomycin (Gibco)
2mM L-glutamine (Gibco)
50μM 2-mercapto-ethanol
1nM Sodium pyruvate
10μM HEPES

IMDM medium was prepared under sterile conditions using the following:

500ml of IMDM + L Glut medium (Lonza)

50ml of FBS

5ml Penicillin/Streptomycin

When prepared for culture of the BHK Cam hCD2-CD52 9aa cell line, the selection agent, G-418 (Invitrogen), was added to a final concentration of 1mg/ml

2.1.3 Tris-buffered ammonium chloride (TBAC)

9 parts of 0.16M NH_4Cl were added to 1 part of 0.17M Tris-HCl and finally adjusted to pH 7.2 followed by 0.2μM filtration.

2.1.4 FACS Buffers

FACS washing buffer:

1 x PBS

0.1% w/v BSA

0.1% w/v NaN3

FACS blocking buffer:

1 x PBS

1% w/v BSA,

5% v/v heat-inactivated (56°C for 30 minutes) normal rabbit serum

0.1% w/v NaN3,

Foxp3 intracellular staining buffers were used according to the menthod provided by eBiosciences.

FACS saponin buffer:

1 x PBS
0.5% w/v saponin
0.5% w/v BSA
0.1% w/v NaN_3

FACS fixation solution was prepared such that 100μl of 1 x PBS + 1% BSA and 100μl 4 % v/v formaldehyde was added to each well on a 96 well plate.

2.1.5 AutoMacs Buffers

AutoMacs running buffer

1 x PBS
2mM EDTA
0.5% w/v BSA

AutoMacs rinsing buffer

1 x PBS

2mM EDTA

2.1.6 GM-CSF for the production of bone marrow derived dendritic cells

GM-CSF was obtained from X-63 myeloma cells transfected with the murine GM-CSF gene (X63 Ag8 12) provided by Dr D. Gray, Royal Postgraduate Medical School, London. Each supernatant batch activity was assessed by comparing its capacity to stimulate proliferation of murine bone-marrow cultures to that of a previous batch, and commercial recombinant GM-CSF (R&D Systems) which was titrated from 20ng/ml to 0.625ng/ml. The lowest concentration of GM-CSF was chosen that was capable of sustaining maximal proliferation of bone marrow cells in culture.

2.1.7 TGFβ

Unless otherwise stated TGFβ (Petrotech) was used in vitro at a final concentration of 2ng/ml from a stock dissolved in 4mM aqueous HCl containing 1mg/ml human serum albumin.

2.2 Antibodies used in this thesis

2.2.1 Flourescently conjugated antibodies commonly used in this thesis

Antibody target	Isotype	Stock Concentration	Manufacturer	Stock Code
Anti-hCD2	R-PE conjugated Mouse IgG1, K, anti-human mAb	100 tests in 2ml	BD Pharmingen	555327
Anti-hCD2	(MEM-65) Dynomics 647	100 tests	AbCam	AB26144
Anti-hCD2 isotype	Mouse IgG1 [PPV-06] (Dyomics 647)	100μl	AbCam	AB76096
Anti CD3	Anti- mouse PerCP (145-2c11)	0.2mg/ml	BD Pharmingen	553067
Anti-CD4	Rat anti-mouse PE (H129.19)	0.2mg/ml	BD Pharmingen	553652
Anti-CD4	anti mouse FITC (L3T4)	0.5mg/ml	BD Pharmingen	553651
Anti-CD4	Rat anti-mouse PerCP (RM4-5)	0.2mg/ml	BD Pharmingen	553052
Anti-CD4	Cy-Chrome anti-mouse (L3T4)	0.2mg/ml	BD Pharmingen	553654
Anti-CD4	Anti-mouse APC (L3T4)	0.2mg/ml	BD Pharmingen	553051
Anti-CD8	Anti-mouse CD8a PE (Ly-2)	0.2mg/ml	BD Pharmingen	553033
Anti-CD8	Anti-mouse CD8a PE-Cy5 (Ly-2)	0.2mg/ml	BD Pharmingen	553034
Anti-CD19	Alexa-flour 647 anti-mouse eBio1D3	0.2mg/ml	eBioscience	51019382
Anti-CD25	Biotin anti-mouse (7D4)	0.5mg/ml	BD Pharmingen	553070
Anti-CD25	Anti-mouse FITC (7D4)	0.5mg/ml	BD Pharmingen	553072
Anti-CD25	Anti-mouse PE (PC61)	0.2mg/ml	BD Pharmingen	553866
Anti-GITR	anti GITR PE IgG2b YGITR765	100 tests	Serotec	MCA2416PE
anti PDL 1	anti mouse PDL-1 PE conjugated		BD Pharmingen	558091
FoxP3	aFoxP3 PE (JFK-16s)		eBioscience	14-5773
FoxP3	aFoxP3 APC (JFK-16s)		eBioscience	77-5775
KI67	PE Mouse Anti-Human Ki-67 Set		BD Pharmingen	556027
Anti-Rat	Anti-Rat IgG FITC whole molecule	stock 1ml	Sigma	F-6258

2.2.2 Antibodies used in vivo

All antibodies used in vivo were produced by the Therapeutic Immunology Group and prepared by ammonium sulphate precipitation as described below under section 2.2.3.

Non lytic, blocking antibodies:

Anti-CD4 - YTS 177 Rat IgG2a (L3T4) (Waldmann, Cobbold et al. 1989)
Anti-CD8 - YTS 105 Rat IgG2a (Waldmann, Cobbold et al. 1989)
Anti-CD40L - MR1 clone, hamster IgG3 (Noelle, Marshall et al. 1992)
A regimen of 3 x 1mg over 7 days of each of these three blocking antibodies was used to induce tolerance to foreign skin in conventional mice unless otherwise stated (Chapter 4)

Lytic antibodies:

Anti-human CD2 - YTH655 clone rat IgG2b (Domain 2) (Qin, Cobbold et al. 1993)
Anti-human CD52 - Campath-1H humanised IgG1 (Waldmann, Polliak et al. 1984)

The following two lytic anti-CD8 antibodies were used to deplete $CD8^+$ cells in vivo prior to purifying $CD4^+$ populations:

YTS 156.7.7 – rat IgG2b (Cobbold, Jayasuriya et al. 1984)
YTS169.4 – rat IgG2b (Cobbold, Jayasuriya et al. 1984)

Isotype control antibodies:

Anti-dog rat IgG2b YKIX 337.217.7 clone

2.2.3 Preparation of monoclonal antibodies

Preparation of Integra Supernatant mAb

All reagents were warmed to 37°C and the following steps were performed under sterile conditions where relevant. 800ml of IMDM + 1% (v/v) Penicillin/Streptomycin was prepared.and added to the large top chamber of a 1 litre Integra supernatant flask. 25ml of cell culture suspended in this IMDM + 5% (v/v) + FCS 1% (v/v) Penicillin/Streptomycin was added to the bottom chamber of the Intergra supernatant flask making sure all bubbles were removed. The culture was incubated at 37°C and 6% CO_2. The bottom chamber was replenished twice a week with fresh cell cultures. After 4 weeks, the supernatant in the top chamber was harvested for antibody purification.

Purification of Endotoxin Free mAbs

Clinical grade, sterile, endotoxin-free disposable plastic containers were used where possible. Otherwise, materials were soaked in 0.5M NaOH and then rinsed thoroughly with endotoxin free water.

Ammonium Sulphate Precipitation

Super-saturated (NH4)2SO4 was prepared using Analar Grade reagent with endotoxin free water in autoclaved and NaOH treated glass bottles. A stock was kept at 4°C. An equal volume of saturated (NH4)2SO4 was added to the hybridoma supernatant (antibody suspension). This was mixed overnight at 4°C. The resulting precipitate was collected following centrifugation for 30 minutes at 3500rpm by discarding the supernatant and re-dissolving the pellet in endotoxin free water. The saturated (NH4)2SO4 precipitation step was repeated up to once more and the re-suspended antibody was dialysed against 1 x PBS. When an antibody solution with greater purity was required, ion exchange purification was performed.

Dialysis of antibody solutions

Endotoxin free 1 x PBS was used at all times for final dialysis. Antibody solutions were carefully injected into Slide-A-Lyzer Dialysis Cassettes or SnakeSkin Dialysis Tubing (Thermo Scientific) depending on the volume to be dialyzed. These were then placed in endotoxin free glass beakers containing >100x v/v 1xPBS with gentle mixing by a magnetic stirrer. Three changes of 100x v/v 1xPBS were performed over 24 hours of incubation at 4°C. The resultant antibody solution was stored at 4°C or frozen at -80°C.The final antibody concentration was measured using NanoDrop OD-280 spectrophotometer.

Ion Exchange Purification

Buffer A Stock (10X)

10x stock of buffer A was made by adding

Malonic Acid	104g
NaOH	60g
Betaine	20g

to a final volume of 2 litres with endotoxin free water. Final pH 5.2 - pH 5.4 at 25°C, passed through a sterile 0.2μm filter and stored at 4°C

Buffer B

1 x Buffer A in PBS + 0.5M NaCl (2.92g/100ml), passed through a sterile 0.2μm filter and used immediately

The (NH4)2SO4 precipitated antibody preparation was dialyzed against 1x Malonate buffer (buffer A) overnight. All solutions and buffers were warmed to room temperature. The preparation was centrifuged at 3000rpm for 10 minutes at room temperature to remove antibody complexes and the supernatant was diluted in 1 x Buffer A so that the total Ig concentration was between 1-20 mg/ml as measured by OD280.

Fast Flow S-Sepharose (Pharmacia) was prepared by first washing once in 0.5M NaOH, followed by buffer B, and then three washes with buffer A (centrifuged at 2000rpm for 2 minutes at each wash stage). 1ml of packed Fast Flow S-Sepharose gel was used for each 20mg of antibody. The antibody solution was added to the appropriate volume of Fast Flow S-Sepharose and incubated for 1 hour at room temperature with gentle rotation. The supernatant was removed and kept in case insufficient antibody had bound to the Fast Flow S-Sepharose gel. The remaining gel was washed three times with buffer A (each wash used at least 5 gel volumes and centrifugation at 2000rpm for 2 minutes). The supernatant from the 1st wash was also kept.

The bound antibody was eluted by adding 1 gel volume of buffer B and rotating at room temperature for 5 minutes. After centrifuging for 2 minutes at 2000rpm, the supernatant was collected. The elution process was repeated once more and the two supernatants pooled. The final solution was passed though a 0.20μm filter and dialyzed using Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific) against three changes of 1xPBS over 24 hours at 4°C with gentle mixing. The final concentration was analyzed using OD280 spectrometer (NanoDrop) and the purity was checked by performing a Phast Gel System (Pharamacia) native and SDS reducing PAGE as per the manufacturers guidelines.

2.2.4 Biotinylation of purified antibody

Regents used were 0.05M Sodium bicarbonate buffer pH 8.5 (7.3g/L NaHCO3 + 8.6g/L Na2CO3) and 1mg/ml Sulfo-NHS-LC-Biotin (Pierce).

2mg of purified antibody diluted to 1mg/ml in 1 x PBS was dialyzed overnight using Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific) in 0.05M sodium bicarbonate buffer at 4°C.

74μl of 1mg/ml Sulfo-NHS-biotin was added to 2ml of antibody solution and left on ice for 2 hours. Following overnight dialysis against 1 x PBS, 0.025% w/v NaN3 and 0.01% v/v BSA as carrier protein was added and stored at 4°C.

2.2.5 Fluorescent conjugation of purified monoclonal antibodies

Alexa Fluor® 488 and 647 Monoclonal Antibody Labeling Kits (Invitrogen) were used as per manufacturer's instructions (http://probes.invitrogen.com/media/pis/mp20181.pdf)

2.2.6 Preparation of YKIX 337.217.7 anti-dog rat IgG2b de-aggregation for tolerance induction of depleting rat IgG2b mAb

To eliminate aggregates of antibodies, ultra-centrifugation was performed for 150 minutes at 150,000g at 4°C (Benjamin, Cobbold et al. 1986). Immediately after centrifugation, the top third of the de-aggregated solution was carefully removed and passed through a sterile 0.22μm filter. The final concentration was analyzed using OD280 spectrometer (NanoDrop).

2.2.7 Analysis of serum concentration of treatment antibody

Blood samples were taken immediately post-mortem from mice by cardiac puncture and collected in 1.5ml Eppendorf tubes. The blood samples were left to stand at room temperature for 45 minutes before being centrifuged for 5 minutes at 2,000 rpm. The serum was carefully removed using a suction pipette and stored at -20°C.

500,000 washed BHK Cam hCD2-CD52 9aa cells were added in blocking buffer to each well of a 96 well, round bottomed, pate. Limiting dilution of the test sample in blocking buffer was added to the appropriate wells and incubated for 60 minutes at 4°C. After washing three times with wash buffer, the plate was incubated at 4°C for a further 60 minutes in the presence of fluorescently conjugated anti-rat Ig antibody in blocking buffer. After washing a further three times, the cells were fixed with FACS fixation solution. FACS anaylsis was performed and the signal intensity of the fluorescently conjugated anti-rat Ig antibody of treatment wells was compared to a standard curve using a known concentration of treatment antibody.

2.2.8 IOC ELISA for detection of anti-rat antibodies in serum

Inhibition of capture enzyme-linked immunosorbent assay (IOC-ELISA) was previously developed to perform an independent measurement of anti-idiotypic and anti-isotypic anti-

globulins in serum samples from animals treated with monoclonal antibodies (Cobbold, Rebello et al. 1990). The protocol was adapted from (Cobbold, Rebello et al. 1990).

The treatment and capture antibody was YTH655 rat IgG2b anti-human CD2 (domain 2). A standard curve was generated using mouse IgG1, anti-rat IgG2b Norig 7.16.42 clone.

Serum samples were harvested from mice as described above. Flexible 96 well microtitre flat bottom plates (Costar 9018) were coated with 50μl of treatment antibody at 10ug/ml diluted in IMDM by incubating overnight at 4°C. Thereafter, all steps were performed at room temperature. The plate was washed three times in 1 x PBS with 0.1% v/v Tween20 before being blocked for 2 hours by the addition of 200μl/well of 1 x PBS with 2% v/v BSA (block buffer). After washing three times in 1 x PBS with 0.1% v/v Tween20, 50μl of the test mouse serum was added to the plates in 1 in 2 limiting dilutions in block buffer and incubated for 1 hour.

The plate was washed three times in 1 x PBS with 0.1% v/v Tween20 and 50μl of 1ug/ml biotinylated treatment antibody in block buffer was added to each well. After 1 hour the plate was washed three times in 1 x PBS with 0.1% v/v Tween20 and 50μl of 1/1000 ExtrAvidin Peroxidase (Sigma) in 1 x PBS + 10% v/v BSA was added to each well for one hour. The pate was washed three times in 1 x PBS with 0.1% v/v Tween20 and developed using o-phenylenediamine dependent photospectrometry. A standard curve was generated using mouse IgG1 anti-rat IgG2b (Norig 7.16.42).

2.3 Flow Cytometry

2.3.1 Surface staining

Staining was performed in FACS tubes or round-bottomed 96-well plates using 0.25-1x10^6 cells/well. Cells were washed in FACS wash buffer and re-suspended in FACS blocking buffer + 1% v/v staining antibody at 4°C for 30 minutes to 1 hour. After washing three times with FACS wash buffer and centrifugation at 1200rpm for 6 minutes, further incubation at 4°C for 30 minutes to 1 hour was performed with a secondary antibody if required. The wells were washed three times with FACS wash buffer, re-suspended and fixed in 200μl per well of FACS fixation solution.

Samples were analyzed using a FACSCalibur (Becton Dickinson) with dual laser excitation (488nm and 633nm). CellQuest software (Becton Dickinson) was used for data acquisition and cross-channel compensation. FlowJo software was used for data analysis (Tree Star Inc). All FACS plots were gated on forward and side scatter to exclude dead cells.

2.3.2 Intracellular Staining

After staining with surface reagents, cells were washed with FACS washing buffer and re-suspended in 50 µl of 1 x Foxp3 fixation/permeabilisation buffer (eBiosciences) for 45-60

minutes at 4°C. Cell samples were then washed twice with 1 x Foxp3 permeabilisation buffer (eBiosciences) and incubated with 50μl of appropriate dilutions of intracellular staining antibodies in 1 x Foxp3 permeabilisation buffer for 45-60 minutes at 4°C. After three washes with 1 x Foxp3 permeabilisation buffer, the cell samples were finally fixed in 200μl per well of FACS fixation solution.

2.4 Cell culture

2.4.1 Specific cell lines used

BHK Cam hCD2-CD52 9aa cell line (expressing GPi linked human CD2-CD52 fusion protein) was cultured in IMDM medium with the selection agent, G-418, added at a final concentration of 1mg/ml

Unless otherwise stated cells were cultured at 37°C in a 6% v/v humid CO2 atmosphere, in R-10 medium and when cells were pelleted by centrifugation this was performed for 5 minutes at 1200 rpm in a Sorvall Legend RT centrifuge.

2.4.2 Preparation of lymphocytes from lymphoid tissue

Lymphoid tissue was dissected post mortem from mice under sterile conditions, if relevant, and placed in appropriate medium for transport from the animal facility. A 2ml syringe plunger was used to mash the splenic tissue against a 70μm cell strainer (BD Falcon) within a 100mm petri dish containing medium. The cell suspension was centrifuged for 6 minutes at 1200rpm and 3ml of TrisNH_4Cl was added to the re-suspended pellet for 3 minutes at room temperature with gentle mixing to lyse any red blood cells. 10 mls of medium was added and the suspension was washed twice before a final re-suspension

2.4.3 Preparation of Bone Marrow-Derived Dendritic Cells

The protocol is based on (Inaba, Inaba et al. 1992). Femoral bones were derived post mortem under sterile conditions from mice. Bone marrow cells were then harvested using lavage and aspiration of long bone marrow with R10 medium. The cell suspension was filtered through a 70μm cell strainer, washed three times using R10 medium. For each mouse used, the cells were plated into 3 x 100mm petri dishes (cell culture quality) in a volume of 15ml of R-10 medium per dish (45ml in total) with GM-CSF at a final concentration of 20ng/ml. Cell cultures were incubated at 37°C, 6% CO_2 for 72 hours. After 72 hours the cell cultures were replenished by gently rotating the dishes before aspirating the medium and any non adherant

cell debris. A further 15ml of R-10 medium and GM-CSF (final concentration: 20ng/ml) was then added to the residual adherent cells.

After a further 72 hours of incubation at 37°C, 6% CO_2 the cells were harvested, washed and re-suspended in R-10 medium, and re-plated into new 100mm petri dishes, reducing the number of plates by 1/3 (e.g. 2 plates instead of 3). Cell cultures were incubated at 37°C, 6% CO_2 for a further 24 hours.

2.4.4 Cryo-preservation of cell suspensions

Cell cultures were washed twice in appropriate medium before being re-suspended in R10 medium and mitomycin C at a final conc. of 25ug/ml (stock is 0.5mg/ml). After 30 minutes of incubation at 37°C, 6% CO_2 the cell suspension was washed twice more with R-10 medium and re-suspended in 8mls of 9 parts FCS (9mls): 1 part DMSO cooled to 4°C (1ml). 1ml of the resultant suspension was added to each cryotube and stored at -80°C.

2.4.5 Thawing of cryo-preserved cells

All reagents were pre-warmed to 37°C. Cryo-tubes were heated to 37°C and immediately added to 10ml of R-10 medium, washed twice with R-10 medium and re-suspended in 10ml R-10 medium under sterile conditions. The final suspension was transferred to a 25ml Corning flask and incubated at 37°C and 6% CO_2.

2.4.6 TGFβ stimulation of Marilyn_foxp3_hCD2 lymphocytes

All reagents were warmed to 37°C and the following steps were performed under sterile conditions. Spleen cells were harvested from Marilyn.Foxp3^{hCD2} mice and prepared as previously described (2.4.2). 5x10^5 Marilyn.Foxp3^{hCD2} cells were cultured at 37°C and 6% CO_2 in 2 mls of R10 medium and TGFβ (2ng/ml), 1x10^5 B6 WT bone marrow dendritic cells and male H-Y peptide (*Dby* H-2A^b - NAGFNSNRANSSRSS 100nM).

2.4.7 Separation of hCD2+ and hCD2- cells using MoFlow sorting

Cells were counted and suspended in 1ml of Running Buffer + 187.1 mAb supernatant (100μl of 1mg/ml) + M5/114 mAb (anti-class II, 100μl of 1mg/ml) for 40 minutes at 4°C. The cell suspension was then washed in running buffer and re-suspended in 4ml of running buffer + washed anti-Rat Ig Dynabeads (1ml of beads/4x10^8 cells) for 45 minutes at 4°C with gentle rotation (Invitrogen). A magnet was used to separate cells (e.g, B-cells and macrophages) bound to the anti-Rat Ig Dynabeads from the cells of interest that were removed with the supernatant after 2 minutes. The resultant cell suspension was counted and 10μl per 150 million cells of fluorescently conjugated antibodies (including anti-hCD2) were added before incubation for a further 45 minutes at 4°C. Single stain samples were also prepared for MoFlow calibration prior to cell sorting. After two washes with running buffer, the cell

suspension was slowly passed through a 70μm sieve and underwent MoFlow cell sorting as per manufacturers guidelines by Mr. Nigel Rust.

2.4.8 Cell counting

10μl of cell suspension was added to 90μl of 0.4% TrypanBlue. 10μl of the final solution was added to a glass Neubauer counting chamber. Four quadrants were counted ignoring the dark blue coloured cells (dead cells).

Formula used: cell count of 4 quadrants/4 x 10 (dilution factor) x 10^4 = number of cells/ml

→ x ml of cell suspension = total number of cells

2.4.9 Osmotic Lysis of RBCs in blood samples

900μl of d.H2O was added to heparinised blood samples and after 10 seconds of gentle mixing a further 100μl of 10x PBS was added. The blood samples were centrifuged at room temperature for 6 minutes at 1200 rpm. Using a glass suction pipette, all but the very lowest layer was aspirated. This process was repeated once more before the residual cells were re-suspended in 1ml of blocking buffer or appropriate medium.

2.4.10 PMA and Ionomycin stimulation

Cells were prepared in R-10 medium as previously described (2.4.2) and 50ng/ml of PMA, 500ng/ml of ionomycin and 10μg/ml of Breveldin A were added. 100μl of cell suspension was added to each well of a 96 well plate and incubated for 5 hours at 37°C and 6% CO_2.

2.5 Experimental animals used in this thesis

All mice were bred and maintained under specific pathogen-free conditions at the Sir William Dunn School of Pathology, University of Oxford, U.K. All experimental procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 and received local ethical committee approval. The mice strains used are described in Table 2.1.

2.5.1 Breeding and genotyping of foxp3_hCD2-CD52 knock in mice

B6.Foxp3^{hCD2} knock in mice (Chapter 3 and (Komatsu, Mariotti-Ferrandiz et al. 2009)) were developed in collaboration with Dr. Shohei Hori et al (Research Unit for Immune Homeostasis, 230-0045 RIKEN RCAI, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, JAPAN).

The initial strain of B6.Foxp3^{hCD2} knock in mice were genotyped using the following protocol:

Primers for *foxp3_hCD2-CD5*2 KI gene:

11a Forward	5' AAGATGAATCTTCCAGTCAAAGAGCCC 3'
11b Reverse	5' GTCTGTATATACACCTATGTGTGATAGTGCC 3'

(PCR products: WT 250bp, KI 1641bp)

PCR conditions for a total 25µl reaction

PCR reaction mix

Primer F 10mM	0.75µl
Primer R 10mM	0.75µl
x10 buffer (Vivantis PI1202)	2.5µl
50mMMgCl2	0.75µl
dNTPs	0.25µl
DMSO	1.5µl
H2O	17.25µl
Taq	0.25µl
DNA	1.0µl

PCR conditions

Denature	94°C 5 minutes
x35 cycles	94°C 30 minutes
	56°C 30 minutes
	72°C 2 minutes
Final extension	72°C 5 minutes

Table 2.1 Mouse strains used in this thesis

Mouse Strain	Haplotype	Genetic manipulation	References
CBA/Ca	H-2^k	-	
C57Bl/6J	H-2^b	-	
CBA.RAG$^{-/-}$	H-2^k	CBA/Ca mice with RAG-$1^{-/-}$	(Zelenika, Adams et al. 1998)
C57Bl/6J (B6) RAG$^{-/-}$	H-2^b	C57Bl/6J (B6) mice with RAG-$1^{-/-}$	(Gorelik and Flavell 2000)
B6.Foxp3^{hCD2}	H-2^b	Transgenic C57Bl/6J (B6) mice with foxp3_hCD2-CD52 KI gene	(Komatsu et al 2010)
A1(M) x RAG1$^{-/-}$ (A1.RAG)	H-2^k	Transgenic TCR against male peptide *Dby* presented by H2E^k on CBA.RAG-$1^{-/-}$ background.	(Zelenika, Adams et al. 1998)
Marilyn.Foxp3^{hCD2}	H-2^b	Transgenic TCR (against male peptide *Dby* on B6.RAG-$1^{-/-}$ background with foxp3_hCD2-CD52 KI.	
(CBA x B6.Foxp3^{hCD2})F1	H-2^k x H-2^b		
CBA.Rag$^{-/-}$ x B6.Rag$^{-/-}$	H-2^k x H-2^b		
A1.RAG$^{-/-}$x B6.RAG$^{-/-}$	H-2^k x H-2^b		

Marilyn.Foxp3^{hCD2} mice were generated from the original B6.Foxp3^{hCD2} strain as follows;

Breeding and genotyping of the working colony was kindly overseen by Elizabeth Adams. B6.Foxp3^{hCD2} mice were crossed to B6.RAG1$^{-/-}$ mice. The subsequent F1 generation was interbred to generate F2 offspring from which B6.Foxp3^{hCD2}RAG1$^{-/-}$ mice were selected. B6.Foxp3^{hCD2}RAG1$^{-/-}$ mice were then crossed to Marilyn RAG1$^{-/-}$ mice for at least three generations selecting Marilyn plus foxp3_hCD2-hCD52 plus RAG1-/- offspring. Once the purity of the colony was established, only female Marilyn.Foxp3^{hCD2} mice were weaned for future use.

2.6 Animal experimental procedures

2.6.1 General Anaesthesia and Peri-operative care

At all times the animals were treated in strict accordance with the project and personal license as verified by the Home Office.

During induction, maintenance and recovery from general anaesthesia, the mice were handled gently, positioned to avoid pressure injuries and placed on a warmed surface or covered when possible to ensure maintenance of normal body temperature.

For a 20g mouse using intra-peritoneally administered anaesthetic the following combination dose of hypnorm and midazolam was used;

250μl of 1/10 hypnorm (stock 10 mg fentanyl and 0.2 mg fluanisone/ml) and 0.05mg of midazolam.

This anaesthetic protocol was used in place of a previous, ketamine based, protocol in order to improve peri-operative morbidity and mortality rates. It was designed and implemented in conjunction with the Oxford University Veterinary services.

Post operative care, as standard, included housing mice in warmed cages, placed on warming mats and covering mice in insulating nesting to help limit heat losses. Water soaked food or jelly was placed within the cage in addition to the standard supply of food and water. Mice were monitored at regular intervals until normal behaviour resumed and they could be returned to normal housing units. In addition, for the first night post-operation, cages were heated gently from below. Any abnormal signs or post operative complications were immediately reported to the NACWO. When temporary sedation was required, 2% isoflurane was administered by a murine anaesthetic gas chamber or mask.

2.6.2 Skin grafting - operative protocol

based on the technique described by (Billingham and Brent 1957)

General anaesthesia performed as outlined above

i) Mouse positioning

Mice were placed prone on table with the flank facing toward the operator. Mice were warmed during the procedure using insulation and heated mats

ii) Skin Preparation

Skin covering the flank region was prepared by electric shaving and alcohol swabs.

iii) Preparation of the graft bed

The flank skin was elevated away from subcutaneous tissue using skin forceps. Curved sharp scissors were used to prepare 1cm^2 graft bed by removing the host skin. Immaculate haemostasis was achieved prior to placement of the skin graft.

iv) Placement of donor skin grafts

Full thickness donor skin was harvested from mice killed and confirmed as dead by methods listed in schedule 1 of the Home Office Guidelines for Animal Experimentation. 1cm^2 skin grafts were placed in sterile 1 x PBS before being placed on the prepared graft bed of the recipient.

v) Closure and dressings

VetBond tissue glue was carefully placed in minute quantities at the four graft corners to help prevent the graft tissue from slipping. Jelonet sterile, non-adherent, dressings were layered

over the graft bed. Mice were then wrapped in approx. 25mm x 100mm sterile guaze before being finally wrapped in 25mm x 100mm Gypsona Plaster of Paris. A single cutaneous clip was used to secure the plaster to the scruff of the neck.

Post procedure (as per anaesthetic protocol)

When performing a two stage transplant experiment, the original (tolerated) graft was surgically excised, cleared of all subcutaneous tissue and then immediately re-grafted onto a prepared graft bed on the second host following the same steps outlined above.

2.6.3 Thymectomy - Operative Protocol

General anaesthesia performed as outline above

i) Mouse positioning

Mice were placed supine on table, head toward the operator (6 o'clock position). They were warmed during the procedure using insulation and heated mats. Dermapore tape was used to gently abduct front and rear paws by affixing them to table (so mice were spread-eagled on the table). A loosely rolled tissue was placed under the mouse's neck and where necessary, the neck was gently extended to improve surgical exposure. Care was taken not to cause cervical hyper-extension

ii) Skin Preparation

A area of skin defined superiorly, by the lower jaw, inferiorly by the costo-chondral border and laterally, by the axillae was prepared by electric shaving and alcohol swabs

iii) Initial Incision

A midline incision measuring 0.75cm was centred on the midpoint of the neck using a size 10 scalpel blade and extending from just inferior to the angle of jaw until the suprasternal notch. Care was taken not to penetrate the subcutaneous layers

iv) Dissection

The superficial cervical musculofascial layer was dissected down to the two salivatory glands that were then parted in the midline revealing the trachea. Fine iris forceps were used to pick up the pre-tracheal fascial which was then longitudinally dissected with 30mm straight sharp scissors and extend towards the superior thoracic aperture

v) Aspiration of thymus

Curved jewellers or Birk's forceps were used to gently expose the thoracic aperture. A blunt glass suction pipette tip was then introduced between the forceps, 3-4mm into the anterior mediastinum. Gentle suction was applied to aspirate both lobes of the thymus

Closure and dressings

Immaculate haemostasis was achieved. With the front paw restraints released to ease tissue tension, the wound was closed by opposing each tissue layer. Skin edges were opposed using tissue glue (VetBond)

Post procedure (as per anaesthetic protocol)

2.6.4 Tail vein phlebotomy

Up to 100 µLwas collected from the lateral tail vein of vaso-dilated mice. 5-10 units of heparin were added when prevention of clotting was required. Commonly, mice were temporarily anaesthetised by 2% isoflurane to help minimise discomfort as well as promoting vasodilatation.

2.6.5 Adoptive transfer of immune cells

Sterile, cell suspensions isolated from AutoMACS or MoFlow sorting were re-suspended in 1 x PBS and kept on ice prior to injection within 60 minutes into the lateral tail vein of mice. Commonly, mice were temporarily anaesthetised by 2% isoflurane to help minimise discomfort as well as resulting in vasodilatation.

2.7 Molecular Techniques

2.7.1 PCR Method

Gloves were worn at all times and filter pipette tips were used. All reagents were either kept on ice or in a freezer set to -20°C. See section 2.5.1 for PCR buffer.

24μl of the master mix was pipetted into each well of a 96 well PCR plate and 1μl of the test sample was added. At all times, a negative, positive and water control were used. The plate was covered with plate sealer, labelled and briefly centrifuged for approximately 15 seconds at 400rpm. It was the placed on a PCR machine and the appropriate heating program was selected

2.7.2 PCR Gel preparation

A 2% (w/v) agarose solution in TBE was prepared, mixed and heated in the microwave for 40 seconds. 1μl of 'SafeView' solution per 20mls of 2% agarose was added, mixed and the cooling solution was placed in mould with a comb in situ. Once set, the samples were added to each well.

2.8 Statistical analysis

Statistical analysis and presentation of data was performed in conjunction with Graphpad Prism software version 4.0. *Log-rank Mantel-Cox Test* was primarily used for analysis of graft survival rates. Where appropriate, Student's t-test and ANOVA post-test were used to determine statistical significance.

CHAPTER 3: Assessing the feasibility of antibody mediated ablation of Foxp3+ T-cells

3.1 Abstract

Foxp3^{+} regulatory T-cells (Treg) are a subset of CD4^{+} T lymphocytes known to be essential for self tolerance. Their role in transplant tolerance remains unproven due to the absence of a known natural cell surface marker by which they can be manipulated in vivo. A transgenic B6.Foxp3^{hCD2} mouse was created to express an artificial GPi-anchored human CD52/CD2 surface fusion protein under the control of the Foxp3 promoter. A number of fluorescently conjugated mAb were tested for their ability to identify and isolate pure populations of live Foxp3^{+} Treg using monoclonal antibodies against the human CD2/CD52 reporter. Different combinations of ablative anti-human CD2 and anti-human CD52 mAb were tested in B6.Foxp3^{hCD2} mice in order to develop an optimal regimen for depleting Foxp3^{+} Treg in vivo in a toxin free environment. Multiple daily injections of low dose YTH655 (rat IgG2b anti-human CD2 mAb) proved the most effective in ablating Foxp3^{+} cells. Circulating Foxp3^{+} cells in B6.Foxp3^{hCD2} mice were far more vulnerable to antibody mediated ablation than those resident in splenic or lymphatic tissue.

3.2 Introduction

The discovery of monoclonal antibodies and their production by hybridoma technology revolutionised the field of immunology (Kohler and Milstein 1975; Kohler, Howe et al. 1976; Kohler and Milstein 1976). Many of the discoveries over the past thirty years have benefited from the ability to identify, stimulate, block or delete particular immune cell subsets with

monoclonal antibodies directed against specific cell surface proteins. At present, the investigation of regulatory T-cells (Treg) is complicated by the description of multiple subtypes that are poorly defined, often by a combination of non specific cell surface molecules or secreted cytokines. Traditionally, these have included $CD25^{+}CD4^{+}$ cells (Kuniyasu, Takahashi et al. 2000), IL-10 secreting Tr1 cells (Levings et al., 2002), TGFβ secreting Th3 cells (Weiner, 2001), $CD4^{+}CD25^{+}$ Treg (which can be functionally sub-divided into $CD4^{+}CD25^{+}CD103^{+}$ cells) and most recently $Foxp3^{neg}$ iT(R)35 cells (Collison et al., 2010). The functional relevance of these various Treg sub-types was called into question following the discovery of the foxp3 gene and the crucial role it plays in immune regulation (Bennett et al., 2001, Fontenot et al., 2003, Hori et al., 2003). This was dramatically demonstrated in transgenic DEREG (DEpletion of Regulatory T-cells) mice in which all $Foxp3^{+}$ cells co-express the high affinity receptor for diphtheria toxin (Kim, Rasmussen et al. 2007). Depletion of $Foxp3^{+}$ cells with diphtheria toxin in previously healthy adult mice led to a 'catastrophic' and fatal immuno-pathology with similar severity to that seen in neonate *foxp3*$^{-/-}$ *scurfy* mice. The implication was that $CD4^{+}Foxp3^{+}$ Treg were required throughout life to maintain self-tolerance and that in their absence all other regulatory cells, regardless of how they are defined, are inadequate.

$Foxp3^{+}CD4^{+}$ were assumed to be the most important Treg and Foxp3 expression became the gold standard against which other markers of Treg were measured. Unfortunately, as an intracellular product, any detection method requires permeabilisation of the cells. Practically this meant that while its expression could therefore be used to assess the 'purity' of, for example, a sample of sorted $CD4^{+}CD25^{+}$ cells, it could not be used to isolate living cells or to manipulate Treg in vivo. To overcome this issue, transgenic Foxp3 knock-in

models were developed in which Foxp3 expression results in co-expression of an artificial cell surface reporter. The first such reporter system (DEREG) employed a sequence coding for the high affinity diphtheria toxin receptor such that all $Foxp3^+$ cells could be selectively depleted in vivo and a green fluorescence protein sequence allowing flow cytometric isolation of live GFP^+Foxp3^+ cells. It was this model that emphasised the critical role that $Foxp3^+$ Treg play in maintaining self-tolerance. However, when investigating the role of $Foxp3^+$ Treg in induced, long term tolerance to foreign tissue, the DEREG model is limited by the severity and rapidity of immune dysregulation in response to diphtheria toxin treatment (Kim, Rasmussen et al. 2007; Lahl, Loddenkemper et al. 2007). There are further concerns that the diphtheria toxin is so effective (Yamaizumi, Mekada et al. 1978) that other cells expressing very low levels of Foxp3, particularly epithelial cells, may also be destroyed and so contribute to the inflammatory pathology (Chang, Zheng et al. 2008; Chen, Chen et al. 2008; Kim, Lahl et al. 2009).

In order to examine the role of $Foxp3^+$ Treg in long term tolerance induced to foreign tissue, a different transgenic reporter mouse was developed containing a sequence coding for a GPi-linked human CD2_CD52 fusion protein in the 3'UTR of the X-linked foxp3 gene; $B6.Foxp3^{hCD2}$ mice (Komatsu, Mariotti-Ferrandiz et al. 2009). This meant that $B6.Foxp3^{hCD2}$ mice were genetically and phenotypically identical to B6 wild type mice with the exception that any cells expressing Foxp3 would also express the human CD52_CD2 reporter protein on their cell surface (figures 3.1 and 3.2). The Therapeutic Immunology Group had previously developed a series of ablative monoclonal antibodies directed against human CD52 (e.g. Campath-1H) and human CD2 (e.g. YTH655). When combined with this experience of

monoclonal antibodies, the transgenic B6.Foxp3^{hCD2} model provided the means to identify and isolate live populations of Foxp3^{+} Treg. Naturally ablative antibody isotypes could be used to deplete specifically Foxp3^{+} in vivo without relying on the use of toxins that might themselves influence the outcome of the investigation.

The initial experiments were aimed at establishing the specificity and sensitivity of the human CD52_CD2 reporter for Foxp3^{+} cells in the B6.Foxp3^{hCD2} model. Thereafter, it was useful to generate a series of fluorescently conjugated monoclonal antibodies (mAb) that could identify human CD52_CD2 positive cells by flow cytometry. Competitive binding studies helped map the various epitopes of the reporter protein bound by the available mAb and established which antibodies could be used in combination without interference.

Antibody mediated ablation of cells is known to be limited by the efficiency of antibody clearance, the anti-globulin response of the treated host (Benjamin, Cobbold et al. 1986; Cobbold, Rebello et al. 1990; Rebello, Hale et al. 1999) and the antibody isotype used, for example rat IgG2b mAb are ablative in vivo (Cobbold and Waldmann 1984). In addition, the formation of antigen-antibody complexes at the cell surface has been shown to down-modulate the target antigen and impair ablation (Glennie and Stevenson 1982). These issues were addressed with the aim of optimising the efficiency of antibody mediated ablation of Foxp3^{+} Treg in B6.Foxp3^{hCD2} mice.

Finally, two ablative mAb that bind to the same target without overlap can synergize, resulting in significantly improved depletion in combination than alone (Hughes-Jones, Gorick et al. 1983; Bindon, Hale et al. 1985). A number of explanations have been offered for this phenomenon. Initiation of the complement cascade was classically shown to require at least two Ig heads binding to C1q. The exposure of Antibody Dependent Cell-mediated Cytoxocity (ADCC) pathways to mAb Fc receptors is also greatly increased (Borsos and Rapp 1965; Borsos, Loos et al. 1980). Firstly because two mAb can bind to a single target and secondly, because antibody-antigen complexes have been shown to group together and form catenaries (Hale, Clark et al. 1985). For these reason, combinations of depleting mAb that could bind the human CD2-CD52 reporter without overlap were initially pursued with the aim of maximising in vivo ablation of $Foxp3^{+}$ cells.

3.3 Results

3.3.1 Assessment of mAb recognition of $Foxp3^{+}CD4^{+}$ cells in B6.$Foxp3^{hCD2}$ mice

Monoclonal antibodies directed against the human CD25_CD2 fusion protein were used to identify and target specifically $Foxp3^{+}$ T-cells in B6.$Foxp3^{hCD2}$ mice. Two commercially available fluorescently conjugated mAb against the two domains of human CD2 were adopted; AbCam Dy647 conjugated anti-hCD2 (MEM-65) recognising domain 1 (figures 3.1, 3.4 and 4.5) and BD Pharminogen PE conjugated anti-hCD2 (RPA-2.10) recognising domain 2 (figures 3.4 and 3.5). When spleen cells from B6.$Foxp3^{hCD2}$ mice were prepared and

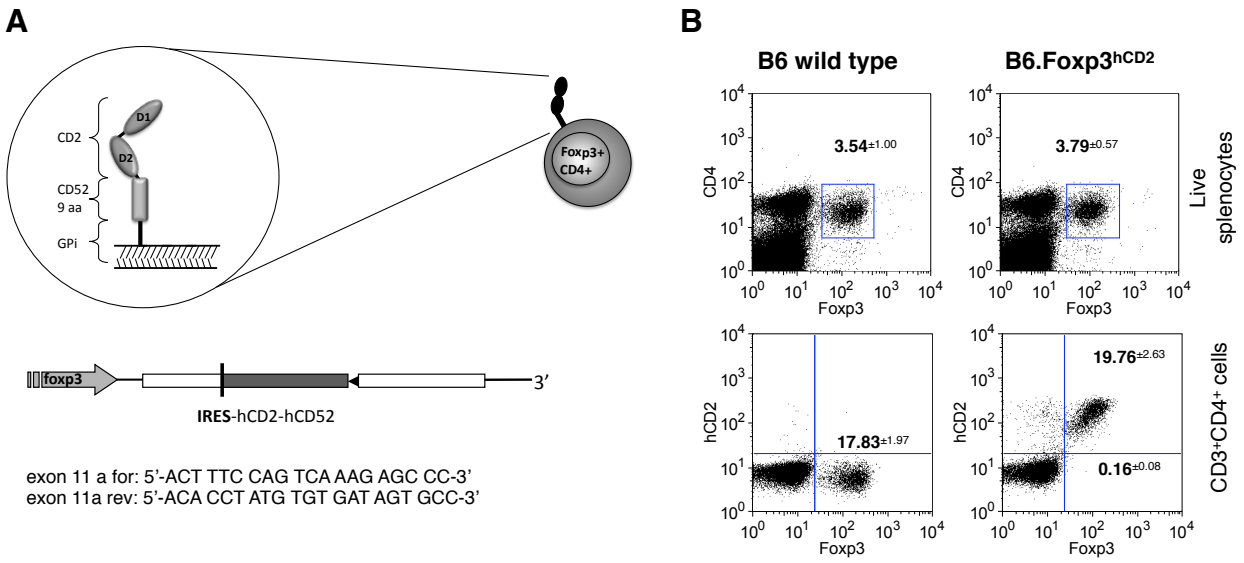


Figure 3.1 The human CD52_CD2 fusion protein is an accurate reporter of Foxp3⁺ T-cells in transgenic B6.Foxp3^{hCD2} mice

(A) B6.Foxp3^{hCD2} mice contain a sequence coding for a GPi-linked human CD52_CD2 fusion protein in the 3' UTR of the X-linked *foxp3* gene. **(B)** A similar percentage of CD4⁺ cells are Foxp3⁺ cells in both the B6 wild type and B6.Foxp3^{hCD2} mice. PE conjugated anti-hCD2 mAb stains over 98% of Foxp3⁺CD4⁺ T-cells in B6.Foxp3^{hCD2} mice. In comparison none of the Foxp3⁺CD4⁺ T-cells in B6 wild type mice are hCD2⁺ ($p<0.0001$). The mean and SEM of three aged matched mice is shown.

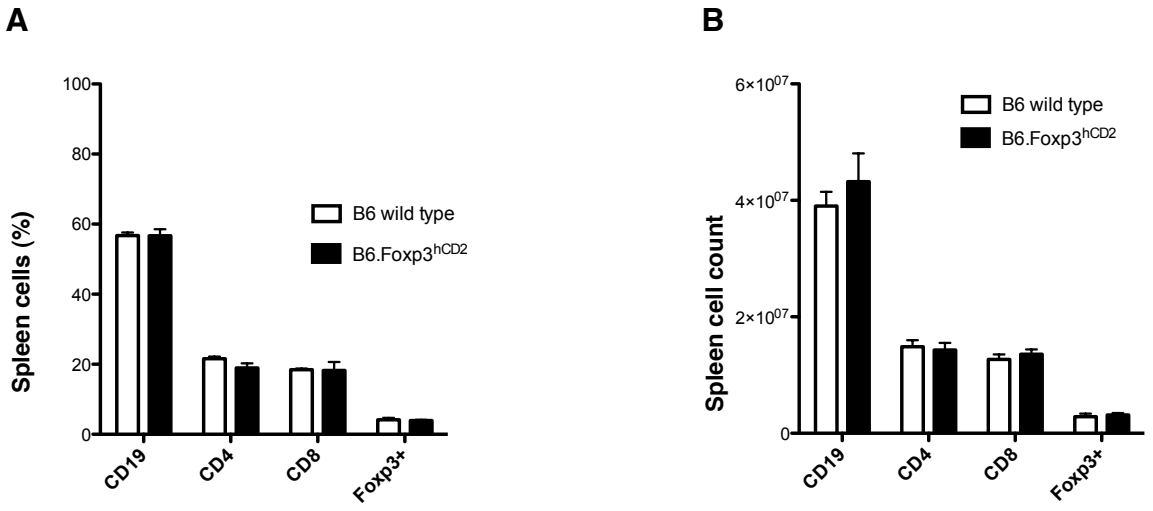


Figure 3.2 B6.Foxp3^{hCD2} mice have similar lymphocyte composition to wild type B6 mice

Flow cytometry analysis of lymphocyte composition of B6.Foxp3^{hCD2} and B6 wild type mice does not reveal a significant difference in the percentage **(A)** and absolute cell counts **(B)** of CD4$^+$, CD8$^+$, CD19$^+$ and CD4$^+$Foxp3$^+$ lymphocytes between the two strains. Each bar represents the mean of three age matched mice with SEM indicated by the error bars. No significant difference was found by t-test.

incubated with these commercial fluorescently conjugated mAb, over 98% of $CD3^{+}CD4^{+}$ cells that stained positive for Foxp3 were also hCD2 positive (figure 3.1). This demonstrated that the human CD2_CD52 reporter fusion protein was a highly accurate reporter for Foxp3 expression in B6.Foxp3^{hCD2} mice, that otherwise had a normal immune repertoire (figure 3.2). Either of these commercial antibodies could therefore be used for isolation of live $hCD2^{+}Foxp3^{+}$ T-cells through fluorescence cytometry (Chapters 5 and 6).

In order to target Foxp3 Treg during transplantation tolerance studies, it was necessary to identify in vivo cell depleting mAb that could bind to the human CD2_CD52 reporter protein. Rat IgG2b mAb have previously been shown to be an ablative isotype in mice (Cobbold, Jayasuriya et al. 1984; Qin, Cobbold et al. 1987). Initially, two depleting monoclonal antibodies were available; Campath-1H (a humanised IgG1 mAb, previously rat IgG2b) that recognises the human CD52 epitope and a rat hybridoma, YTH655, producing IgG2b mAb capable of depleting lymphocytes expressing human CD2 (Friend, Tighe et al. 1987; Qin, Cobbold et al. 1993; Friend, Rebello et al. 1995). The ability of the commercial and depleting antibodies to bind the human CD52_CD2 fusion protein found in B6.Foxp3^{hCD2} mice was verified using flow cytometry. In order to limit the use of animals, a BHK cell line (BHK-Cam-9aa-12C) known to express constitutively the same hCD2_hCD52 fusion protein, was used in all assays assessing the binding properties of the putative anti-hCD2 and anti-hCD52 mAb. 500,000 BHK cells were incubated in 96 round bottom well plates with limiting dilutions of Campth-1H, YTH655, anti-hCD2 PE and anti-hCD2 Dy647 mAb. Bound mAb was analysed using direct and indirect flow cytometry to generate a saturation binding curve (figure 3.3). All four mAb were able to bind the human CD2_CD52 fusion protein found in B6.Foxp3^{hCD2} mice.

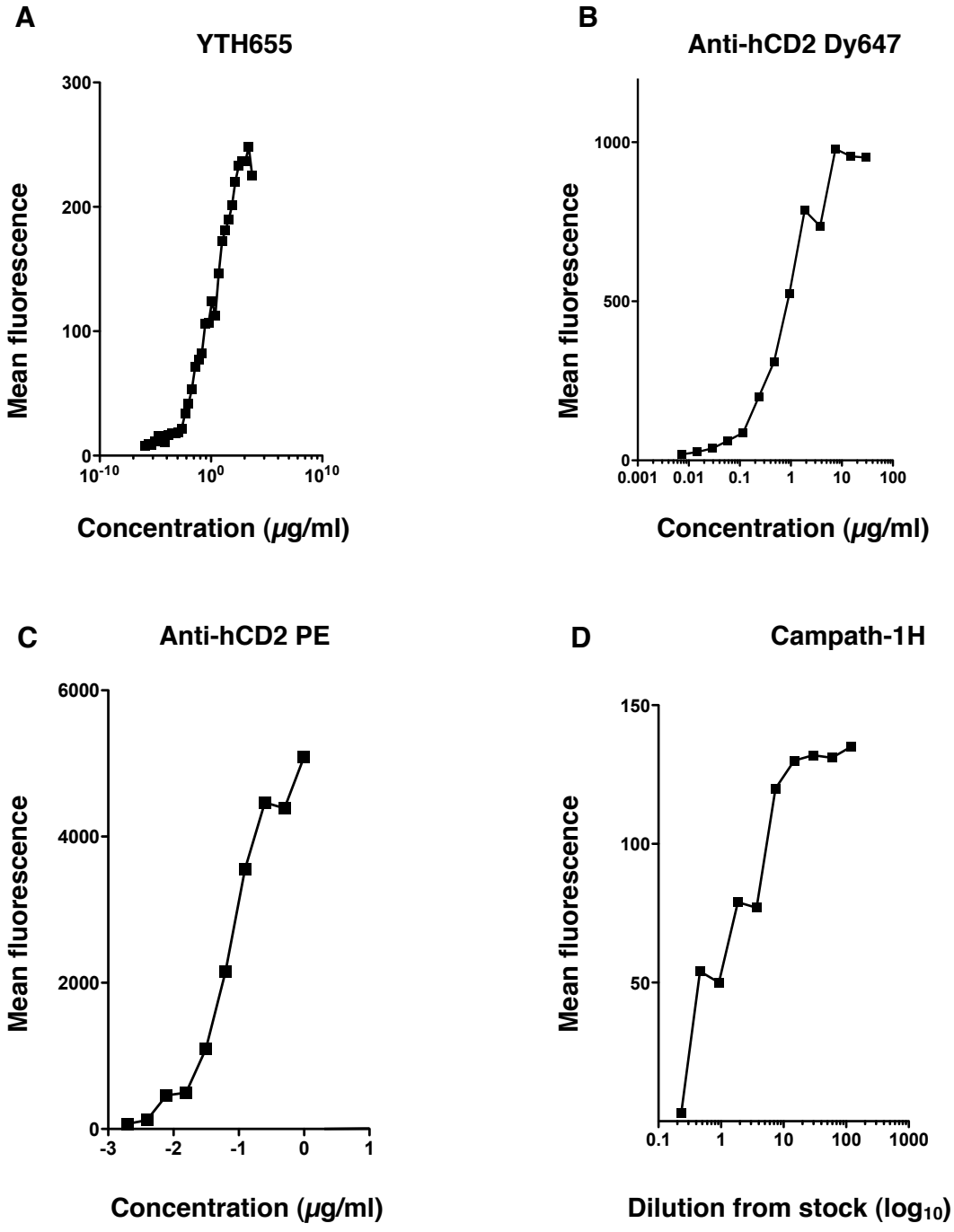


Figure 3.3 Binding affinity curves for anti-human CD52 and CD2 monoclonal antibodies

A cell line (BHK Cam hCD2-CD52 9aa) that constitutively expresses the same human CD52_CD2 fusion protein found in Foxp3^{+} cells of B6.Foxp3^{hCD2} mice was used to investigate the binding affinity of candidate anti-human CD52 and CD2 mAb. The mean fluorescence intensity was measured by flow cytometry for limiting dilutions of three conjugated anti-human CD2 mAb; **(A)** YTH655, **(B)** AbCam anti-CD2 and **(C)** BD bioscience anti-CD2. **(D)** A binding curve for humanised Campath-1H, an anti-CD52 mAb, was also generated.

A

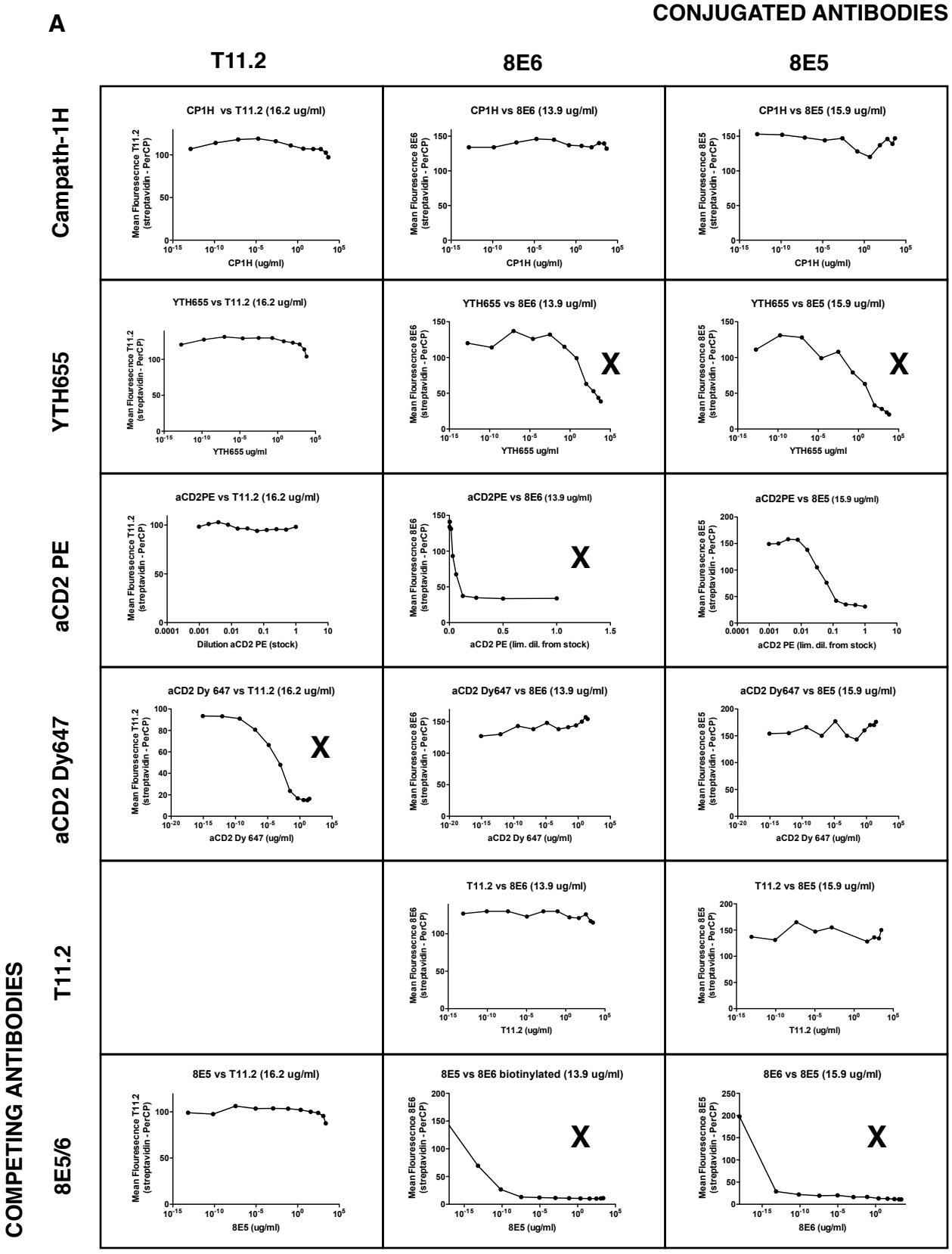

CONJUGATED ANTIBODIES
T11.2
8E6
8E5
COMPETING ANTIBODIES
Campath-1H
YTH655
aCD2 PE
aCD2 Dy647
T11.2
8E5/6
CP1H vs T11.2 (16.2 ug/ml)
CP1H vs 8E6 (13.9 ug/ml)
CP1H vs 8E5 (15.9 ug/ml)
YTH655 vs T11.2 (16.2 ug/ml)
YTH655 vs 8E6 (13.9 ug/ml)
YTH655 vs 8E5 (15.9 ug/ml)
aCD2PE vs T11.2 (16.2 ug/ml)
aCD2PE vs 8E6 (13.9 ug/ml)
aCD2PE vs 8E5 (15.9 ug/ml)
aCD2 Dy 647 vs T11.2 (16.2 ug/ml)
aCD2 Dy647 vs 8E6 (13.9 ug/ml)
aCD2 Dy647 vs 8E5 (15.9 ug/ml)
T11.2 vs 8E6 (13.9 ug/ml)
T11.2 vs 8E5 (15.9 ug/ml)
8E5 vs T11.2 (16.2 ug/ml)
8E5 vs 8E6 biotinylated (13.9 ug/ml)
8E6 vs 8E5 (15.9 ug/ml)
X

Figure 3.4 (A)

B

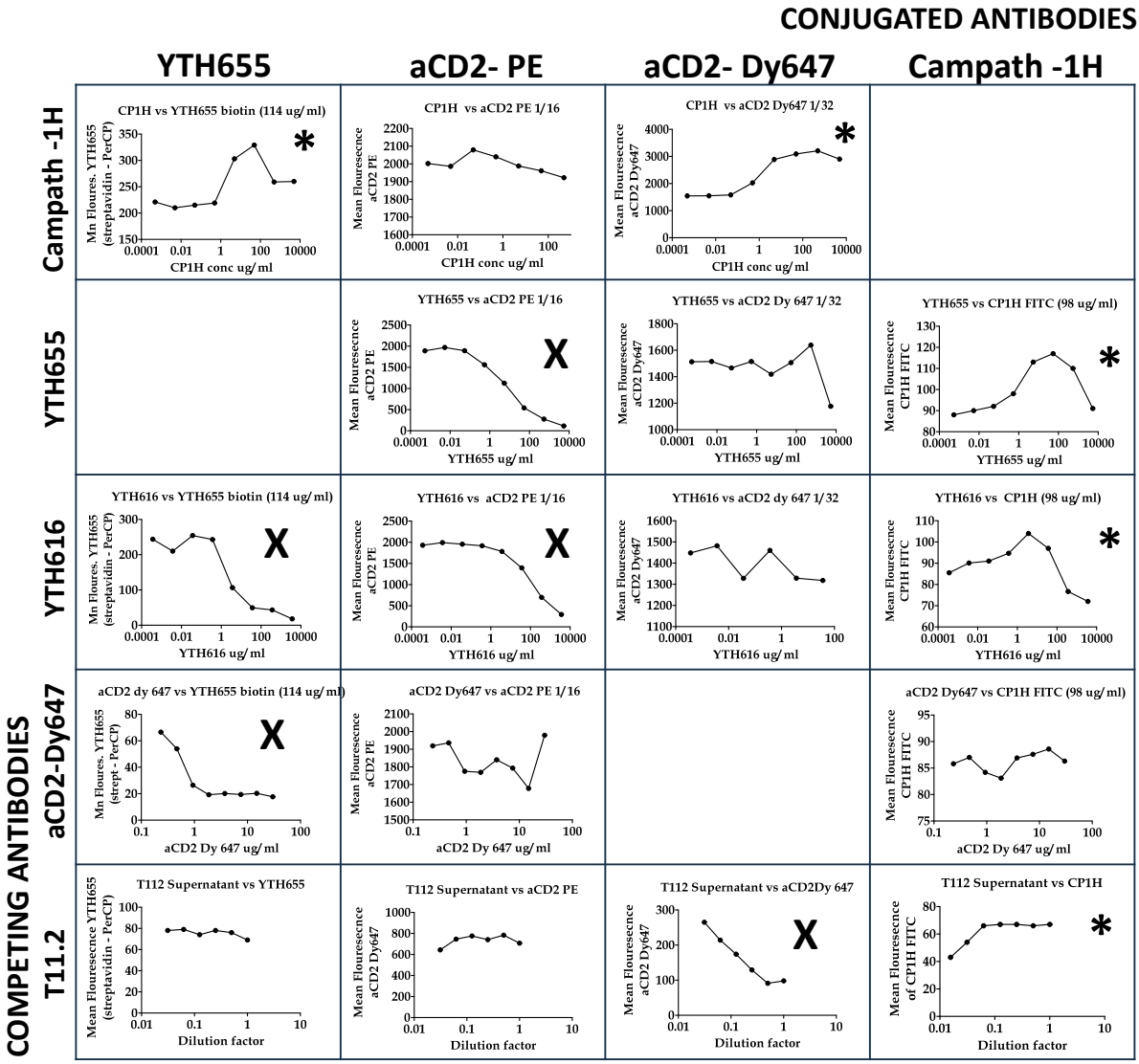


Figure 3.4 Anti-human CD52 and CD2 mAb competition studies

(A) and (B) BHK Cam hCD2-CD52 9aa cells were incubated with limiting dilutions of a test antibody before the addition of a competing conjugated antibody at a concentration known to achieve ~50% saturation (**Figure 3.3**). Mean fluorescence intensity was determined by flow cytometry. **X** - marks where two antibodies compete for binding. ✶ - indicates where the first antibody enhanced the binding of the second antibody. Those antibody combinations that had no reciprocal effect on binding are left unmarked.

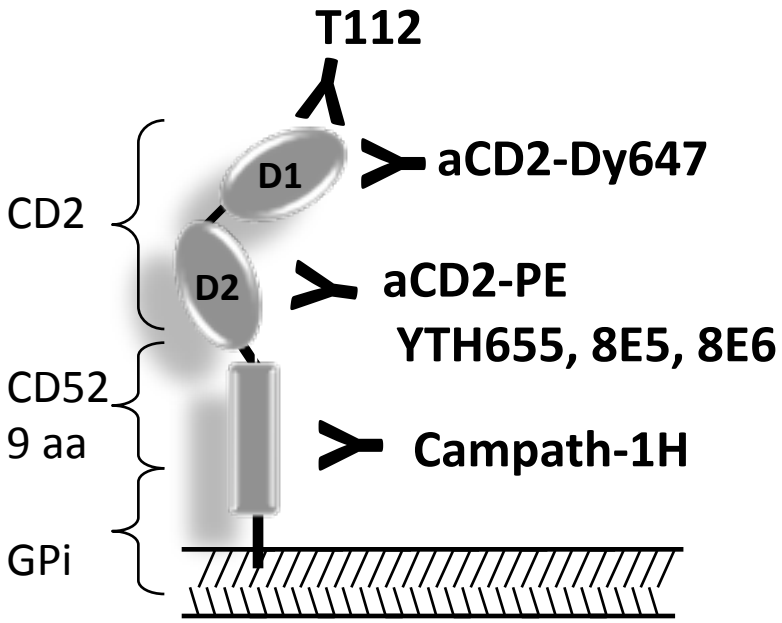


Figure 3.5 Proposed schematic of anti-human CD52 and CD2 mAb binding sites to B6.Foxp3^{hCD2} reporter protein

Based on the results of competitive binding assays (**Figure 3.4**) it was possible to construct a schematic illustrating the relative binding sites of various anti-human CD52 and CD2 monoclonal antibodies to the GPi-anchored human CD52_CD2 fusion protein in B6.Foxp3^{hCD2} mice. T11.2 (a mouse IgG2a mAb) and AbCam aCD2-Dy647 both bind to the distal CD2 subunit (D1). Several other anti-CD2 mAb (BD bioscience aCD2-PE, YTH655 and mouse 8E5/6) bind to the proximal CD2 subunit (D2). Finally, Campath-1H binds to the human CD52 epitope. These guidelines proved useful in determining various antibody combinations. For example, if YTH655 were used in vivo for ablation, aCD2-Dy647 could be used to identify residual hCD2^{+}Foxp3^{+} cells.

The concentration of fluorescently conjugated mAb that achieved 50-70% saturation was used in further cross competition assays to determine which anti-hCD2 and anti-hCD52 could bind to the fusion protein without overlap and which competed. 500,000 washed BHK cells were incubated in limiting dilutions of the test antibody in blocking buffer for one hour. Without further washing, the second mAb (fluorescently conjugated) was then added at a concentration previously shown to achieve 50-70% saturation for a further hour (figure 3.3). After washing three times, the cell samples were fixed and analysed by flow cytometry. Where possible, the reciprocal assay was performed. Figure 3.4 shows a summary of these cross competition studies of anti-hCD2 and anti-hCD52 mAb. Included are YTH616 (another rat anti-human CD2, IgG2b mAb) and three mouse anti-hCD2 IgG2a mAb; T11.2, 8E5 and 8E6. Three patterns of binding were found; reduced mean fluorescence of the second antibody with increasing concentration of the first (competition), no change in mean fluorescence of the second antibody with increasing concentration of the first (both mAb bind without overlap) and increased mean fluorescence of the second antibody with increasing concentration of the first at a certain range (augmentation; discussed further below). This suggested a binding schematic of the putative anti-human CD2 and CD52 mAb as illustrated in figure 3.5 and determined the best combinations of staining and depleting mAb. For example, when YTH655 was used for in vivo ablation of $hCD2^{+}Foxp3^{+}$ (see below), the percentage of surviving $Foxp3^{+}$ cells could be determined by flow cytometry using anti-hCD2 Dy647 that binds to a different domain of the human CD2 than YTH655. Equally, administration of two depleting antibodies that bind without overlap was hoped to result in synergistic ablation (see 3.3.3).

3.3.2 Multiple small doses of YTH655 rat IgG2b anti-human CD2 mAb achieved optimal ablation of Foxp3^{+} T cells in B6.Foxp3^{hCD2} mice

Having established that YTH655 and Campath-1H could bind without overlap to the human CD2_CD52 fusion reporter protein, both were administered in combination, i/p, into B6.Foxp3^{hCD2} mice to assess their ablative properties in vivo. After 24 hours the mice were killed, their spleen cells harvested and prepared for flow cytometry. Fluorescently conjugated anti-mouse Foxp3 mAb and anti-human CD52 or CD2 mAb, depending on which ablative mAb was given, were used to identify the percentage of residual CD4^{+}Foxp3^{+} cells compared to untreated control B6.Foxp3^{hCD2} mice. A single dose of 1mg YTH655 ablated over 40% of splenic Foxp3^{+} T-cells in B6.Foxp3^{hCD2} mice (figure 3.6). Whereas the same dose of Campath-1H ablated on average only 25% of splenic Foxp3^{+} T-cells.

Having shown that Campath-1H and YTH655 bind to different epitopes of the human CD52_CD2 fusion protein without overlap (section 3.3.1 and figure 3.4) it was hoped that co-administration would achieve synergistic cell lysis and greatly improve cell depletion (Bindon, Hale et al. 1985; Waldmann, Cobbold et al. 1987). This proved not to be the case (figure 3.6).

When the same dose of 1mg of Campath-1H and YTH655 was split into two intra-peritoneal injections administered over 48 hours, the degree of splenic cell ablation was significantly improved to 45% and 50% respectively (figure 3.6). However, no synergy was seen when both mAb were co-administered. There was no significant improvement when

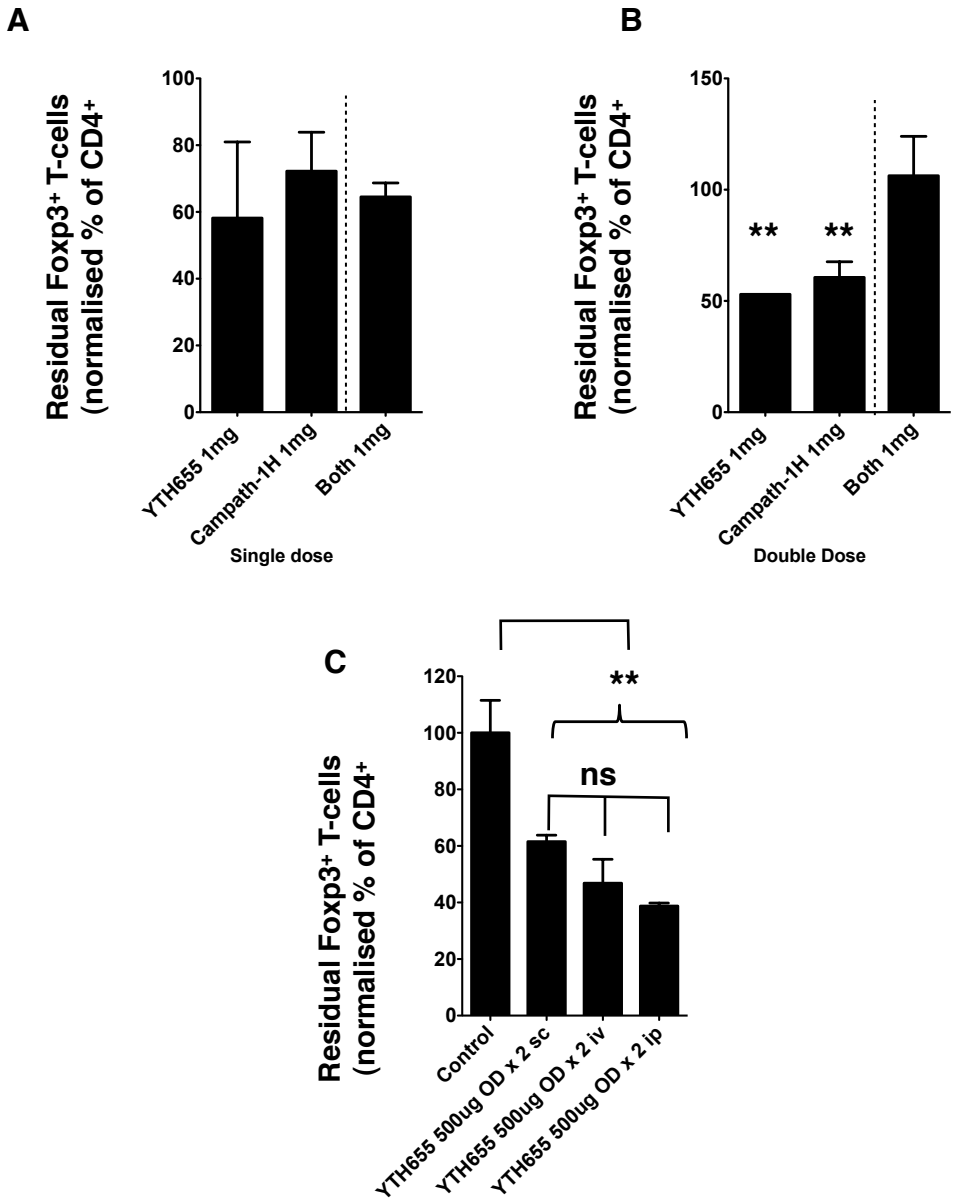


Figure 3.6 YTH655 and Campath-1H ablate Foxp3+ in B6.Foxp3^{hCD2} mice

B6.Foxp3^{hCD2} mice were killed 24 hours after ip injection of mAb and flow cytometry of prepared spleen cells was used to determine the percentage of residual Foxp3+ cells compared to control mice **(A)** A single 1mg dose of YT655 or Campath-1H did not result in significant ablation of Foxp3+ cells when injected i/p into B6.Foxp3^{hCD2} mice. **(B)** In contrast, two daily doses of 1mg YTH655 or Campath-1H resulted in significant ablation, $p<0.01$. Synergy was not found on combining the two. **(C)** Subcutaneous and intravenous administration of YTH655 failed to improve the degree of ablation achieved by intra-peritoneal injection. None of these regimens achieved more than 60% ablation of splenic Foxp3+ T-cells. Each bar represents the mean of up to three age matched mice with SEM indicated by the error bars. Statistical significance was analysed by ANOVA post-test.

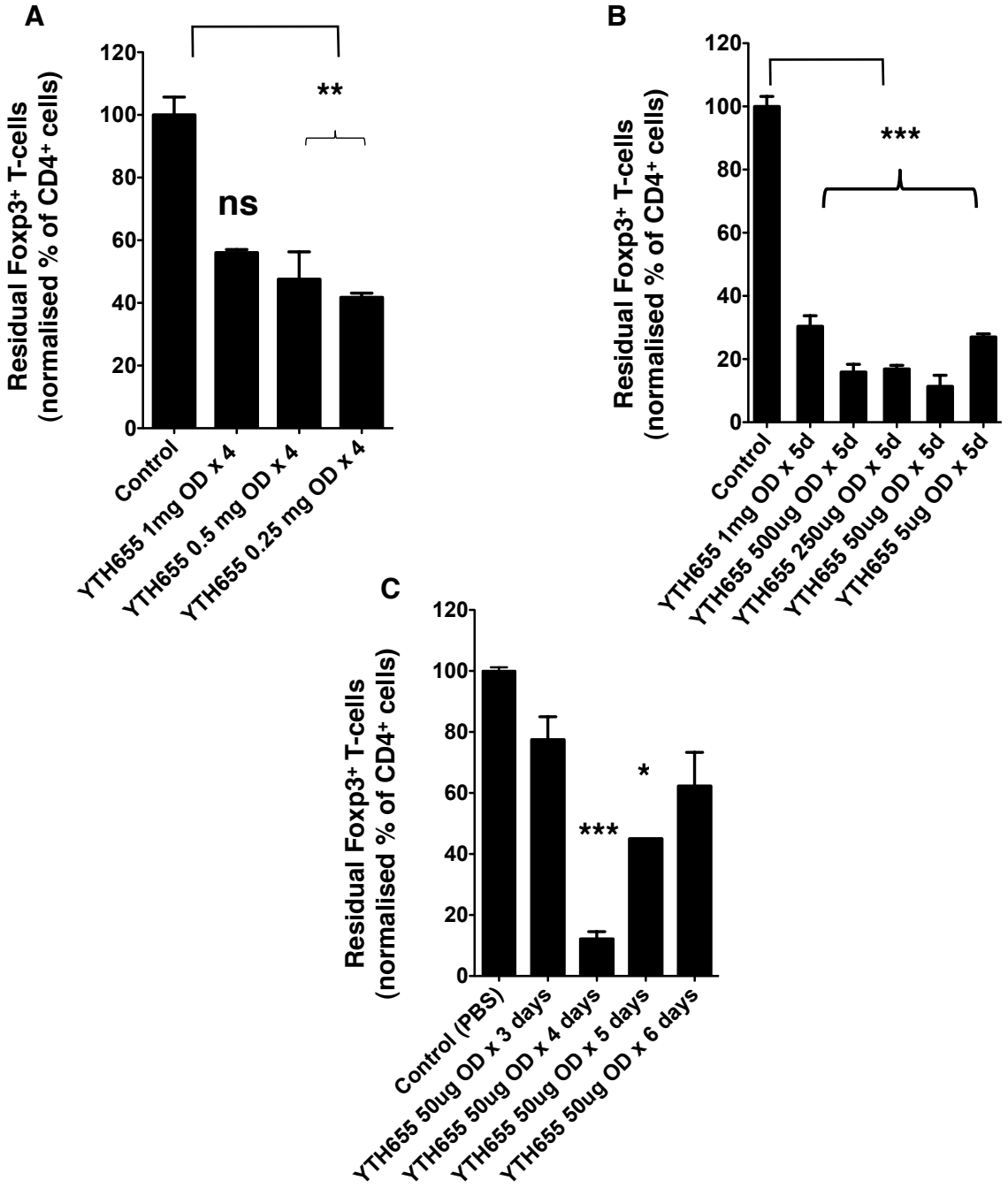


Figure 3.7 Multiple smaller doses of YTH655 result in improved ablation of Foxp3⁺ cells in B6.Foxp3^{hCD2} mice

(A) 4 daily i.p. injections of 500μg and 250μg of YTH655 resulted in significant depletion of 50% and 60% of splenic Foxp3⁺ cells respectively ($p<0.01$) **(B)** 5 daily injections of 50-500μg YTH655 resulted in ablation of over 80% of splenic Foxp3⁺ cells ($p<0.001$) **(C)** Treatment regimens using multiple doses of only 50μg of YTH655 proved unreliable. Each bar represents the mean of up to three age matched mice with SEM indicated by the error bars. Statistical significance was analysed by ANOVA post-test.

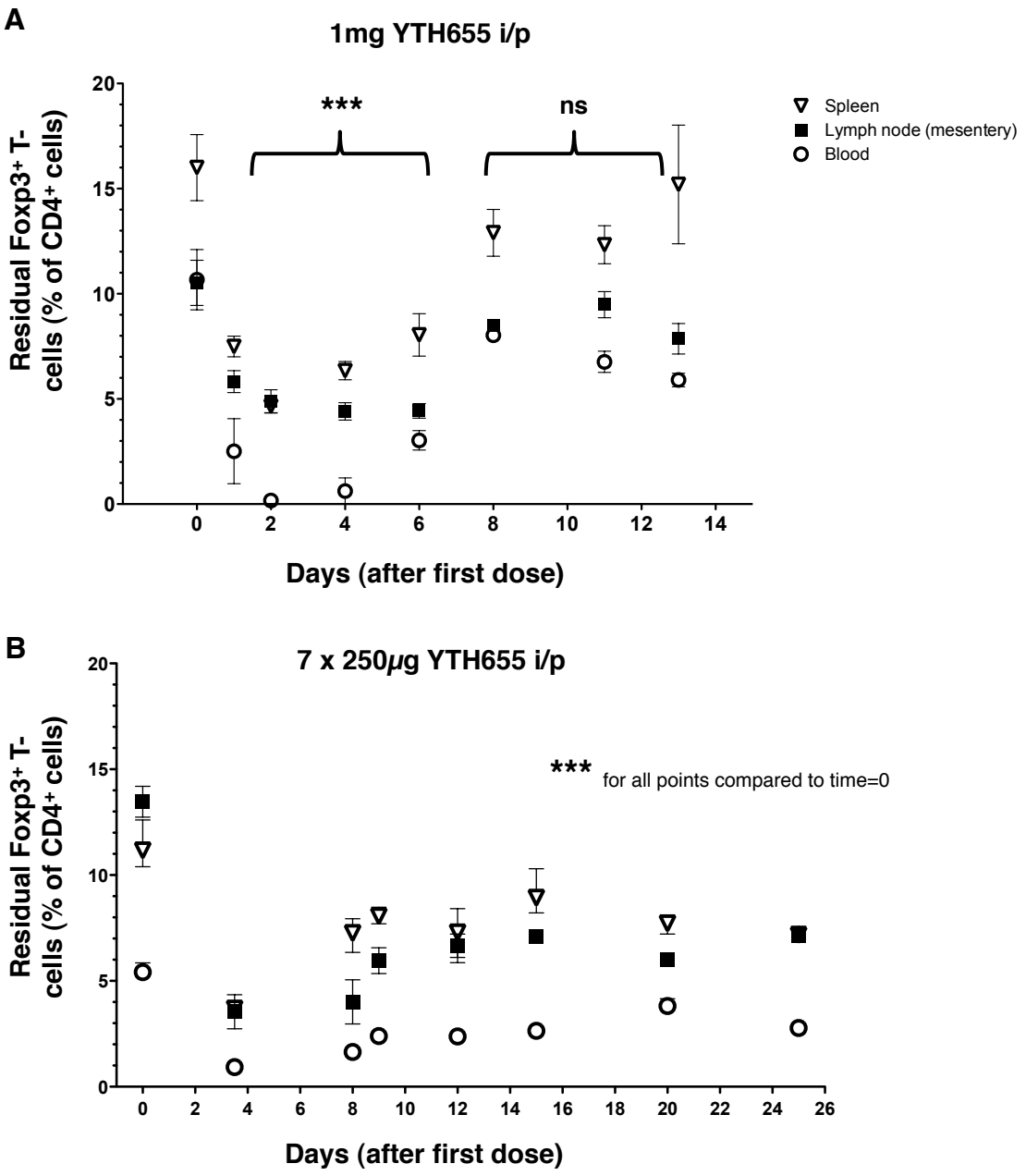


Figure 3.8 Seven daily injections of low dose YTH655 enhances the extent and duration of Foxp3+ cell ablation compared to a single high dose

(A) Groups of B6.Foxp3^{hCD2} mice were killed at various time points following a single i.p injection of 1mg YTH655. Residual Foxp3+ cells were detected by flow cytometry and compared to age matched untreated mice. Maximal ablation was found after 3 days. Almost all Foxp3+ cells in the blood were ablated. Over 30% of splenic and 50% of lymph node Foxp3+ cells survived. After 6 days there was no significant difference in Foxp3+ cells (as percentage of CD4+ cells) between treated and control mice. **(B)** Seven daily i.p. injections of 250μg YTH655 delayed the return of pre-treatment levels. 25 days after the first dose of YTH655, treatment mice still had significantly less Foxp3+ cells (as percentage of CD4+ cells) than control mice. Each point represents the mean of three age matched mice with SEM indicated by the error bars. ANOVA post-test was used.

YTH655 was given either sub cutaneously or intravenously compared to intraperitoneally (figure 3.6C). As a result of these two early findings, regimens consisting of multiple small doses of YTH655 given i/p were explored as a means to deplete a higher percentage of $Foxp3^+$ T-cells.

Four daily doses of 500μg and 250μg of YTH655 resulted in significant ablation of 50% and 60% of $Foxp3^+CD4^+$ T-cells respectively (figure 3.7A). Five consecutive daily doses of YTH655 (between 50μg to 1mg) resulted in depletion of approximately 70-80% of $Foxp3^+$ cells (figure 3.7B). These results demonstrated that multiple daily doses of YTH655 achieved a significantly greater degree of $Foxp3^+$ cell ablation compared to a single high dose injection. Although multiple injections of YTH655 at very low concentration (i.e. 50μg) could achieve up to 80% depletion of $Foxp3^+$ T-cells, this result was not consistent when repeated (figure 3.7C), suggesting that this dose was too low for reliable use.

The chosen regimen of 7 daily i/p doses of 250ug YTH655 was therefore a practical compromise between maximising the depletion of $Foxp3^+$ cells and minimising the overall protein load, handling and stress to which the mice were subjected. Figure 3.8 shows the degree of $Foxp3^+$ cell ablation and recovery over time in response to either 7 daily injections of 250μg or to a single high dose of 1mg YTH655. Flow cytometry was used to detect any residual $Foxp3^+$ cells in the spleen, lymph node and blood of treated mice. Three important similarities of the two treatment regimens were found.

- In both cases, ablation was maximal at least 2 days after the first injection.

- Foxp3^{+} cells in the blood stream were far more susceptible to ablation than those resident in the spleen or lymph nodes with fewer than 1% being detected after 3 days (discussed in 3.3.4).

- Finally, Foxp3^{+} cells returned to pre-treatment levels following cessation of treatment

In comparison to a single 1mg dose, 7 daily injections of 250μg YTH655 improved the maximal degree of splenic and lymph node Foxp3^{+} cells and significantly delayed the recovery of pre-treatment levels from 6 days to at least 25 days in total (figure 3.8B). In summary, multiple smaller doses improved the window of opportunity, by increasing the degree and duration of hCD2^{+}Foxp3^{+} cell ablation.

3.3.3 Mouse anti-human CD2 IgG2a mAb also ablated hCD2^{+} cell in B6.Foxp3^{hCD2} mice

Mouse IgG2a antibodies are known, in general, to be ablative in vivo (Herlyn and Koprowski 1982). For this reason, three mouse hybridomas producing anti-human CD2 IgG2a mAb, called T11.2, 8E5 and 8E6, were kindly obtained from Prof. Stefan Meuer so that their lytic properties in B6.Foxp3^{hCD2} mice could also be assessed.

Purified mAb was isolated from the integra supernatant of the three hybridoma cell lines (Chapter 2). In order to assess their ability to bind to human CD2, the purified antibody was then incubated in blocking buffer with washed BHK Cam hCD2-CD52 9aa cells that

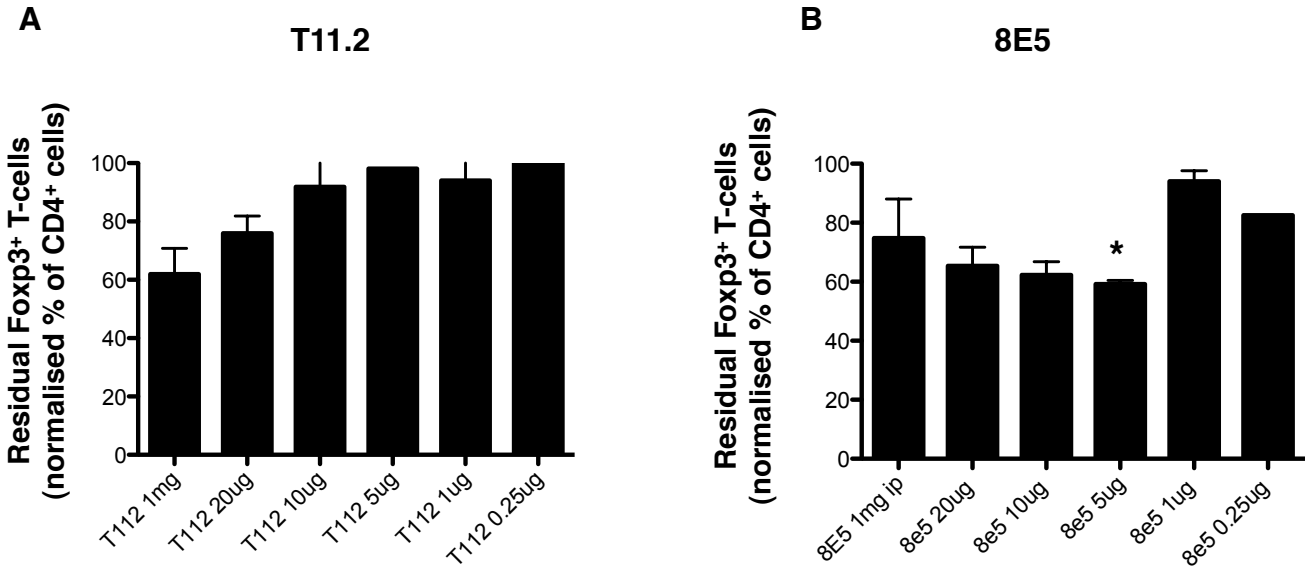


Figure 3.9 Mouse IgG2a anti-human CD2 mAb deplete hCD2^{+}Foxp3^{+} cells in B6.Foxp3^{hCD2} mice

Two mouse IgG2a anti-human CD2 mAb (**A** - T11.2 and **B** - 8E5) were injected intra-peritoneally into B6.Foxp3^{hCD2} mice at various concentration. The experimental mice were killed 24 hours later and residual Foxp3^{+} spleen cells expressed as a percentage of all CD4^{+} cells and normalised to control untreated mice. **(A)** T11.2 titrated from 1mg to 0.25μg failed to significantly ablate Foxp3^{+} cells. **(B)** 5μg of 8E5 mAb was the most effective of the doses used in this experiment and ablated approximately 40% of Foxp3^{+} spleen cells ($p>0.05$). Each bar represents the mean of two or three age matched mice with SEM indicated by the error bars. No significant difference was found by ANOVA post-test.

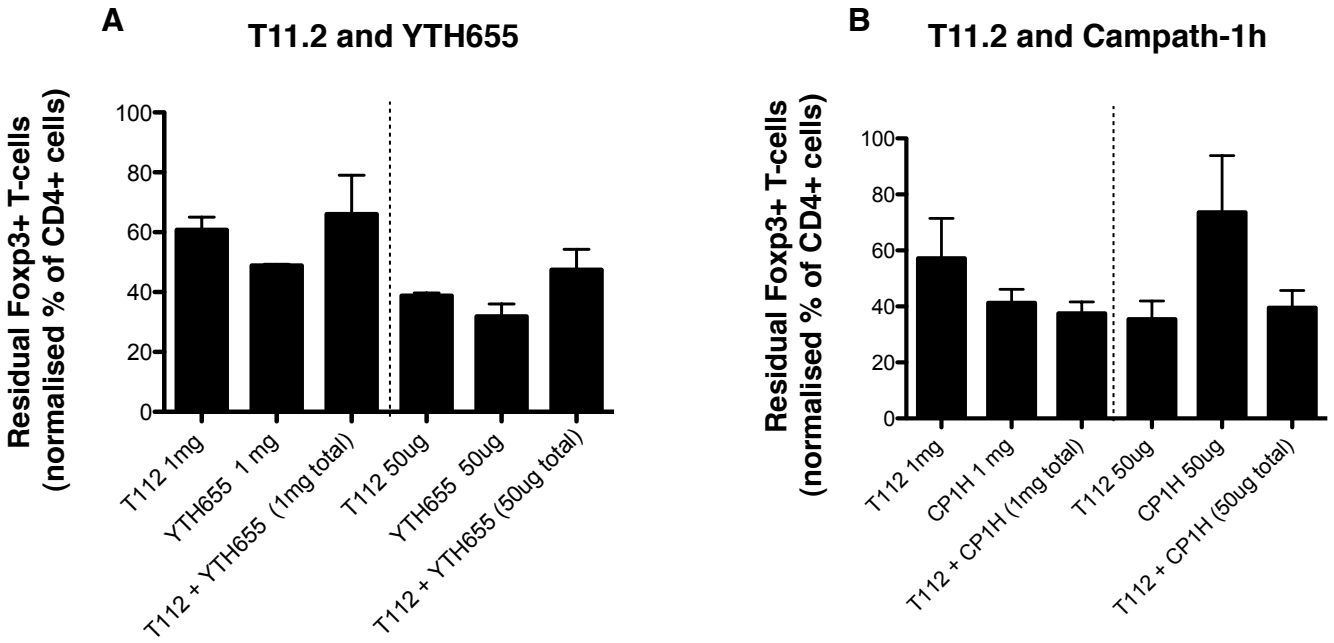


Figure 3.10 Mouse IgG2a anti-human CD2 mAb fail to surpass or synergise with rat IgG2b mAb in B6.Foxp3^{hCD2} mice

(A) High (1mg) and low (50μg) dose T11.2 failed to ablate significantly more Foxp3^{+} cells than the equivalent doses of YTH655. No synergy was found when both antibodies were used in combination. **(B)** Similarly, high (1mg) and low (50μg) dose T11.2 failed to ablate significantly more Foxp3^{+} cells than the equivalent doses of Campath-1H (CP1H). No synergy was found when both antibodies were used in combination. Each bar represents the mean of three age matched mice with SEM indicated by the error bars. Statistical significance was analysed by ANOVA Dunnett post-test.

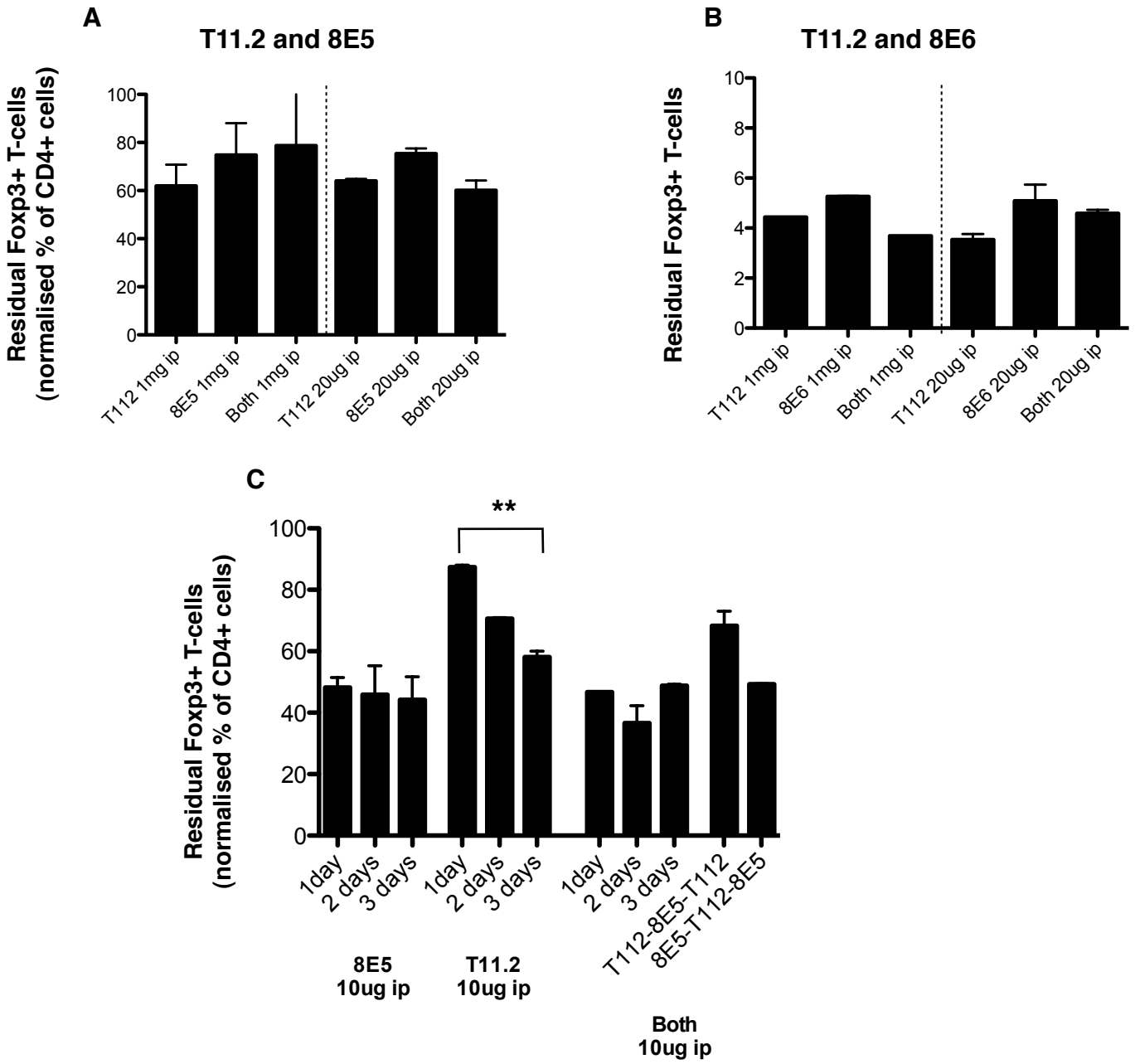


Figure 3.11 Failure of two mouse IgG2a anti-human CD2 mAb to achieve ablative synergy in vivo

(**A**)A combination of high dose (1mg) and low dose (20μg) T11.2 with 8E5 failed to significantly improve the ablation of Foxp3$^+$ cells compared to single use of each mAb. (**B**) A combination of high dose (1mg) and low dose (20μg) T11.2 with 8E6 failed to significantly improve the ablation of Foxp3$^+$ cells compared to single use of each mAb. (**C**) 3 daily doses of 10μg T11.2 ablated significantly more Foxp3$^+$ cells than a single dose. No such improvement with consecutive daily injections was found for 10μg 8E5. Regimens that combine the two mAb (e.g T11.2 then 8E5 then T11.2 over 3 days) also fail to result in significant synergistic ablation. Each bar represents the mean of three age matched mice with SEM indicated by the error bars. Statistical significance was analysed by ANOVA post-test.

constitutively express the same human CD52_CD2 fusion protein as Foxp3^{+} cells in B6.Foxp3^{hCD2} mice. Bound mAb was detected by flow cytometry using fluorescently conjugated anti-mouse IgG. Figure 3.4 shows that T11.2, 8E5 and 8E6 all bind to the human CD52_CD2 fusion protein. Cross competition studies against known anti-human CD2 and CD52 mAb demonstrated that 8E6 and 8E5 bind to the proximal domain (D2) of human CD2 and compete with YTH655 (figure 3.4). Whereas, T11.2 binds to the distal domain of CD2 (D1) and competes with AbCam anti-hCD2 Dy647 (figures 3.4 and 3.5).

The ability of T11.2, 8E5 and 8E6 anti-human CD2 IgG2a mAb to deplete hCD2^{+} cells in vivo was assessed by intra-peritoneal injection of the respective antibody solutions into B6.Foxp3^{hCD2} mice. As before, spleen cells were harvested from mice after 24 hours and the percentage of residual Foxp3^{+} cells was compared to untreated control B6.Foxp3^{hCD2} mice. 1mg of T11.2 i/p removed approximately 40% of Foxp3^{+} splenic T-cells (figure 3.9). Whereas, 1mg of 8E5 depleted approximately 25% of splenic Foxp3^{+} cells in B6.Foxp3^{hCD2} mice. These statistically insignificant levels of ablation were inferior to that achieved by an equivalent dose of YTH655 and Campath-1H (figure 3.6).

Previous structural studies of human CD2 have demonstrated that the binding site for human CD58 (a natural ligand for human CD2) is found at the tip of the distal domain (D1) (Li, Smolyar et al. 1996). Binding of CD58 causes a conformational change, effectively straightening up the CD2 dimer to expose a linker region called CD2R between domain 1 and 2. Antibodies that bind near the CD58 binding site of D1, such as T11.2, also result in the

same conformational change of human CD2 and might improve the binding affinity of those mAb that recognise D2 (e.g. 8E5, 8E6 and YTH655) by exposing the CD2R. T11.2 and anti-CD2 Dy647 both bind to the distal D1 and compete with each other (figure 3.4). Both enhance the binding of Campath-1H and YTH655 as measured by mean fluorescence intensity on flow cytometry (figure 3.4). It was therefore hoped that combining two non competing anti-human CD2 mAb would lead to synergistic depletion in vivo, particularly given that the binding of one (T11.2) could enhance that of the second (YTH655, 8E5, or 8E6). However, no synergistic cell depletion was found when combinations of T11.2 **plus** YTH655, 8E5 or 8E6 respectively were tried at high (1mg) and low (50μg) dose (figure 3.10 and figure 3.11). A similar result was observed when T11.2 was combined in vivo with Campath-1H (figure 3.10B). Multiple dose combinations of anti-human CD2 mAb directed against D1 and D2 failed to ablate more than 60% of splenic Foxp3$^+$ cells (figure 3.11C).

Collectively these findings did not support the future use of mouse anti-hCD2 mAb in depleting Foxp3$^+$ cells in B6.Foxp3^{hCD2} mice.

3.3.4 Circulating Foxp3$^+$ T-cells are vulnerable to mAb mediated ablation

In comparison to splenic hCD2$^+$Foxp3$^+$ cells, those circulating in the blood stream appeared more susceptible to mAb mediated ablation. Whereas no Foxp3$^+$ cells were found in the blood of mice 3 days after treatment with 1mg YTH655 i/p, at least a third of splenic and lymph node Foxp3$^+$ cells survived (figure 3.8); multiple daily doses of 50μg were not

consistent in depleting splenic Foxp3$^+$ cells but reliably ablated at least 80% of those found in the blood (figure 3.12B); and a significantly higher percentage of Foxp3$^+$ cells in the blood were ablated by high dose (1mg) or low dose (20μg) YTH655 than in the spleen (figure 3.12A, $p<0.05$). This phenomenon was not restricted to YTH655 because Campth-1H and T11.2 also achieved better ablation, dose for dose, of blood Foxp3$^+$ cells than those resident in the spleen (figure 3.12C).

There are many potential explanations for the observation that circulating hCD2$^+$Foxp3$^+$ cells are more susceptible to ablation that those resident in lymphatic tissue,

i) An inability to achieve a high enough antibody concentration in tissue compartments to adequately saturate the target cells

ii) Local factors within the tissue (but not in the circulation) that protected cells against mAb mediated lysis

iii) The necessity for antibody coated cells to be transported to a site favouring cell lysis

iv) Failure to sufficiently engage lytic machinery before the antibody was lost from the surface particularly in light of the observed down-modulation of surface antibody plus hCD52_hCD2 complexes (see below - 3.3.7)

v) Phenotypic differences between those Foxp3$^+$ cells found in lymphatic tissue and those that circulate which make the former more resistant to ablation than the latter

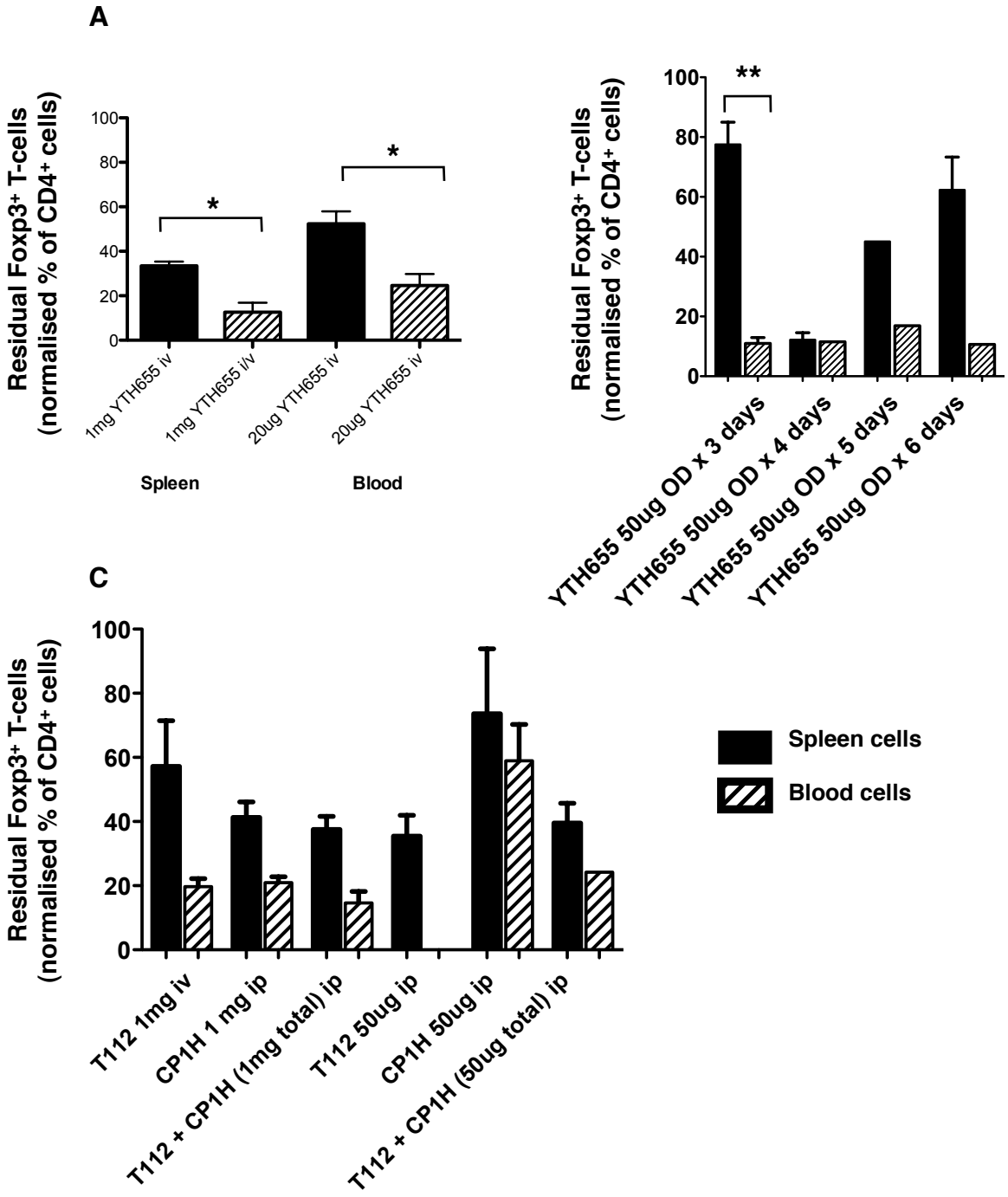


Figure 3.12 Circulating Foxp3+ cells are more susceptible to mAb ablation than those in the spleen

Regardless of which regimen of YTH655 (**A, B**), T11.2 or Campath-1H (**C**) is used, consistently more Foxp3+ cells circulating in the blood are ablated than those resident in the spleen. Each bar represents the mean of up to three age matched mice with SEM indicated by the error bars. Statistical significance was analysed by ANOVA Dunnett post-test.

There may be further explanations for the relative vulnerability of Foxp3^{+} cells in different compartments, but it was largely beyond the scope of the thesis to pursue, in depth, all the factors responsible for limiting cell ablation in this specific transgenic model. Nonetheless, it was important to investigate the last factor; is there something particular to splenic hCD2^{+}Foxp3^{+} cells that makes them resistant to ablation or would they be just as vulnerable if forced to circulate?

Spleen cells were prepared from B6.Foxp3^{hCD2} mice and washed before being injected intravenously into lymphocyte deficient B6 RAG$^{-/-}$ mice with or without 1mg YTH655. 4 weeks later, the mice were killed and flow cytometry was used to detect any remaining Foxp3^{+} cells in the spleen, lymph nodes and blood. The control group of B6 RAG$^{-/-}$ mice that received 2 million B6.Foxp3^{hCD2} spleen cells alone were found to have a normal contingent of Foxp3^{+} cells in all three compartments. In comparison, infusing B6 RAG$^{-/-}$ mice with cells plus 1mg YTH655 resulted in the elimination of >98% of Foxp3^{+} cells (figure 3.13A).

Even spleen cells that had survived the initial round of systemic ablation could be depleted when infused into lymphocyte deficient mice with 1mg YTH655 (figure 3.13B). Spleen cells were harvested form B6.Foxp3^{hCD2} mice 24 hours after the last dose of 7 x 250μg of YTH655, prepared and infused into B6 RAG$^{-/-}$ recipient mice with or without 1mg YTH655. When the surviving spleen cells alone were injected intravenously into lymphocyte deficient mice, a normal percentage of Foxp3^{+} cells was detected. Whereas very few, if any, Foxp3^{+} cells were found in those mice that received surviving cells plus 1mg YTH655 (figure 3.13B,

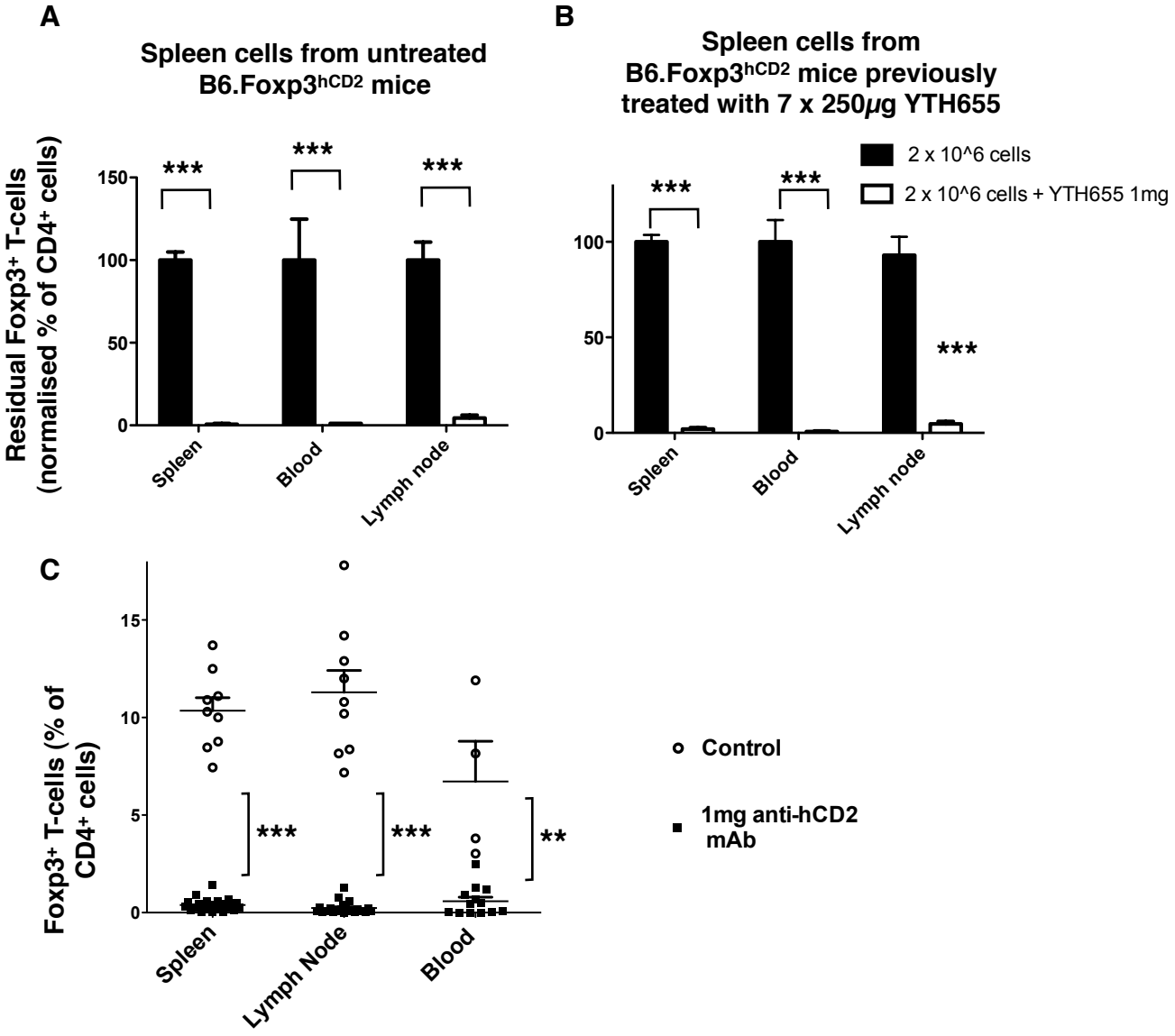


Figure 3.13 Adoptive transfer of B6.Foxp3^{hCD2} cells coated with anti-hCD2 mAb efficiently ablates Foxp3^{+} cells

(A) 2x10^6 B6.Foxp3^{hCD2} spleen cells with or without 1mg YTH655 were injected i.v. into lymphocyte deficient hosts. 4 weeks later, fewer than 2% of Foxp3^{+} cells were detected in the spleen, blood and lymph nodes compared to control mice (2x10^6 B6.Foxp3^{hCD2} cells alone). (**B**) Even spleen cells from mice harvested 24 hours after previous treatment with 7x250μg YTH655 were efficiently ablated. Each bar represents the mean of three age matched mice with SEM indicated by the error bars. (**C**) A summary of at least 4 separate adoptive transfer assays of B6.Foxp3^{hCD2} spleen cells plus 1mg YTH655, n>9. Error bars indicate SEM. Statistical significance was analysed by t-test.

$p<0.0001$). Collectively these results demonstrated that 'forced' circulation of $hCD2^{+}Foxp3^{+}$ cells rendered them more susceptible to antibody mediated ablation and importantly, offered a more efficient method of depleting $Foxp3^{+}$ cells by exploiting adoptive cell transfer protocols (figure 3.13B).

3.3.5 Detecting residual functional anti-hCD2 mAb in the serum of treated mice

It was possible to determine the concentration of residual YTH655 mAb by incubating the serum of treated mice with BHK Cam hCD2-CD52 9aa cells that express the human CD2_CD52 reporter protein. A fluorescently conjugated anti-rat IgG2b mAb was used to detect bound YTH655 and the resultant mean fluorescence on flow cytometry was compared to that of a known standard of YTH655 (figure 3.14). This method of detecting the concentration of residual mAb in a treated mouse has the advantage of only detecting functional mAb still capable of binding to the target reporter protein. An average of 7500ng/ml residual YTH655 was detected 4 days after the first of 7 daily doses of 250μg YTH655. No residual antibody was detected by this method 8 days after the first dose.

The failure to detect residual YTH655 8 days after the first treatment dose does not necessarily mean that there is no residual functional YTH655. Drawing that conclusion is limited by the sensitivity of the assay. The assay also fails to determine whether $hCD2^{+}$ cells that survive antibody mediated ablation are adequately saturated with ablative mAb. At best, it therefore serves as a comparative guide. For example, 5 daily injections of 500μg resulted in

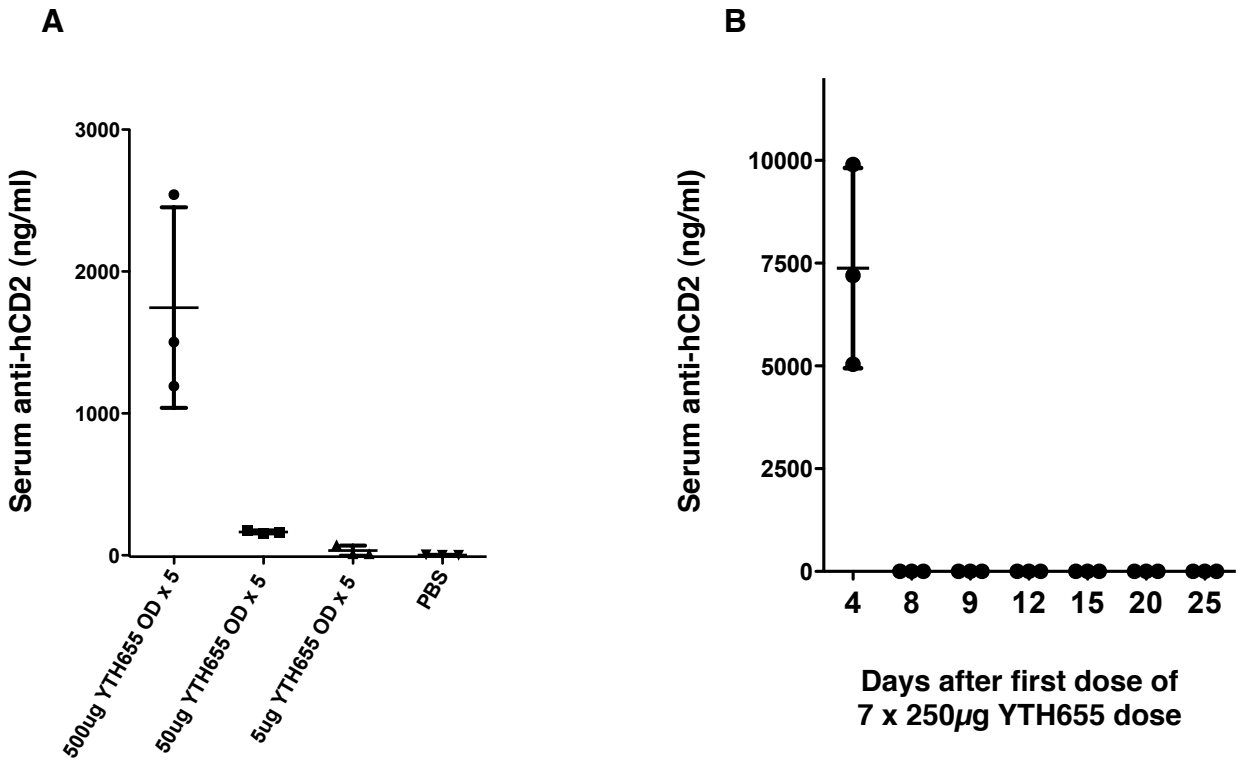


Figure 3.14 Serum concentration of YTH655 in treated B6.Foxp3^{hCD2} mice

The concentration of unbound, functional, YTH655 in treated B6.Foxp3^{hCD2} mice was detected by flow cytometry. The resultant signal intensity of fluorescently conjugated anti-rat IgG2b mAb was compared to a known standard. (**A**) 5 daily injections of 500μg YTH655 resulted in at least 1μg/ml of residual functional antibody detectable after 24 hours. In comparison, very little excess YTH655 was detected after regimens consisting of lower doses (e.g. 5 x <50μg). (**B**) Over 5μg/ml excess YTH655 was detected after 4 of the 7 daily injections of 250μg YTH655. After the last dose no residual YTH655 was detected by this method. Error bars indicate SEM.

over 1μg/ml of detectable YTH655 in the serum on day 6, as compared to <150ng/ml in mice treated with much lower doses of 5 x 50μg, or 5 x 5μg (figure 3.14). This result was another reason for choosing a lower dose regimen of 7 x 250μg in vivo.

3.3.6 B6.Foxp3^{hCD2} mice develop anti-globulin in response to treatment antibody

Although there are may be several physiological routes for clearing treatment antibody, the neutralising anti-globulin response is one that is susceptible to manipulation (Benjamin, Cobbold et al. 1986).

Mice treated with depleting rat IgG2b antibodies produce a neutralising antibody response to the foreign protein that can ultimately limit the efficacy of the depleting treatment antibody (Benjamin, Cobbold et al. 1986) and indeed, this was a driving force in developing a humanised form of anti-human CD52 mAb Campath-1H therapy (Rebello, Hale et al. 1999). The anti-globulin response to YTH655 was detected by double capture IOC-ELISA (Chapter 2) of serum collected from treated B6.Foxp3^{hCD2} mice and compared to a mouse anti-rat IgG2b mAb standard (Norig 7.16.2) of known concentration (Cobbold, Rebello et al. 1990). The first detectable anti-globulin response was found 14 days after a single i/p injection of 1mg YTH655, peaked after 28 days to over 15μg/ml and was undetectable by this method after 40 days (figure 3.15A). A similar anti-globulin response was produced by B6.Foxp3^{hCD2} mice that received 7 daily doses of 250μg i/p, rising for 4 weeks after the first dose (figure 3.15B).

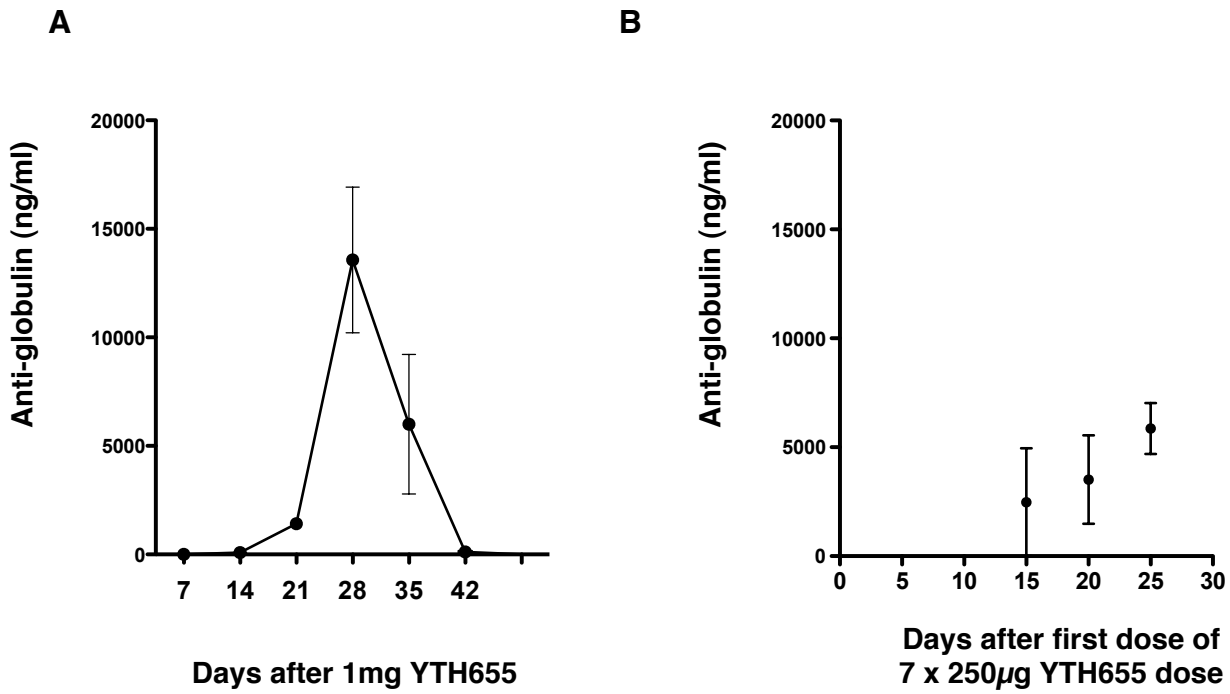


Figure 3.15 Anti-globulin response of B6.Foxp3^{hCD2} cells to single and multiple doses of YTH655

Serum samples from B6.Foxp3^{hCD2} mice treated with 1mg YTH655 were prepared and analysed for specific (anti-YTH655) anti-globulin by double capture IOC ELISA. (**A**) Peak anti-globulin response was detected 4 weeks following a single injection of 1mg YTH655 and was undetectable by this technique after 6 weeks. (**B**) Anti-globulin response to 7x250μg YTH655. Each point represents the mean of three age matched mice with SEM indicated by the error bars.

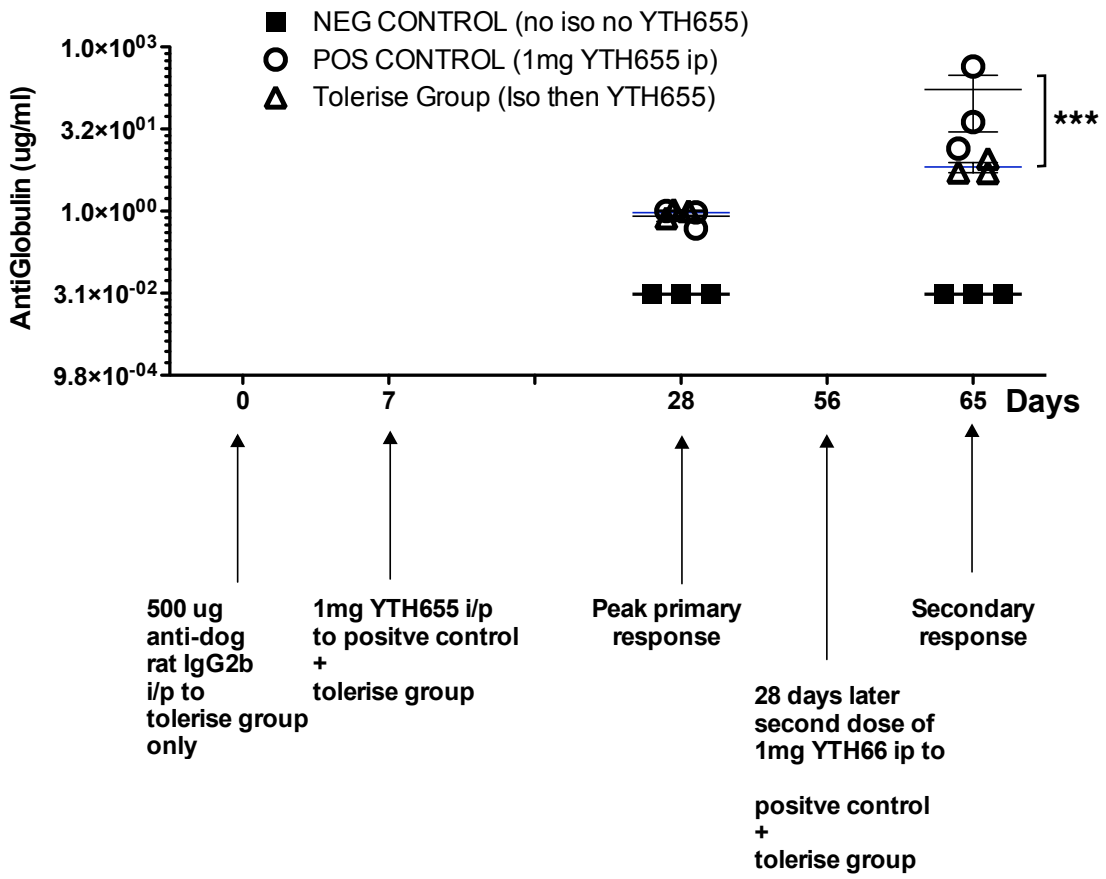


Figure 3.16 B6.Foxp3^{hCD2} mice can be tolerised to YTH655 mAb

1mg de-aggregated, non cell-binding mAb of the same isotype as YTH655 (YKIX 337.217.7, rat IgG2b) was given i.p to the 'tolerise group' of B6.Foxp3^{hCD2} mice. 7 days later, 1mg YTH655 was given to these mice and B6.Foxp3^{hCD2} mice in the 'positive control group'. Serum anti-YTH655 anti-globulin was measured using double capture IOC ELISA on day 28 and compared to the age matched, untreated 'negative control group'. A second i.p injection of 1mg YTH655 was given 28 days later and the secondary anti-globulin response measured after a further 9 days. The peak primary anti-globulin response was similar between the 'tolerise group' and 'positive control group'. The secondary response was significantly reduced in the 'tolerised group' compared to the 'positive control group,' $p<0.0001$. Error bars indicate SEM. t-test used to determine statistical significance.

It has previously been possible to tolerise and limit this anti-globulin response in hosts by pre-treatment with a monoclonal immunoglobulin of similar isotype that importantly does not bind to the cell surface (Benjamin, Cobbold et al. 1986). In a similar experiment, a cohort of B6.Foxp3^{hCD2} mice were pre-treated with 500μg of YKIX 337.217.7 (Chapter 2), a rat IgG2b mAb monoclonal immunoglobulin that does not bind to mouse cells, followed by a priming dose of 1mg YTH655. The initial anti-globulin response was once more screened on a weekly basis using double capture IOC ELISA. There was no significant difference in the peak primary anti-globulin response to YTH655 between mice pre-treated with the non-binding mAb compared to the positive control group that received 1mg YTH655 alone (figure 3.16). In keeping with adaptive immune memory, the mice were re-challenged with a second injection of 1mg YTH655 28 days later and the peak secondary anti-globulin response was measured a further 9 days later. This secondary anti-globulin response was significantly reduced, but still detectable, in mice pre-treated with tolerising non-binding mAb (figure 3.16).

The above results indicated that the anti-globulin response to YTH655 would ultimately limit its long term use. If a protocol required chronic treatment of B6.Foxp3^{hCD2} mice with anti-hCD2 mAb, pre-treatment with non-binding rat IgG2b mAb monoclonal immunoglobulin could attenuate the anti-globulin response and so prolong its effects.

3.3.7 YTH655 results in down-modulation of surface CD52_CD2 fusion protein in vivo

Treatment of B6.Foxp3^{hCD2} with YTH655 anti-hCD2 mAb results in down-modulation of the GPi-linked human CD52_CD2 reporter protein off the surface of hCD2^{+}Foxp3^{+} cells. In many of the initial ablation assays, spleen cells that were harvested from YTH655 treated mice were incubated with anti-human CD2 Dy647; a fluorescently conjugated mAb that binds to a different domain of the human CD2 reporter protein without competition or overlap with YTH655 (figures 3.4 and 3.5). In those mice that were treated with YTH655, the mean fluorescence intensity of anti-hCD2 Dy647 of surviving Foxp3^{+} was reduced by at least 70% compared to Foxp3^{+} cells from control B6.Foxp3^{hCD2} mice (figure 3.17). The down modulation of the reporter protein also occurred in response to Campath-1H, demonstrating that this phenomenon is not restricted to YTH655 treatment (figure 3.17E). The route of antibody administration did not affect the degree of down-modulation (figure 3.17D).

As well as potentially limiting the ablation of hCD2^{+} cells in this model (see 3.4 Discussion), down-modulation of the reporter meant that staining for Foxp3 by flow cytometry proved the most reliable method for determining the number of residual Foxp3^{+} Treg after ablation.

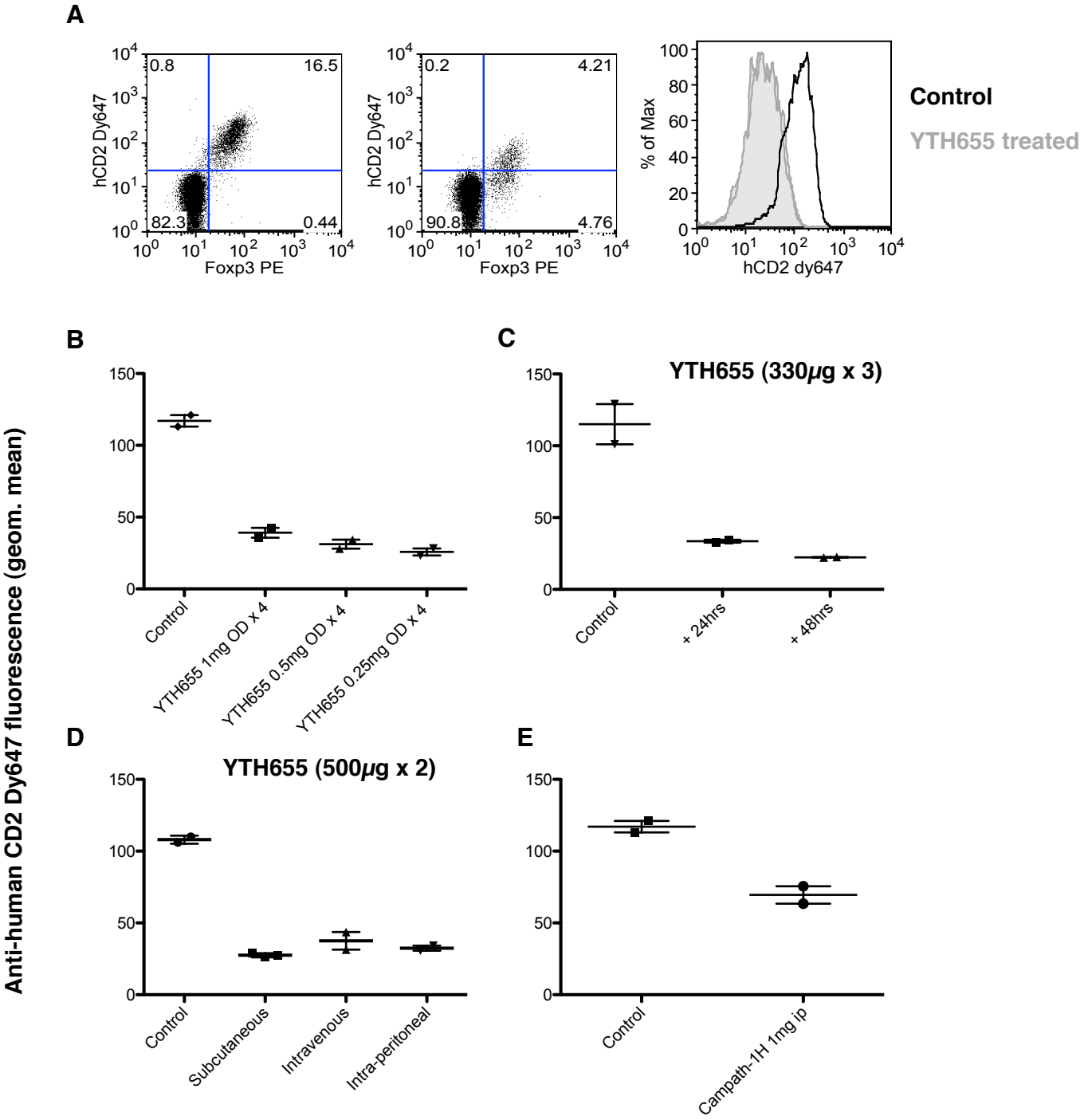


Figure 3.17 In vivo YTH655 mAb results in down modulation of hCD52_hCD2 membrane protein

Foxp3^{+} cells from B6.Foxp3^{hCD2} mice that were treated with YTH655 were stained with anti-hCD2 Dy647 mAb that binds to a separate epitope from the ablative YTH655. (**A**) Flow cytometry revealed a decrease in the anti-CD2 Dy647 signal intensity of cells from YTH655 treated mice compared to untreated control mice (**B**) This down-modulation of hCD52_hCD2 surface protein was found following 4 daily doses of 250μg-1mg YTH655, continued for at least 5 days following the first dose (**C**) and occurred independently of the route of YTH655 administration (**D**). Campath-1H also resulted in down-modualtion of the reporter protein (**E**). Error bars indicate SEM.

3.4 Discussion

The transgenic B6.Foxp3^{hCD2} knock-in model offers a way of manipulating specifically Foxp3^{+} cells based on their co-expression of a cell surface human CD2_CD52 fusion protein. A variety of monoclonal antibodies directed against human CD2 and human CD52 epitopes allow accurate identification, isolation and ablation of live Foxp3^{+} cells.

The human CD52_CD2 fusion protein is an accurate cell surface reporter of intracellular Foxp3 expression in B6.Foxp3^{hCD2} mice. Over 98% of Foxp3^{+}CD4^{+} cells are hCD2 positive and less than 1% of hCD2^{neg} cells are Foxp3^{+}, meaning that any protocol which removes all the hCD2^{+} cells would effectively remove all Foxp3^{+} cells. Fluorescently conjugated monoclonal antibodies directed against the reporter protein can also be used by flow cytometry to separate cells into live effector and regulatory populations based on their respective expression of Foxp3.

Approximately 10% of hCD2^{+} cells are Foxp3 negative at the time of testing (figure 3.1). This apparent discrepancy is not explained by the weaker signal intensity of the fluorescently conjugated anti-Foxp3 mAb compared to that of the anti-hCD2 mAb since titration of two different anti-Foxp3 mAb to higher concentrations failed to eliminate the population (data not shown). Alternatively, our collaborators have shown that a minor population of hCD2^{+}CD25^{-} cells demonstrate transient expression of Foxp3 (Komatsu, Mariotti-Ferrandiz et al. 2009). Some retain Foxp3 expression, some lose it only to regain it

later under favourable conditions and some go on to exhibit T-helper cell activity. It is therefore possible that within lymphocyte-replete mice there exists a small population of $CD4^+$ cells that exhibit a degree of plasticity and may only temporarily express the *foxp3* gene. If the half life of the reporter protein is greater than that of Foxp3, such cells would stain positively for hCD2 while being $Foxp3^{negative}$. From an operational point of view this is advantageous because removal of $hCD2^+$ cells, either by cell sorting or ablation, will also eliminate these $CD4^+$ cells that have transiently expressed Foxp3 and retain the potential to be Treg in the future.

None of the mouse or rat anti-human CD2 and CD52 ablative antibodies were able to completely deplete the $Foxp3^+$ cells in B6.$Foxp3^{hCD2}$ mice. Although mouse IgG2a mAb are generally known to be ablative in vivo, it was not known whether mouse anti-hCD2 mAb, in particular, would be ablative (Herlyn and Koprowski 1982). T11.2 mouse anti-hCD2 was able to remove up to 50% of splenic $Foxp3^+$ cells at high (1mg) and low (50μg) dose (figure 3.10 and 3.11). It performed better than the other two mouse anti-hCD2 mAb (8E5, 8E6), but was less effective than YTH655, a rat IgG2b anti-CD2 (domain 1) mAb. Indeed, multiple small doses of YTH655 achieved the best result by ablating over 60% of splenic and over 95% of blood $Foxp3^+$ cells (figure 3.8).

The same YTH655 mAb achieved over 98% ablation of splenic $hCD2^+$ cells in a previous transgenic model in which all lymphocytes expressed hCD2 on their cell surface (Qin, Cobbold et al. 1993). Although, it is beyond the scope of this project to pursue

mechanism of antibody mediated ablation in vivo, it is interesting to speculate why the same antibody was more ablative in the previous model compared to the new B6.Foxp3^{hCD2} mice. Perhaps there is a lower density of target hCD2 on the surface of B6.Foxp3^{hCD2} cells, in which case one would expect a relative decrease in mean fluorescence intensity on flow cytometry.

Down-modulation of the target hCD2_hCD52 reporter in response to surface antigen-antibody complex formation may also impair depletion; particularly if this further reduces the density of bound antibody below the threshold necessary to result in cell lysis (figure 3.17). Interestingly it also occurs in response to Campath-1H, suggesting that this response is not specific to YTH655 (figure 3.17). A number of different mechanisms for down-modulation have classically been described for various cell surface receptors and include internalisation or surface shedding of immune receptors (Robb and Greene 1987; Huizinga, van der Schoot et al. 1988). Alteration of the binding site by the treatment mAb can inhibit binding of the staining antibody and so cause an apparent decrease in signal intensity. In this case, previous competition assays failed to show inhibition of staining anti-hCD2 Dy647 by bound YTH655 treatment antibody and so do not support this last explanation (figure 3.4). Admittedly, this assumes that the human CD2/CD52 protein expressed by the BHK cell line used in the competition studies is identical to that displayed on the surface of Foxp3^{+} cells in B6.Foxp3^{hCD2} mice.

There may be a critical time period during which antibody coated Foxp3^{+} cells need to interact with lytic pathways before down-modulation of antibody bound surface targets limits

ablation. Hu et al. found that Campath-1H mediated depletion of immune cells in a CD52 transgenic mouse was far more dependent on ADCC (antibody-dependent cell-mediated cytotoxity), particularly through NK cells and neutrophils, than complement activation (Hu, Turner et al. 2009). hCD2^{+}Foxp3^{+}cells circulating in the blood may be more likely to engage these ADCC mechanisms before significant reporter down-modulation and this may be one explanation for their increased susceptibility to depletion by both anti-hCD2 and Campath-1H (figures 3.12 and 3.13). It may also explain why even splenic Foxp3^{+} cells that had survived one round of systemic anti-hCD2 mediated ablation could be completely ablated if injected intravenously into lymphocyte deplete mice (figure 3.13C). Of note, Hu et al. also found that splenic Treg were more resistant to Campath-1H ablation than circulating Treg and, in general, Treg were harder to ablate than Th cells.

The efficiency of antibody mediated ablation can be limited by the rate of antibody clearance (Scully, Qin et al. 1994). In order to investigate this, a BHK cell line expressing the same hCD2_hCD52 reporter protein was used to screen plasma, sampled from treated mice, for residual YTH655 that could still bind hCD2. Beyond 4 days after the first of 7 x 250μg doses, no functional YTH655 was detected by this method (figure 3.15), a time point that coincides with peak ablation of Foxp3^{+} cells (figure 3.8B). Thereafter, Foxp3^{+} Treg returned to pre-treatment levels over a period of two weeks, presumably through the expansion of surviving Treg in lymphoid tissue (figure 3.8B).

One way of overcoming the limited depletion and subsequent reconstitution following a short course of treatment is to increase the duration of antibody therapy. However, the anti-globulin response mounted by recipients often limits the efficacy of long term regimens by neutralising the treatment antibody (Cobbold, Rebello et al. 1990; Rebello, Hale et al. 1999). The peak primary anti-globulin response to YTH665 occurred in B6.Foxp3^{hCD2} mice after 4 weeks and was undetectable after 6 weeks (figure 3.15A). Exposure to a non cell-binding antibody has been shown to reduce the anti-globulin response to a subsequent treatment antibody of the same isotype (Benjamin, Cobbold et al. 1986). A similar reduction in the secondary anti-globulin response, but not the primary response, was found in mice that were pre-treated with a non binding rat IgG2b mAb (figure 3.16). Unlike lytic mAb, the tolerising mAb does not bind the cell surface and so is less likely to cluster and engage inflammatory signalling pathways. It also does not result in cell lysis that can further evoke an inflammatory response. This tolerising protocol therefore shares many characteristics with tolerance induction by high dose antigen administered continuously by an osmotic pump (discussed further in Chapter 5) (Kretschmer, Apostolou et al. 2005).

Initially it seemed that the inability to find an antibody regimen that could deplete all Foxp3^{+} Treg for a sustained period of time would preclude B6.Foxp3^{hCD2} mice from being a useful model to study transplant tolerance. In retrospect, sub-optimal ablation of Foxp3^{+} Treg was operationally sufficient in many transplant experiments to overcome induced tolerance (Chapters 4 and 5). Moreover, enough residual Foxp3^{+} Treg survived to prevent the mice from developing the severe, wasting immuno-pathology described in many systems devoid of Foxp3^{+} cells. Their role in transplant tolerance could therefore be examined in the context of

an otherwise healthy mouse. Furthermore, the discovery that adoptive transfer of B6.Foxp3^{hCD2} spleen cells mixed with 1mg YTH655 resulted in over 95% ablation of Foxp3^{+} cells offered an alternative protocol for any study requiring near total depletion of Foxp3^{+} Treg. This protocol proved useful in two main areas; examining the residual capacity of an otherwise tolerant population of lymphocytes to reject a graft when stripped of its Foxp3^{+} Treg (Chapter 4) and investigating under what circumstances an immune system devoid of Foxp3^{+} Treg could achieve tolerance (Chapter 6).

CHAPTER 4: Foxp3+ regulatory T-cells are crucial to transplant tolerance induced by co-receptor and co-stimulatory blockade

4.1 Abstract

Foxp3^{+} regulatory T-cells (Treg) are a subset of CD4^{+} T lymphocytes thought to be essential in transplant tolerance. However, without the discovery of a natural cell surface marker by which they can be manipulated in vivo, it has not been possible to establish their role definitively. Foxp3^{+} cells in B6.Foxp3^{hCD2} reporter mice co-express a Gpi-anchored human CD2_CD52 fusion protein on their cell surface. Ablative anti-human CD2 mAb is used here to target hCD2^{+}Foxp3^{+} cells in reporter mice tolerised to foreign skin grafts. For the first time, it has been possible to prove that Foxp3^{+} Treg are crucial to therapeutic transplant tolerance generated by short term co-receptor and co-stimulatory blockade. Effector T cells capable of rejecting the graft remain in an otherwise tolerant host and require constant suppression by Foxp3^{+} Treg.

4.2 Introduction

It has become increasingly clear that immune homeostasis is indispensable, self sustaining and incorporates multiple levels of regulation. The hope is that a better understanding of these processes will provide therapeutic targets for short term intervention to result in long lasting benefit. The ability to re-program an immune system therefore promises significant gains in the management of autoimmune disease, graft rejection and cancer. Moreover, it represents a fundamental therapeutic shift away from chronic treatments

which indiscriminately penalise the whole immune system and whose side effects cause significant morbidity.

Transplantation models of immune tolerance have classically been used to investigate the mechanisms underlying regulation, partly because they offer the practical advantage of control over a number of important factors. These include the timing of tolerance induction and subsequent intervention, the type and site of tissue, the antigenic burden, and the type of immune cells under investigation. They also provide an attractive translational route to improving clinical graft survival rates.

It has previously been established that short-term co-receptor (CD4, CD8) and co-stimulatory (CD40L) blockade with non depleting monoclonal antibodies can induce long lived tolerance to foreign tissue (Waldmann and Cobbold 1998). Furthermore, regulatory elements within the $CD4^+$ population carry that tolerance with them when adoptively transferred into secondary hosts (Qin, Cobbold et al. 1993; Scully, Qin et al. 1994). What is unknown is which $CD4^+$ regulatory cells are critical to this antibody mediated transplant tolerance. Several $CD4^+$ cell subtypes have been shown to have a regulatory phenotype and these include $Foxp3^+$ T-cells (Bennett, Christie et al. 2001; Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003), IL-10 secreting Tr1 cells (Levings, Sangregorio et al. 2002), TGFβ secreting Th3 cells (Weiner 2001) and most recently $Foxp3^{neg}$ iT(R)35 cells (Collison, Chaturvedi et al. 2010). Of these, $Foxp3^+$ Treg have been found to be essential to self-tolerance (Kim, Rasmussen et al. 2007; Lahl, Loddenkemper et al. 2007).

In contrast, the role of Foxp3$^+$ Treg in tolerance to foreign tissue has not been confirmed despite a large collection of favourable circumstantial evidence. This data typically falls into three categories; (i) the enhancement of graft survival by adoptive transfer of Foxp3$^+$ rich, but impure, CD25$^+$CD4$^+$ populations (Golshayan, Jiang et al. 2007; Feng, Wood et al. 2008; Joffre, Santolaria et al. 2008; Xia, Shah et al. 2009), (ii) the induction of Foxp3$^+$ Treg as a result of tolerogenic protocols (Cobbold, Castejon et al. 2004; Battaglia, Stabilini et al. 2006; Turnquist, Raimondi et al. 2007) and (iii) the detection of small numbers of Foxp3$^+$ Treg in tolerated grafts (Cobbold, Castejon et al. 2004; Lee, Wang et al. 2005; Fan, Spencer et al. 2010; Semiletova, Shen et al. 2010). However, simply demonstrating their presence, regardless of how well it is performed, fails to confirm a genuine operational role for Foxp3$^+$ Treg. Achieving that conclusive evidence has been hampered by the lack of a natural cell surface marker by which Foxp3$^+$ Treg can be specifically manipulated in vivo. In addition, although diphtheria toxin ablation of Foxp3$^+$ cells in DEREG models has dramatically demonstrated the critical role of Foxp3$^+$ Treg in self tolerance, the resultant systemic immunopathology is so severe that it limits the investigation of long-term induced tolerance to foreign antigen (Kim, Rasmussen et al. 2007). Few mice survive beyond three weeks and any destruction of foreign graft tissue within that time period would have to be interpreted in the context of global tissue disruption (whether foreign or host) by an unregulated immune system.

By combining classical transplant tolerance studies with the transgenic B6.Foxp3^{hCD2} model it is possible to selectively target Foxp3^{+} Treg and so investigate their exclusive roles in tolerance to foreign tissue. Using ablative anti-human CD2 antibodies, Foxp3^{+} Treg are confirmed necessary for therapeutic tolerance induced by co-receptor/co-stimulatory blockade across full MHC, multiple minors and single minor histocompatibility barriers. Interestingly, T-cells capable of rejecting the tolerated graft remain in even a tolerant host and are therefore constantly suppressed by Foxp3^{+} Treg.

4.3 Results

4.3.1 Antibody mediated co-receptor and co-stimulatory blockade induces long term graft acceptance

The B6.Foxp3^{hCD2} knock in mice were crossed with CBA/Ca mice to generate hybrid offspring known to be more susceptible to tolerance induction through antibody mediated blockade of co-receptors and co-stimulatory molecules (Daley, Ma et al. 2007). Since the *foxp3_hCD2* knock in gene is X-linked, female B6.Foxp3^{hCD2} mice were mated with male CBA/Ca mice. This ensured that all of the Foxp3^{+} T-cells in male F1 progeny (hence referred to as CBAxB6^{hCD2} by default) would express the human CD2_CD52 fusion reporter protein and therefore would be vulnerable to anti-hCD2 mAb mediated ablation. In comparison, the female (CBAxB6^{hCD2})F1 progeny would inherit two X-chromosomes, one containing the wild type *foxp3* gene (from their male CBA/Ca parent) and one containing the *foxp3_hCD2* knock

in gene (from their female B6.Foxp3^{hCD2} parent). As a result of random X-inactivation, about 50% of the Foxp3^{+} T-cells in female (CBAxB6^{hCD2})F1 progeny would be hCD2^{+} and hence susceptible to ablation by anti-hCD2 mAb. Although the majority of the transplant tolerance experiments described below used male CBAxB6^{hCD2} mice, there were circumstances in which it was advantageous to have a female strain in which no more than 50% of Foxp3^{+} cells could be ablated by anti-hCD2 mAb at any one time (see 4.3.2).

Male CBAxB6^{hCD2} mice are haplotype H-2^{k}xH2^{b} and are mismatched by multiple minor antigens to BALB/K mice of H-2^{k} haplotype. As a result, skin grafts from BALB/K donors were rapidly rejected by CBAxB6^{hCD2} recipients with a median survival time (MST) of 12 days (figure 4.1A). In contrast, graft tolerance could be achieved if the BALB/K skin was allowed to heal while preventing the normal host rejection response with short-term, antibody mediated blockade of co-receptor and co-stimulatory signals. CBAxB6^{hCD2} mice that received non-depleting anti-CD4, anti-CD8 and anti-CD40L monoclonal antibodies (3 x 3mg i.p. injections during the first week post-transplant) accepted their BALB/K grafts long term compared to untreated mice (figure 4.1A, $p<0.0001$).

Therapeutic antibody levels have been found to naturally decay over a period of 5-6 weeks, such that any graft transplanted beyond this time would not be protected by co-receptor or co-stimulatory blockade (Scully, Qin et al. 1994). After 60 days, a second, ‘challenge’ BALB/K skin graft was placed on the same CBAxB6^{hCD2} recipient mice (without any

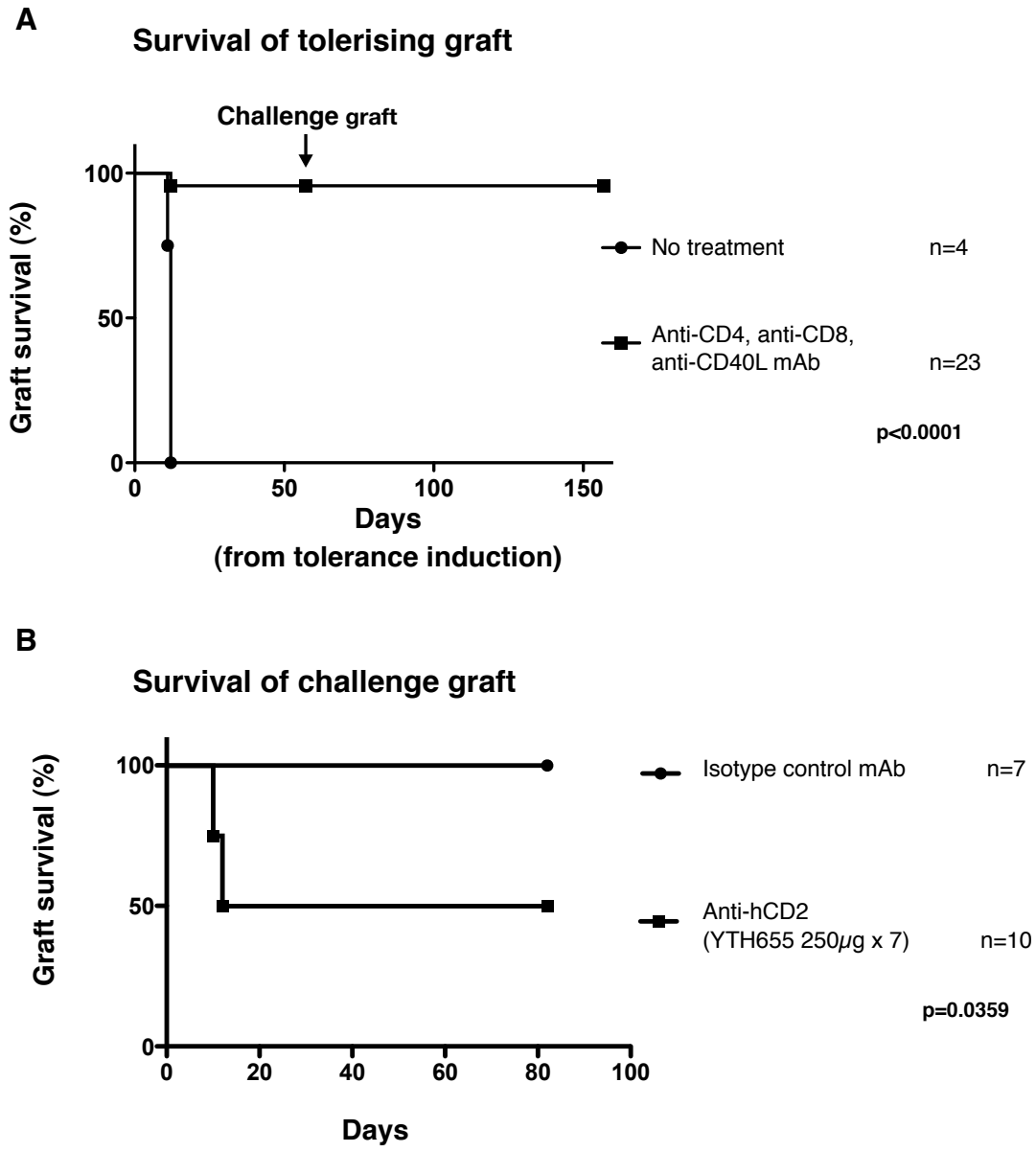


Figure 4.1 Co-receptor/co-stimulatory blockade induces long term tolerance

Untreated CBAxB6^{hCD2} recipient mice (H-2^{b} x H-2^{k}) acutely rejected BALB/K (H-2^{k}) skin mismatched by multiple minor antigens. The same recipients accepted BALB/K skin long term if grafted under the cover of non depleting anti-CD4, CD8 and CD40L mAb (3mg x 3 over 7 days, p<0.0001). Graft survival was not affected by the transplant of a second 'challenge' BALB/K graft at day 60 (arrow). (**B**) The challenge BALB/K graft was also accepted long-term in isotype mAb control mice. In contrast, half the challenge BALB/K grafts are rejected when hCD2^{+}Foxp3^{+} T-cells are ablated by anti-hCD2 (250μg x 7) p=0.0359.

antibody mediated blockade). The 'challenge' graft re-exposes the immune system to the same donor antigens under the inflammatory conditions of a healing wound, and so tests the tolerance previously induced by short term co-receptor/co-stimulatory blockade. Continued long term survival of both the original 'tolerising' and the 'challenge' graft demonstrated that the tolerance induced by co-receptor/co-stimulatory blockade was active, self-sustaining and able to resist the challenge of re-exposure to the same foreign antigens in an inflammatory context (figure 4.1B). Such 'dominant tolerance' across multiple minor antigens was thought to be dependent on the activity of immune regulatory elements within the $CD4^+$ cell population after adoptive transfer of $CD4^+$ cells from tolerant to naive animals was shown sufficient to transfer tolerance (Qin, Cobbold et al. 1993; Davies, Martin et al. 1996; Marshall, Cobbold et al. 1996). The human CD2_CD52 reporter protein in male $CBAxB6^{hCD2}$ mice made it possible to investigate whether $Foxp3^+$ cells are the crucial ($CD4^+$) regulatory cells by selectively ablating them with anti-hCD2 mAb. The time point chosen for ablation was the first seven days following transplantation of the 'challenge' graft, because it was initially thought that this period of re-exposure to antigen would be the most critical for putative regulatory cells.

$CBAxB6^{hCD2}$ mice that had accepted a BALB/K skin graft for more than 60 days were divided into two groups, the first received a challenge BALB/K graft and 7 daily i.p injections of 250μg anti-hCD2 mAb (YTH655) to target $hCD2^+Foxp3^+$ cells (Chapter 3) and the second group received a challenge graft with an equivalent regimen of an isotype control mAb (YKIX 337.217.7 - Chapters 2 and 3). In this first experiment, half the mice treated with anti-hCD2 mAb rejected their challenge graft compared to none in the control group (figure 4.1B, $p=0.0359$). It was possible that the survival of half the challenge skin grafts in the treatment

group was due to the inability of YTH655 to ablate all Foxp3$^+$ Treg, allowing a significant proportion to remain and maintain tolerance (Chapter 3). For this reason, an adoptive transfer model, in which >95% ablation of Foxp3$^+$ Treg can be achieved (Chapter 3), was used to explore further the role of Foxp3$^+$ Treg in transplant tolerance.

4.3.2 Foxp3$^+$ regulatory cells are essential for adoptive transfer of transplant tolerance

Classical adoptive transfer studies have previously demonstrated that tolerant CD4$^+$ cells can suppress both naive and primed lymphocytes from rejecting a foreign graft when transferred intravenously into lymphocyte deficient murine recipients (Marshall, Cobbold et al. 1996). Using a similar cell transfer protocol was advantageous because intravenous injection of cells forced them to circulate and under these conditions, 1mg YTH655 anti-hCD2 was found to ablate >95% of Foxp3$^+$ cells (Chapter 3). It was therefore possible to re-visit these pioneering studies and investigate whether Foxp3$^+$ Treg, in particular, were the regulatory CD4$^+$ cells responsible for suppressing rejection by naive or primed lymphocytes.

20 million tolerant CBAxB6^{hCD2} splenocytes were harvested from CBAxB6^{hCD2} mice that had previously been treated with anti-CD4, anti-CD8 and anti-CD40L mAb, and had therefore accepted a BALB/K graft for over 60 days. These were transferred into lymphocyte deficient RAG$^{-/-}$ recipient mice (CBA.RAG$^{-/-}$ x B6.RAG$^{-/-}$) with 5 million naive splenocytes. On the same day, all the RAG$^{-/-}$ recipients were transplanted with BALB/K skin. Whereas the mice that received isotype control mAb accepted their grafts long term, those that were treated with

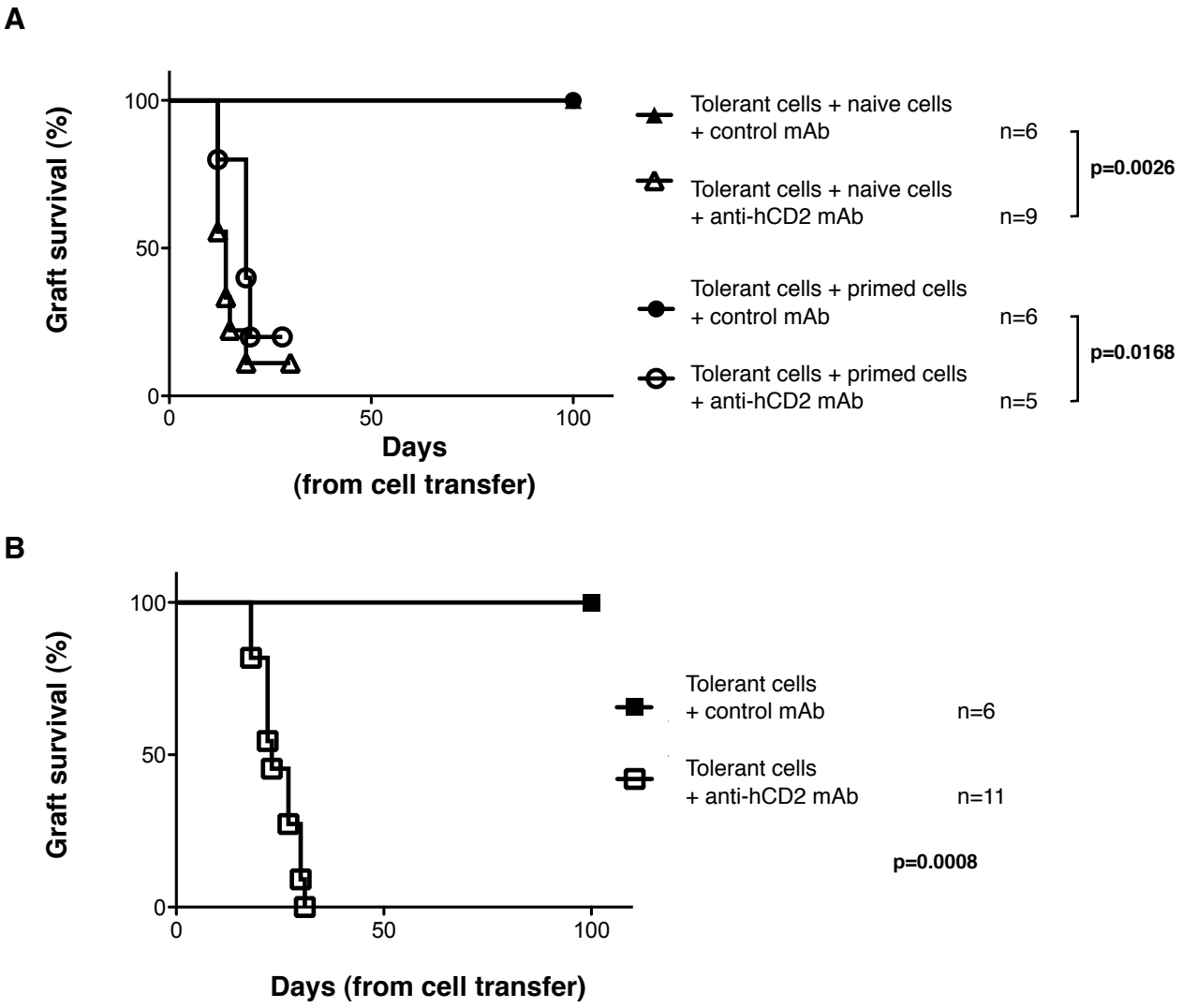


Figure 4.2 Loss of transplantation tolerance following adoptive transfer with anti-CD2 mAb

(A) 20 million tolerant splenocytes from male (CBAxB6^{hCD2})F1 mice that accepted the BALB/K graft (Fig 4.1) suppressed the addition of 5 million naive splenocytes (triangles) and 1 million primed splenocytes (circles) when transferred into CBA.RAG$^{-/-}$ x B6.RAG$^{-/-}$ mice. This suppression was dependent on Foxp3^{+} T-cells, since anti-hCD2 mAb led to rapid rejection of the skin grafts, p=0.0026 and p=0.0168, respectively (one mouse in each group was euthanized due to severe distress). **(B)** 20 million tolerant splenocytes from male (CBAxB6^{hCD2})F1 mice that accepted the first BALB/K graft also accepted a BALB/K skin graft when adoptively transferred into lymphocyte deplete (RAG$^{-/-}$) recipients. When 1mg anti-hCD2 was co-administered, the grafts were rejected, p=0.0008.

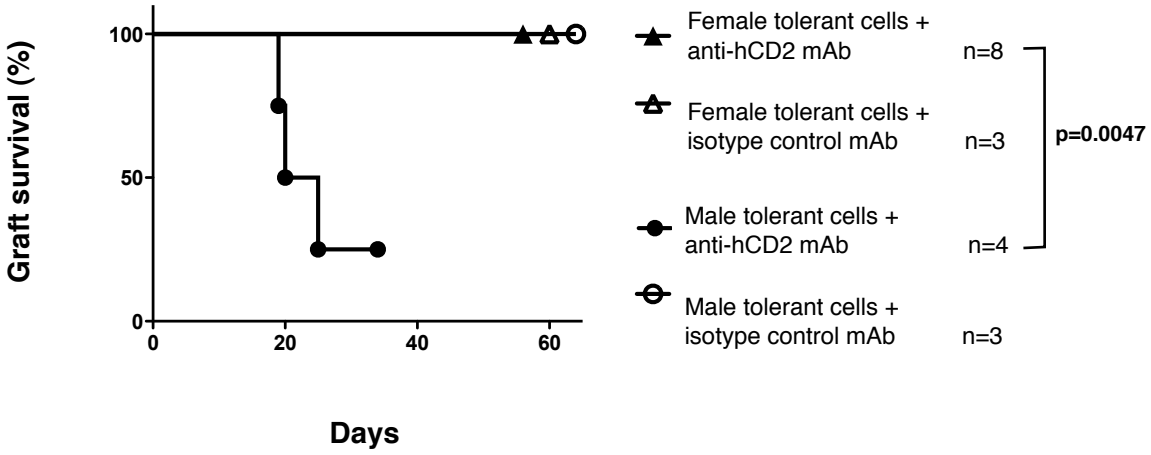


Figure 4.3 Female (CBAxB6^{hCD2})F1 tolerant cells maintain suppression despite anti-hCD2 mAb

20 million tolerant splenocytes from female (CBAxB6^{hCD2})F1 mice that accepted the first BALB/K graft also accepted a BALB/K skin graft when adoptively transferred into lymphocyte deplete (RAG$^{-/-}$) recipients with (open triangles) or without (closed triangles) anti-hCD2 mAb. In the same experiment, 20 million tolerant splenocytes from male (CBAxB6^{hCD2})F1 mice rejected the second BALB/K graft when co-administered with anti-hCD2 mAb (open circles, P=0.0047 one mouse was euthanized due to severe distress).

1mg YTH655 at the time of cell transfer rapidly rejected their grafts (figure 4.2A, open triangles, p=0.0026). This demonstrated that 20 million tolerant spleen cells could suppress 5 million naive spleen cells from rejecting a graft and that this suppression was dependent on Foxp3^{+} Treg. Similarly, 20 million tolerant spleen cells also prevented 1 million 'primed' spleen cells (from untreated CBAxB6^{hCD2} mice that had previously rejected BALB/K skin) from rejecting a subsequent BALB/K graft when transferred into RAG-/- mice. Once more, this suppression was dependent on Foxp3^{+} Treg since intravenous infusion of 1mg YTH655 resulted in graft rejection compared to isotype control mAb (figure 4.2A closed and open circles, p=0.0168). Taken together, these results demonstrated for the first time, that Foxp3^{+} Treg are essential for the suppression found in therapeutic transplant tolerance

Remarkably, 20 million tolerant CBAxB6^{hCD2} splenocytes alone (i.e. without the addition of naive or primed lymphocytes known to cause graft rejection) also rejected BALB/K skin when Foxp3^{+} cells were ablated by anti-hCD2 mAb (figure 4.2B open vs closed squares of control, p=0.0008). The rate of graft rejection in this case was slower in the absence of additional naive effector cells (figure 4.2A, MST = 23d vs 14d respectively, p=0.0019). The finding that a tolerant population, when stripped of its Foxp3^{+} Treg, could nonetheless reject grafts demonstrated that rejection competent T-cells were still present despite the state of operational tolerance. This challenges pre-conceptions that regulatory cells are at their most active during tolerance *induction* by rendering any potential rejecting effector cells anergic at this early stage (Alters, Shizuru et al. 1991; Wekerle, Kurtz et al. 2002). It is also consistent with previous findings that CD8^{+} cells from primed, yet tolerised, hosts retained the ability to reject foreign grafts once all CD4^{+} cells (including regulators) were removed (Marshall,

Cobbold et al. 1996). Collectively, these experiments suggest that rejecting T-cells remain in tolerant hosts and the survival of the grafted tissue therefore requires that they are constantly kept in check by Foxp3^{+} Treg.

It has recently been demonstrated in a tumour model of immune regulation that cell lysis can override the suppressive effects of regulators through the release of factors such as nicotinamide adenine dinucleotide (NAD) (Hubert, Rissiek et al. 2010). Given that all adoptively transferred Foxp3^{+}hCD2^{+} cells from *male* (CBAxB6^{hCD2})F1 mice were susceptible to ablation by anti-hCD2 mAb, it was conceivable that the resulting cell destruction was a major contributor to the loss of tolerance, independent of specifically targeting Foxp3^{+} cells. To exclude this, spleen cells from tolerant *female* (CBAxB6^{hCD2})F1 mice were adoptively transferred into lymphocyte deficient hosts, with and without anti-hCD2 mAb. Since both the natural and knocked in *foxp3* gene are X-linked, female (CBAxB6^{hCD2})F1 mice would inherit both copies of the gene and random X-inactivation would ensure that approximately half the Foxp3^{+} cells would be hCD2^{+}, and so lysed by anti-hCD2 antibody, while the rest would express the wild type *foxp3* gene and survive. This scenario seemingly achieved the best of both worlds since substantial cell lysis was generated while leaving at least half the Foxp3^{+} cells in tact to preserve tolerance. If cell lysis alone was indeed contributing to a loss of tolerance in this model, one would expect the grafts to be rejected. However, tolerant female (CBAxB6^{hCD2})F1 splenocytes failed to reject a BALB/K skin graft when transferred into lymphocyte deficient RAG-/- recipients, even when ablative anti-hCD2 mAb was added (figure 4.3). Whereas, in the same experiment, mice that received male (CBAxB6^{hCD2})F1 splenocytes did reject the grafts, since all Foxp3^{+} cells are hCD2^{+} and therefore vulnerable to anti-hCD2

mAb mediated ablation (figure 4.2, p=0.0008). These results demonstrated that cell lysis alone was unlikely to contribute significantly to the abrogation of tolerance in this model.

4.3.3 Foxp3$^+$ Treg maintain tolerance through constant suppression of cells capable of rejection

A current dogma of therapeutic tolerance is that the induction process incapacitates effector cells to such an extent that very little, if any, subsequent regulation is required to maintain that tolerance (Alters, Shizuru et al. 1991; Wekerle, Kurtz et al. 2002). This is contradicted by the above discovery that even a previously tolerant population could reject skin from the original donor strain when Foxp3$^+$ cells are ablated (figure 4.2B). The data suggests that sufficient rejection competent T-cells survive the induction phase of tolerance and remain capable of rejecting a graft when unleashed from suppression by Foxp3$^+$ Treg. However, a major limitation of adoptive transfer studies of this kind is that homeostatic expansion of cells injected into a lymphocyte deficient host may artificially magnify the potency of an otherwise insignificant population of effector T-cells. It was therefore conceivable that the loss of tolerance was an artefact of the adoptive transfer process whereas, in 'whole' lympho-replete mice there would be too few rejection competent cells to destroy the foreign graft. For these reasons, it was important to investigate whether such effector function was revealed when Foxp3$^+$ Treg were ablated in the more physiologically relevant model of a naive mouse with a replete immune system.

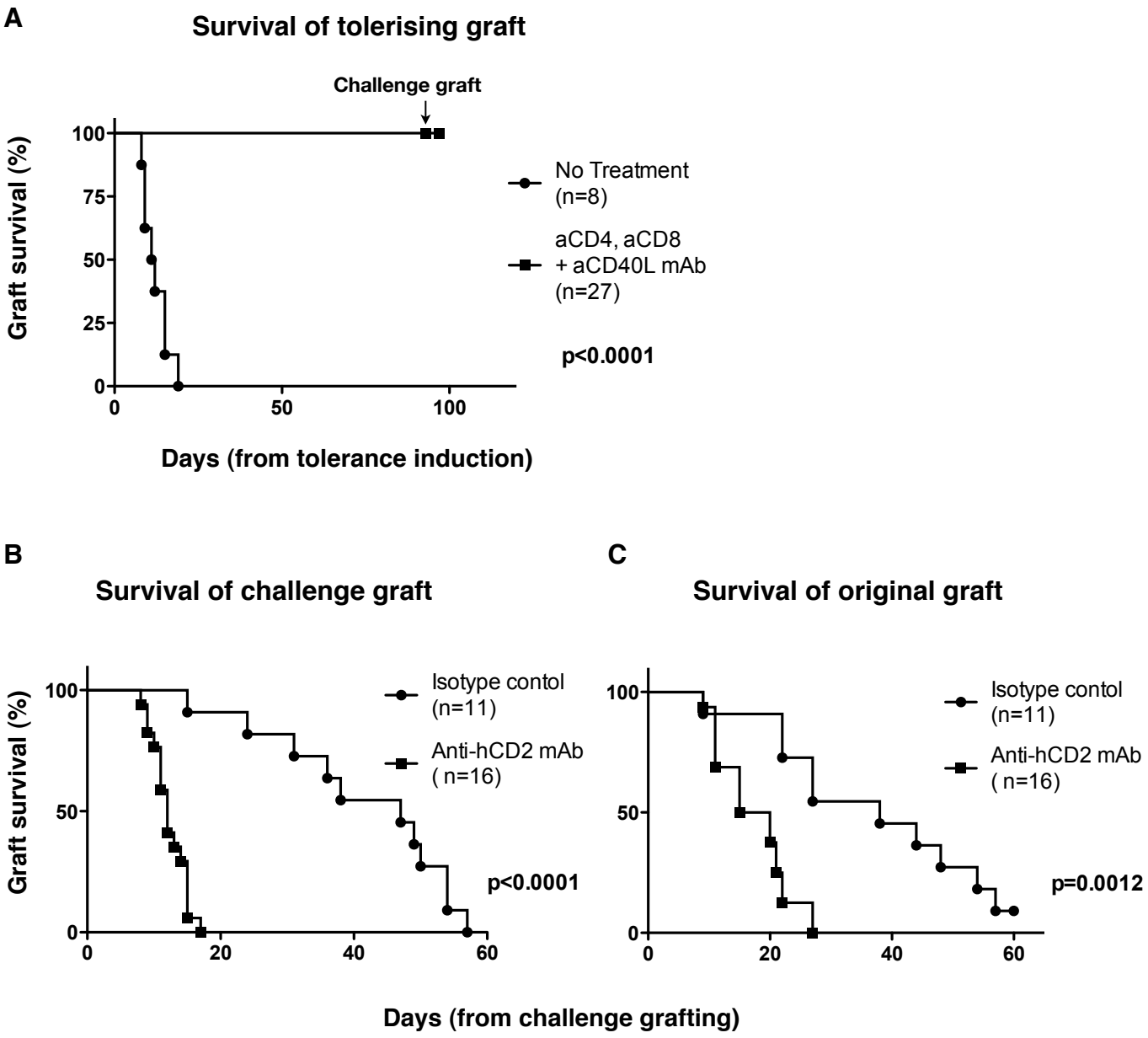


Figure 4.4 Foxp3⁺ Treg are crucial for inducing and maintaining transplant tolerance

(**A**) Male (CBAxB6^{hCD2})F1 mice treated with anti-CD4/CD8/CD40L blocking mAb (3 x 3 mg doses over 7 days) accepted a MHC mismatched BALB/C skin, p<0.0001 After 100 days a challenge BALB/C skin graft was transplanted (arrow). (**B**) Mice that received ablative anti-hCD2 mAb to deplete Foxp3⁺ T-cells rapidly rejected the challenge graft, MST 12.0 vs 47.0 days of control group, p<0.0001. (**C**) The original tolerising BALB/C graft was also rapidly rejected when mice received anti-hCD2 mAb, MST (+100d) 17.5 vs 38.0 of control group, p=0.0012.

CBAxB6^{hCD2} (H-2^{k}xH2^{b}) accepted MHC mismatched skin grafts from BALB/C (H-2^{d}) mice long-term, when those grafts were transplanted under the cover of non depleting anti-CD4, anti-CD8 and anti-CD40L mAb blockade (figure 4.4A, $p<0.0001$). Once therapeutic antibody levels had naturally decayed, the mice also accepted second donor 'challenge' grafts long-term. In this stringent graft model of full MHC mismatch, Foxp3^{+} Treg also proved necessary for therapeutic, long-term graft survival as secondary 'challenge grafts' in mice receiving ablative anti-hCD2 mAb were promptly rejected (figure 4.4B, MST 12 vs 47 days of isotype control mAb, $p<0.0001$). Somewhat surprisingly, the original tolerated graft was also rejected, despite appearing well healed and healthy for over 100 days (figure 4.4C, MST (+100d) 17 vs 38 days of isotype control, $p=0.0012$).

Following the above observation that the original, well accepted, grafts were also rejected when Foxp3^{+} Treg were ablated, the protocol was once more adapted to investigate the role of Foxp3^{+} regulation during steady state conditons. Naive CBAxB6^{hCD2} mice were tolerised to BALB/C skin with short-term depleting anti-CD4, anti-CD8 and anti-CD40L mAb blockade, as previously described. 50 days following tolerance induction, the mice were separated into 4 groups;

(i) 7 x 250μg i.p. injections of anti-hCD2 mAb (no further skin grafting)

(ii) 7 x 250μg i.p. injections of isotype control mAb (no further skin grafting)

(iii) 7 x 250μg i.p. injections of anti-hCD2 mAb + syngeneic graft

(iv) 7 x 250μg i.p. injections of isotype control mAb + syngeneic graft

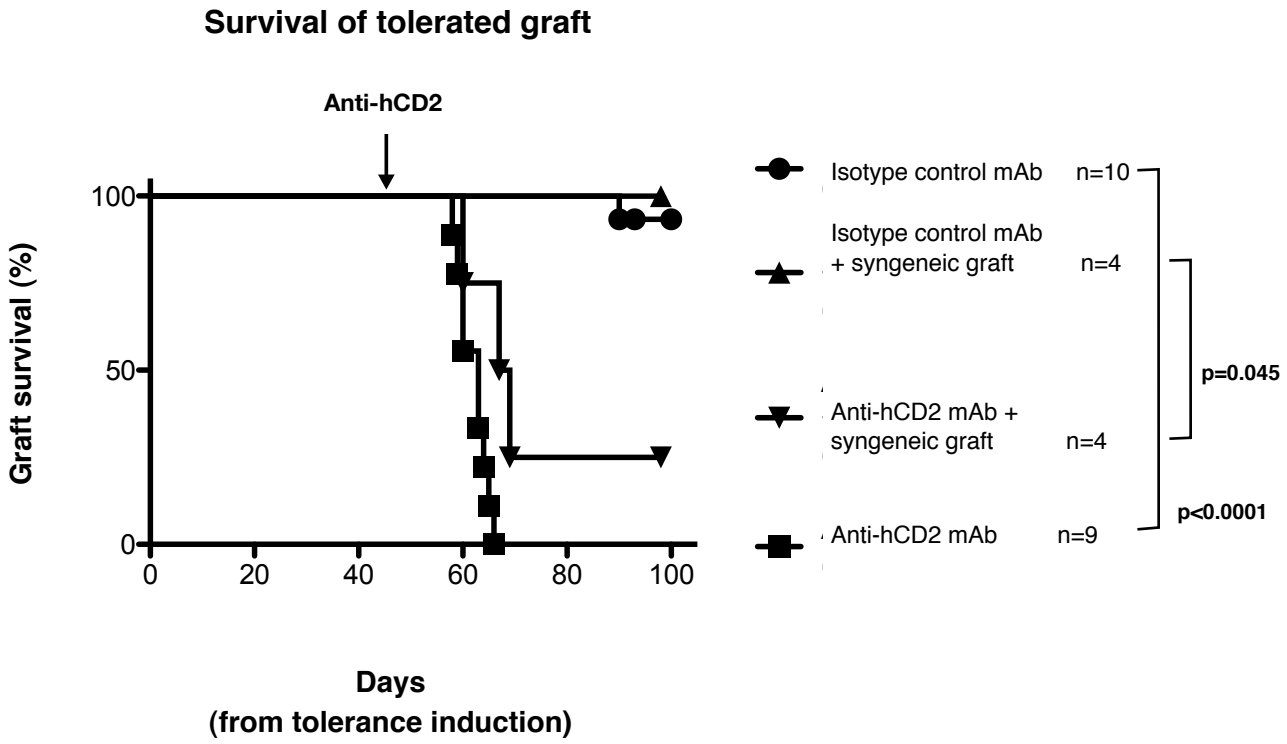


Figure 4.5 Foxp3⁺ Treg are constantly required to suppress T-cells still capable of rejection

Male CBAxB6^{hCD2} mice were tolerised to BALB/C skin with anti-CD4/CD8/CD40L blocking mAb (Figure 4.4). 50 days later. mice that received a seven day course of anti-hCD2 mAb to ablate Foxp3⁺ T-cells rejected the BALB/C graft. Rejection of the previously well tolerated graft occurred with ($p=0.045$) or without ($p<0.0001$) a co-transplanted syngeneic graft and did not occur in the control groups that received a non-ablative isotype mAb.

Importantly, none of the experimental mice received a challenge skin graft and therefore none of the mice were re-exposed to the original donor antigen under inflammatory conditions. Mice that received anti-hCD2 mAb, in the absence of any further treatment, rapidly rejected skin grafts that had appeared healthy and well accepted for over 50 days (figure 4.5, MST (+50) 16, $p<0.0001$ vs isotype control mAb). This finding that rejection can occur without re-exposing the immune system to antigen under inflammatory conditions (i.e. challenge grafting) or a nearby inflammatory insult from a syngeneic graft (figure 4.5) demonstrates that effector T-cells, still capable of rejecting a graft, remain in a tolerised host and graft survival therefore depends on their continuous restraint by Foxp3$^+$ Treg. These results indicate that short-term antibody mediated immune blockade achieves long-term tolerance through promoting constant immune vigilance by Foxp3$^+$ Treg, not simply by incapacitating effector T-cells at the outset.

4.3.4 The role Foxp3$^+$ Treg within the graft tissue

Having demonstrated the critical role of systemic Foxp3$^+$ iTreg, I next investigated whether tissue specific regulation occurs within conventional, lymphocyte replete mice. Regulatory T-cells found within tolerated skin grafts have previously been shown to recolonize a lymphocyte deficient mouse and thereafter suppress a subsequent transfer of naive T-cells from rejecting a graft (Graca, Cobbold et al. 2002). More recently, it was shown that tolerated graft tissue is enriched for natural Foxp3$^+$ cells (Fan, Spencer et al. 2010). A two step

transplantation study was therefore used to investigate whether there were any *functional* Foxp3^{+} Treg in graft tissue.

CBAxB6^{hCD2} mice were first tolerised by co-receptor/co-stimulatory blockade to BALB/C or BALB/K skin grafts that were mismatched by full MHC or multiple minor antigens respectively (as shown in figures 4.1 and 4.4). After 2 months, the well tolerated skin grafts were carefully excised and then immediately re-transplanted onto lymphocyte deficient (CBA.RAG-$^{-/-}$ x B6.RAG$^{-/-}$) mice. This process ensured that any functional T cells in the new recipient (whether effector or regulator) would be derived from the original CBAxB6^{hCD2} mice and could only have been transferred within the re-transplanted skin (figure 4.6). In both cases, treatment with anti-human CD2 mAb failed to result in significant rejection compared to control mice (figure 4.6).

The principal limitation of this experiment is that YTH655 anti-hCD2 fails to deplete all hCD2^{+}(Foxp3^{+}) cells (Chapter 3) and therefore it is possible that sufficient Treg remain to maintain tolerance. Interestingly, a similar experiment using TCR transgenic mice did result in significant graft rejection and the implications of these two differing findings are discussed in more detail in Chapter 5.

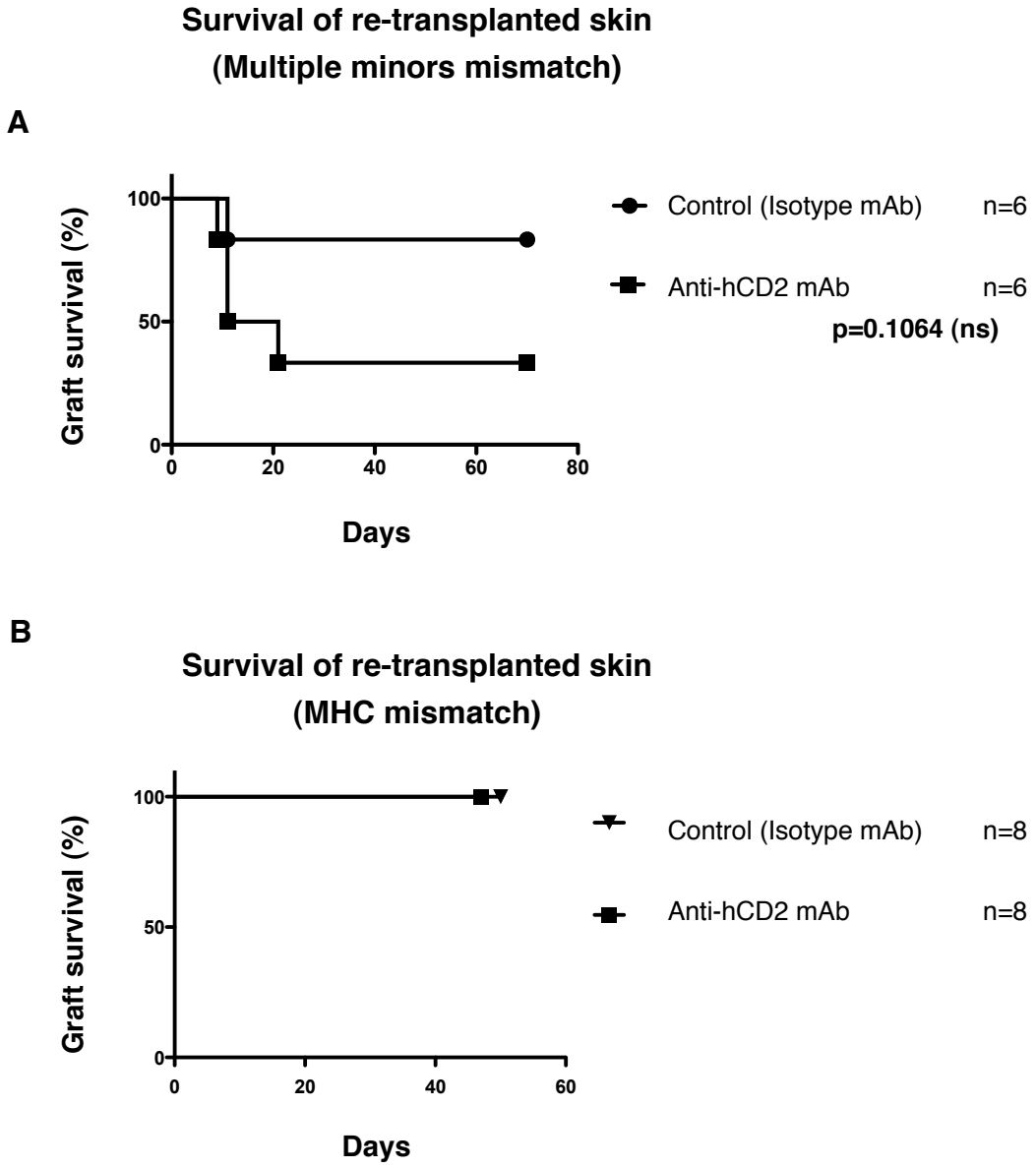


Figure 4.6 Anti-hCD2 depletion of polyclonal Foxp3+ Treg within the graft fails to cause rejection

(**A**) BALB/K (H-2k) skin grafts that had been tolerated by CBAxB6^{hCD2} recipient mice (H-2^{b} x H-2^{k}) for over 60 days following co-receptor/co-stimulatory blockade were surgically removed and re-transplanted onto lymphocyte deficient (CBA.RAG$^{-/-}$ x B6.RAG$^{-/-}$) mice. Treatment with ablative anti-hCD2 mAb (7 x 250μg YTH655) failed to result in significant graft rejection compared to control mice. (B) BALB/C (H-2^{d}) skin grafts that had been tolerated by CBAxB6^{hCD2} mice despite full MHC mismatch for over 60 days were surgically removed and re-transplanted onto lymphocyte deficient (CBA.RAG$^{-/}$ x B6.RAG$^{-/-}$) mice. Treatment with ablative anti-hCD2 mAb (7 x 250μg YTH655) failed to result in significant graft rejection compared to control mice.

4.4 Discussion

One of the main challenges to investigating the biology of regulatory T-cells has been the inability to define them accurately. Many review papers will cite studies of $CD25^+$ cells as evidence in favour of a critical role for regulatory T-cells in transplant tolerance (Wood and Sakaguchi 2003; Wood, Bushell et al. 2011). There are good practical reasons for this early reliance on $CD25^+$ cells; before the discovery of foxp3, CD25 was the best indicator of $CD4^+$ cells with a regulatory phenotype (Kuniyasu, Takahashi et al. 2000) and as a surface protein, there are clear benefits when pursuing live cell based therapies. Studies in which they were adoptively transferred were often successful in preventing immunopathology, reducing tumour immunity and improving graft survival rates in vivo (Read, Malmstrom et al. 2000; Graca, Thompson et al. 2002; Jonuleit, Schmitt et al. 2002; Mottet, Uhlig et al. 2003; Karim, Feng et al. 2005; Cao, Cai et al. 2007). The better transplant studies even demonstrated a loss of tolerance when $CD25^+$ cells were ablated with PC61 antibody (Benghiat, Graca et al. 2005; Jiang, Morita et al. 2006; Francis, Feng et al. 2011). Unfortunately, CD25 is neither specific nor sensitive for 'regulatory T-cells'. There is evidence that it is upregulated in most $CD4^+$ T-cells upon activation, irrespective of phenotype, and CD25- populations can also prevent graft rejection (Graca, Thompson et al. 2002), perhaps in part because up to a third of $Foxp3^+$ cells are CD25 negative.

When developing live, Treg cell based therapies, there is an understandable need to overcome the current lack of a single specific marker by using a combination of natural cell

surface proteins (Jiang, Tsang et al. 2006; Peters, Preijers et al. 2008; Chen, Delgado et al. 2009; Trzonkowski, Bieniaszewska et al. 2009). In comparison, the development of therapies aimed at exploiting host regulation ultimately cannot proceed without a clear description and understanding of the underlying mechanisms. $Foxp3^+$ Treg are known to be essential for self-tolerance (Kim, Rasmussen et al. 2007), but as yet they have not been proven essential to graft tolerance. Several studies have adoptively transferred either sorted GFP^+Foxp3^+ (Lee, Wang et al. 2005; Xia, Shah et al. 2009) or even sorted CD25+ cells, inaccurately described as $Foxp3^+$ cells (Turnquist, Raimondi et al. 2007; Joffre, Santolaria et al. 2008), into lymphocyte deplete mice to demonstrate transplant tolerance. Only one recent study actually depleted $Foxp3^+$ cells in the DEREG mouse and demonstrated that they are responsible for the ability of certain strains to accept MHC mismatched renal grafts without immunosuppression (Miyajima, Chase et al. 2011). Importantly, the mice only received two days of diphtheria toxin because longer regimens result in such profound depletion of $Foxp3^+$ Treg that mice rapidly succumb to a fatal immunopathology (Kim, Rasmussen et al. 2007; Lahl, Loddenkemper et al. 2007). This limits the ability to investigate the exclusive long term roles of $Foxp3^+$ Treg in transplant tolerance. In comparison, the ablative anti-hCD2 mAb regimen given to $(CBAxB6^{hCD2})F1$ mice in the transplant experiments described above is known to be sub-optimal, sparing at least a third of splenic and lymphatic $Foxp3^+$ Treg (Chapter 3). Surprisingly, while this degree of ablation was insufficient to result in immuno-pathology (the mice remained well and gained weight throughout the duration of the experiment) it nonetheless resulted in significant graft rejection (figures 4.1 and 4.4). Perhaps because induced non-self tolerance has a higher regulatory threshold or is more dependent on circulating Treg.

For the first time it has therefore been possible to demonstrate that Foxp3^{+} cells are critical to long-term therapeutic tolerance to foreign antigen induced by clinically relevant protocols of antibody mediated co-receptor and co-stimulatory blockade. Foxp3^{+} Treg are required for tolerance induction across all three levels of histocompatibility mismatch; full MHC, multiple minor and single minor antigen.

Adoptive transfer of spleen cells from tolerant mice into naive lymphocyte-deficient recipients demonstrated that once tolerance is established, Foxp3^{+} Treg maintain that state despite challenges from naive and primed lymphocytes (figure 4.2). A recent study has demonstrated that non specific cell lysis may release extracellular factors (e.g. NAD^{+}) that could themselves override suppression (Scheuplein, Schwarz et al. 2009). Given that all adoptively transferred Foxp3^{+}hCD2^{+} cells from *male* (CBAxB6^{hCD2})F1 mice were susceptible to ablation by anti-hCD2 mAb, it was conceivable that the resulting cell destruction was a major contributor to the loss of tolerance, independent of specifically targeting Foxp3^{+} cells. To exclude this, spleen cells from tolerant *female* (CBAxB6^{hCD2})F1 mice were adoptively transferred into lymphocyte deficient hosts, with and without anti-hCD2 mAb. Since both the natural and knocked in *foxp3* gene are X-linked, female (CBAxB6^{hCD2})F1 mice would inherit both copies and random X-inactivation would ensure that approximately half the Foxp3^{+} cells would be hCD2^{+}, and so lysed by anti-hCD2 antibody, while the rest would express the wild type *foxp3* gene and survive (figure 4.3). The failure of tolerant female (CBAxB6^{hCD2})F1 spleen cells plus ablative anti-hCD2 to reject any grafts demonstrated that cell lysis alone was unlikely to contribute significantly to the abrogation of tolerance in this model.

A surprising finding was that even a population of tolerant cells, without the addition of naive or primed cells, could still reject a graft if stripped of its Foxp3^{+} Treg (figure 4.2). This implied, firstly, that tolerance induction fails to incapacitate all cells capable of rejection at the outset, as was previously assumed (Alters, Shizuru et al. 1991; Wekerle, Kurtz et al. 2002), and secondly, compensates for this inadequacy by promoting sustained regulation by Foxp3^{+} Treg. An important limitation of adoptive transfer studies of this kind is that homeostatic expansion of newly transferred cells may lead to 'false' rejection by artificially magnifying the effect of an otherwise insignificant population of effector T-cells. A second concern is that the lympho-deficient RAG$^{-/-}$ recipients developed a wasting immunopathology around 3-4 weeks after transfer of spleen cells devoid of Foxp3^{+} Treg. As previously stated, the onset of systemic immune dysregulation not only shortens the time period during which graft experiments can be conducted but also means that graft tolerance cannot be assessed independently of global immune attack. For these reasons, we turned to conventional lymphocyte-replete CBAxB6^{hCD2} mice which did not develop wasting immunopathology in response to anti-hCD2 (figure 4.4 and 4.5). Once again, Foxp3^{+} Treg were found to be responsible for maintaining transplant tolerance by suppressing effector cells (figure 4.4). Indeed, short-term ablation of Foxp3^{+} cells 50 days after the induction of tolerance to a foreign skin graft resulted in rejection. In this setting, the rapid destruction of healthy, well healed and well integrated skin dramatically demonstrated the rejection potential of residual effector T cells otherwise suppressed by Foxp3^{+} Treg (figure 4.5).

Collectively, these findings demonstrate that Foxp3$^+$ Treg are critical to the generation of long term tolerance by short term immune blockade with non depleting antibodies. The rejection capability of residual effector cells in the absence of Foxp3$^+$ cells implies that short term immune blockade potentiates Foxp3$^+$ dependent regulation. What is not clear, is how this occurs. 'Potentiation' remains undefined and could include expansion of existing thymic nTreg or the generation of new Foxp3$^+$ recruits in the periphery (explored in Chapters 5 and 6). It may not even be necessary to increase Treg numbers, merely redistribute the existing population to critical sites. An increase in the number of Foxp3$^+$ cells in the draining lymph nodes (Chapter 5) supports previous findings that Treg can acquire gut homing receptors such as a combination of CCR9, FucT-VII and the α4β7 integrin when stimulated by dendritic cells; particularly CD103$^+$ DCs (Siegmund, Feuerer et al. 2005). Interestingly, the tissue microenvironment seems to play a role in grooming the right kind of Treg cell, in that Treg cell induction in peripheral lymph nodes led to the expression of a set of Treg integrins enabling migration into inflamed skin, while activation within mesenteric lymph nodes led to induction of the gut mucosal homing integrin α4β7 (Christiane Siewert 2007). Arthritides' models have also shown that Treg cells isolated from inflamed synovium display greater suppressor capacity than those isolated from the blood (Jocea M. R. van Amelsfort 2004).

As to whether tolerance induction requires expansion of natural Treg or the generation of new converts in the periphery; a polyclonal model such as this is limited by its inability to distinguish between the two populations. The same is true of investigating whether Foxp3$^+$ Treg need to be antigen specific or not. These questions are addressed in the next chapter using a TCR transgenic model in which there are no natural Treg at the outset, and all CD4

cells are monoclonal for a specific graft peptide (Chapter 5). As will be discussed, the relevance of a TCR transgenic mouse to a polyclonal replete immune system is often questioned. A useful compromise might therefore be reporter mice in which effector T-cells, natural Foxp3^{+} cells and those that have only recently expressed Foxp3 can be distinguished by different flouroforms (Fan, Spencer et al. 2009). Now that depletion studies have proven the essential nature of Foxp3^{+} Treg, such in vivo tracking is well placed to follow Treg and effector cell interactions under different circumstances, for example local or systemically induced inflammation, blockade of putative chemokine receptors, or re-exposure to the graft antigen under inflammatory conditions. This pancreatic islet transplantation model has already shown that tolerance induction is more closely correlated with a change in the ratio of Treg: effector T-cells than absolute numbers of Treg (Fan, Spencer et al. 2009).

Lastly, it is not clear whether other agents known to induce long term tolerance, such as rapamycin, also require Foxp3^{+} cells. If Foxp3^{+} Treg do prove to be the master regulator, irrespective of the mode of tolerance induction, then current reporter mice might identify pharmacological targets for the development of pro-regulatory drugs.

CHAPTER 5: The role of peripherally induced Foxp3^{+} iTreg in Infectious Tolerance

5.1 Abstract

In order to investigate the role of antigen specific induced Foxp3^{+} iTreg in transplant tolerance, B6.Foxp3^{hCD2} reporter mice were back crossed onto TCR transgenic Marilyn mice to generate Marilyn.Foxp3^{hCD2}. The lymphocyte population in these naive female reporter mice consists of monoclonal CD4^{+} cells that recognise the male Dby peptide and initially lack any natural Foxp3^{+} Treg. This model demonstrated that new, antigen specific, Foxp3^{+} Treg are produced in the periphery when tolerance is induced to male grafts using anti-CD4 mAb immune blockade. Well tolerated skin grafts were found to harbour effector T-cells capable of causing their rejection, but were prevented from doing so by tissue resident Foxp3^{+} iTreg that had been generated de novo during tolerance induction. Finally, induced Foxp3^{+} iTreg are shown here to recruit new Foxp3^{+} cells, in vivo, by converting naive CD4^{+} cells. This finding provides one potential mechanism by which the classical phenomenon of infectious tolerance may operate in vivo.

5.2 Introduction

In the previous chapter, the generation of transplant tolerance by short term immune blockade was shown to be dependent on Foxp3^{+} Treg (Chapter 4). Furthermore, even if some T-cells capable of rejection were eliminated during tolerance induction, sufficient cells nonetheless survived and rejected the graft as soon as Foxp3^{+} Treg were ablated. This

implied that co-receptor blockade does not just involve incapacitation of reactive T-cells but also somehow enhances Foxp3 dependent regulation. A number of pathways for preferentially promoting regulation were discussed, one of which was a relative increase in the number of Foxp3$^+$ Treg. At present, two potential sources of Treg are described; thymic derived natural Foxp3$^+$ Treg and induced iTreg that can be generated in the periphery from naive CD4$^+$ cells. The relative operational importance of these two populations remains a controversial issue (reviewed in (Curotto de Lafaille and Lafaille 2009)).

Natural Foxp3$^+$ Treg are known to develop in the thymus in response to high avidity binding of TCR to antigen presented by MHC class II (Apostolou, Sarukhan et al. 2002; Fontenot, Rasmussen et al. 2005; Larkin, Rankin et al. 2008). The resultant Foxp3 expression is essential to their thymic development, continued survival and regulatory function (Fontenot, Rasmussen et al. 2005; Williams and Rudensky 2007). Although their role in self tolerance has been dramatically demonstrated (Kim, Rasmussen et al. 2007; Lahl, Loddenkemper et al. 2007), it is still not clear how a population of nTreg with a limited TCR repertoire skewed toward self antigen could adapt to maintain tolerance to new or foreign antigen, for example in transplant tolerance (Jordan, Boesteanu et al. 2001; Apostolou, Sarukhan et al. 2002; Hsieh, Liang et al. 2004; Larkin, Rankin et al. 2008). In addition, thymic development of nTreg was initially thought to be independent of TGF-β signaling since a normal percentage of thymic nTreg were found in TGF-β1 deficient mice (Marie, Letterio et al. 2005) and TGF-βRII deficient mice (Fahlen, Read et al. 2005; Li, Sanjabi et al. 2006). In comparison, induction of peripheral transplant tolerance does depend on TGF-β and was impaired in dominant negative TGF-βRII mice (Daley, Ma et al. 2007). This finding followed a number of reports that

Foxp3+ iTreg could be generated in vitro from naive CD4+ cells cultured in the presence of APCs and TGF-β (Chen, Jin et al. 2003; Cobbold, Castejon et al. 2004; Fantini, Becker et al. 2004; Nishimura, Sakihama et al. 2004). Collectively they suggested that induced tolerance to foreign antigen requires regulation beyond nTreg through the generation of a different group of (TGF-β dependent) iTreg. This might also explain the phenomenon of infectious tolerance (Qin, Cobbold et al. 1993), in which one population of tolerant cells could pass on that tolerance to a naive population in the periphery.

The original assumption that iTreg preferentially rely on TGF-β, and therefore represent a functionally distinct class from nTreg, has since been challenged by a reduction in early post natal Foxp3+ nTreg seen in mice with a T-cell selective deletion of TGF-βRI (Liu, Zhang et al. 2008). The inability to tolerise TGF-βRII-/- mice to foreign skin could therefore be explained by defective nTreg in the absence of normal TGF-beta signaling. Similarly a role for nTreg in infectious tolerance has not been properly excluded. Indeed, some groups have shown that expansion of nTreg alone is sufficient for therapeutic tolerance (Nishimura, Sakihama et al. 2004; Vukmanovic-Stejic, Zhang et al. 2006) and the discovery that nTreg can be induced by peripheral dendritic cells that migrate to the thymus offered a mechanism by which the nTreg TCR repertoire could adapt in response to new antigen exposure (Watanabe, Wang et al. 2005; Proietto, van Dommelen et al. 2008).

The difficulty in examining the relative importance of iTreg and nTreg, or whether iTreg even exist in conventional mice, stems from our current inability to distinguish reliably

between the two populations in vivo. Even in Foxp3 reporter mice, it is almost impossible to detect newly generated iTreg against a backdrop of natural nTreg. For this reason, $RAG^{-/-}$ TCR transgenic mice have traditionally been used because they have no natural, thymic derived, $Foxp3^{+}$ Treg. In addition, all the lymphocytes present are monoclonal $CD4^{+}$ cells with a single TCR specific to a defined peptide, including any new $Foxp3^{+}$ Treg. Such models have already described the generation of antigen specific $Foxp3^{+}$ iTreg in the periphery in tolerant animals (Cobbold, Castejon et al. 2004; Kretschmer, Apostolou et al. 2005). This chapter aims to expand these findings and examine whether these in vivo generated iTreg actually perform an active regulatory role or whether they are incidental bystanders.

TCR transgenic Marilyn.$Foxp3^{hCD2}$ mice were created by backcrossing B6.$Foxp3^{hCD2}$ reporter mice onto Marilyn mice. These mice have the following important features for investigating antigen specific $Foxp3^{+}$ iTreg

- All lymphocytes in female mice are monoclonal $CD4^{+}$ T-cells expressing a single TCR recognising the male H-Y antigen (male mice would negatively select their T-cells)

- There are no natural $Foxp3^{+}$ Treg (as opposed to conventional 'wild type' mice)

- Any identifiable $Foxp3^{+}$ Treg can only have been produced in the periphery in response to a given tolerogenic stimulus

- All $Foxp3^{+}$ Treg are identifiable by their surface expression of $hCD2^{+}$ and susceptible to anti-hCD2 mAb mediated ablation (Chapter 3).

5.3 Results

5.3.1 Induction of transplant tolerance in Marilyn.Foxp3^{hCD2} mice

Female Marilyn.Foxp3^{hCD2} mice contain monoclonal CD4^{+} lymphocytes that recognise male H-Y peptide and rapidly rejected lymphocyte deficient male B6 RAG$^{-/-}$ skin grafts (figure 5.1, MST 11 days). Despite this, long-term graft acceptance could be achieved if the male skin was transplanted in conjunction with short term treatment of anti-CD4 blocking mAb (figure 5.1, $p<0.0001$). The induced tolerance was dominant in that it resisted a 'challenge' male B6 RAG$^{-/-}$ graft transplanted at least 60 days later when the blocking antibody had been cleared (figure 5.1B). In comparison, untreated female Marilyn.Foxp3^{hCD2} not only rejected the original graft (MST 11 days, $p<0.0001$) but also rejected the challenge graft (figure 5.1B, MST 12 days, $p=0.0160$). In this model, anti-CD2 mAb failed to result in rejection of the challenge graft (figure 5.1B circles).

In the first tolerance experiment using female Marilyn.Foxp3^{hCD2} mice, three out of ten mice failed to accept male B6 RAG$^{-/-}$ skin when treated with 250μg anti-CD4 blocking mAb (figure 5.1A). Such rates of long-term graft survival were poor in comparison to our groups previous experience with TCR transgenic female A1.RAG$^{-/-}$ mice, in which lower doses of anti-CD4 mAb achieved higher rates of graft acceptance. Furthermore, female Marilyn.Foxp3^{hCD2} mice also proved resistant to tolerance induction to male CBA.RAG$^{-/-}$ skin via indirect antigen presentation (figure 5.2B). Subsequent assays therefore explored various regimens of anti-CD4 mAb with the goal to improve graft acceptance rates in this particular strain (figure 5.2A).

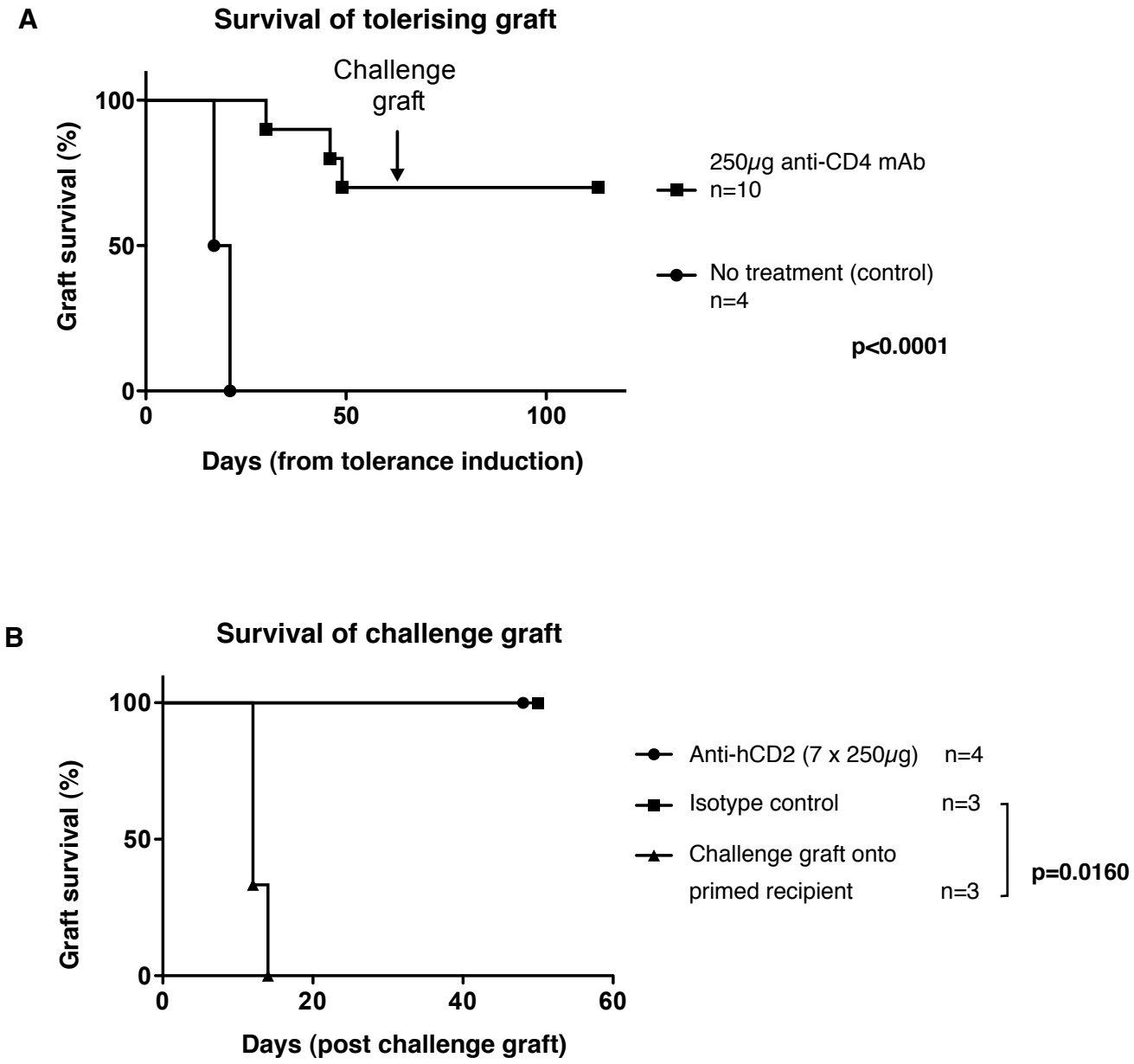


Figure 5.1 Anti-CD4 mAb induces graft tolerance in Marilyn.Foxp3^{hCD2} mice

(**A**) Female transgenic Marilyn.Foxp3^{hCD2} mice can be tolerised to male B6 RAG$^{-/-}$ skin by 250μg anti-CD4 blocking mAb, p=0.0001. Once induced, the tolerance is resistant to a challenge graft (arrow). **(B)** The challenge graft is itself accepted long term by Marilyn.Foxp3^{hCD2} mice previously treated with anti-CD4 mAb. Anti-hCD2 treatment does not result in graft rejection in this model (circles). In contrast challenge grafts are rapidly rejected by untreated Marilyn.Foxp3^{hCD2} mice that had previously rejected the original male graft (p=0.016).

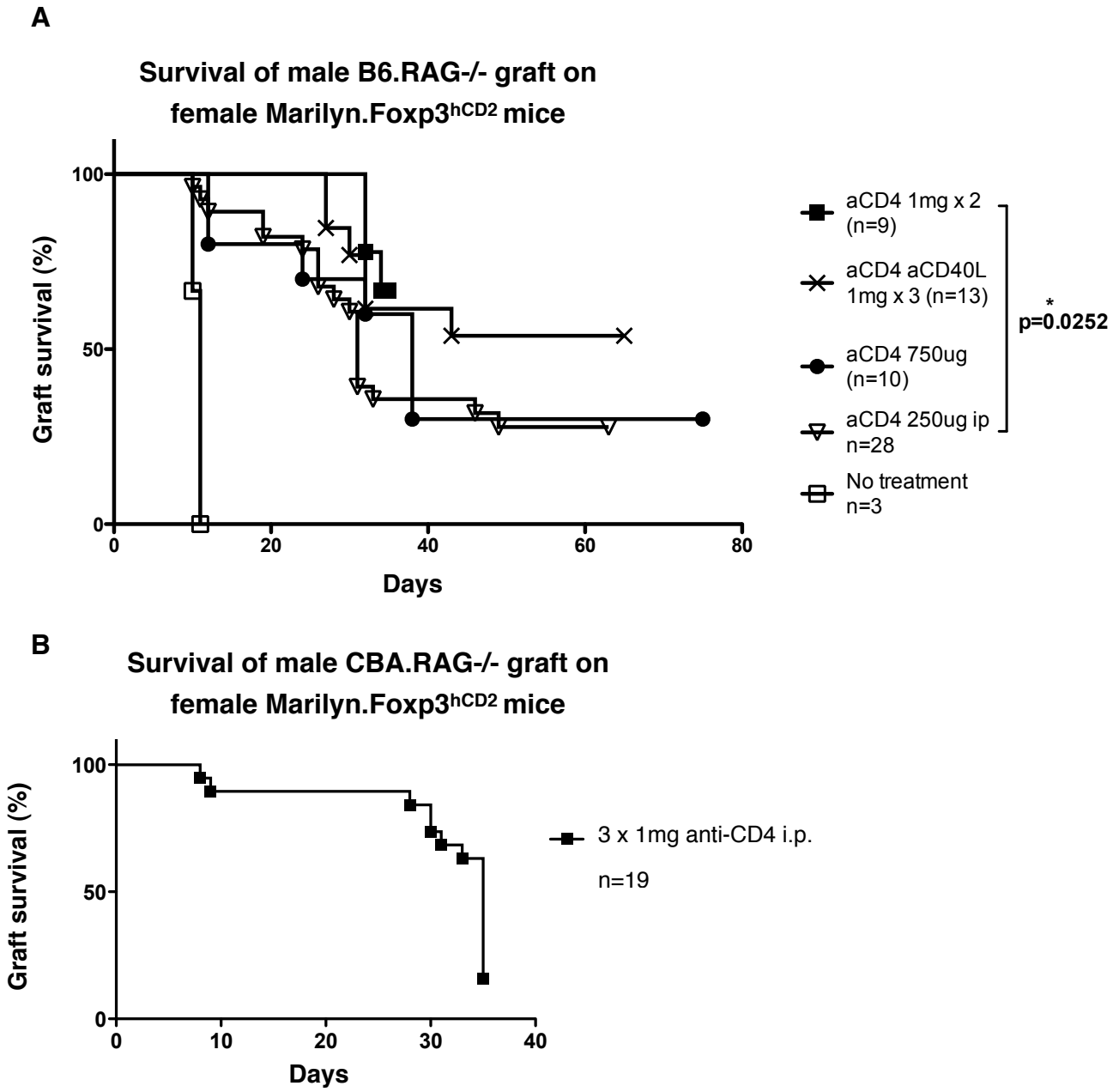


Figure 5.2 Delayed rejection of male RAG$^{-/-}$ grafts by female Marilyn.Foxp3^{hCD2} mice despite anti-CD4 mAb treatment

(**A**) Various anti-CD4 mAb tolerising regimens were tested with the aim of increasing male B6.RAG$^{-/-}$ grafts survival rates on female Marilyn.Foxp3^{hCD2} mice. The grafts typically underwent delayed rejection after 5-7 weeks (**B**) Male CBA.RAG$^{-/-}$ skin grafts also underwent a crisis 5-7 weeks after administration of anti-CD4 mAb.

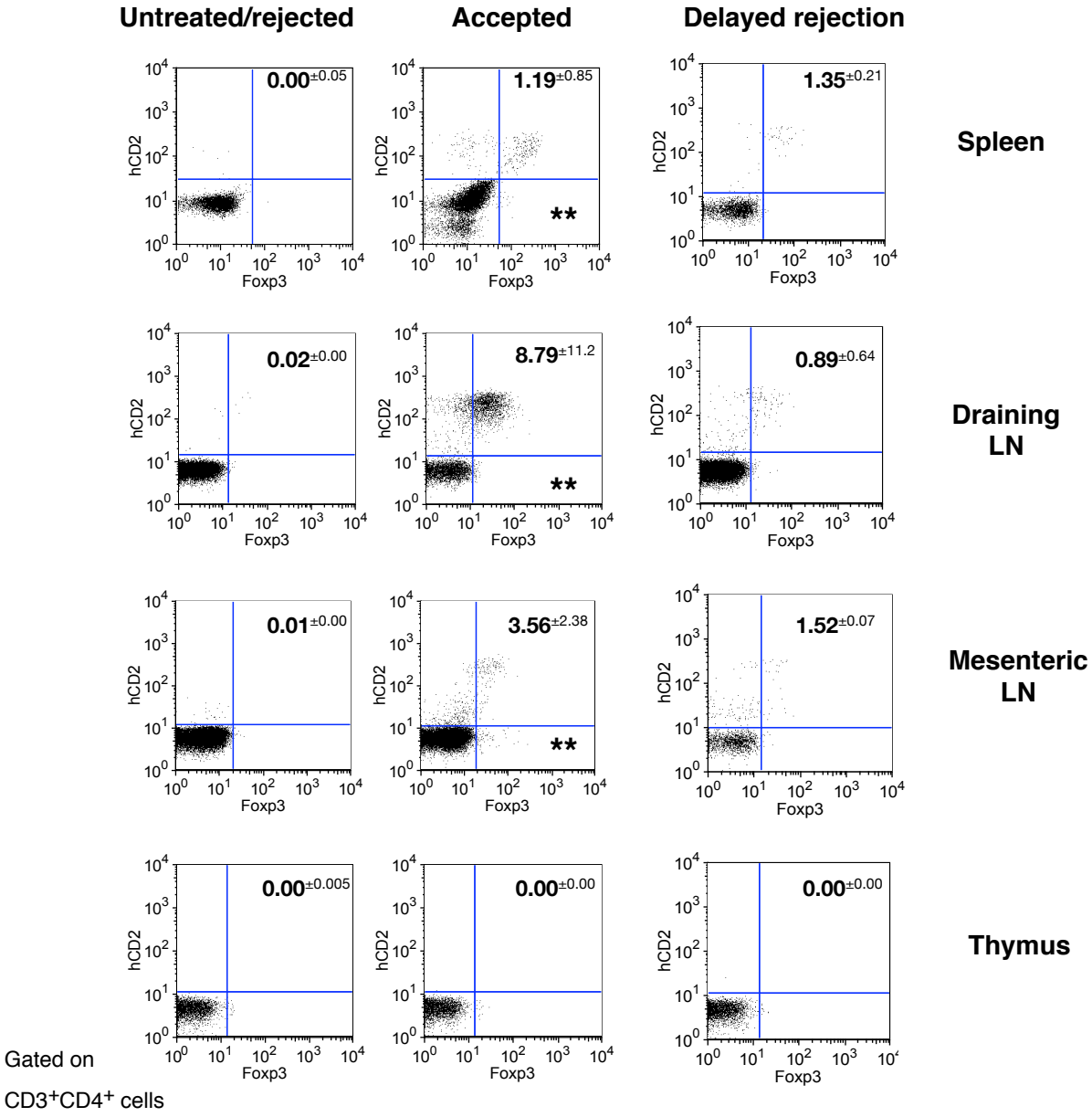


Figure 5.3 De novo Foxp3⁺ iTreg are generated in the periphery of anti-CD4 treated mice

De novo hCD2⁺Foxp3⁺ Treg were found in the spleen, draining and mesenteric lymph nodes, but not the thymus, of Marilyn.Foxp3^{hCD2} mice that accepted a male graft for at least 60 days (middle column) but not in untreated, rejecting Marilyn.Foxp3^{hCD2} mice (left hand column), $p<0.01$ in all cases. A smaller percentage of Foxp3⁺ cells were found in the periphery of those mice that exhibited delayed rejection within 5-7 weeks after anti-CD4 mAb treatment (right hand column). Data is representative of three independent experiments of at least 4 mice per group. Mean and SEM are shown. Statistical significance was analysed by t-test.

When the results of several transplant experiments using the same batch of anti-CD4 mAb were combined, it became clear that at least half the recipient mice treated with 250μg or 750μg of anti-CD4 mAb ultimately rejected their male B6.RAG$^{-/-}$ skin grafts. In the majority of cases, the grafts were rejected between 4-6 weeks following intraperitoneal administration of anti-CD4 mAb; a time period known to coincide with reduction in the functional levels of treatment antibody (Scully, Qin et al. 1994). Two and three intraperitoneal injections of 1mg anti-CD4 mAb given within the first seven days after transplantation resulted in some improvement but nonetheless failed to achieve graft survival rates greater than 70% (figure 5.2). The addition of anti-CD40L blocking mAb also failed to achieve any significant improvement.

5.3.2 Transplant tolerance generates antigen specific hCD2^{+}Foxp3^{+} iTreg

Female Marilyn.Foxp3^{hCD2} mice that had accepted male skin for at least two months were sacrificed and splenic, draining lymph node, mesenteric lymph node and thymic samples were harvested. Flow cytometry was used to detect any de novo generated hCD2^{+}Foxp3^{+} cells within these samples. No hCD2^{+}Foxp3^{+} cells were found in the the spleen, draining lymph node and mesenteric lymph nodes of female mice that had rejected the male skin acutely (figure 5.3). In contrast, hCD2^{+}Foxp3^{+} Treg were found in the equivalent tissue samples of mice that had accepted a male graft long term (figure 5.3, $p<0.0001$). Those female recipients that initially accepted, but then rejected their male grafts approximately 5-6

weeks following anti-CD4 mAb also had detectable, albeit few, hCD2^{+}Foxp3^{+} cells in the spleen, draining lymph nodes and mesenteric lymph nodes (figure 5.3, right hand column). No hCD2^{+}Foxp3^{+} were found in the thymus of any of the experimental animals.

Although an association between long term graft acceptance and generation of peripheral hCD2^{+}Foxp3^{+} cells was demonstrated, such correlative data fails to prove a necessary role for hCD2^{+}Foxp3^{+} iTreg in therapeutic tolerance. As previously discussed, their presence could, for example, be a bystander consequence, and not the driving force of tolerance induction. In addition, past work has shown that in vitro TGFβ conditioned CD4^{+} T-cells suppress graft rejection in vivo (Graca, Thompson et al. 2002), but because such populations were mixtures of Foxp3^{+} and Foxp3^{neg} T-cells it has not yet been established which are the active components. The Marilyn.Foxp3^{hCD2} mouse model offered a way to investigate whether Foxp3^{+} iTreg, in particular, are the cells responsible for suppression. In collaboration with Dr. Y. Chen and Dr. Frederico Regateiro, I first asked if TGFβ-induced iTreg, generated in vitro, could suppress graft rejection, and if this was dependent on Foxp3 expression.

Antigen specific Foxp3^{+} iTreg were derived by culturing Marilyn.Foxp3^{hCD2} splenocytes in vitro with dendritic cells, TGFβ and the cognate male peptide for 7 days. The cultured cells were then separated into hCD2^{neg} (Foxp3-) and hCD2^{+} (Foxp3^{+}) cell populations by fluorescent flow cytometry (figure 5.4A). When these were adoptively transferred into lymphocyte deficient female RAG$^{-/-}$ mice, those populations devoid of hCD2^{+}Foxp3^{+} Treg,

whether by MoFlow sorting of hCD2^{negative} cells or opsonisation with ablating anti-hCD2 mAb, rejected a male RAG$^{-/-}$ graft (figure 5.4B; p=0.0003). In contrast, recipients of purified hCD2^{+}Foxp3^{+} Treg tolerated their grafts. In a similar experiment, in vitro generated female hCD2^{+} cells not only failed to reject the male graft (as before) but also resisted the challenge of a second male graft transplanted 30 days later in conjunction with a further 1x10^5 monoclonal naïve T-cells (figure 5.5); a number at least 10 fold in excess of the minimum required to reject male skin (Dr. Y. Chen, DPhil Thesis, 2010). This demonstrated that adoptive transfer of artificially-induced Foxp3^{+} iTreg can suppress graft rejection in vivo. The next step was to ascertain whether this was true of Foxp3^{+} cells generated in vivo by antibody blockade.

5.3.3 In vivo generated Foxp3^{+} iTreg prevent rejection by graft resident T-cells

It has previously been shown that regulatory T-cells found within tolerated skin grafts can recolonize a lymphocyte deficient mouse and thereafter suppress graft rejection in spite of subsequent transfer of naive T-cells (Graca, Cobbold et al. 2002). More recently, it was shown that tolerated graft tissue is enriched for Foxp3^{+} mRNA (Cobbold, Castejon et al. 2004) and Foxp3^{+} cells (Fan, Spencer et al. 2010). Using Marilyn.Foxp3^{hCD2} mice it was possible to supplement these early findings by asking whether specifically Foxp3^{+} iTreg *in the tissue* are actually responsible for safeguarding tolerance from the threat of infiltrating effector cells. A two stage transplantation study involving the harvesting and subsequent re-transplantation of

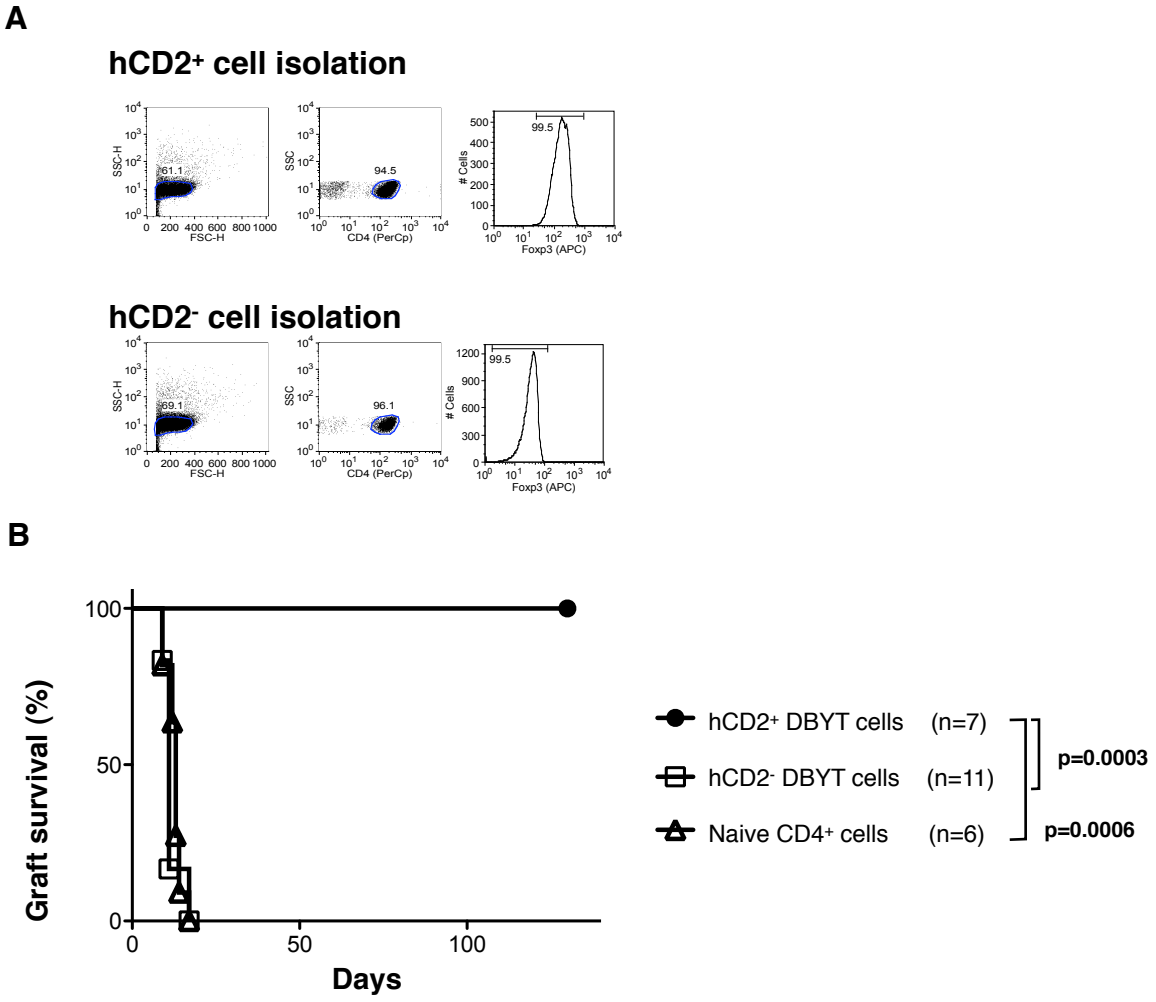


Figure 5.4 Antigen specific induced female Foxp3^{+} iTreg fail to reject a male graft

(**A**) FACS analysis of MoFlow sorted female CD3^{+}CD4^{+}**hCD2$^{+/pos}$** Marilyn.Foxp3^{hCD2} DBYT cells and CD3^{+}CD4^{+}**hCD2$^{-/neg}$** Marilyn.Foxp3^{hCD2} DBYT cells following 7 days of in vitro culture with TGFβ, dendritic cells and male H-Y antigen prior to transfer into male B6 RAG$^{-/-}$ recipients. (**B**) 5x10^5 TGFβ conditioned male H-Y antigen specific **hCD2**$^{+}$ CD4^{+} Marilyn.Foxp3^{hCD2} (DBYT) cells failed to reject a male CBA.Ca RAG$^{-/-}$ graft when injected into B6 RAG$^{-/-}$ recipients compared to 5x10^5 **hCD2**$^{-}$ DBYT cells, p=0.0003, or 1x10^5 *naive* CD4^{+}Marilyn.Foxp3^{hCD2} cells, p=0.0006. (hCD2^{-} DBYT group consists of mice injected with MoFlow sorted hCD2- DBYT cells; alone (n=3), plus anti-hCD2 mAb (n=4), plus isotype control mAb (n=4)).

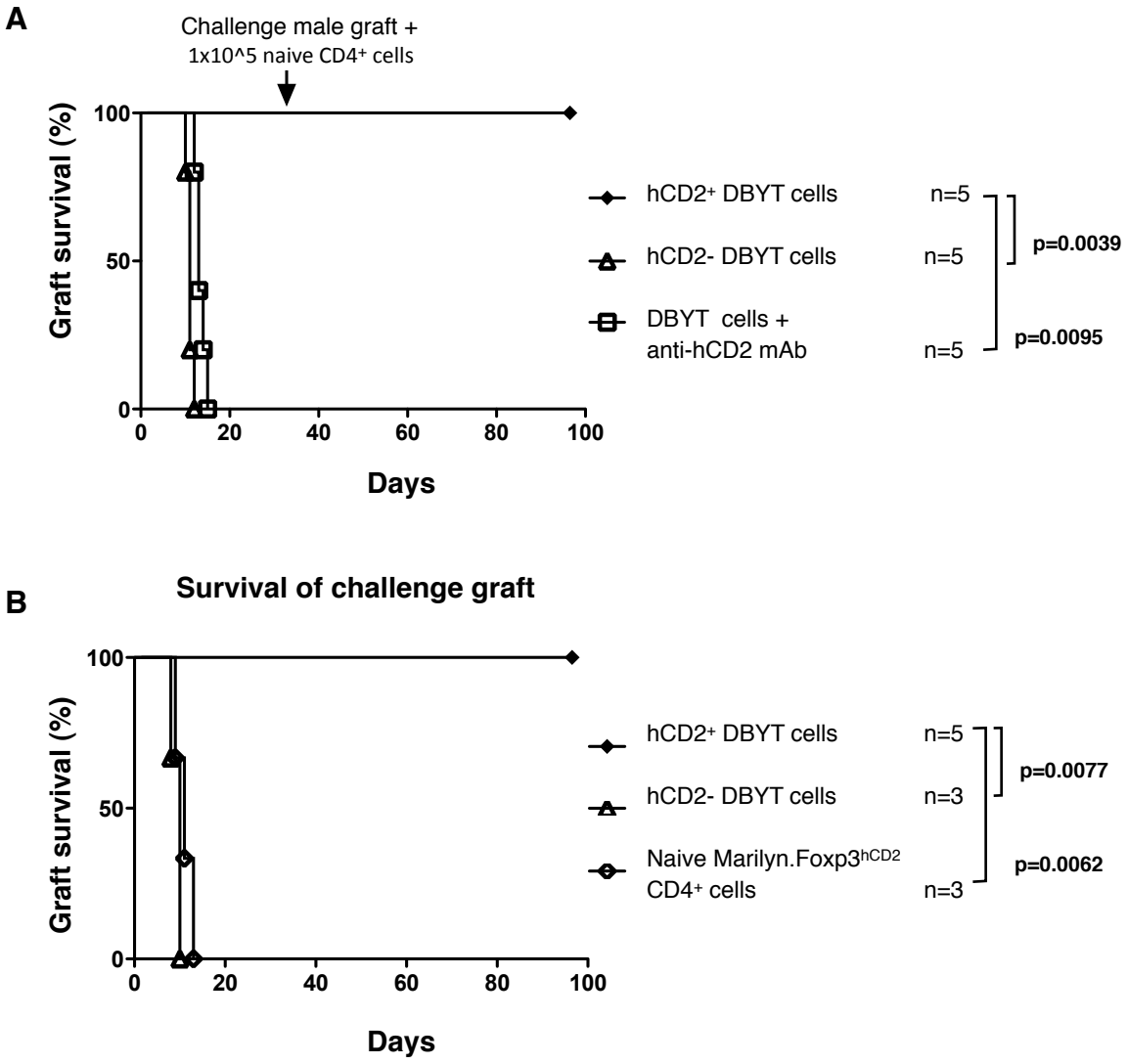


Figure 5.5 Foxp3+ iTreg actively suppress T-cells from rejecting the graft

(**A**)1x10^6 TGFβ conditioned male H-Y antigen specific hCD2+CD4+ (DBYT) cells fail to reject a male CBA.Ca RAG-/- graft when injected into B6 RAG-/- recipients. Elimination of hCD2+Foxp3+ T-cells, either by anti-hCD2 mAb (squares, p=0.0095), or MoFlow cell sorting of hCD2- cells (triangles, p=0.0039), results in graft rejection. A challenge graft co-transplanted with 1x10^5 naive CD4+ cells after 30 days (arrow) fails to break tolerance. **(B)** The challenge graft is rejected by mice that received hCD2- cells (p=0.0077) or naive cells (p=0.0062), but not mice that originally received hCD2+ cells.

well tolerated male skin grafts from female Marilyn.Foxp3^{hCD2} mice was employed to examine the role of Foxp3^{+} iTreg in the graft tissue itself. Lymphocyte deficient male B6 RAG$^{-/-}$ skin grafts were first placed on female Marilyn.Foxp3^{hCD2} mice and tolerance was induced through short-term anti-CD4 mAb blockade (figures 5.1 and 5.2). After 2 months, the accepted male skin grafts were carefully excised and then immediately re-transplanted onto female lymphocyte deficient hosts (female B6 RAG$^{-/-}$ mice). This process ensured that any functional T cells in the new host (whether effector or regulator) would be derived from the original female Marilyn.Foxp3^{hCD2} mice and could only have been transferred within the re-transplanted skin. Ablation of Foxp3^{+} Treg by anti-human CD2 mAb resulted in rapid rejection of the majority of these previously well accepted grafts when compared to control mice, demonstrating that a healthy graft can still harbor T-cells capable of causing its own rejection (figure 5.6, p=0.0040).

Taken together, the data described above establish a number of key findings. Firstly, de novo antigen specific Foxp3^{+} iTreg are produced in the periphery during tolerance induction through co-receptor blockade. Secondly, these Foxp3^{+} iTreg are actively suppressive and prevent potential effectors from rejecting grafts. Thirdly, because the tolerated graft tissue itself contains CD4^{+} effector T-cells fully capable of rejection, its survival depends on constant suppression by Foxp3^{+} iTreg generated de novo in response to the tolerising protocol.

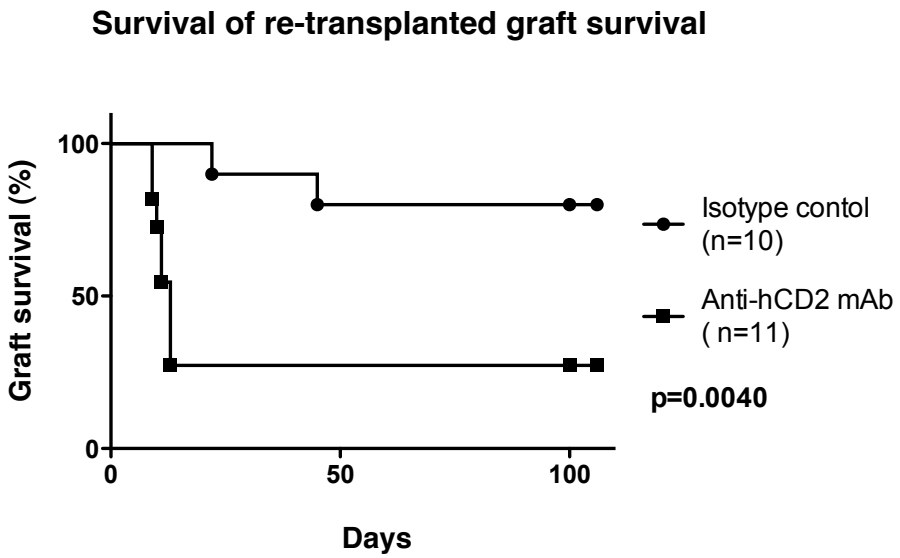


Figure 5.6 In situ Foxp3^{+} iTreg prevent rejection by tissue resident T-cells

Male B6 RAG$^{-/-}$ grafts that were accepted for over 60 days by anti-CD4 mAb treated female Marilyn.Foxp3^{hCD2} mice (**Fig. 5.1**) were then re-transplanted onto lymphocyte deficient female B6 RAG$^{-/-}$ recipients. Ablation with anti-hCD2 mAb resulted in rapid graft rejection compared to isotype control

5.3.4 Foxp3$^+$ cells are required for Infectious Tolerance in vivo

Infectious tolerance was first described in 1993 as a process by which a population of tolerant immune cells could pass on that tolerance to a naive population (Qin, Cobbold et al. 1993). What turned out to be a landmark paper confirmed that short term immune blockade could generate long term tolerance and went on to explore how such induced tolerance could be self sustaining. Providing the tolerant and naive population co-existed in a tolerant host for a period of at least 2 weeks, infectious tolerance could occur.

The data outlined in the previous chapter is consistent with a crucial role for Foxp3$^+$ cells in generating and maintaining tolerance to foreign tissue in a polyclonal immune system. Furthermore, antigen specific Foxp3$^+$ iTreg, produced in vivo in response to anti-CD4 mAb, actively prevent graft rejection by tissue resident T-cells. What is not clear, is whether new Foxp3$^+$ Treg are generated from within a naive cell population following co-existence with other Foxp3$^+$ cells i.e. as a result of infectious tolerance.

Exploring the role of Foxp3$^+$ Treg in infectious tolerance requires an *in vivo* model with the following

- A reliable method for distinguishing between the original tolerant population and the naive population
- An absence of Foxp3$^+$ cells in the naive population at the start of the experiment

- Accurate identification of any new Foxp3^{+} cells that develop in response to infectious tolerance

In the original infectious tolerance experiments, *all lymphocytes* in the reporter CBA.Ca mouse expressed the human CD2 reporter protein (Qin, Cobbold et al. 1993). Once these mice had been tolerised to a foreign graft, a challenge graft was transplanted in conjunction with a transfusion of naive spleen cells from wild type CBA.Ca mice. Anti-hCD2 mAb was then used to deplete all the original tolerant lymphocytes leaving only the 'naive' population after an appropriate hand over period (at least 2 weeks). A similar strategy could now be employed using Marilyn.Foxp3^{hCD2} mice to assess the roles of specifically Foxp3^{+} iTreg in infectious tolerance.

Antigen specific Foxp3^{+} iTreg were generated from spleen cells of female Marilyn.Foxp3^{hCD2} mice cultured in vitro for seven days with dendritic cells, cognate male Dby peptide and TGFβ. Foxp3^{+} cells were isolated by flow cytometry based on their co-expression of hCD2 and 500,000 cells were infused intravenously into lymphocyte deficient female B6.RAG$^{-/-}$ recipient mice. A second group received 100,000 naive CD4^{+} cells from Marilyn^{WT} mice. The third group received a mixture of 500,000 hCD2^{+}Foxp3^{+} cells and 100,000 naive Marilyn^{WT} CD4^{+} cells. All groups were grafted with male CBA.RAG$^{-/-}$ skin after 24 hours. None of the mice that received 500,000 hCD2^{+}Foxp3^{+} cells rejected their male grafts. In comparison, all the mice receiving 100,000 naive CD4^{+} cells rejected their grafts (figure 5.7, p=0.0066). Half of the mice that received a mixture of tolerant hCD2^{+}Foxp3^{+} cells

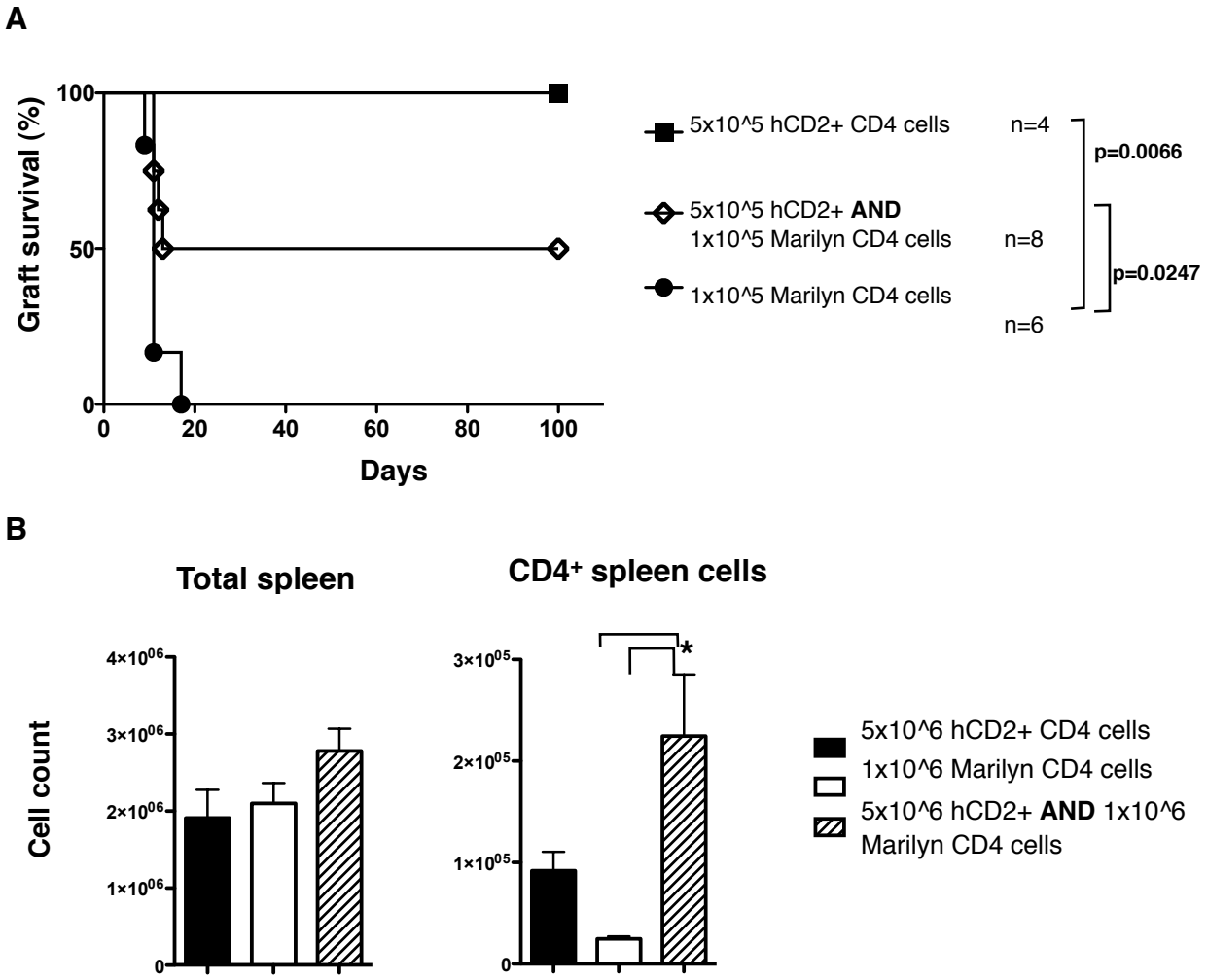


Figure 5.7 Foxp3⁺ iTreg are necessary for infectious tolerance

(**A**) Female B6.RAG$^{-/-}$ received a male CBA.RAG$^{-/-}$ skin graft in conjunction with either 5x10^5 in vitro cultured female **hCD2⁺** Marilyn.Foxp3^{hCD2} DBYT cells, 1x10^5 naïve CD4⁺ cells from Marilyn^{WT} mice, or a mixture of both. All of the mice that received 1x10^5 naïve CD4⁺ cells rejected the male skin compared to none of those that received 5x10^5 hCD2⁺ cells (p=0.0066) and half that received a mixture (p=0.0247). After 100 days, the mice were killed and Foxp3⁺ cell composition was analyzed. **(B)** Total white cell spleen counts were similar between the three groups, but there were significantly more CD4⁺ cells in mice that received a mixture of **hCD2⁺** and naïve CD4⁺ cells. Two independent experiments were combined. Bars show mean of 6 or 8 individuals, SEM indicated by error bars. ANOVA test used to determine statistical significance.

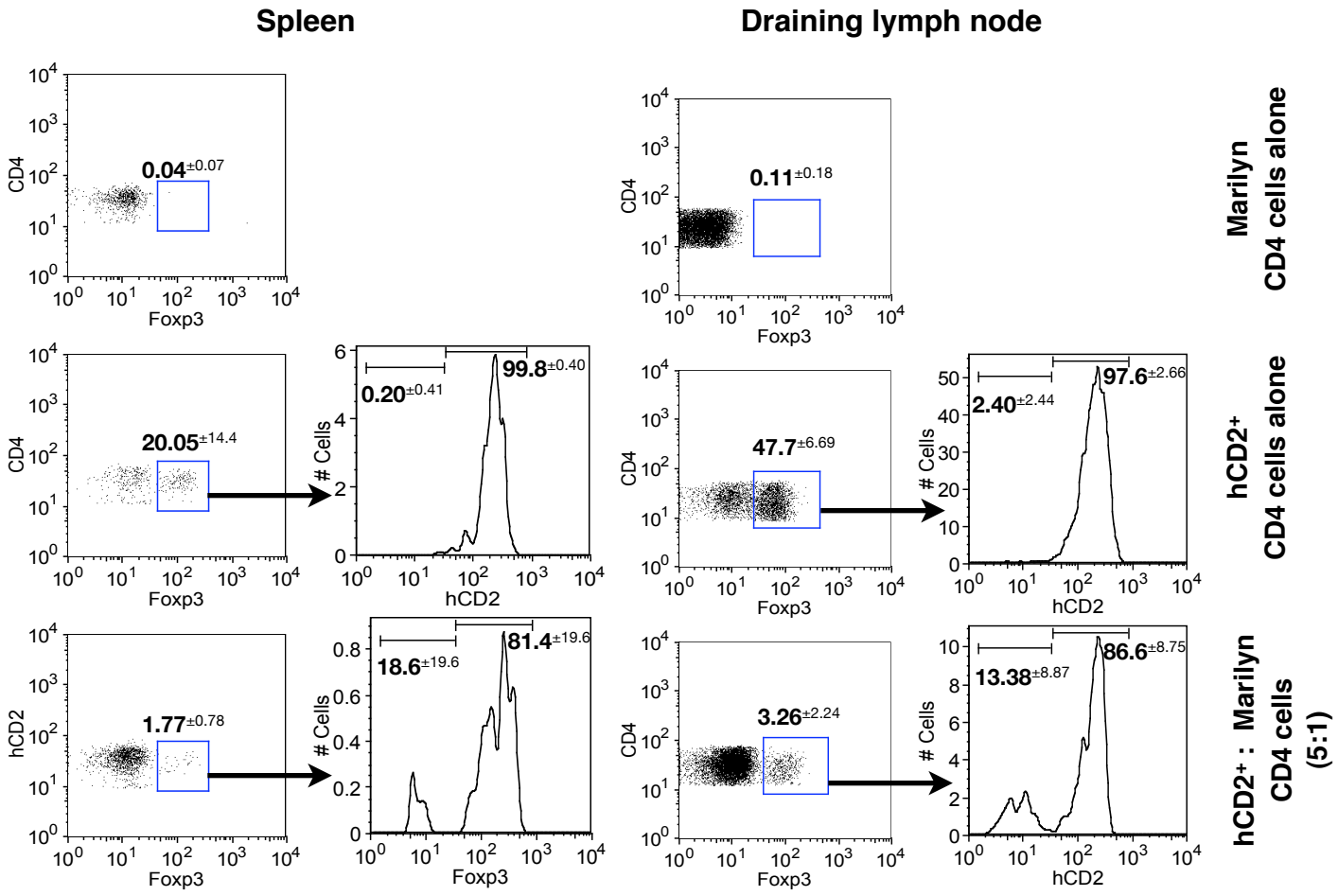


Figure 5.8 Infectious tolerance converts naive CD4⁺ cells into Foxp3⁺ cells in vivo

Experimental mice from figure 5.7 were analyzed by flow cytometry for conversion of naïve CD4⁺ cells into Foxp3⁺ T-cells. Over 98% of Foxp3⁺ cells in mice that received 5x10^5 isolated hCD2⁺ Marilyn.Foxp3^{hCD2} cells were hCD2⁺. Whereas 13% of Foxp3⁺ cells in the draining lymph and 18% in the spleen of mice that received a 5:1 mixture (and had accepted the male graft) were hCD2 negative (p=0.0052 and p=0.0056 respectively on t-test). Data figures represent the mean and SEM of 4 to 8 mice from two independent experiments.

and naive $CD4^+$ cells accepted the male graft (figure 5.7, p=0.0247 compared to positive control).

Those mice that accepted the male skin for over 100 days were sacrificed and analysed for their cell profile. Figure 5.7B shows a similar total spleen white cell count in all three groups, but a significantly larger number of $CD4^+$ cells in the group that received a mixture of $hCD2^+$ and naive $CD4^+$ cells compared to the other two groups (figure 5.7B, $p<0.01$).

Very few, if any, $Foxp3^+$ cells were found in mice that received 100,000 naive $CD4^+$ cells alone and consequently rejected the male skin (figure 5.8). In contrast, $Foxp3^+$ cells were found in the spleen and draining lymph nodes of mice that had accepted the male graft for over 100 days. In those mice that received a 5:1 mixture of $hCD2^+Foxp3^+CD4^+$ cells to naive $CD4^+$ cells, 13% of $Foxp3^+$ cells in the draining lymph nodes and 18% in the spleen were hCD2 negative (figure 5.8, p=0.0052 and p=0.0056 respectively). Whereas over 98% of $Foxp3^+$ cells in mice that received 500,000 isolated $hCD2^+Foxp3^+$ Marilyn.$Foxp3^{hCD2}$ cells were $hCD2^+$.

The presence of $hCD2^{neg}Foxp3^+$ cells in only those mice that received the 5:1 mixture demonstrates that a proportion of the naive cells $Foxp3^-$ $CD4^+$ cells from Marilyn^{WT} mice have developed into $Foxp3^+$ cells in response to co-existence with tolerant $hCD2^+Foxp3^+$ cells from Marilyn.$Foxp3^{hCD2}$ mice. This finding that $Foxp3^+$ Treg not only suppress but enable

conversion of naive T-cells into new Foxp3$^+$ cells provides one mechanism by which infectious tolerance may occur.

5.4 Discussion

5.4.1 Functional Foxp3$^+$ iTreg in the absence of nTreg

In Chapter 4 the induction of long term tolerance to foreign tissue by short term immune blockade was shown to require the enhancement of Foxp3$^+$ cell dependent regulation, potentially by increasing the relative numbers of Foxp3$^+$ Treg. This could be accomplished in vivo by the expansion of pre-existing natural Foxp3$^+$ Treg from the thymus or through the conversion of naive CD4$^+$ cells into Foxp3$^+$ iTreg in the periphery. The relative importance of iTreg remains unclear with some groups demonstrating that nTreg alone can maintain therapeutic tolerance (Nishimura, Sakihama et al. 2004; Vukmanovic-Stejic, Zhang et al. 2006) as well as pointing to a lack of evidence in support of peripheral conversion of naive cells in conventional mice (Wong, Mathis et al. 2007).

Historically, the situation has not been helped by the inability to identify and manipulate iTreg independently of nTreg. For this reason, TCR transgenic mice have traditionally been used because they lack thymic derived nTreg meaning that new Foxp3$^+$ cells would be induced in the periphery. Such models have already demonstrated that exposure to antigen under sub-optimal conditions for T-cell activation results in generation of new, peripheral

Foxp3^{+} Treg. For example, constant delivery of antigen via an osmotic pump led to the conversion of naive Foxp3 negative CD4^{+} cells into Foxp3^{+} cells (Kretschmer, Apostolou et al. 2005). Targeting tolerogenic peptide to dendritic cells using an antibody against CD205 (DEC-205) also led to an increase in antigen specific Foxp3^{+} cells that could prevent immunopathology (Mahnke, Qian et al. 2003; Bruder, Westendorf et al. 2005). Our group has gone further and found Foxp3 induction actually within tolerated graft tissue in response to co-receptor and co-stimulatory blockade (Cobbold, Castejon et al. 2004). The implication is that when naive CD4^{+} cells encounter antigen presented by immature or poorly activated dendritic cells, they have an increased probability of becoming Foxp3^{+} Treg and, indeed, this is one effective way of generating antigen specific Foxp3^{+} Treg in vitro (Chen, Jin et al. 2003; Peters, Preijers et al. 2008; Chen, Delgado et al. 2009). Although these studies have described the generation of Foxp3^{+} cells in response to certain tolerogenic conditions, it has been harder to prove that these cells are actively participating in regulation in vivo.

Using Marilyn.Foxp3^{hCD2} model, I was able to show that transfer of MoFlow sorted hCD2^{+}(Foxp3^{+}) cells generated in vitro not only failed to reject a graft but actively prevented naive cells from doing so (figure 5.5 and 5.6). More importantly, ablation of newly generated hCD2^{+}(Foxp3^{+}) cells residing in the graft led to rejection. This proved that antigen specific Foxp3^{+} iTreg, produced in response to anti-CD4 therapy, were operational and normally prevent rejection by residual effector T-cells living within the graft tissue itself (figure 5.6).

5.4.2 Foxp3^{+} iTreg and Infectious Tolerance

Given that Foxp3^{+} iTreg are so crucial to graft survival in Marilyn.Foxp3^{hCD2} mice, it seemed likely that they would need to establish a constant presence, particularly given that tolerated grafts harbor effector T-cells capable of rejection. Once again, it was important to ask whether sufficient numbers are maintained through proliferation of the original iTreg population; in this case found in the periphery, (and not the thymus - figure 5.3), or by further recruits from naive CD4^{+} cells? The discovery of 'Infectious Tolerance,' in which tolerance can be passed from one population of lymphocytes to another, suggests that the latter might be possible (Qin, Cobbold et al. 1993). The mechanisms underlying infectious tolerance, or indeed whether it has any natural relevance, are still unknown. hCD2^{+}Foxp3^{+} cells have been shown here not only to suppress, but to actively recruit new Foxp3^{+} converts from the naive lymphocyte population, providing one potential mechanism by which infectious tolerance may operate in vivo (figures 5.7 and 5.8). In this case, the converted Foxp3^{+} iTreg were distinguished from the original tolerant population by the absence of the hCD2_hCD52 reporter protein.

An important feature of infectious tolerance is that the newly tolerant population is able to maintain tolerance in the absence of the original regulators. Qin et al. originally used YTH655 to deplete over 98% of the first lymphocyte population, *all* of which expressed high levels of hCD2 reporter. In this Foxp3-hCD2_CD52 knock in model, systemic ablation of hCD2^{+}Foxp3^{+} cells by anti-hCD2 mAb is far less effective (Chapter 3), and so could not be reliably used to expose the new iTreg. Intravenous infusion of hCD2^{+}Foxp3^{+} cells into RAG$^{-/-}$

recipients in the presence of YTH655 was shown to improve depletion, removing >95% of cells (Chapter 3). However, a fresh round of homeostatic expansion in the new $RAG^{-/-}$ recipient could have a significant effect on the balance of effector vs Treg.

A number of groups have also found it difficult to demonstrate that the new Treg are suppressive in vivo. Certain laboratories have claimed to demonstrate infectious tolerance *in vitro*, where $CD25^{+}$ T-cells were able to induce further $CD25^{+}$ Treg that could suppress lymphocyte proliferation, again in vitro (Jonuleit, Schmitt et al. 2002), or could suppress a population of naive cells from causing colitis on adoptive transfer (Andersson, Tran et al. 2008). The latter paper suggested that new $Foxp3^{+}$ cells developed in the co-transferred naive population, but also failed to confirm them as suppressive. Finally, a recent paper has highlighted that human Treg require IL-35 for infectious tolerance, but once again, suppression is only demonstrated in vitro (Chaturvedi et al., 2011). Potential mechanisms of infectious tolerance are discussed further in Chapter 7.

Although $Foxp3^{+}$ iTreg have been shown here to generate new $Foxp3^{+}$ Treg from naive cells, the ability of the new recruits to suppress has still not been established.

5.4.3 Does transplant tolerance in conventional mice require iTreg?

Antigen specific, Foxp3^{+} iTreg have been confirmed here as crucial for induced transplantation tolerance in Marilyn.Foxp3^{hCD2} mice. However, the TCR transgenic model is necessarily so different to a conventional, lymphocyte replete mouse that the relevance of the above findings to normal immune homeostasis is debatable.

Similar transplant tolerance protocols have differing results in conventional compared to TCR transgenic mice. For example, although depletion of Marilyn.Foxp3^{hCD2} hCD2^{+} cells within re-transplanted graft tissue resulted in rejection (figure 5.6), when the same two stage transplant study was performed on conventional mice using tolerated skin grafts that were mismatched by full MHC or multiple minor antigens, anti-hCD2 mAb treatment failed to result in significant graft rejection (Chapter 4). Systemic administration of the YTH655 anti-CD2 mAb is known to result in suboptimal ablation of Foxp3^{+} T-cells (Chapter 3). But it is unclear why the same depleting regimen should cause rejection in the TCR transgenic (Marilyn.Foxp3^{hCD2}) system and not CBAxB6^{hCD2} mice. It might be that there is a greater proportion of graft specific rejecting T-cells in the TCR transgenic model since all of these CD4 cells can bind the male graft antigen. In comparison, the number of T-cells able to reject the graft in conventional polyclonal mice could be far less. The impact of depleting only a proportion of Treg might therefore be greater in the former than the latter.

In addition, antigen specific iTreg have been shown to lose their Foxp3 expression when transferred in vivo (Kretschmer, Apostolou et al. 2005; Floess, Freyer et al. 2007), with some reverting back to a T-helper cell phenotype (Zhou, Bailey-Bucktrout et al. 2009). Although the presence of a tolerated graft limits this loss (Dr. Y. Chen, DPhil Thesis 2010), the putative instability of Foxp3 expression of iTreg in the TCR transgenic mouse may result in a further increase in the ratio of effector to regulatory cells. As a result the Marilyn.Foxp3^{hCD2} system may be more closely poised to rejection, even before depleting anti-hCD2 mAb is given. It is important to acknowledge here that a significant role for iTreg has so far not been proven in conventional mice with a full repertoire of nTreg. The loss of Foxp3 expression in iTreg may therefore be less of a limiting factor in conventional mice which could still rely on their expanded population of natural Treg.

Cells capable of rejection that survive despite tolerance induction in CBAxB6^{hCD2} mice (Chapter 4), may reside more in areas such as the draining lymph node, than the graft tissue itself. In which case, very few would be transferred within the re-transplanted skin and this might explain why these grafts were not rejected when anti-hCD2 mAb was administered to ablate Treg. One way of testing this would be to investigate whether there are even sufficient cells carried over to reject a third party graft. If nTreg are actually found in more 'central' lymphatic tissue compared to iTreg, would this difference underpin a division of labour in conventional immune regulation? It could also explain why the same anti-hCD2 mAb regimen resulted in rejection in conventional mice during challenge grafting (Chapter 4) but failed to do so in TCR transgenic mice (figure 5.1), perhaps because the majority of effector cells in the latter are detained and suppressed within the original graft.

Finally, antigen specificity is required for induction of Foxp3$^+$ Treg, but it may not be an absolute requirement of their action. For example, could in vivo expanded monoclonal Foxp3$^+$ iTreg suppress immunopathology in a wild type polyclonal immune system? Alternatively, are Foxp3$^+$ iTreg ever produced de novo, in the periphery of tolerant *conventional* mice, irrespective of nTreg? The difficulty in answering this last question is that it requires a polyclonal immune model without any Foxp3$^+$ Treg at the start of the experiment and this is currently limited by the rapid onset of severe immunopathology (Chapter 3 and (Kim, Rasmussen et al. 2007)). Chapter 6 describes my attempts to generate such a model and prevent immunopathology so that immune regulation beyond natural, thymic, Foxp3$^+$ Treg can be explored.

CHAPTER 6: Tolerance in the absence of Foxp3+ regulatory T-cells

6.1 Abstract

A protocol was developed to investigate whether antibody mediated co-receptor blockade could induce tolerance in a conventional, polyclonal immune system devoid of Foxp3^{+} natural nTreg. Lymphocytes from B6.Foxp3^{hCD2} mice were adoptively transferred into B6.RAG$^{-/-}$ recipients in the presence of anti-hCD2 mAb to deplete Foxp3^{+} nTreg. Recipients typically developed a severe wasting immunopathology that limited the investigation of long term tolerance. However, when smaller numbers of purified hCD2^{neg} CD4^{+} lymphocytes were transferred, the disease phenotype was milder and mice survived many months despite the absence of Foxp3^{+} nTreg. Anti-CD4 mAb treatment of these recipients resulted in prolonged survival of MHC mismatched grafts without the generation of new Foxp3^{+} iTreg.

6.2 Introduction

Scurfy f*oxp3*$^{-/-}$ mice develop early onset, fatal immunopathology, characterised by lymphocyte infiltrating inflammation of predominantly epithelial and endocrine tissue (Godfrey, Wilkinson et al. 1991; Brunkow 2001; Appleby and Ramsdell 2008). A similar phenotype is found in patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome who lack a functional foxp3 gene. Typically male patients present within the first 6 months of age with a clinical triad of watery diarrhoea, eczematous dermatitis, and endocrinopathy; most commonly insulin-dependent diabetes mellitus and/or thyroiditis (Gambineri, Torgerson et al. 2003). The initial explanation that these characteristics were the

result of abnormal immune development in the absence of foxp3 expression was challenged by the demonstration that even well developed, healthy, adult DEREG mice also developed severe immunopathology when treated with diphtheria toxin to target Foxp3$^+$ Treg (Kim, Rasmussen et al. 2007; Lahl, Loddenkemper et al. 2007). From these results, it was argued that Foxp3$^+$ Treg were necessary for immune homeostasis throughout life and that in their absence there was no regulation of any practical worth (Kim, Rasmussen et al. 2007).

The challenge for those who believe in the importance of other regulatory cell sub-types is to overcome the profound effect that regulation of, particularly gut, epithelia has on mouse survival. The majority of models involving conventional mice lacking or stripped of Foxp3$^+$ Treg rapidly develop a wasting colitis and die within 4 weeks (Lahl, Loddenkemper et al. 2007). At present, the problem is therefore not so much whether Foxp3$^+$ Treg are the only regulators, but that in their absence, wasting immunopathology limits the examination of other potential regulators or the additional roles of Foxp3$^+$ Treg themselves in other tissues.

As discussed in Chapter 5, a necessary protective role for newly induced Foxp3$^+$ iTreg in conventional mice remains unproven, mainly because it has been technically difficult to identify and then interrogate specifically iTreg against a background of nTreg. TCR transgenic models have traditionally been used to investigate the function of iTreg, but it is not clear whether these findings are applicable to conventional mice or are just a transgenic artefact. One way of overcoming this problem would be to induce transplant tolerance in a

conventional immune system that from the outset lacks Foxp3^{+} nTreg. This requires not only an immune system devoid of these cells but an animal that can survive their loss.

The aim was therefore to develop a model of a polyclonal (conventional) immune system where the host could survive without Foxp3^{+} nTreg. In Chapter 3, Foxp3^{+} cells harvested from the spleens of B6.Foxp3^{hCD2} mice were serendipitously found to be highly vulnerable to ablative anti-hCD2 mAb on adoptive transfer into RAG$^{-/-}$ recipients. While this experimental protocol could achieve near total depletion of Foxp3^{+} Treg, the mice are shown here to develop a wasting colitis of comparable dynamic to that perviously found on transfer of CD4^{+}CD25^{-}CD45RB^{high} cells (Mottet, Uhlig et al. 2003; Izcue, Coombes et al. 2006; Uhlig, Coombes et al. 2006). In this chapter, variations in the number and type of lymphocyte population were found to impact on the incidence of wasting colitis. This enabled me to establish protocols where colitis was significantly delayed with a favourable effect on host survival. Short term co-receptor blockade from the time of cell transfer could also prevent wasting immunopathology.

A protocol was developed in which murine recipients of Foxp3^{neg} lymphocytes could nonetheless survive beyond ten weeks so providing the opportunity to investigate whether short term antibody mediated co-receptor/co-stimulatory blockade could generate tolerance in the absence of natural Foxp3^{+} nTreg. If tolerance could be induced, would this result in the generation of new Foxp3^{+} iTreg and would these iTreg be actively suppressive? If no Foxp3^{+}

iTreg were generated, then what are the alternative regulatory elements achieving tolerance in their absence?

6.3 Results

6.3.1 Foxp3- lymphocytes generate systemic immunopathology when transferred into lymphocyte deficient hosts

Circulating Foxp3^{+}hCD2^{+} in the blood of B6.Foxp3^{hCD2} mice were found to be more susceptible to antibody mediated ablation by YTH655 than those resident in splenic or lymphatic tissue (see Chapter 3). This lead to a proof of principal assay in which spleen cells were harvested from B6.Foxp3^{hCD2} mice that had already been treated with anti-hCD2 mAb (24 hours after 7 daily intra-peritoneal injections of 250μg YTH655) and injected intravenously into lymphocyte deficient B6.RAG$^{-/-}$ mice in the presence of an additional 1mg YTH655. In comparison to the untreated control group, over 95% of Foxp3^{+}hCD2^{+} cells were ablated (figure 6.1). This demonstrated that even splenic cells which had survived systemic ablation could be removed if they were forced to circulate in the presence of YTH655. By ensuring very efficient ablation of Foxp3^{+} cells, adoptive transfer studies of this kind provided an alternative protocol for examining tolerance in the absence of Foxp3^{+} Treg (Chapter 3, 4 and 5). The elimination of Foxp3^{+} nTreg could be further improved by isolating hCD2- cells prior to the anti-hCD2 mAb opsonisation step (section 6.3.3).

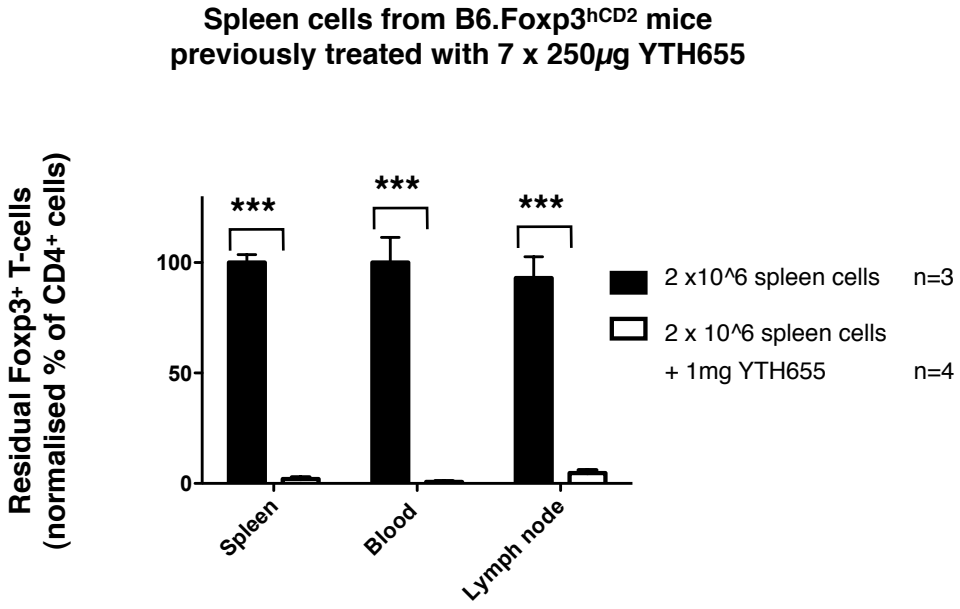


Figure 6.1 Adoptive transfer of B6.Foxp3^{hCD2} cells + anti-hCD2 mAb depletes Foxp3^{+} cells

2x10^6 spleen cells were harvested from B6.Foxp3^{hCD2} mice 24 hours after previous treatment with 7x250μg YTH655 and injected into B6.RAG$^{-/-}$ mice intravenously with 1mg YTH655. 4 weeks later, fewer than 2% of Foxp3^{+} cells were detected in the spleen, blood and lymph nodes compared to control mice that received 2x10^6 B6.Foxp3^{hCD2} cells alone (Chapter 3). Each bar represents the mean of three or four age matched mice with SEM indicated by the error bars. Statistical significance was determined by t-test.

DISTRESS SCORING SHEET – ADAPTED FROM WOLFENSOHN AND LLOYD, 1998

ANIMAL IDENTIFICATION	Score	**Date/Time**	**Date/Time**	**Date/Time**	**Date/Time**	**Date/Time**
APPEARANCE						
Normal	0					
General lack of grooming	1					
Coat staring, ocular and nasal discharges	2					
Piloerection, hunched up	3					
FOOD AND WATER INTAKE						
Normal	0					
Uncertain: body weight ↓<5% or Body Condition Score = BC2 (Appendix I)	1					
↓intake: body weight ↓10-15% or Body Condition Score = BC1 (Appendix I)	2					
No food or water intake or polydipsic	3					
CLINICAL SIGNS						
Normal colour and movement	0					
Slight changes: e.g. pallor, diminished activity, or increased water intake (polydipsia)	2					
Moderate changes: e.g. wet or blood on bedding (i.e. polyuria, diarrhoea or gastro-intestinal bleeding), urine scalding, visible abcess, ulceration or granuloma formation	4					
Severe changes: e.g. immobility, abnormal gait, lameness, fractures, paralysis, fitting	6					
NATURAL BEHAVIOUR						
Normal	0					
Minor changes	1					
Less mobile and alert, isolated	2					
Vocalisation, self mutilation, restless or very still	3					
PROVOKED BEHAVIOUR						
Normal	0					
Minor depression or exaggerated response	1					
Moderate change in expected behaviour	2					
Reacts violently, or very weak and precomatose	3					
SCORE ADJUSTMENT						
If you have scored >= 3 more than once, score an extra point for each	2-5					
TOTAL	0-23					

JUDGEMENT

0-4 Normal

5-8 Monitor frequently – at least 3x daily. If scored by NACWO they should inform personal licensee or deputy.

9+ Humane end point. Terminate by Schedule 1 method.

Figure 6.2 Clinical distress score sheet

Foxp3_diphtheria toxin-GFP reporter mice develop a severe wasting immunopathology of similar phenotype to *foxp3-/-* scurfy mice when treated with diphtheria toxin to target Foxp3^{+}(DT receptor^{+}) cells in vivo (Kim, Rasmussen et al. 2007; Lahl, Loddenkemper et al. 2007). Such immune dysregulation was not found in B6.Foxp3^{hCD2} (or CBAxB6^{hCD2}) mice systemically treated with YTH655, presumably because the residual, surviving Foxp3^{+} Treg were able to maintain self tolerance (see Chapters 3 and 4). In contrast, given that very few Foxp3^{+}hCD2^{+} cells survived anti-hCD2 mAb ablation when adoptively transferred into B6.RAG-/- recipients, it was important to validate this degree of ablation by establishing whether this too resulted in a wasting immunopathology.

CD4^{+} T-lymphocytes were isolated from the spleens of B6.Foxp3^{hCD2} mice by AutoMacs. However, because this resulted in a large proportion of CD8^{+} cell contaminants, the sorted cell population is hereafter referred to as 'T-lymphocytes'. Cells were prepared and then transfused intravenously into lymphocyte deficient B6.RAG-/- recipients. Initially, there were three experimental groups;

- intravenous infusion of 2 million B6.Foxp3^{hCD2} T-cells pre-mixed with 1mg YTH655 in 1 x PBS
- intravenous infusion of 2 million B6.Foxp3^{hCD2} T-cells plus a simultaneous intra-peritoneal injection of 1mg YTH655
- a control group receiving 2 million B6.Foxp3^{hCD2} T-cells plus isotype control mAb intravenously

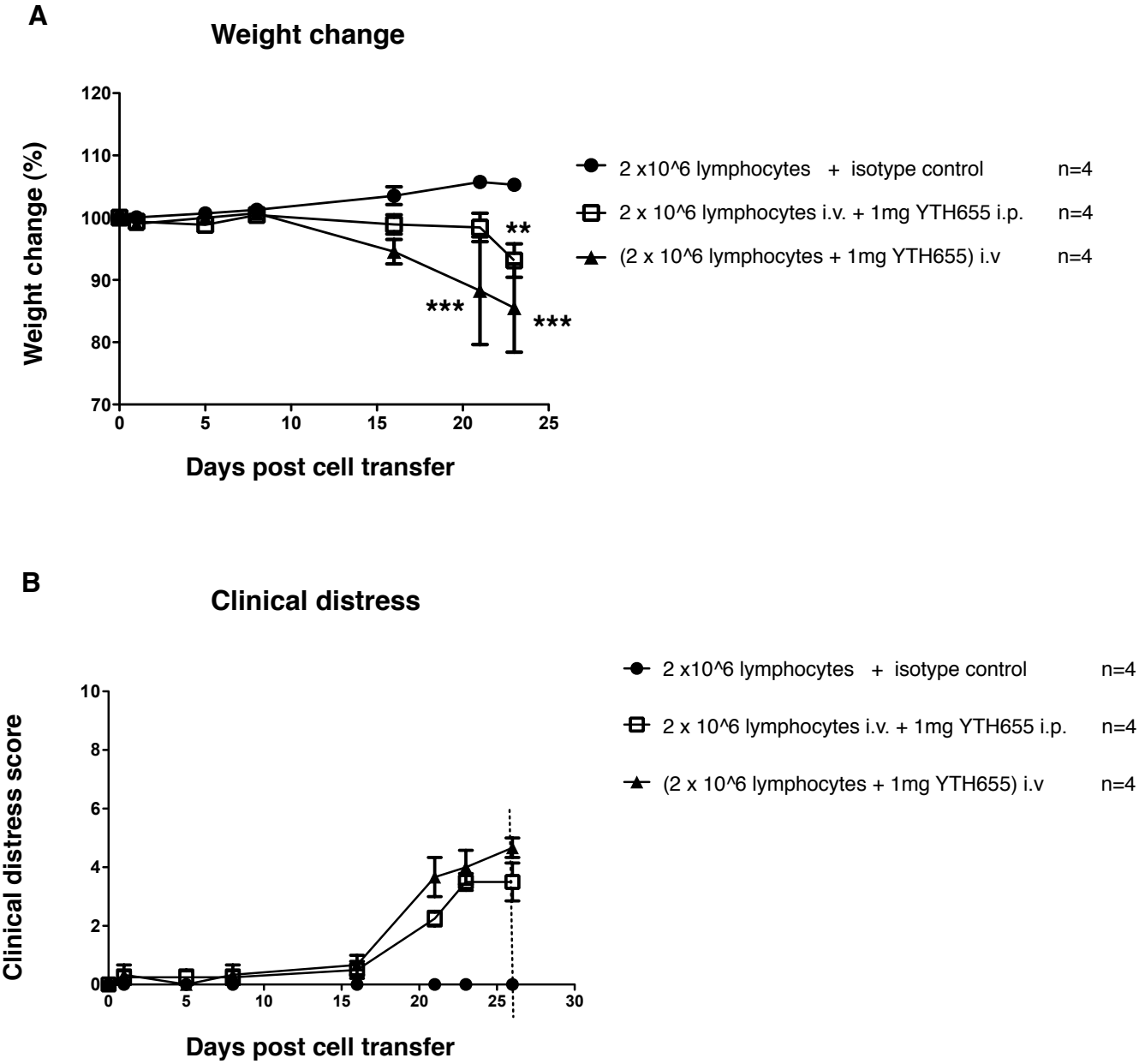


Figure 6.3 Foxp3^{+} Treg prevent immunopathology (1)

B6.RAG$^{-/-}$ developed severe weight loss (**A**) and signs of clinical distress (**B**) 14 days following intravenous infusion of 2x10^6 AutoMacs isolated splenic lymphocytes from B6.Foxp3^{hCD2} mice + 1mg YTH655 (i.v. and i.p). Control mice receiving 2x10^6 B6.Foxp3^{hCD2} lymphocytes + isotype control mAb remained well. For humane reasons, the experiment was terminated at 25 days. Each point represents the mean of four age matched mice with SEM indicated by the error bars. Statistical significance analysed by ANOVA Dunnett post-test.

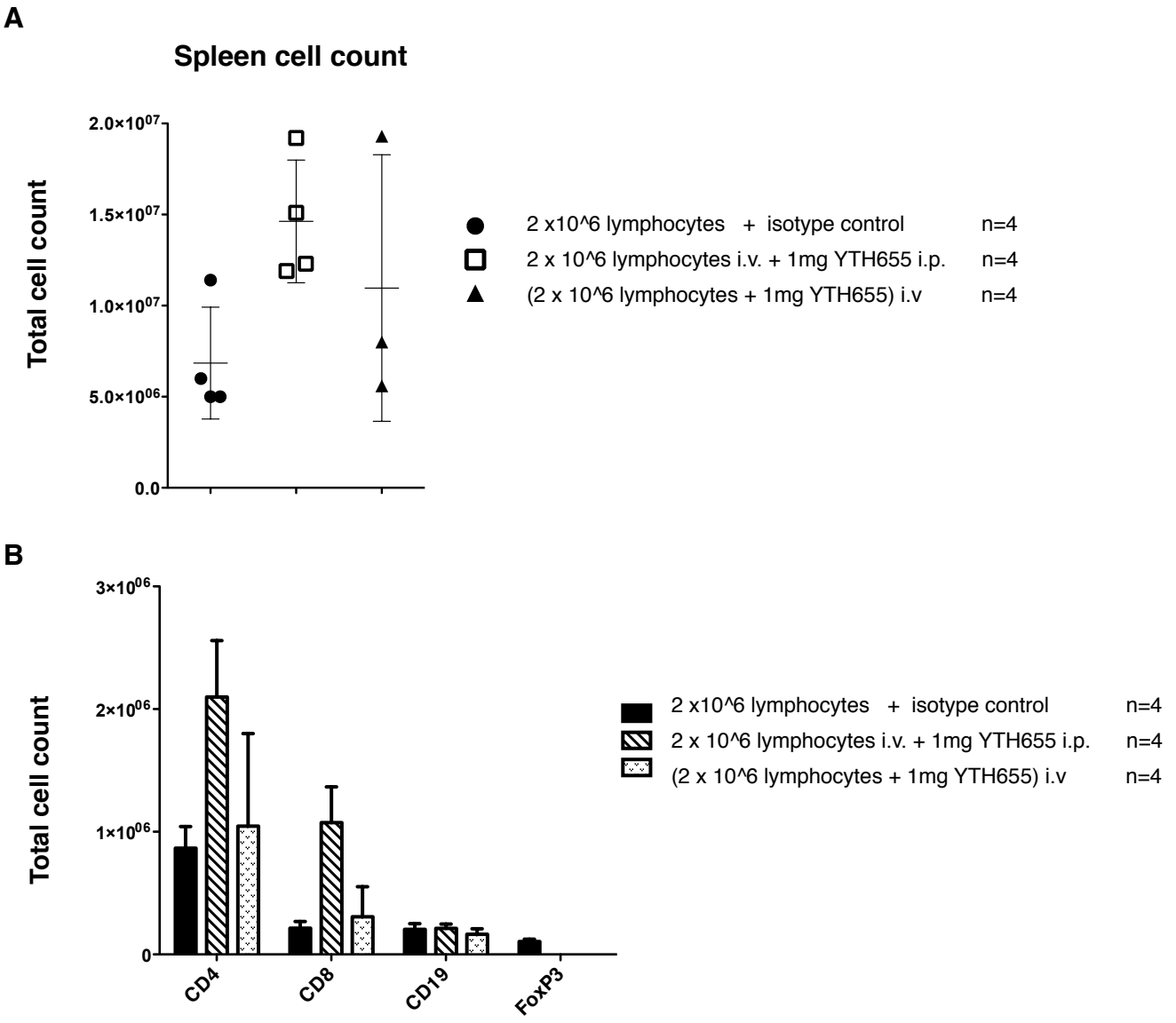


Figure 6.4 Foxp3⁺ Treg prevent immunopathology (2)

Flow cytometry analysis of spleen cells harvested from experimental B6.RAG$^{-/-}$ on termination (see **Figure 6.3**) was combined with the total spleen cell count (**A**) in order to estimate the number of CD4⁺, CD8⁺, CD19⁺ and Foxp3⁺ cells in each group (**B**). The adoptively transferred T-lymphocytes were able to expand in all three groups, as did the small proportion of contaminating (CD19⁺) B-cells. No statistical significance was found for CD4⁺ and CD8⁺ cells between the three groups (t-test). Foxp3⁺ cells were only observed in the control group (displayed more clearly in figure 6.5). Each bar represents the mean of four age matched mice. SEM are indicated by the error bars.

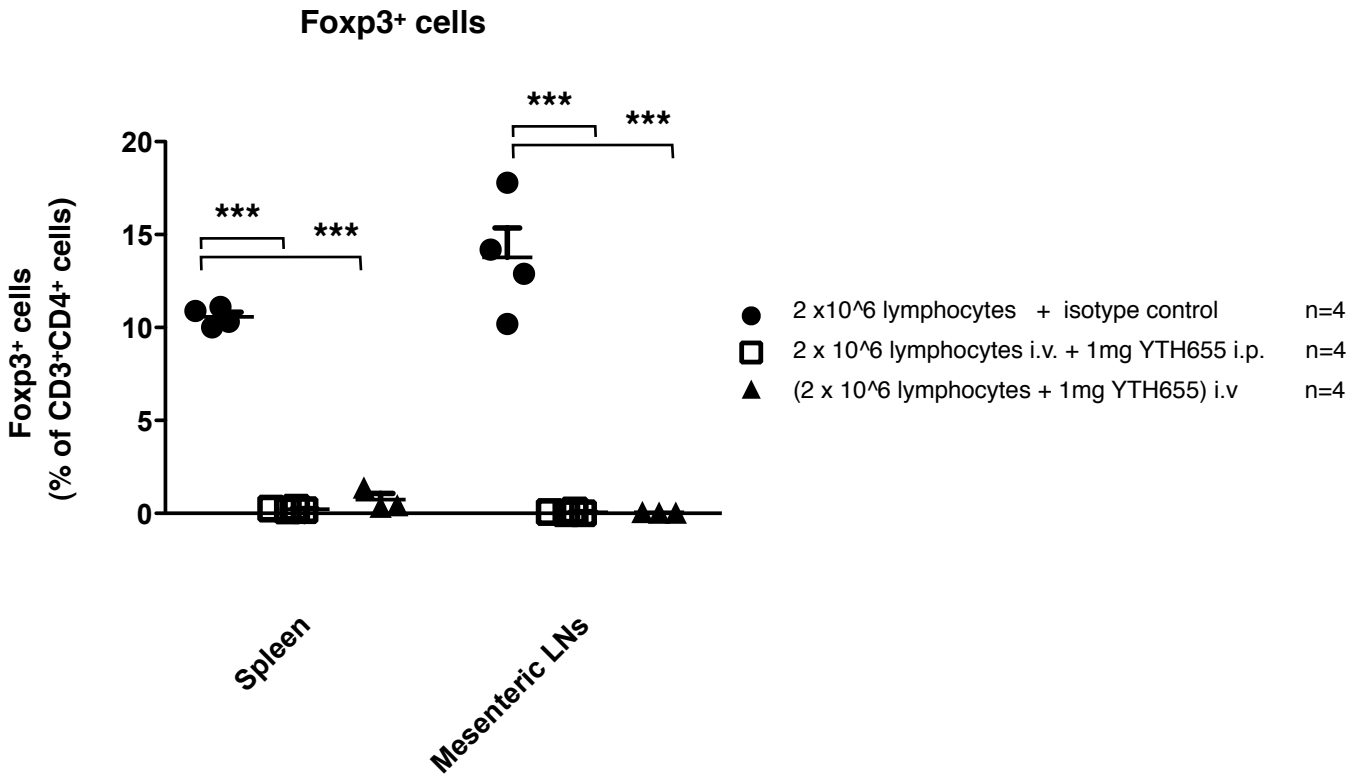


Figure 6.5 Foxp3+ Treg prevent immunopathology (3)

Between 10-18% of CD4+ cells were Foxp3+ in the spleen and mesenteric lymph nodes of control mice that received 2x10^6 B6.Foxp3^{hCD2} T-cells + isotype control mAb. In comparison, fewer than 1.5% of CD4+ cells were Foxp3+ cells when 1mg YTH655 (i.v. or i.p.) was added on adoptive transfer ($p<0.0001$ by t-test). Each point represents an individual mouse with SEM indicated by the error bars.

Experimental mice were carefully monitored for signs of clinical distress as outlined in the Home Office Guidelines for Animal Experimentation (figure 6.2) and regularly weighed. Any animal that lost at least 15% of their starting body weight or achieved a clinical distress score of 9 or more was euthanised in accordance with the Home Office Guidelines for Animal Experimentation. Cells were harvested from the blood, mesenteric lymph nodes and spleen for analysis by flow cytometry. The absolute cell counts of whole spleen cells were compared across all experimental groups as representative of the degree of homeostatic expansion. The total spleen cell count was combined with flow cytometric analysis of the percentage of lymphocyte subgroups such that, for example, the total number $CD4^+$ spleen cells could be estimated for each experimental mouse. Post-mortem samples were also taken, initially from a combination of the large bowel and liver, and thereafter from small bowel, stomach, skin, lungs, liver, pancreas, spleen, adrenal glands, gonads, thyroid, salivary glands, kidneys, cardiac muscle and skeletal muscle for histological analysis.

B6.RAG-/- mice that received an intravenous infusion of 2 million B6.Foxp3^{hCD2} T-cells pre-mixed with 1mg YTH655 anti-hCD2 mAb began to show clinical signs of distress and rapidly lost weight after 7 days such that they were euthanized after 25 days (figure 6.3). To allow comparison between the treatment and control groups, all experimental mice were sacrificed at this time point and tissue samples were harvested for analysis. The second treatment group that received 2 million cells intravenously plus 1mg anti-hCD2 mAb intra-peritonally, also showed clinical signs of distress and lost weight, but after at least 18 days. In comparison, the control group that was infused with 2 million cells in the presence of 1mg of an isotype control mAb remained healthy (figure 6.3).

Figure 6.4 shows that the number of spleen cells was greater, but not statistically significant, in the two treatment groups that either received 1mg YTH655 intravenously (mean = 14.6 x 10^6) or intra-peritoneally (mean = 10.9 x 10^6) compared to the control group that received 2 x 10^6 lymphocytes and control mAb (mean = 6.85 x 10^6), Similarly, there were more splenic $CD4^+$ cells in the two experimental groups (2.1 x 10^6 and 1.0 x 10^6 respectively) compared to the control group (8.6 x 10^5). The total number of $CD8^+$ cells (contaminants from the original AutoMacs sort) were also greater in the treatment groups compared to the control group. Very few $Foxp3^+$ cells were detected by flow cytometry in the spleen or mesenteric lymph nodes of the two groups treated with 1mg YTH655 as opposed to 10-15% of all $CD3^+CD4^+$ cells in control mice (figure 6.5).

A repeat experiment produced a similar outcome (figure 6.6). Two million AutoMacs enriched lymphocytes were injected intravenously into B6.RAG-/- recipient mice with isotype mAb (control), concomitantly with 1mg anti-hCD2 mAb i/p or having been mixed with 1mg anti-hCD2. Once again, those mice that received a mixture of lymphocytes and 1mg anti-hCD2 rapidly lost weight after 7 days, showed clinical signs of distress and the experiment was terminated in accordance with Home Office guidelines after 23 days (figure 6.6A-B). The group that received 2 million lymphocytes i/v and a separate intraperitoneal injection of 1mg anti-hCD2 once more demonstrated a delay in the onset of weight loss. These mice only started to lose weight after 18 days. The mice in the control group that received 2 million lymphocytes alone remained healthy throughout, failed to lose any weight and showed no signs of distress (figure 6.6A-B). Very few $Foxp3^+$ cells were found in the spleen, mesenteric

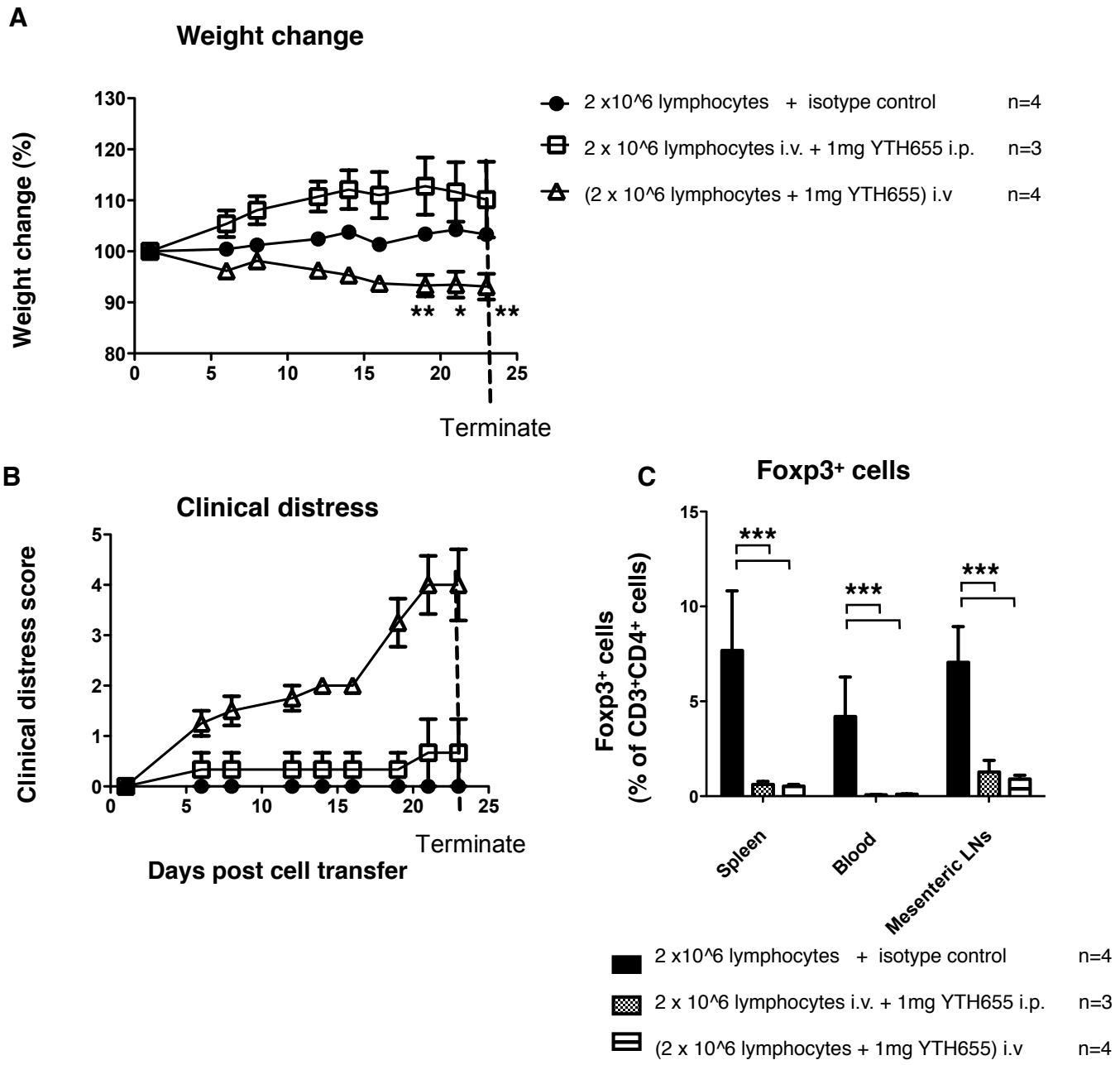


Figure 6.6 Adoptive transfer of Foxp3^{neg} T-cells reliably leads to immunopathology

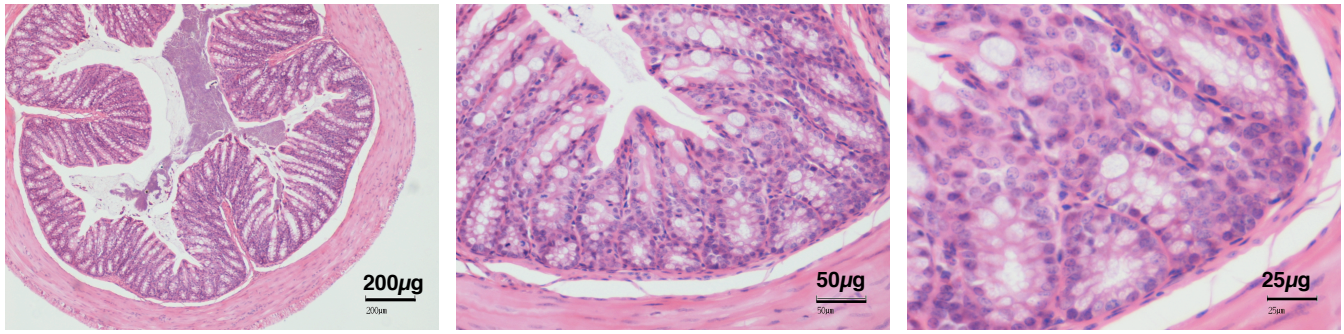
A second set of B6.RAG$^{-/-}$ developed significant weight loss (**A**) and signs of clinical distress (**B**)18 days following intravenous infusion of 2x10^6 AutoMacs isolated splenic T-cells from B6.Foxp3^{hCD2} mice + 1mg YTH655 (i.v. triangles, ANOVA test). Once again, there was a delay in the onset of disease when YTH655 aCD2 mAb was given intra -peritoneally instead of intravenously. (**C**) In comparison to control mice, very few Foxp3^{+} cells were found in recipients of 2x10^6 B6.Foxp3^{hCD2} T-cells + YTH655 mAb ($p<0.0001$ by t-test). Further tissue samples were taken for histological analysis (see below). Each point or bar represents the mean of three or four age matched mice with SEM indicated by the error bars.

lymph node or blood of mice that received 1mg anti-hCD2 (whether intra-peritoneal or intravenous) compared to control mice (figure 6.6C, $p<0.0001$ in all cases).

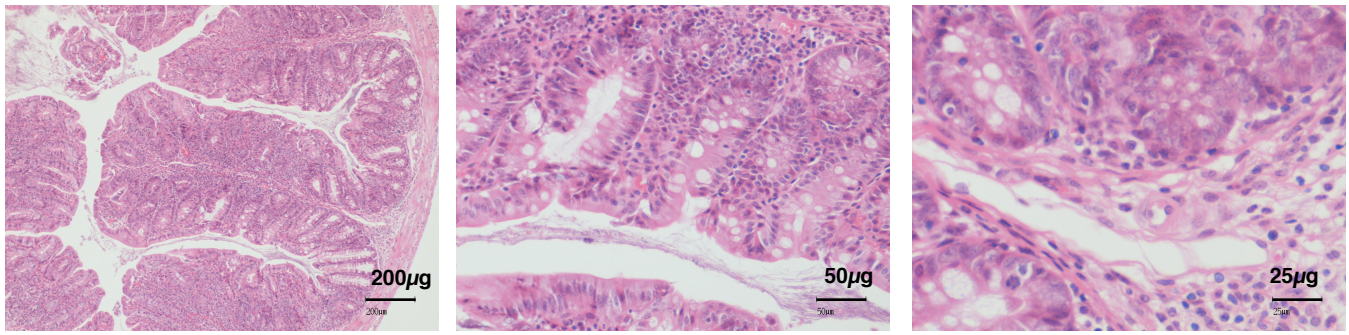
Histological analysis of haematoxylin and eosin (H&E) stained sections of the large bowel demonstrated marked thickening of the mucosal wall, disruption of the normal epithelial architecture characterised predominantly by a reduction in the number of goblet cells and villi, and infiltration of inflammatory cells within the lamina propria (figure 6.7). Immuno-histochemistry of similar sections demonstrated that the inflammatory cell infiltrate consisted primarily of $CD3^+$ lymphocytes interspersed in places by $CD19^+$ cells. There were several examples of direct contact between $CD3^+$ and $CD19^+$ cells in keeping with $CD4^+$ T helper cell interaction with $CD19^+$ B-cells (figure 6.7).

H&E stained liver sections demonstrated clusters of intra-parenchymal and peri-lobular infiltration of inflammatory cells that were shown predominantly to be $CD3^+$ lymphocytes based on further immuno-histology. Once again, there were examples of direct contact between $CD3^+$ and $CD19^+$ cells within these inflammatory cell collections (figure 6.7). Histological evidence of lymphocyte infiltrating inflammatory pathology was found in other sections of the gastrointestinal tract (including small bowel and stomach), as well as the lungs and skin (data not included). Endocrine organs including the adrenals, the thyroid gland, pancreas and gonads were also affected, consistent with early reports of f*oxp3-/- scurfy* mice (Godfrey, Wilkinson et al. 1991; Brunkow 2001), and depletion of $Foxp3^+$ Treg in adult mice (Kim, Rasmussen et al. 2007).

(A) Control - Lymphocytes + Isotype control mAb (H&E)



(B) Lymphocytes + 1mg YTH655 (H&E)



(C) Lymphocytes + 1mg YTH655 (CD3 brown, CD19 blue)

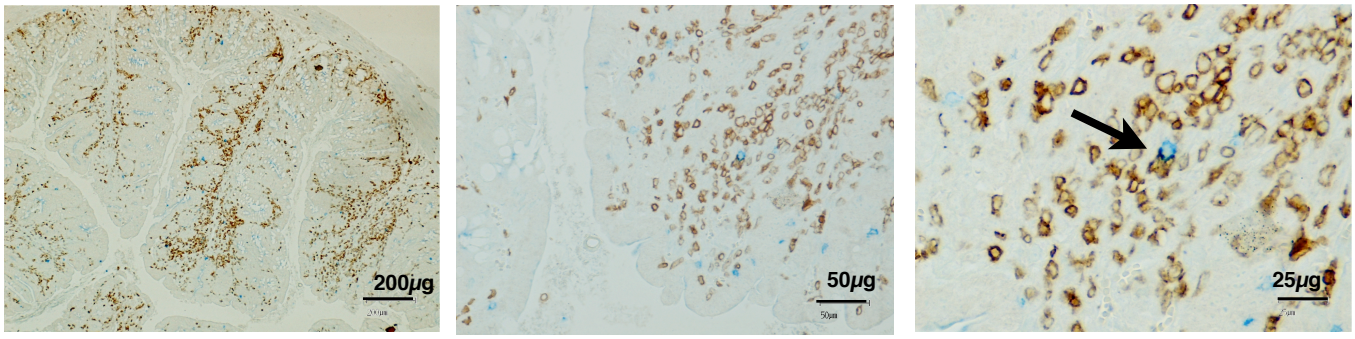
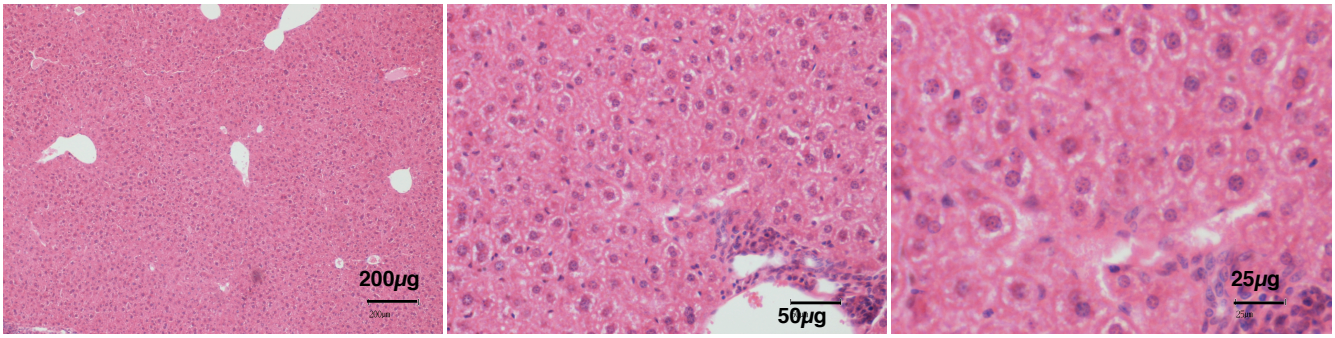


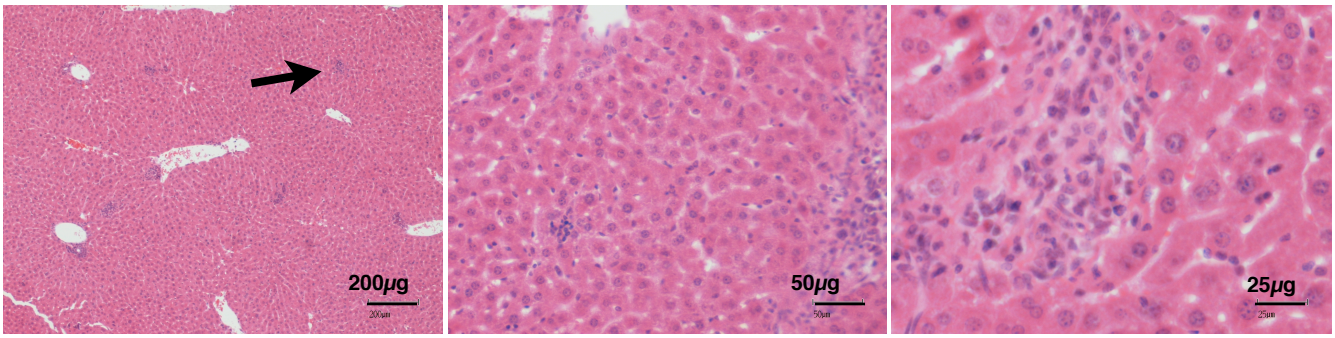
Figure 6.7 Foxp3⁺ Treg prevent lymphocyte infiltrating inflammatory pathology; (1) - Large bowel

Compared to the H&E prepared sections of large bowel from the control animals that received lymphocytes and control mAb (**A**), those mice that received lymphocytes + 1mg YTH655 (**B**) demonstrated thickening of the bowel wall, reduction in the number of goblet cells and infiltration of lymphocytes into the lamina propria. (**C**) Immuno-histology of the same samples revealed infiltration of $CD3^+$ cells (brown stain) into the lamina propria of YTH655 treated mice. The black arrow indicates a contaminating $CD19^+$ cell (in blue) interacting with a $CD3^+$ T-helper cell.

(A) Control - Lymphocytes + isotype control mAb (H&E)



(B) Lymphocytes + 1mg YTH655 (H&E)



(C) Lymphocytes + 1mg YTH655 (CD3 brown, CD19 blue)

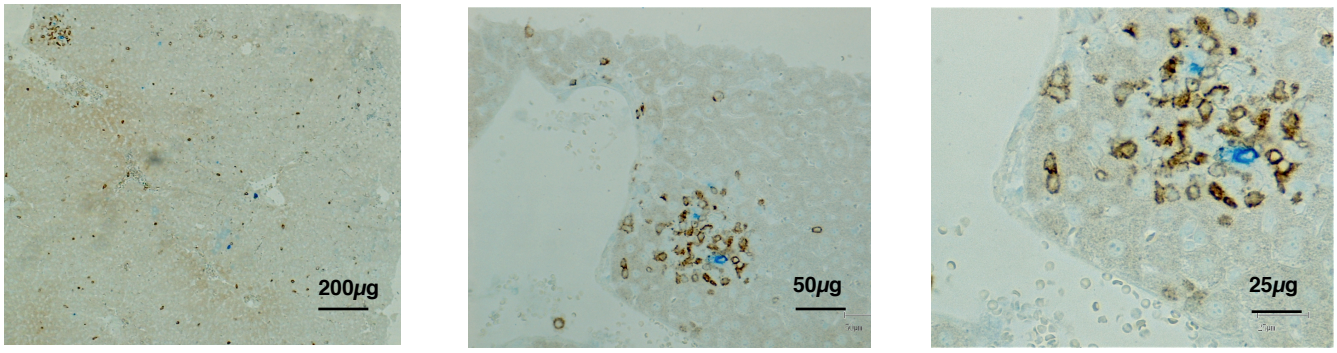


Figure 6.7 Foxp3⁺ Treg prevent lymphocyte infiltrating inflammatory pathology; (2) - Liver

Compared to the H&E prepared sections of liver from the control animals that received lymphocytes and control mAb (**A**), those mice that received lymphocytes + 1mg YTH655 (**B**) demonstrated peri-portal and intra-parenchymal infiltration of lymphocytes (black arrow). (**C**) Immuno-histology of the same samples revealed that these infiltrates were comprised of predominantly CD3⁺ cells (brown stain) and some contaminating CD19⁺ cells (blue stain).

6.3.2 Attenuating immune mediated enteropathy

Weight loss associated with widespread immune mediated enteropathy was one of the earliest clinical features in mice that received lymphocytes stripped of their Foxp3^{+} cells. The immunopathology affecting the bowel was so severe that, for humane reasons, mice were typically euthanised within 4 weeks of cell transfer. The development of colitis was therefore the major limiting factor to the survival of the experimental mice. In order to investigate the long term effects of Foxp3^{+} cells or the role of other regulatory cells in their absence, it was necessary to increase the survival rate by reducing the severity of colitis. The original experimental protocol was modified such that only (MoFlow sorted) CD4^{+} lymphocytes were adoptively transferred, as opposed to a mixed lymphocyte population. When 10 million B6.Foxp3^{hCD2} CD4^{+} cells were transferred in conjunction with 1mg anti-hCD2 mAb, B6.RAG-/- recipient mice rapidly lost weight, exhibited signs of clinical distress and were subsequently euthanised after 22 days (figure 6.8A - triangles). In comparison, adoptive transfer of 2 million CD4^{+} cells plus anti-hCD2 mAb resulted in a much milder phenotype (figure 6.8A - squares). There was no significant difference in weight change between this treatment group and the control group and the average clinical distress score remained below 4 (i.e within previously defined normal limits; see figure 6.2). After 70 days, the experiment was terminated. Splenic and mesenteric lymph node cell samples were prepared for flow cytometry.

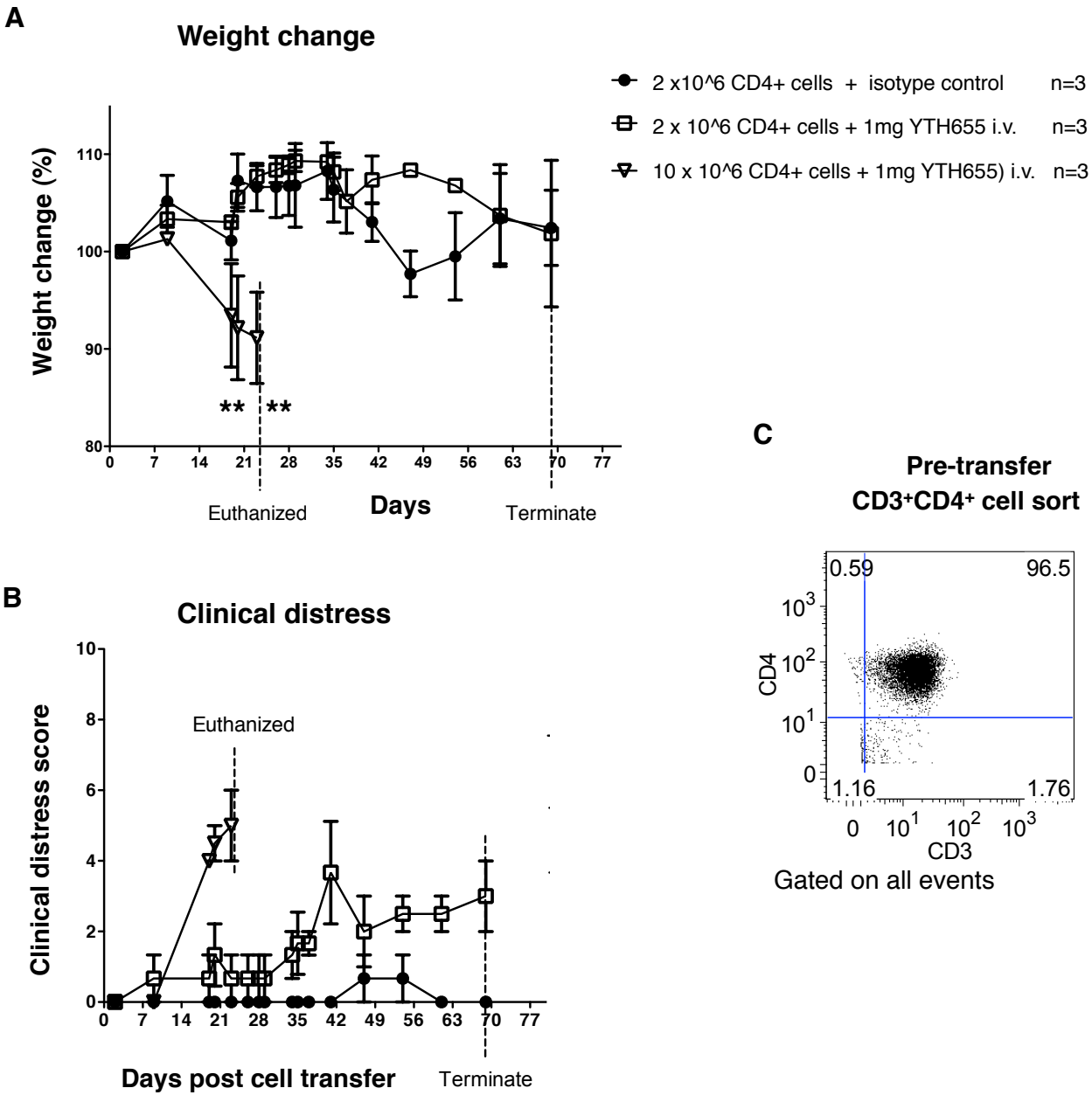


Figure 6.8 Adoptive transfer of purified CD4+ B6.Foxp3^{hCD2} cells + aCD2 mAb (1)

In order to minimise the effect of contaminating cells, CD3+CD4+ splenic B6.Foxp3^{hCD2} cells were isolated by MoFlow cytometry before transfer into B6.RAG$^{-/-}$ recipients. (**A,B**) 10x10^6 CD4+ cells + 1mg YTH655 resulted in significant weight loss after 18 days and distress from 7 days onwards (ANOVA test). Mice that received 2x10^6 CD4+ cells (with or without 1mg YTH655) remained healthy for 70 days, (**C**) Flow cytometry demonstrated >96% of the MoFlow sorted cells were CD3+CD4+ prior to adoptive transfer. Each point represents the mean of three age matched mice with SEM indicated by the error bars.

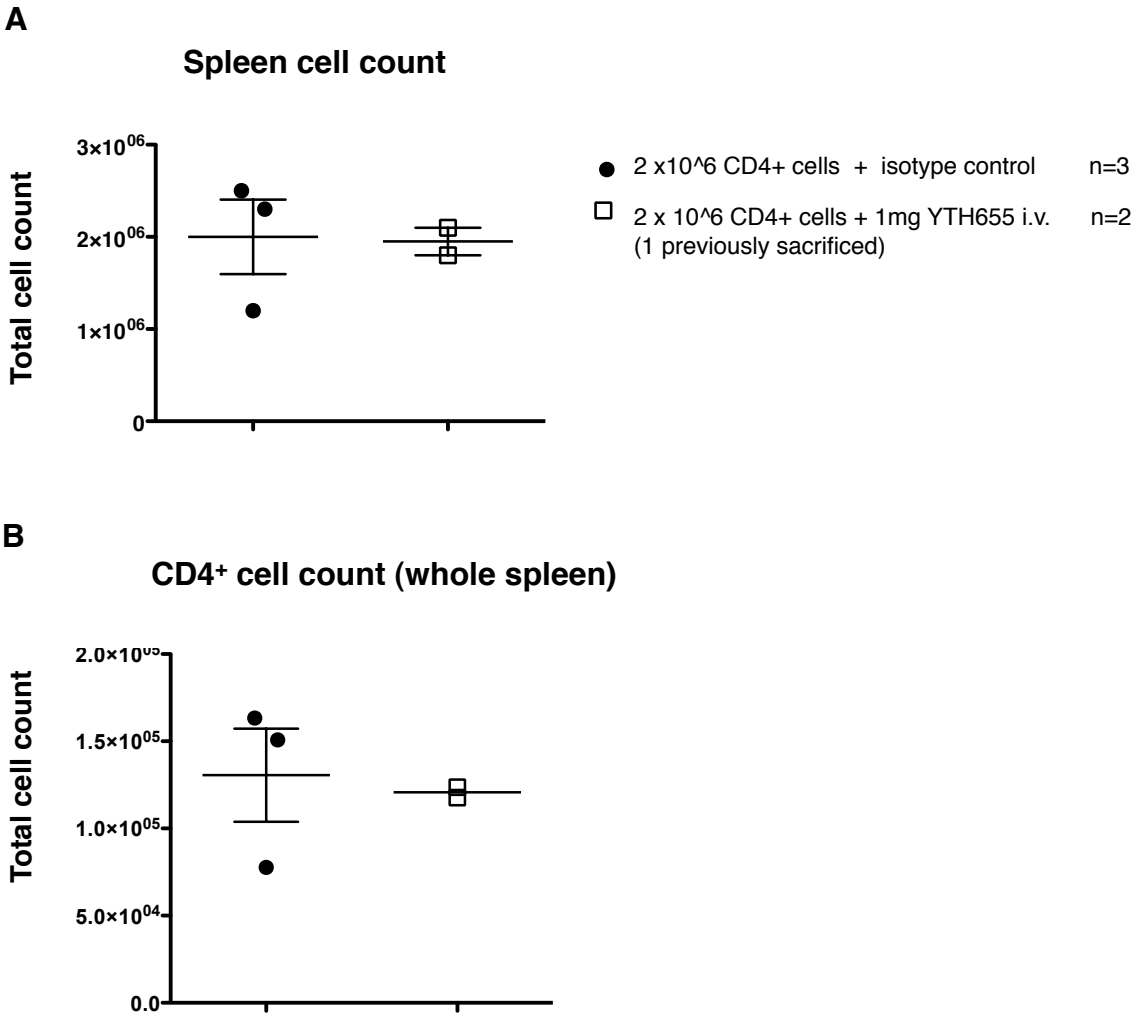


Figure 6.9 Adoptive transfer of purified CD4+ B6.Foxp3^{hCD2} cells + aCD2 mAb (2)

There was no significant difference 70 days after cell transfer in the total spleen white cell count (**A**) and CD4+ spleen cell count (**B**) between the group that received 2x10^6 CD4+ cells + 1mg YTH655 and the control group that received 2x10^6 CD4+ cells + 1mg isotype control mAb (t-test). Each point represents an individual mouse with SEM indicated by the error bars.

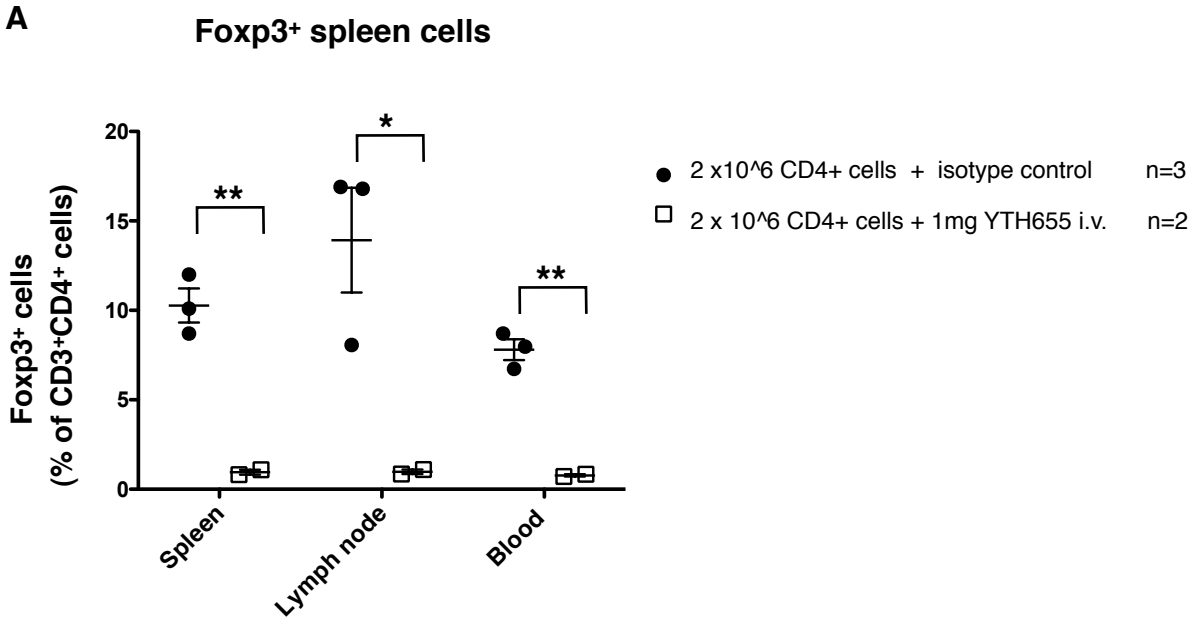


Figure 6.10 Adoptive transfer of purified CD4+ B6.Foxp3^{hCD2} cells + aCD2 mAb (3)

Between 10-18% of CD4+ cells were Foxp3+ in the control mice that received 2x10^6 B6.Foxp3^{hCD2} CD4+ cells + isotype control mAb. In comparison, fewer than 2% of CD4+ cells were Foxp3+ cells when cells were mixed with 1mg YTH655 prior to intravenous infusion. Each point represents an individual mouse with SEM indicated by the error bars, t-test used to assess statistical significance.

There was no statistically significant difference between the total number of spleen cells counted in the treatment group that received 2 million B6.Foxp3^{hCD2} CD4^{+} cells plus anti-hCD2 mAb compared to the control group that received 2 million CD4^{+} cells plus isotype control mAb (figure 6.9A; mean 1.9 x 10^6 vs 2.0 x 10^6 respectively). The total number of splenic CD4^{+} cells was also similar between the control and treatment groups (figure 6.9B; mean 1.2 x 10^5 vs 1.3 x 10^5). Flow cytometric analysis of spleen, mesenteric lymph node and blood cell samples revealed that Foxp3^{+} cells constituted approximately 10-15% of all CD4^{+} cells in the control mice (figure 6.10). In comparison, very few Foxp3^{+} cells were detectable in mice that received 2 million B6.Foxp3^{hCD2} CD4^{+} cells plus anti-hCD2 and constituted less than 1.5% of all CD4^{+} cells ($p<0.01$).

6.3.3 Anti-CD4 mAb prevents cell mediated immunopathology

B6.RAG$^{-/-}$ mice were infused with hCD2^{-}(Foxp3^{-}) spleen cells isolated by MoFlow cytometry from B6.Foxp3^{hCD2} mice. Based on previous findings that 10 million (hCD2^{-})CD4^{+} cells caused severe wasting disease with rapid onset while 2 million cells resulted in a much milder phenotype (figure 6.8), an initial compromise of 4 million CD4^{+}hCD2^{-} cells were injected in this first experiment. The mice were divided into two groups. All three mice in the control group that received hCD2^{-} cells alone began to lose weight after only 14 days (figure 6.11A - each triangle represents one mouse), increasingly showed clinical signs of distress and had to be euthanised within 4 weeks (figure 6.11B). In comparison, the treatment group that received 4 x 1mg anti-CD4 mAb over the first 10 days following hCD2^{-} cell transfer,

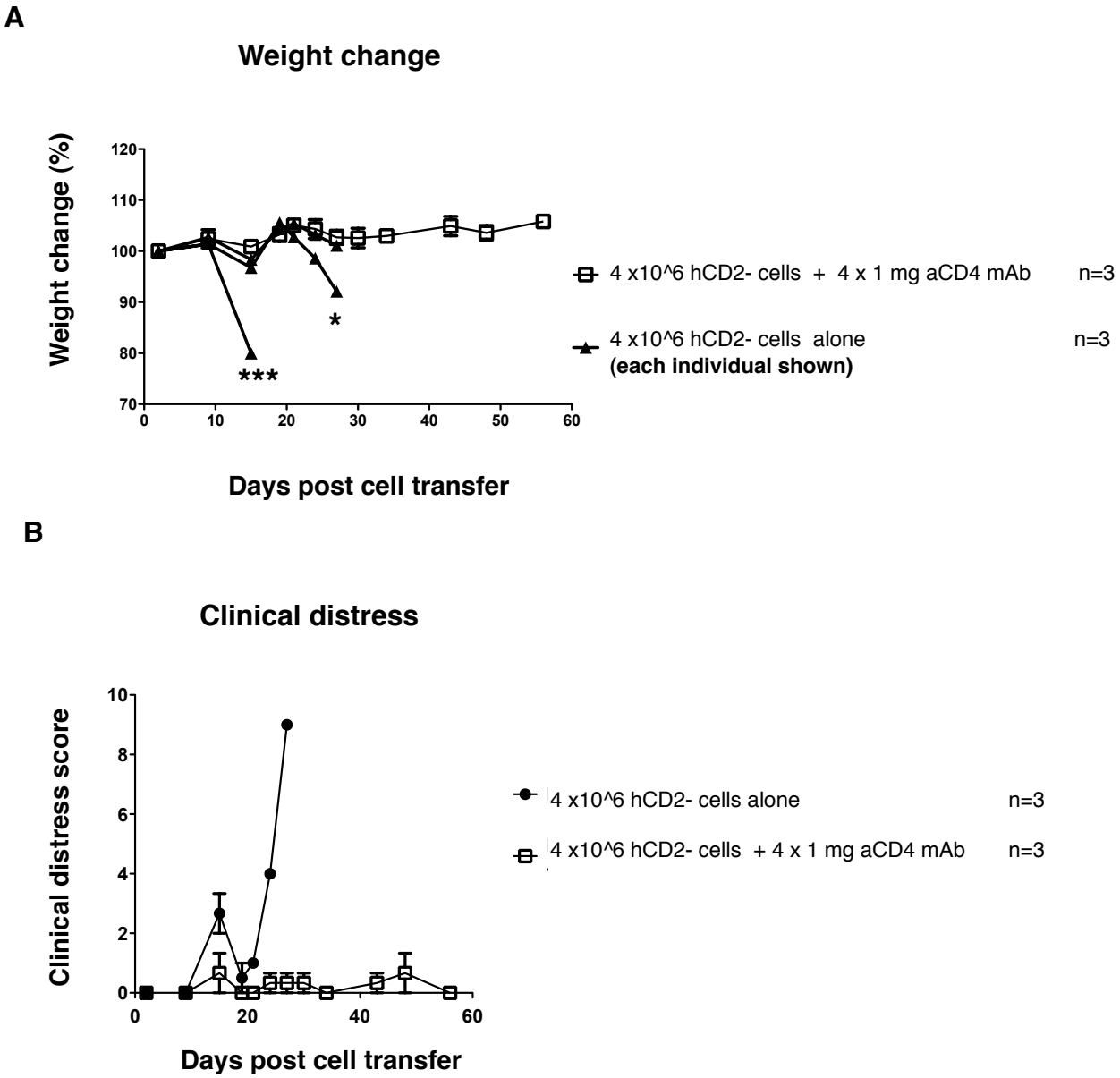


Figure 6.11 Anti-CD4 mAb prevents immunopathology by Foxp3- T-cells (1)

4 x 10^6 MoFlow sorted CD4^{+}hCD2^{-}(Foxp3^{+}) cells were intravenously infused into B6.RAG$^{-/-}$ mice. One group was treated with 4 x 1mg i.p. injections of non depleting anti-CD4 mAb over 10 days (black circles). In comparison to the untreated group (all of which were euthanised), those that received anti-CD4 mAb did not lose weight (**A**) and showed no clinical distress (**B**) for at least 50 days. Unless stated, each point represents the mean of three age matched mice with SEM indicated by the error bars. Statistical significance was determined by ANOVA test.

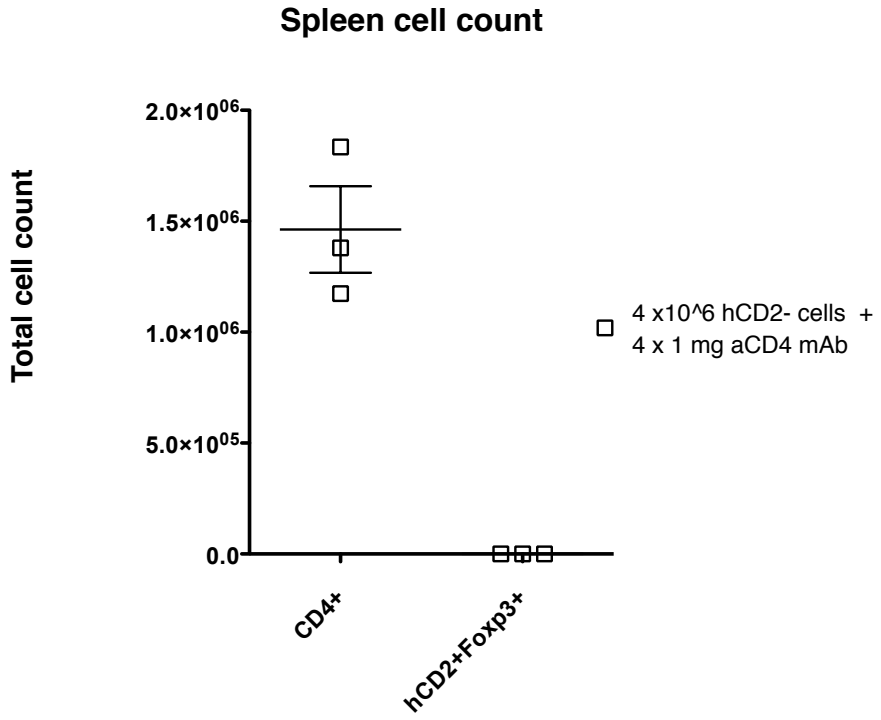


Figure 6.12 Anti-CD4 mAb prevents immunopathology by Foxp3- T-cells (2)

The three B6.RAG$^{-/-}$ mice that received 4 x10^6 hCD2- cells and 4 x 1 mg aCD4 mAb i.p. were killed after 60 days and the total spleen cell, CD4^{+} and Foxp3^{+} cell count was derived using flow cytometry. On average, 1.4 x 10^6 CD4^{+} cells were found in the spleen of treated mice. No hCD2^{+}Foxp3^{+} cells were detected. Each point represents an individual mouse with SEM indicated by the error bars. A positive control for conjugated anti-Foxp3 mAb was included to verify the detection method.

(B6.RAG$^{-/-}$ mice that received 4 x10^6 hCD2- cells alone were previously euthanised in accordance with Home Office guidelines and could not be used for comparison at this time point.)

remained healthy and gained weight for over 60 days. These mice were found to have on average 1.5 x 10^6 $CD4^+$ spleens cell, of which, none were $Foxp3^+$ cells (figure 6.12).

Taken together, the above findings demonstrated that B6.$RAG^{-/-}$ mice infused with 2 million B6.$Foxp3^{hCD2}$ $CD4^+$ cells plus anti-hCD2 mAb were able to survive for at least 10 weeks despite a lack of $Foxp3^+$ Treg. This protocol could therefore be used to examine the ability of co-receptor blockade (which itself prolongs survival) to induce long term graft tolerance in an animal lacking Foxp3 nTreg at the outset. If tolerance can be achieved, would this result in de novo production of $Foxp3^+$ Treg in the periphery, similar to that only previously found in TCR transgenic animals (Chapter 5)? If not, it would suggest that other, Foxp3 independent, mechanisms of regulation could compensate in the absence of $Foxp3^+$ Treg.

6.3.4 Transplant tolerance without $Foxp3^+$ Treg?

In order to eliminate as many of the natural $Foxp3^+$ nTreg as possible, $CD4^+$hCD2-($Foxp3^-$) spleen cells were isolated from B6.$Foxp3^{hCD2}$ mice by MoFlow and then incubated for thirty minutes with 1mg YTH655 anti-hCD2 mAb to target any $hCD2^+$ cells that might have escaped the initial sort. The cells were washed to remove any unbound YTH655 before being infused into B6.$Rag^{-/-}$ recipients. This last step was included to prevent excess YTH655 ablating any new $hCD2^+$($Foxp3^+$) that might be generated in response to tolerance induction. All B6.$Rag^{-/-}$ recipients received a MHC mismatched BALB/K skin graft on the day of cell transfer. The mice were divided into two groups, one received $hCD2^-$ cells (as above) and 3 x

1mg anti-CD4 blocking mAb and the other received only $hCD2^-$ cells. A third, control group of 2 million $CD4^+$ cells, plus BALB/K graft, plus 3 x 1mg anti-CD4 was included to ensure that the anti-CD4 blocking mAb was able to induce transplant tolerance across a MHC barrier in this system (figure 6.13).

All of the mice that received 2 million hCD2- cells alone rejected the BALB/k skin graft (MST 12 days, figure 6.13, triangles). In comparison, treatment with 3 x 1mg anti-CD4 mAb over 7 days prevented graft rejection in the groups that received 2 x 10^6 $CD4^+$ (figure 6.13, circles, p=0.0043) or $hCD2^-$ cells (squares, p=0.0103). The two groups treated with anti-CD4 mAb did not shows signs of clinical distress for 80 days and actually gained weight (figure 6.13B-C). Four out of five mice in the group that received hCD2- cells alone had to be euthanised between 30 to 42 days after cell transfer, in keeping with the milder disease phenotype previously found when 2 million $CD4^+$ cells were co-transferred with 1mg YTH655 (figure 6.8).

The experiment was terminated after 80 days. Splenic and draining lymphatic tissue was harvested from the remaining animals and prepared for analysis by flow cytometry. There was no significant difference in the number of $CD4^+$ spleen cells between mice that received 2 x10^6 $CD4^+$ cells and anti-CD4 mAb (figure 6.14A, circles) compared to mice that received 2 x 10^6 $hCD2^-$ cells plus anti-CD4 mAb (figure 6.14A, squares).

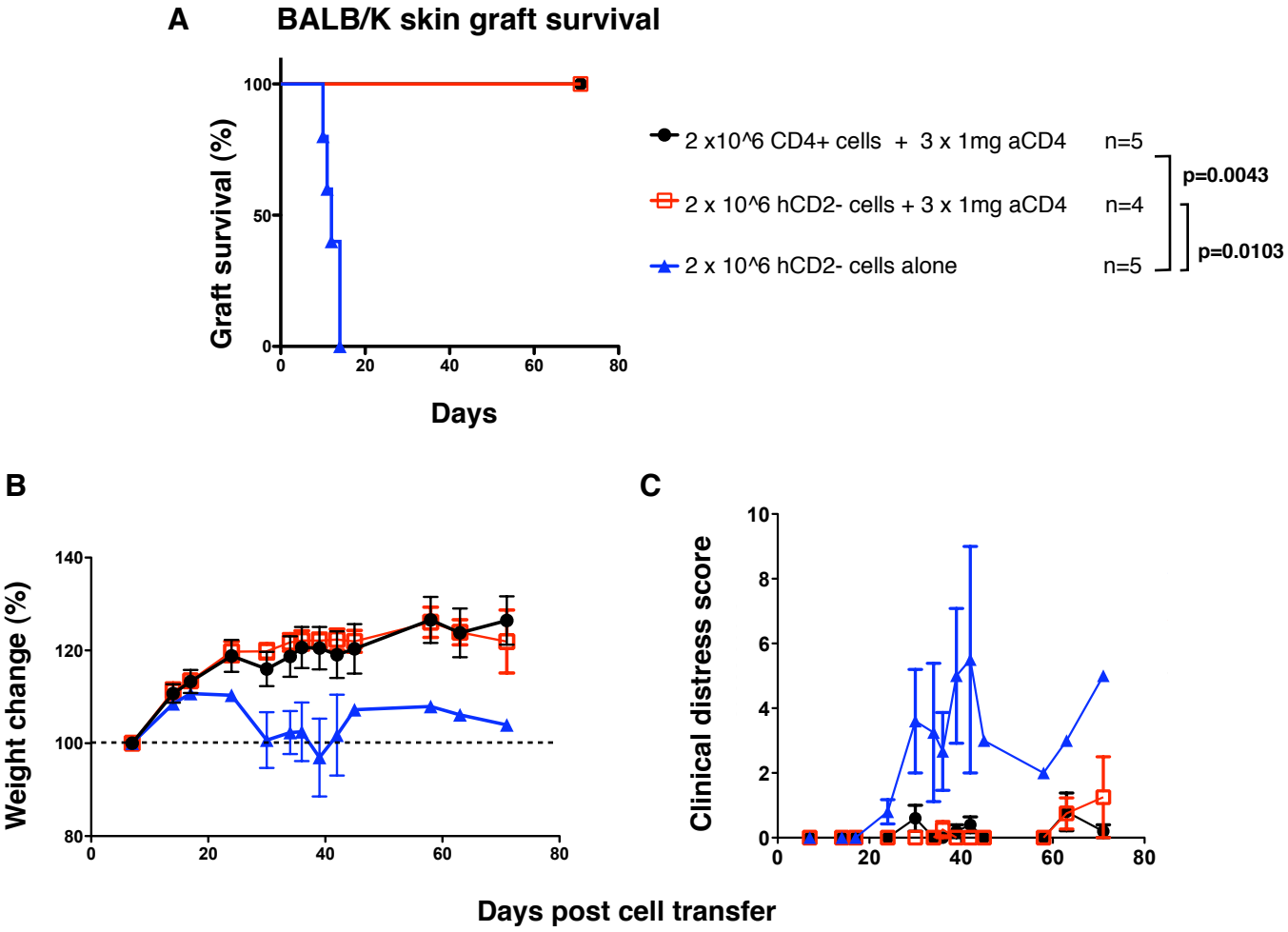


Figure 6.13 Anti-CD4 mAb prevents graft rejection by adoptively transferred Foxp3- T-cells (1)

2 x 10^6 MoFlow sorted CD4^{+}hCD2^{-}(Foxp3^{+}) cells from B6.Foxp3^{hCD2} mice were intravenously infused into B6.RAG$^{-/-}$ mice. A BALB/K skin graft was transplanted on the same day. (**A**) All the mice that received 2 x 10^6 MoFlow sorted CD4^{+}hCD2^{-}(Foxp3^{+}) cells alone rejected the skin grafts (triangles, MST 12 days). In comparison, treatment with 3 x 1mg aCD4 mAb over 7 days prevented graft rejection in the groups that received 2 x 10^6 CD4^{+} (circles, p=0.0043) or hCD2^{-} cells (squares, p=0.0103). The two groups treated with anti-CD4 mAb gained weight (**B**) and did not shows signs of clinical distress (**C**) for 80 days. 4 mice in the group that received hCD2- cells alone, were euthanised. Each point represents the mean of four or five age matched mice with SEM indicated by the error bars.

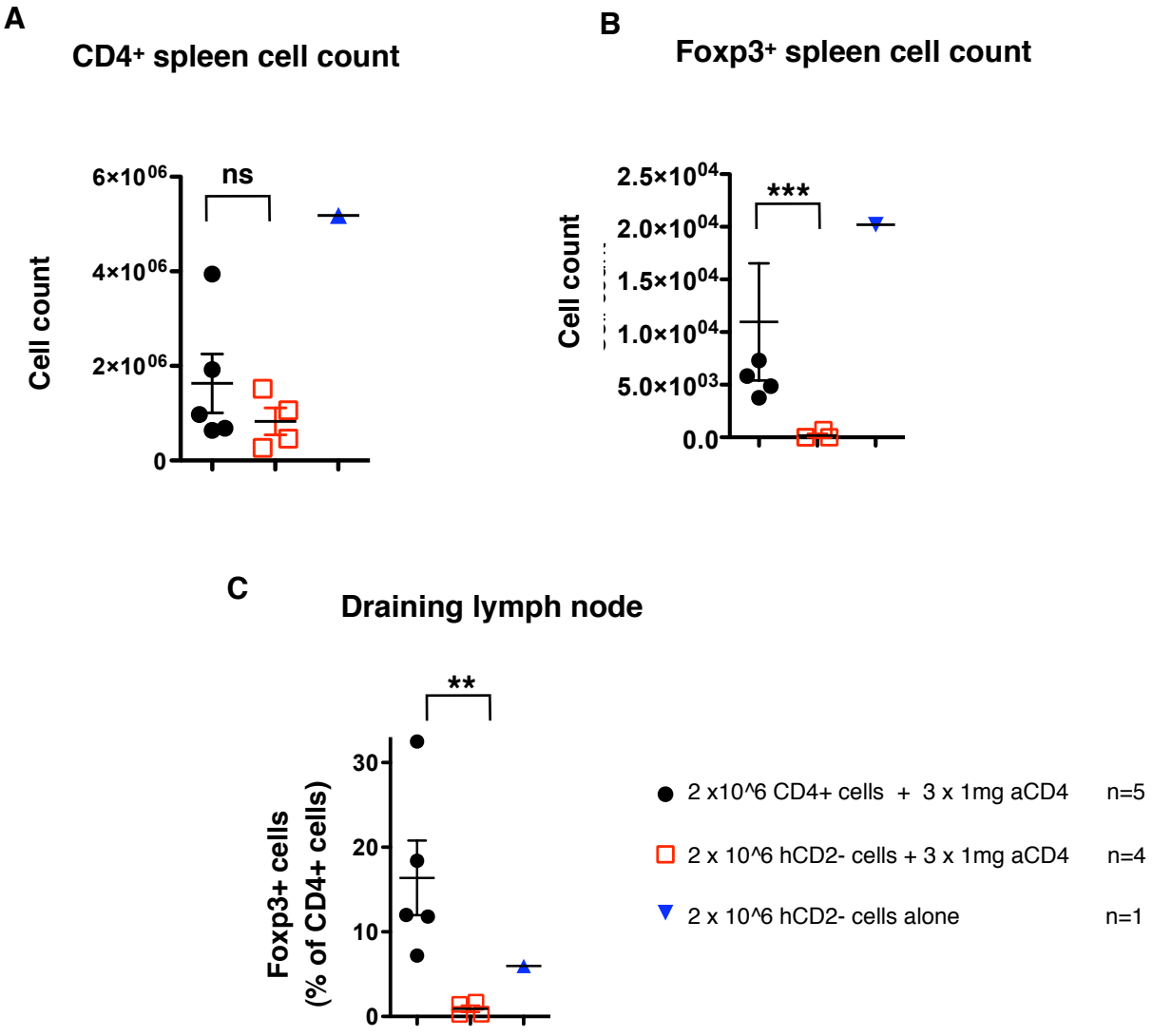


Figure 6.14 Anti-CD4 mAb prevents graft rejection by adoptively transferred Foxp3- T-cells (2)

After 80 days, the experiment was terminated so that splenic and draining lymphatic tissue could be analysed by flow cytometry. (**A**) On average, 1.6 x 10^6 CD4+ cells were found in the spleen of mice that received 2 x10^6 CD4+ cells + 3 x 1mg aCD4 (circles) compared to 0.8 x 10^6 in mice that received 2 x 10^6 hCD2- cells + 3 x 1mg aCD4 (squares). (**B**) 1.1 x 10^4 Foxp3+ cells were found in the spleen of mice receiving CD4+ cells and aCD4 mAb (circles). Very few, if any, Foxp3+ cells were found in mice that received hCD2- cells with aCD4 mAb (squares, $p<0.0001$). (**C**) Similarly, <1% of all CD4+ cells in the draining lymph node were Foxp3+ (squares), compared to >15% in mice that received CD4+ cells and aCD4 mAb (circles, $p=0.0014$). One mouse from the group that received hCD2- cells alone survived (triangle). This individual was found to have 5 x 10^6 CD4+ spleen cells, of which 2 x 10^4 were Foxp3+ cells. Each point represents an individual mouse with SEM indicated by the error bars. Statistical significance was determined by t-test.

Whereas 1.1 x 10^4 Foxp3$^+$ cells were found in the spleen of mice receiving CD4$^+$ cells and aCD4 mAb, very few, if any, Foxp3$^+$ cells were found in mice that received hCD2- cells plus aCD4 mAb (figure 6.14B, $p<0.0001$). Similarly, <1% of all CD3$^+$CD4$^+$ cells in the draining lymph node were Foxp3$^+$ (squares), compared to >15% in mice that received CD4$^+$ cells and aCD4 mAb (figure 6.14C, $p=0.0014$).

Acceptance of MHC mismatched grafts as a result of anti-CD4 mAb therapy was not accompanied by the generation of a new population of Foxp3$^+$ cells in this model (figure 6.14).

One mouse from the group that received only hCD2- cells (without anti-CD4 mAb) survived the full 80 days (figure 6.13, triangle). This individual was found to have 5 x 10^6 CD4$^+$ spleen cells, of which 2 x 10^4 were Foxp3$^+$ cells and 6% of its CD3$^+$CD4$^+$ cells in the draining lymph node were Foxp3$^+$ cells (figure 6.14).

6.4 Discussion

The aim of these experiments was to develop a protocol to examine whether new Foxp3$^+$ iTreg (previously found in TCR transgenic models - Chapter 5) are also induced in response to transplant tolerance in a conventional polyclonal immune system. An important

criterion is to be able to distinguish the new Foxp3 iTreg from the nTreg. One way is to ensure that there are no natural Treg at the start of the experiment, so that any detectable $Foxp3^{+}$ cells would be produced de novo in response to tolerance induction. It is possible to deplete >95% of $Foxp3^{+}$ cells from B6.$Foxp3^{hCD2}$ mice by adoptively transferring them into lymphocyte deficient mice with 1mg anti-hCD2 mAb (figure 6.1) and this can be further improved by initially MoFlow sorting only $hCD2^{negative}$ lymphocytes. The main limitation of this approach is that the mice develop immune dysregulation characterised clinically by wasting enteropathy, skin lesions and distress, and histologically by lymphocyte infiltrating inflammation affecting epithelia, endocrine tissue and latterly solid organs (figures 6.3 to 6.8).

Serendipitously, it was found that transfer of 2 million $CD4^{+}$ cells resulted in a milder phenotype with mice surviving long enough to be useful in transplant tolerance experiments (figure 6.8). Under these conditions, treatment with anti-CD4 prevented MHC mismatched graft rejection without generating new $Foxp3^{+}$ iTreg. A similar result has been reported following transfer of lymphocytes from $foxp3^{-/-}$ *scurfy* mice into B6.RAG-/- mice with DBA/2 skin grafts in conjunction with anti-CD4 mAb (Kim, O'Connor M et al. 2011). The mice failed to reject the graft and no new $Foxp3^{+}$ Treg were demonstrated. In contrast, delayed rejection was seen when rapamycin was given instead of anti-CD4 mAb and this did result in the generation of $Foxp3^{+}$ (i)Treg.

Collectively, the two results may appear to suggest that tolerance induction by anti-CD4 therapy does not necessarily require the conversion of naive cells into iTreg, or even the

presence of nTreg. But that is because the immune system, in both cases, was not challenged with re-exposure to the original antigen under inflammatory conditions. In other words, failure to reject a transplant is not synonymous with active transplant tolerance. Before investigating whether other regulatory cells have compensated for the absence of $Foxp3^+$ Treg in this model, it is necessary to exclude global immune compromise as a result of anti-CD4 mAb treatment. In these cell transfer experiments, anti-CD4 mAb during the first seven days may have resulted in general immune suppression by limiting the expansion of the infused $CD4^+$ cells or perhaps forcing the majority into a state of anergy. Alternatively, it may have preferentially incapacitated only those clones with TCR specific for graft antigens (discussed below). None of these outcomes necessarily require extrinsic regulation e.g. regulatory cells and may instead rely on the collective effect of anergic cells, particularly since they can have an immuno-suppressive role (Lechler, Chai et al. 2001; Chen, Cobbold et al. 2004). In order to distinguish between these two possibilities, recipients of $CD4^+$ cells and anti-CD4 mAb will be challenged with a MHC mismatched graft from BALB/C ($H\text{-}2^d$) mice. This donor was chosen because it shares very few histocompatibility antigens with the original Babl/K ($H\text{-}2^K$) and one would expect little cross over tolerance in the form of linked suppression (Davies, Leong et al. 1996; Wise, Bemelman et al. 1998; Honey, Cobbold et al. 1999). A failure to reject the graft would therefore imply that anti-CD4 mAb therapy resulted in global immune incompetence in this model. Of note, Kim et al. did not challenge their model with a third party graft once anti-CD4 mAb had cleared (Kim, O'Connor M et al. 2011).

If the mice reject the BALB/C graft, the next step would be to see if they could prevent an infusion of naive $CD4^+$ cells from rejecting a challenge graft from the original BALB/K

donor. This would show that there are graft specific suppressive elements within the tolerised $CD4^+$ population that are Foxp3 negative and can nonetheless maintain graft tolerance. It would be interesting to pursue the characteristics of those $Foxp3^{neg}$ regulators; are they Tr1 cells, or perhaps the newly described IL-35 dependent $Foxp3^{neg}$ iT(R)35 cells that have been shown to be suppressive in almost every model of immune regulation except transplant tolerance (Collison et al., 2010)?

An alternative method for identifying the generation of induced iTreg against the backdrop of nTreg in conventional immune systems using a combination of colour coded reporter cells has recently been described (Fan, Spencer et al. 2009). $B6.RAG^{-/-}$ mice were reconstituted with a 1:9 ratio of nTreg to naive cells. The nTreg were sorted based on their expression of GFP prior to transfer. Whereas, the naive population were $DsRed^+CD4^+GFP^{neg}$ cells that are constitutively red under fluorescent microscopy until they express Foxp3 (and GFP) when they turn yellow. This allowed the authors to follow nTreg (green), Foxp3-effectors (red) and recently converted iTreg (yellow) in vivo in response to islet transplant tolerance induced by anti-CD40L and rapamycin. iTreg were found in both rejecting and tolerated grafts, in fact there were more in the grafts that ultimately rejected. However, the ratio of both nTreg:Teff and iTreg:Teff was greater in tolerated grafts. The descriptive data is compelling, particularly since it includes in vivo microscopy through a tiny endoscopic port and even live flow cytometry of cells passing through aural blood vessels. However, the operational capability of iTreg is understandably not addressed. For example, in vitro generated iTreg are notorious for losing Foxp3 expression (Kretschmer, Apostolou et al. 2005; Zhou, Bailey-Bucktrout et al. 2009) so questioning the stability of these iTreg generated in

vivo. In addition, the experimental readout of iTreg in their model is the presence of yellow cells (i.e. red cells that co-express Foxp3-GFP). This means that a series of naive cells only need express Foxp3 transiently, and at different times, to generate sufficient yellow cells to give the illusion of a constant presence of iTreg when viewed infrequently. Even if the expression of Foxp3 is found to be stable in these iTreg, what is the evidence that they are performing an active role in regulation, as opposed to being incidental bystanders?

Fan et al's approach of using two reporter systems to mix pure $Foxp3^{+}$ nTreg with naive cells is a good one. A similar transplant experiment could be designed so that MoFlow sorted GFP^{+} nTreg are mixed with naive $hCD2^{neg}$ cells from B6.$Foxp3^{hCD2}$ mice. In this case, any generated iTreg would be $hCD2^{+}$ and could therefore be sorted and adoptively transferred into secondary transplant assays (given sufficient numbers), so providing one way of verifying their ability to suppress. Although this kind of model might demonstrate that the action of iTreg can be independent of nTreg, it would not exclude a role for nTreg in the development of iTreg. Such necessary instruction might also explain why no new iTreg were found once all nTreg were depleted on adoptive transfer (figures 6.13 and 6.14)

Finally, it would be interesting to explore how differences in immuno-suppressive conditions might determine the induction of active regulation versus anergy. In order to differentiate them from active regulatory cells, anergic cells are typically characterised in vitro by their decreased proliferation, reduced production of IL-2 and potential reversal of their hypo-responsive state by IL-2 (Lechler, Chai et al. 2001; Schwartz 2003). A number of

different ways of inducing anergy have been described including presentation of peptide partial agonists (Sloan-Lancaster, Evavold et al. 1993), antigen recognition in the absence of proliferative cytokines such as IL-2 (DeSilva, Urdahl et al. 1991), or in the presence of anti-inflammatory cytokines such as IL-10 (Groux, Bigler et al. 1996) and blockade of co-stimulatory signals (Judge, Wu et al. 1999). The inability of anti-CD4 mAb treated hCD2$^-$ cells to reject a MHC mismatched graft (figure 6.13) may therefore reflect clonal anergy as result of graft antigen recognition in the absence of co-receptor signals. Donor specific hypo-responsiveness is known to occur in vivo and is associated with prolonged graft survival in transplant patients (Mason, Robinson et al. 1996; Baker, Hernandez-Fuentes et al. 2001). In which case, one would still expect the anti-CD4 treated mice to reject the BALB/C (third party) graft as outlined above (Ng, Baker et al. 2001) but accept a second graft from the original BALB/K donor.

In comparison, it is interesting that the only mouse (out of five) that survived transfer of 2 million hCD2- cells alone was found to have 2 x 10^4 splenic Foxp3$^+$ cells (control mice had an average of 1.1 x 10^4 Foxp3$^+$ cells) and 6% of its CD4$^+$ cells in the draining lymph node were Foxp3$^+$ cells. Accepting that this is just one mouse, and that this phenomenon was not seen in other adoptive transfer assays of hCD2- cells, it is conceivable that when foreign tissue is encountered without co-receptor blockade there is a greater drive to develop Treg in response to an escalating inflammatory response; even if regulation ultimately fails. Others have also shown that Foxp3$^+$ Treg are found in *rejecting* grafts, often in greater numbers than in tolerated tissue (Cobbold, Castejon et al. 2004; Fan, Spencer et al. 2010; Batsford, Duermueller et al. 2011).

It is possible that circumstances favouring the development of anergy, for example immune suppression when adoptively transferred cells are expanding, are not the same as those that drive the generation of Foxp3^{+} iTreg. Perhaps in response to increasing inflammation, the likelihood of anergy diminishes and Foxp3^{+} Treg are required to compensate. If this was the case, examination of mice that received hCD2- cells alone at an earlier stage, before they were euthanised, should enable me to detect more Foxp3^{+} iTreg in comparison to those treated with anti-CD4 mAb from the start. Similarly, would an inflammatory challenge of repeat grafting with an infusion of hCD2- cells be sufficient to induce iTreg in tolerant mice previously treated with anti-CD4 mAb?

CHAPTER 7: General Discussion

The discovery that natural mechanisms of immune regulation exist in the periphery and can be harnessed to generate self sustaining tolerance to foreign antigen offered the opportunity of significantly improving the management of autoimmune disease and organ transplantation (Cobbold and Waldmann 1984; Cobbold and Waldmann 1998). Many groups have tried to exploit peripheral regulation by pursuing Treg cell based therapies, particularly following the identification of $CD25^+$ regulatory T-cells that can suppress immunopathology or confer transplant tolerance on adoptive transfer (Kuniyasu, Takahashi et al. 2000). Given that CD25 has proved an imperfect marker of Treg (Graca, Thompson et al. 2002) most groups have explored a collection of surface proteins that best define the most effective Treg population (Jiang, Tsang et al. 2006; Golshayan, Jiang et al. 2007; Peters, Preijers et al. 2008; Chen, Delgado et al. 2009; Trzonkowski, Bieniaszewska et al. 2009). A number of issues including the practicalities of treatment standardization and how in vitro generated Treg might perform their role have not yet been resolved. Nonetheless there is an understandable impatience to develop cell based therapies, given the burden of disease they aim to improve. An alternative approach is to place a greater emphasis on identifying those specific regulatory mechanisms that are most crucial to induced tolerance in vivo with the aim of then developing targeted therapies. The first step is to establish which regulatory cells are critical.

$Foxp3^+CD4^+$ cells, in particular, have already been shown to be essential to the maintenance of self tolerance (Kim, Rasmussen et al. 2007; Lahl, Loddenkemper et al. 2007). This thesis has established that they are also crucial to non-self tolerance induced by co-receptor and co-stimulatory blockade. Ablative anti-hCD2 mAb has revealed a non redundant role for $Foxp3^+$ Treg in the generation and maintenance of non-self tolerance induced by short

term co-receptor and co-stimulatory blockade in B6.Fxop3^{hCD2} reporter strains (Chapter 4). Indeed, constant vigilance of Foxp3^{+} Treg seems to be required, since their ablation during steady state conditions resulted in the rapid rejection of previously well accepted and healthy skin grafts.

Having established that Foxp3^{+} Treg are crucial to induced tolerance, it was important to explore how co-receptor/co-stimulatory blockade might potentiate Foxp3 dependent regulation and prevent graft rejection. One possible mechanism was the conversion of naive CD4^{+} cells into Foxp3^{+} iTreg found in the periphery of TCR transgenic mice. Such iTreg were shown to actively prevent tissue resident T-cells from causing rejection (Chapter 5). Finally, Foxp3^{+} iTreg not only prevented an infusion of naive cells from rejecting a graft, but enabled their conversion into new Foxp3^{+} cells. This suggested a way in which iTreg could self-sustain their numbers and also provided one mechanism by which infectious tolerance may operate in vivo.

Although peripherally induced Foxp3^{+} iTreg were found to be active regulators in the TCR transgenic, Marilyn.Foxp3^{hCD2} mice, a similar role was not shown in conventional mice (Chapter 6). It is not known whether a functional distinction between nTreg and iTreg actually exists in natural, conventional immune systems. Originally, nTreg were thought to develop through TCR recognition of predominantly self-antigen presented centrally in the thymus and so it was unclear how induction of peripheral tolerance could instruct their development to incorporate new antigens. In comparison, the peripheral conversion of naive CD4^{+} cells into

iTreg in response to tolerogenic conditions offered a much more plausible explanation. This concern that nTreg with a TCR repertoire skewed to self antigen might not be able to contribute to transplant tolerance has partially been addressed by the discovery that peripheral DCs can migrate to the thymus and influence nTreg selection (Watanabe, Wang et al. 2005; Proietto, van Dommelen et al. 2008). It is possible that co-receptor blockade may also operate through this pathway. In either case, once antigen specific Treg have been generated by co-receptor blockade, how might they prevent graft rejection?

Admittedly many of the regulatory mechanisms currently assigned to Treg have been observed in vitro. However it is still worthwhile considering how they might integrate to achieve Foxp3 dependent tissue tolerance on multiple levels, particularly if such mechanisms generate therapeutic opportunities. Beginning with direct cell to cell contact; human $CD25^{+}CD4^{+}$ cells stimulated in vitro with anti-CD3 beads in the presence of IL-2 were found to exhibit perforin and granzyme A dependent cytotoxicity on co-culture with effector T-cells, monocytes and B-cells (Grossman, Verbsky et al. 2004; Zhao, Thornton et al. 2006). Others have also demonstrated granzyme B dependent cytotoxicity (Gondek, Lu et al. 2005). The finding that a barrier prevented the suppression of $CD25^{-}CD4^{+}$ by $CD25^{+}$ cells also suggested that Treg operate through direct cell contact with effector cells. Although, the membrane was semi-permeable to allow diffusion of cytokines, it is still possible that suppression is dependent on secreted factors that require a narrow distance for their optimal effect.

It is clear that dendritic cells play an important intermediary role. Culturing naive $CD4^+$ cells with cognate antigen, dendritic cells and TGF-β has become the standard method for generating iTreg in vitro (Chapter 5) (Chen, Jin et al. 2003; Cobbold, Castejon et al. 2004; Nishimura, Sakihama et al. 2004) and tolerogenic dendritic cells alone can attenuate immunopathology when transferred into murine models of autoimmune disease (Menges, Rossner et al. 2002; Verginis, Li et al. 2005; Hadeiba, Sato et al. 2008). Antigen pulsed APCs that have been chemically fixed with ethylenecarbodimide can also prevent and treat autoimmune EAE (Turley and Miller 2007). Interestingly, the finding that MHC deficient fixed APCs could also attenuate delayed-type hypersensitivity responses and that the majority were apoptotic by 8 hours suggests these fixed Ag-APCs may also be acting as a tolerogenic peptide delivery system, perhaps regardless of their co-stimulatory signal profile (Farquhar, Paterson et al. 2010). Based on this collective evidence, it is likely that transplanting male skin onto female Marilyn.$Foxp3^{hCD2}$ recipients in conjunction with anti-CD4 blocking mAb generates $Foxp3^+$ iTreg through a similar process of antigen presentation in the absence of co-stimulatory signals (Cobbold, Castejon et al. 2004). Once the graft is healed and inflammation settles, the graft tissue could effectively become a natural antigen 'pump' for presentation by dendritic cells, perhaps even in the same way that host tissue antigen is sampled and displayed by quiescent dendritic cells (Wise, Bemelman et al. 1998).

Understanding how Treg and APCs interact to promote tolerance within a tissue has already helped develop an important therapeutic target; Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4). Whereas most effector cells only express CTLA-4 on activation, the majority of $Foxp3^+$ Treg constitutively express it and indeed, it is upregulated on forced ectopic

expression of foxp3 in naive cells (Hori, Nomura et al. 2003). The importance of CTLA-4 dependent regulation is demonstrated by the severe lymphoproliferative disease found in deficient mice (Waterhouse, Penninger et al. 1995). Moreover, anti-CTLA-4 mAb therapy results in significant increase in survival rates of patients with advanced metastatic melanoma (Hodi, O'Day et al. 2010). It is impressive that in both settings, negating a single regulatory signaling pathway can have such a profound effect of T-cell activation. It is still not know how CTLA-4 signaling affects the interplay between Treg, APCs, naive and effector T-cells. Possible mechanisms include down regulating co-stimulatory molecules such as CD80 and CD86 (Paust, Lu et al. 2004; Schmidt, Wang et al. 2009), and upregulating LFA-1 to enhance the binding of APCs to Treg (Schneider, Valk et al. 2005; Onishi, Fehervari et al. 2008).

The early finding that transplant tolerance could operate through indirect antigen recognition implied an important role for host APCs (Wise, Bemelman et al. 1998) potentially through a tolerogenic pathway dependent on cross-presentation (reviewed in (Lin, Zhan et al. 2008). DCs have been found to present self antigen taken up from apoptotic bodies (Huang, Platt et al. 2000). So long as these dendritic cells present the antigen in the absence of activatory signals, it is unlikely that they will favour the activation and proliferation of $CD8^+$ Tc cells. Given that DCs can also take up and cross present pathogenic antigen by the same pathway, a major concern is how systemic immunity against pathogens will be conserved in spite of tolerance induction (Albert, Sauter et al. 1998; Inaba, Turley et al. 1998; Yrlid and Wick 2000). For example, if a tolerated graft was infected by a virus, would tolerance be extended to viral antigens (Bedoui, Whitney et al. 2009)? What if there is significant cross over between graft and pathogenic antigens? The ultimate impact of Treg inadvertently

directed against pathogenic antigen is likely to be dependent on their relative numbers, the residual potency of systemic immunity (both adaptive and innate) and the local environment of the tolerated tissue. Indeed, it may well be confined to the tolerated graft tissue alone.

The local tissue is increasingly emerging as an important micro-environment and immune cells appear to be sensitive to changes in the availability of metabolic resource, a sensitivity that is exploited by regulatory T-cells. The interaction between Treg and DCs activates enzymes to increase the consumption of essential amino acids. The resulting fall in extracellular amino acids limits the activation and proliferation of effector cells and promotes the conversion of naive $CD4^+$ cells into new $Foxp3^+$ Treg through the inhibition of mTOR signaling pathways (Cobbold, Adams et al. 2009). Treg may also inhibit the proliferation of near by T-cells by raising the extracellular redox potential through a reduction in the secretion of glutathione by APCs (Yan, Garg et al. 2009). The conversion of inflammatory adenosine into anti-inflammatory AMP may be another way in which inflammation can be restricted by Treg (Dr. F. Regateiro, DPhil Thesis 2009) (Mandapathil, Hilldorfer et al. ; Kobie, Shah et al. 2006).

It is conceivable that immune regulation exploits conserved pathways that normally limit cellular processes which threaten to exhaust the tissue. Indeed, Medzhitov has argued that tissue stress, in general, determines the type and degree of immune response (Medzhitov 2008). Regardless of the original stressor (e.g. infection, ischaemia, trauma) activatory and regulatory immune elements must work in concert to help return the particular

tissue to its original structure and function. Foxp3^{+} Treg may be the most effective regulators because they restore homeostasis by promoting and harnessing regulatory pathways that are most relevant to a given tissue. For example, TGF-beta (Cobbold, Castejon et al. 2004; Daley, Ma et al. 2007) and IL-10 have all been shown to be important to Treg function (Asseman, Mauze et al. 1999), but neither are specific to Foxp3^{+} Treg (Chapter 5). Indeed, IL-10 production was found to be the crucial factor in curing cell mediated colitis regardless of whether Foxp3^{+} Treg were producing it (Vieira, Christensen et al. 2004; Uhlig, Coombes et al. 2006; Anderson, Oukka et al. 2007). It turns out this is one pathway that pathogens can exploit to undermine host responses and establish a chronic infection (Belkaid, Piccirillo et al. 2002).

Skin transplantation causes a significant stress to the tissue. The resulting inflammatory response is fueled by immune competent lymphocytes that are activated through recognition of graft antigen and results in such severe tissue damage that the graft fails. The fact that this process nonetheless occurs in the absence of cytotoxic Tc-cells and B-cells in RAG$^{-/-}$ TCR transgenic mice (containing only CD4^{+} cells) again emphasises that a global loss of tissue homeostasis drives rejection. In contrast, when this initial immune response is inhibited by co-receptor and co-stimulatory blockade during healing, antigen recognition by potential effector cells no longer sustains the inflammatory process. The concerted immune response therefore shifts to ensure healing of a temporarily traumatized tissue and resolution of normal homeostasis.

Clearly the tissue remains foreign and cells capable of rejection have been shown to remain in tolerant hosts (Chapter 4), however it is possible that during resolution, Foxp3$^+$ Treg establish a number of regulatory check points both outside and within that tissue. This may include an expansion of resident Treg that remain in situ to suppress potential effectors both directly and indirectly via APCs, local secretion of anti-inflammatory cytokines that inhibit lymphocyte proliferation and endothelial changes which limit the migration of effector cells or incapacitate any that pass through the walls. Treg may also act beyond the local tissue by migrating to draining lymph nodes and inhibiting the activation of effector cells (Zhang, Schroppel et al. 2009). Such mechanisms of tissue dependent regulation naturally exist and at extremes manifest as sites of 'immune privilege,' such as the eye, where the barriers to immune cell activation permit foreign grafts to be accepted without any additional immunosuppression. Furthermore, local tolerance of alloantigen injected into the anterior chamber of the eye can spread beyond the local environment to influence systemic tolerance, so called anterior chamber associated immune deviation (ACAID) (Streilein 2003). Micro-environments of immune regulation are not restricted to 'normal' healthy tissue as demonstrated by their discovery in many tumour models and these may prove a significant obstruction to cancer therapy (Colombo and Piconese 2007).

Antibody mediated co-receptor and co-stimulatory blockade inhibits the normal rejection response long enough for tissue homeostasis to return. I would argue that short term immune blockade therefore exploits the role that Foxp3$^+$ Treg naturally perform in maintaining tissue homeostasis so that graft survival is protected by limiting the exposure of rejection competent cells to the foreign antigen.

Translating these encouraging in vivo findings of co-receptor blockade to clinical benefit has been hampered by a number of practical limitations. For example, current legislation is against trialing multiple new agents simultaneously, i.e. anti-CD4, CD8 and CD40L mAb blockade. One way to overcome this is to use anti-CD3 mAb instead to block both CD4 and CD8 T-cells and this has already revealed encouraging results in renal and liver transplants, autoimmune arthritides and particularly in Type 1 Diabetes (T1D) (Cosimi, Jenkins et al. 1990; Grino, Castelao et al. 1992; Utset, Auger et al. 2002; Herold, Gitelman et al. 2005). In a recent phase II trial of new onset T1D, patients treated for 6 days with anti-CD3 (ChAglyCD3) required significantly less insulin replacement and maintained greater endogenous insulin secretion compared to the placebo group (Keymeulen, Walter et al. 2010). It was encouraging that 75% of younger patients who began antibody treatment earlier in their disease, as measured by residual pancreas beta cell mass, did not require insulin therapy at 18 months and their initial beta cell function was preserved for 3 years. In these patients, short term therapy had achieved long term benefit. Unfortunately, early studies with anti-CD3 were hampered by temporary flu-like symptoms brought on by the release of TNF-alpha, IL-2 and IFN-gamma (Abramowicz, Schandene et al. 1989). Although these can be attenuated by oral administration of anti-CD3 (Ilan, Zigmond et al. 2010) or humanizing the antibody and mutating it so that it cannot bind Fc receptors (Bolt, Routledge et al. 1993), a preference for modern therapeutics with minimal side effects may ultimately limit wider clinical application.

The best compromise might prove to be the development of alternative ways of exploiting infectious tolerance. Given the challenge of investigating this phenomenon in vivo, it is understandably even harder to demonstrate its role in clinical therapies. One group has come very close in reporting, at the least, a proof of principle study in man. Simultaneous bone marrow transplantation achieved long term graft acceptance in four out of five patients who underwent renal transplantation, despite cessation of immunosupressants after 9 months (Kawai, Cosimi et al. 2008). The high risk of this treatment excludes it from all but the most desperate of patients, but it does show that co-transfer of a graft with donor immune regulatory elements has the potential to achieve long-term tolerance.

Similarly, so called 'physician guided reconstitution' of a depleted immune system may achieve therapeutic regulation by deliberately favouring Foxp3$^+$ Treg. For example, the early success of Alemtuzumab (Campath-1H) in ablating T cells in leukaemia paved its way to clinical trials of transplantation and latterly, multiple sclerosis (Friend, Tighe et al. 1987; Friend, Rebello et al. 1995; Coles, Compston et al. 2008). Alemtuzumab is profoundly depleting and it can take over 5 years for lymphocytes to return to pre-treatment levels. Once T cell numbers are reduced, factors found to promote specifically Foxp3$^+$ Treg in vivo could be used to boost their regulatory effect (Bloom, Chang et al. 2008). One such agent could be rapamycin, an immunosuppressant already used in renal transplantation after it was found to improve graft survival when added to cyclosporine regimens (MacDonald 2001). More recently, it has been shown to promote Treg cell dependent graft tolerance in vivo (Turnquist, Raimondi et al. 2007; Horibe, Sacks et al. 2008), perhaps by preferentially favouring the survival of Foxp3$^+$ Treg (Strauss, Whiteside et al. 2007).

In theory, infectious tolerance has always promised the ability to generate self-perpetuating therapeutic tolerance from short-term intervention. By contributing to what was previously known of Foxp3^{+} Treg, this thesis hopefully serves as encouragement for fledging therapies aimed at exploiting natural regulatory mechanisms - even despite the harsh realities of developing commercially viable therapeutics.

Bibliography

Abramowicz, D., L. Schandene, et al. (1989). "Release of tumor necrosis factor, interleukin-2, and gamma-interferon in serum after injection of OKT3 monoclonal antibody in kidney transplant recipients." Transplantation **47**(4): 606-608.

Albert, M. L., B. Sauter, et al. (1998). "Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs." Nature **392**(6671): 86-89.

Alters, S. E., J. A. Shizuru, et al. (1991). "Anti-CD4 mediates clonal anergy during transplantation tolerance induction." J Exp Med **173**(2): 491-494.

Anderson, C. F., M. Oukka, et al. (2007). "CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis." J Exp Med **204**(2): 285-297.

Anderson, M. S., E. S. Venanzi, et al. (2002). "Projection of an immunological self shadow within the thymus by the aire protein." Science **298**(5597): 1395-1401.

Andersson, J., D. Q. Tran, et al. (2008). "CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner." J Exp Med **205**(9): 1975-1981.

Apostolou, I., A. Sarukhan, et al. (2002). "Origin of regulatory T cells with known specificity for antigen." Nature Immunology **3**(8): 756-763.

Appleby, M. W. and F. Ramsdell (2008). "Scurfy, the Foxp3 locus, and the molecular basis of peripheral tolerance." Curr Top Microbiol Immunol **321**: 151-168.

Asseman, C., S. Mauze, et al. (1999). "An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation." J Exp Med **190**(7): 995-1004.

Ayliffe, W., Y. Alam, et al. (1992). "Prolongation of rat corneal graft survival by treatment with anti-CD4 monoclonal antibody." Br J Ophthalmol **76**(10): 602-606.

Baker, R. J., M. P. Hernandez-Fuentes, et al. (2001). "The role of the allograft in the induction of donor-specific T cell hyporesponsiveness." Transplantation **72**(3): 480-485.

Batsford, S., U. Duermueller, et al. (2011). "Protein level expression of Toll-like receptors 2, 4 and 9 in renal disease." Nephrol Dial Transplant **26**(4): 1413-1416.

Battaglia, M., A. Stabilini, et al. (2006). "Rapamycin promotes expansion of functional CD4(+)CD25(+)FOXP3(+) regulatory T cells of both healthy subjects and type 1 diabetic patients." Journal of Immunology **177**(12): 8338-8347.

Bedoui, S., P. G. Whitney, et al. (2009). "Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells." Nature Immunology **10**(5): 488-495.

Belkaid, Y., C. A. Piccirillo, et al. (2002). "CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity." Nature **420**(6915): 502-507.

Benghiat, F. S., L. Graca, et al. (2005). "Critical influence of natural regulatory CD25+ T cells on the fate of allografts in the absence of immunosuppression." Transplantation **79**(6): 648-654.

Benjamin, R. J., S. P. Cobbold, et al. (1986). "Tolerance to rat monoclonal antibodies. Implications for serotherapy." J Exp Med **163**(6): 1539-1552.

Benjamin, R. J. and H. Waldmann (1986). "Induction of Tolerance by Monoclonal-Antibody Therapy." Nature **320**(6061): 449-451.

Bennett, C. L., J. Christie, et al. (2001). "The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3." Nat Genet **27**(1): 20-21.

Bennett, C. L. and H. D. Ochs (2001). "IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and a variety of autoimmune phenomena." Curr Opin Pediatr **13**(6): 533-538.

Billingham, R. E. and T. Boswell (1953). "Studies on the problem of corneal homografts." Proc R Soc Lond B Biol Sci **141**(904): 392-406.

Billingham, R. E. and L. Brent (1957). "A Simple Method for Inducing Tolerance of Skin Homografts in Mice." Transplantation Bulletin **4**(2): 58-71.

Billingham, R. E., L. Brent, et al. (1953). "Actively acquired tolerance of foreign cells." Nature **172**(4379): 603-606.

Bindon, C. I., G. Hale, et al. (1985). "Therapeutic Potential of Monoclonal-Antibodies to the Leukocyte-Common Antigen - Synergy and Interference in Complement-Mediated Lysis." Transplantation **40**(5): 538-544.

Bloom, D. D., Z. Chang, et al. (2008). "CD4+ CD25+ FOXP3+ regulatory T cells increase de novo in kidney transplant patients after immunodepletion with Campath-1H." Am J Transplant **8**(4): 793-802.

Boivin, G. P., I. Ormsby, et al. (1997). "Germ-free and barrier-raised TGF beta 1-deficient mice have similar inflammatory lesions." Transgenic Res **6**(3): 197-202.

Bolt, S., E. Routledge, et al. (1993). "The generation of a humanized, non-mitogenic CD3 monoclonal antibody which retains in vitro immunosuppressive properties." Eur J Immunol **23**(2): 403-411.

Borsos, T., M. Loos, et al. (1980). "A novel way of relating the structure of Cl1 to the hemolytic activity of the first component of complement." Mol Immunol **17**(11): 1415-1421.

Borsos, T. and H. J. Rapp (1965). "Complement fixation on cell surfaces by 19S and 7S antibodies." Science **150**(3695): 505-506.

Bouillet, P., D. Metcalf, et al. (1999). "Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity." Science **286**(5445): 1735-1738.

Bruder, D., A. M. Westendorf, et al. (2005). "On the edge of autoimmunity: T-cell stimulation by steady-state dendritic cells prevents autoimmune diabetes." Diabetes **54**(12): 3395-3401.

Brunkow, M. E. (2001). "Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse." Nat. Genet. **27**: 68-73.

Buch, T., F. L. Heppner, et al. (2005). "A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration." Nat Methods **2**(6): 419-426.

Burchill, M. A., J. Yang, et al. (2007). "IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells." J Immunol **178**(1): 280-290.

Cao, X., S. F. Cai, et al. (2007). "Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance." Immunity **27**(4): 635-646.

Cazac, B. B. and J. Roes (2000). "TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo." Immunity **13**(4): 443-451.

Chang, X., P. Zheng, et al. (2008). "Homeostatic proliferation in the mice with germline FoxP3 mutation and its contribution to fatal autoimmunity." Journal of Immunology **181**(4): 2399-2406.

Chen, G. Y., C. Chen, et al. (2008). "Cutting edge: Broad expression of the FoxP3 locus in epithelial cells: A caution against early interpretation of fatal inflammatory diseases following in vivo depletion of FoxP3-expressing cells." Journal of Immunology **180**(8): 5163-5166.

Chen, L. C., J. C. Delgado, et al. (2009). "Direct Expansion of Human Allospecific FoxP3(+)CD4(+) Regulatory T Cells with Allogeneic B Cells for Therapeutic Application." Journal of Immunology **183**(6): 4094-4102.

Chen, T. C., S. P. Cobbold, et al. (2004). "Generation of anergic and regulatory T cells following prolonged exposure to a harmless antigen." Journal of Immunology **172**(10): 5900-5907.

Chen, W., W. Jin, et al. (2003). "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3." J Exp Med **198**(12): 1875-1886.

Christiane Siewert, A. M., Jan Dudda, Kerstin Siegmund, Uta Lauer, Stefan Floess, Daniel J. Campbell, Alf Hamann, Jochen Huehn, (2007). "Induction of organ-selective CD4+ regulatory T cell homing." European Journal of Immunology **37**(4): 978-989.

Cobbold, S. and H. Waldmann (1998). "Infectious tolerance." Current Opinion in Immunology **10**(5): 518-524.

Cobbold, S. P., E. Adams, et al. (2009). "Infectious tolerance via the consumption of essential amino acids and mTOR signaling." Proceedings of the National Academy of Sciences of the United States of America **106**(29): 12055-12060.

Cobbold, S. P., E. Adams, et al. (2009). "Infectious tolerance via the consumption of essential amino acids and mTOR signaling." Proc Natl Acad Sci U S A **106**(29): 12055-12060.

Cobbold, S. P., R. Castejon, et al. (2004). "Induction of foxP3(+) regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants." Journal of Immunology **172**(10): 6003-6010.

Cobbold, S. P., R. Castejon, et al. (2004). "Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants." J Immunol **172**(10): 6003-6010.

Cobbold, S. P., A. Jayasuriya, et al. (1984). "Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo." Nature **312**(5994): 548-551.

Cobbold, S. P., A. Jayasuriya, et al. (1984). "Therapy with Monoclonal-Antibodies by Elimination of T-Cell Subsets Invivo." Nature **312**(5994): 548-551.

Cobbold, S. P., P. R. U. B. Rebello, et al. (1990). "A Simple Method for Measuring Patient Anti-Globulin Responses against Isotypic or Idiotypic Determinants." Journal of Immunological Methods **127**(1): 19-24.

Cobbold, S. P. and H. Waldmann (1984). "Therapeutic potential of monovalent monoclonal antibodies." Nature **308**(5958): 460-462.

Coles, A. J., D. A. S. Compston, et al. (2008). "Alemtuzumab vs. Interferon beta-1a in early multiple sclerosis." New England Journal of Medicine **359**(17): 1786-1801.

Collison, L. W., V. Chaturvedi, et al. (2010). "IL-35-mediated induction of a potent regulatory T cell population." Nature Immunology.

Colombo, M. P. and S. Piconese (2007). "Regulatory T-cell inhibition versus depletion: the right choice in cancer immunotherapy." Nature Reviews Cancer **7**(11): 880-887.

Coombes, J. L., K. R. R. Siddiqui, et al. (2007). "A functionally specialized population of mucosal CD103(+) DCs induces Foxp3(+) regulatory T cells via a TGF-beta- and retinoic acid-dependent mechanism." Journal of Experimental Medicine **204**(8): 1757-1764.

Cosimi, A. B., R. L. Jenkins, et al. (1990). "A randomized clinical trial of prophylactic OKT3 monoclonal antibody in liver allograft recipients." Arch Surg **125**(6): 781-784; discussion 785.

Curotto de Lafaille, M. A. and J. J. Lafaille (2009). "Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor?" Immunity **30**(5): 626-635.

Daley, S. R., J. Ma, et al. (2007). "A key role for TGF-beta signaling to T cells in the long-term acceptance of allografts." Journal of Immunology **179**(6): 3648-3654.

Daley, S. R., J. Ma, et al. (2007). "A key role for TGF-beta signaling to T cells in the long-term acceptance of allografts." J Immunol **179**(6): 3648-3654.

Danke, N. A., D. M. Koelle, et al. (2004). "Autoreactive T cells in healthy individuals." Journal of Immunology **172**(10): 5967-5972.

Davies, J. D., L. Y. Leong, et al. (1996). "T cell suppression in transplantation tolerance through linked recognition." J Immunol **156**(10): 3602-3607.

Davies, J. D., G. Martin, et al. (1996). "T-cell regulation in adult transplantation tolerance." Journal of Immunology **157**(2): 529-533.

Derynck, R. and Y. E. Zhang (2003). "Smad-dependent and Smad-independent pathways in TGF-beta family signalling." Nature **425**(6958): 577-584.

DeSilva, D. R., K. B. Urdahl, et al. (1991). "Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation." Journal of Immunology **147**(10): 3261-3267.

Fahlen, L., S. Read, et al. (2005). "T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells." J Exp Med **201**(5): 737-746.

Fan, Z. G., J. Spencer, et al. (2009). "In vivo tracking of "color-coded" effector, natural and induced regulatory T cells in allograft response." Xenotransplantation **16**(5): 326-327.

Fan, Z. G., J. A. Spencer, et al. (2010). "In vivo tracking of 'color-coded' effector, natural and induced regulatory T cells in the allograft response." Nature Medicine **16**(6): 718-U125.

Fantini, M. C., C. Becker, et al. (2004). "Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7." J Immunol **172**(9): 5149-5153.

Farquhar, C. A., A. M. Paterson, et al. (2010). "Tolerogenicity is not an absolute property of a dendritic cell." European Journal of Immunology **40**(6): 1728-1737.

Feng, G., K. J. Wood, et al. (2008). "Interferon-gamma conditioning ex vivo generates CD25(+)CD62L(+)Foxp3(+) regulatory T cells that prevent allograft rejection: Potential avenues for cellular therapy." Transplantation **86**(4): 578-589.

Floess, S., J. Freyer, et al. (2007). "Epigenetic control of the foxp3 locus in regulatory T cells." Plos Biology **5**(2): 169-178.

Fontenot, J. D., M. A. Gavin, et al. (2003). "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." Nature Immunology **4**(4): 330-336.

Fontenot, J. D., J. P. Rasmussen, et al. (2005). "Regulatory T cell lineage specification by the forkhead transcription factor foxp3." Immunity **22**(3): 329-341.

Francis, R. S., G. Feng, et al. (2011). "Induction of transplantation tolerance converts potential effector T cells into graft-protective regulatory T cells." European Journal of Immunology **41**(3): 726-738.

Friend, P. J., P. Rebello, et al. (1995). "Successful Treatment of Renal-Allograft Rejection with a Humanized Antilymphocyte Monoclonal-Antibody." Transplantation Proceedings **27**(1): 869-870.

Friend, P. J., H. Tighe, et al. (1987). "The use of monoclonal antibodies against activated human T cells following renal allografting in the baboon." Transplant Proc **19**(5): 4317-4318.

Gambineri, E., T. R. Torgerson, et al. (2003). "Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis." Current Opinion in Rheumatology **15**(4): 430-435.

Glennie, M. J. and G. T. Stevenson (1982). "Univalent antibodies kill tumour cells in vitro and in vivo." Nature **295**(5851): 712-714.

Godfrey, V. L., J. E. Wilkinson, et al. (1991). "X-linked lymphoreticular disease in the scurfy (sf) mutant mouse." Am J Pathol **138**(6): 1379-1387.

Golshayan, D., S. Jiang, et al. (2007). "In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance." Blood **109**(2): 827-835.

Gondek, D. C., L. F. Lu, et al. (2005). "Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism." Journal of Immunology **174**(4): 1783-1786.

Graca, L., S. P. Cobbold, et al. (2002). "Identification of regulatory T cells in tolerated allografts." J Exp Med **195**(12): 1641-1646.

Graca, L., S. Thompson, et al. (2002). "Both CD4(+)CD25(+) and CD4(+)CD25(-) regulatory cells mediate dominant transplantation tolerance." Journal of Immunology **168**(11): 5558-5565.

Grino, J. M., A. M. Castelao, et al. (1992). "Antilymphocyte globulin versus OKT3 induction therapy in cadaveric kidney transplantation: a prospective randomized study." Am J Kidney Dis **20**(6): 603-610.

Grossman, W. J., J. W. Verbsky, et al. (2004). "Human T regulatory cells can use the perforin pathway to cause autologous target cell death." Immunity **21**(4): 589-601.

Groux, H., M. Bigler, et al. (1996). "Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells." J Exp Med **184**(1): 19-29.

Gutstein, N. L., W. E. Seaman, et al. (1986). "Induction of immune tolerance by administration of monoclonal antibody to L3T4." J Immunol **137**(4): 1127-1132.

Hadeiba, H., T. Sato, et al. (2008). "CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease." Nature Immunology **9**(11): 1253-1260.

Hale, G., M. Clark, et al. (1985). "Therapeutic potential of rat monoclonal antibodies: isotype specificity of antibody-dependent cell-mediated cytotoxicity with human lymphocytes." Journal of Immunology **134**(5): 3056-3061.

Harrington, L. E., R. D. Hatton, et al. (2005). "Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages." Nature Immunology **6**(11): 1123-1132.

Hedrick, S. M., D. I. Cohen, et al. (1984). "Isolation of cDNA clones encoding T cell-specific membrane-associated proteins." Nature **308**(5955): 149-153.

Hedrick, S. M., E. A. Nielsen, et al. (1984). "Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins." Nature **308**(5955): 153-158.

Herbelin, A., J. M. Gombert, et al. (1998). "Mature mainstream TCR [alpha][beta]+CD4+ thymocytes expressing L-selectin mediate [ldquo]active tolerance[rdquo] in the nonobese diabetic mouse." J. Immunol. **161**: 2620-2628.

Herlyn, D. and H. Koprowski (1982). "IgG2a monoclonal antibodies inhibit human tumor growth through interaction with effector cells." Proc Natl Acad Sci U S A **79**(15): 4761-4765.

Herold, K. C., S. E. Gitelman, et al. (2005). "A single course of anti-CD3 monoclonal antibody hOKT3 gamma 1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes." Diabetes **54**(6): 1763-1769.

Hodi, F. S., S. J. O'Day, et al. (2010). "Improved survival with ipilimumab in patients with metastatic melanoma." N Engl J Med **363**(8): 711-723.

Honey, K., S. P. Cobbold, et al. (1999). "CD40 ligand blockade induces CD4+T cell tolerance and linked suppression." Journal of Immunology **163**(9): 4805-4810.

Hori, S., T. Nomura, et al. (2003). "Control of regulatory T cell development by the transcription factor Foxp3." Science **299**(5609): 1057-1061.

Horibe, E. K., J. Sacks, et al. (2008). "Rapamycin-conditioned, alloantigen-pulsed dendritic cells promote indefinite survival of vascularized skin allografts in association with T regulatory cell expansion." Transpl Immunol **18**(4): 307-318.

Hsieh, C.-S., Y. Liang, et al. (2004). "Recognition of the Peripheral Self by Naturally Arising CD25+ CD4+ T Cell Receptors." Immunity **21**(2): 267-277.

Hu, Y., M. J. Turner, et al. (2009). "Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model." Immunology **128**(2): 260-270.

Huang, F. P., N. Platt, et al. (2000). "A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes." J Exp Med **191**(3): 435-444.

Hubert, S., B. Rissiek, et al. (2010). "Extracellular NAD(+) shapes the Foxp3(+) regulatory T cell compartment through the ART2-P2X7 pathway." Journal of Experimental Medicine **207**(12): 2561-2568.

Hughes-Jones, N. C., B. D. Gorick, et al. (1983). "The mechanism of synergistic complement-mediated lysis of rat red cells by monoclonal IgG antibodies." European Journal of Immunology **13**(8): 635-641.

Huizinga, T. W., C. E. van der Schoot, et al. (1988). "The PI-linked receptor FcRIII is released on stimulation of neutrophils." Nature **333**(6174): 667-669.

Ilan, Y., E. Zigmond, et al. (2010). "Oral Administration of OKT3 Monoclonal Antibody to Human Subjects Induces a Dose-Dependent Immunologic Effect in T Cells and Dendritic Cells." Journal of Clinical Immunology **30**(1): 167-177.

Inaba, K., M. Inaba, et al. (1992). "Generation of Large Numbers of Dendritic Cells from Mouse Bone-Marrow Cultures Supplemented with Granulocyte Macrophage Colony-Stimulating Factor." Journal of Experimental Medicine **176**(6): 1693-1702.

Inaba, K., S. Turley, et al. (1998). "Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells." J Exp Med **188**(11): 2163-2173.

Izcue, A., J. L. Coombes, et al. (2006). "Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation." Immunological Reviews **212**: 256-271.

Jenkins, M. K., C. A. Chen, et al. (1990). "Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody." Journal of Immunology **144**(1): 16-22.

Jiang, S., J. Tsang, et al. (2006). "Generation and expansion of human CD4+ CD25+ regulatory T cells with indirect allospecificity: Potential reagents to promote donor-specific transplantation tolerance." Transplantation **82**(12): 1738-1743.

Jiang, X., M. Morita, et al. (2006). "The importance of CD25+ CD4+ regulatory T cells in mouse hepatic allograft tolerance." Liver Transpl **12**(7): 1112-1118.

Jocea M. R. van Amelsfort, K. M. G. J., Johannes W. J. Bijlsma, Floris P. J. G. Lafeber, Leonie S. Taams, (2004). "CD4+CD25+ regulatory T cells in rheumatoid arthritis: Differences in the presence, phenotype, and function between peripheral blood and synovial fluid." Arthritis & Rheumatism **50**(9): 2775-2785.

Joffre, O., T. Santolaria, et al. (2008). "Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes." Nat Med **14**(1): 88-92.

Johnston, J. A., C. M. Bacon, et al. (1996). "Signaling by IL-2 and related cytokines: JAKs, STATs, and relationship to immunodeficiency." J Leukoc Biol **60**(4): 441-452.

Jonuleit, H., E. Schmitt, et al. (2002). "Infectious tolerance: human CD25(+) regulatory T cells convey suppressor activity to conventional CD4(+) T helper cells." J Exp Med **196**(2): 255-260.

Joo, C. K., J. S. Pepose, et al. (1995). "T-cell mediated responses in a murine model of orthotopic corneal transplantation." Invest Ophthalmol Vis Sci **36**(8): 1530-1540.

Jordan, M. S., A. Boesteanu, et al. (2001). "Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide." Nature Immunology **2**(4): 301-306.

Judge, T. A., Z. Wu, et al. (1999). "The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival." Journal of Immunology **162**(4): 1947-1951.

Kappler, J. W., N. Roehm, et al. (1987). "T cell tolerance by clonal elimination in the thymus." Cell **49**(2): 273-280.

Karim, M., G. Feng, et al. (2005). "CD25(+)CD4(+) regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific reactivation in vivo is critical for bystander regulation." Blood **105**(12): 4871-4877.

Kaufmann, D. E. and B. D. Walker (2009). "PD-1 and CTLA-4 Inhibitory Cosignaling Pathways in HIV Infection and the Potential for Therapeutic Intervention." Journal of Immunology **182**(10): 5891-5897.

Kawai, T., A. B. Cosimi, et al. (2008). "Brief report: HLA-mismatched renal transplantation without maintenance immunosuppression." New England Journal of Medicine **358**(4): 353-361.

Keymeulen, B., M. Walter, et al. (2010). "Four-year metabolic outcome of a randomised controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass." Diabetologia **53**(4): 614-623.

Khattri, R., T. Cox, et al. (2003). "An essential role for Scurfin in CD4+CD25+ T regulatory cells." Nat Immunol **4**(4): 337-342.

Kim, J., K. Lahl, et al. (2009). "Cutting Edge: Depletion of Foxp3(+) Cells Leads to Induction of Autoimmunity by Specific Ablation of Regulatory T Cells in Genetically Targeted Mice." Journal of Immunology **183**(12): 7631-7634.

Kim, J. I., R. O'Connor M, et al. (2011). "Generation of adaptive regulatory T cells by alloantigen is required for some but not all transplant tolerance protocols." Transplantation **91**(7): 707-713.

Kim, J. M., J. P. Rasmussen, et al. (2007). "Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice." Nat Immunol **8**(2): 191-197.

Kim, J. M., J. P. Rasmussen, et al. (2007). "Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice." Nature Immunology **8**(2): 191-197.

Kobie, J. J., P. R. Shah, et al. (2006). "T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine." Journal of Immunology **177**(10): 6780-6786.

Koenecke, C., N. Czeloth, et al. (2009). "Alloantigen-specific de novo-induced Foxp3(+) Treg revert in vivo and do not protect from experimental GVHD." European Journal of Immunology **39**(11): 3091-3096.

Kohler, G., S. C. Howe, et al. (1976). "Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines." European Journal of Immunology **6**(4): 292-295.

Kohler, G. and C. Milstein (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity." Nature **256**(5517): 495-497.

Kohler, G. and C. Milstein (1976). "Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion." European Journal of Immunology **6**(7): 511-519.

Komatsu, N., M. E. Mariotti-Ferrandiz, et al. (2009). "Heterogeneity of natural Foxp3(+) T cells: A committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity." Proceedings of the National Academy of Sciences of the United States of America **106**(6): 1903-1908.

Kretschmer, K., I. Apostolou, et al. (2005). "Inducing and expanding regulatory T cell populations by foreign antigen." Nature Immunology **6**(12): 1219-1227.

Kuniyasu, Y., T. Takahashi, et al. (2000). "Naturally anergic and suppressive CD25(+)CD4(+) T cells as a functionally and phenotypically distinct immunoregulatory T cell subpopulation." International Immunology **12**(8): 1145-1155.

Lahl, K., C. Loddenkemper, et al. (2007). "Selective depletion of Foxp3(+) regulatory T cells induces a scurfy-like disease." Journal of Experimental Medicine **204**(1): 57-63.

Laouar, Y., F. S. Sutterwala, et al. (2005). "Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma." Nat Immunol **6**(6): 600-607.

Larkin, J., 3rd, A. L. Rankin, et al. (2008). "CD4+CD25+ regulatory T cell repertoire formation shaped by differential presentation of peptides from a self-antigen." Journal of Immunology **180**(4): 2149-2157.

Leach, D. R., M. F. Krummel, et al. (1996). "Enhancement of antitumor immunity by CTLA-4 blockade." Science **271**(5256): 1734-1736.

Lechler, R., J. G. Chai, et al. (2001). "The contributions of T-cell anergy to peripheral T-cell tolerance." Immunology **103**(3): 262-269.

Lechler, R., J. G. Chai, et al. (2001). "T-cell anergy and peripheral T-cell tolerance." Philos Trans R Soc Lond B Biol Sci **356**(1409): 625-637.

Lee, I., L. Q. Wang, et al. (2005). "Recruitment of Foxp3(+) T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor." Journal of Experimental Medicine **201**(7): 1037-1044.

Letterio, J. J., A. G. Geiser, et al. (1996). "Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression." J Clin Invest **98**(9): 2109-2119.

Levings, M. K., R. Sangregorio, et al. (2002). "Human CD25(+) CD4(+) T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells." Journal of Experimental Medicine **196**(10): 1335-1346.

Li, J., A. Smolyar, et al. (1996). "Ligand-induced conformational change within the CD2 ectodomain accompanies receptor clustering: Implication for molecular lattice formation." Journal of Molecular Biology **263**(2): 209-226.

Li, M. O., S. Sanjabi, et al. (2006). "Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms." Immunity **25**(3): 455-471.

Li, M. O., Y. Y. Wan, et al. (2006). "Transforming growth factor-beta regulation of immune responses." Annual Review of Immunology **24**: 99-146.

Liblau, R., E. Tournier-Lasserve, et al. (1991). "T cell response to myelin basic protein epitopes in multiple sclerosis patients and healthy subjects." Eur J Immunol **21**(6): 1391-1395.

Lin, M. L., Y. Zhan, et al. (2008). "The cell biology of cross-presentation and the role of dendritic cell subsets." Immunol Cell Biol **86**(4): 353-362.

Liston, A., S. Lesage, et al. (2003). "Aire regulates negative selection of organ-specific T cells." Nature Immunology **4**(4): 350-354.

Liu, Y., P. Zhang, et al. (2008). "A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells." Nature Immunology **9**(6): 632-640.

MacDonald, A. S. (2001). "A worldwide, phase III, randomized, controlled, safety and efficacy study of a sirolimus/cyclosporine regimen for prevention of acute rejection in recipients of primary mismatched renal allografts." Transplantation **71**(2): 271-280.

MacDonald, H. R., S. Baschieri, et al. (1991). "Clonal expansion precedes anergy and death of V beta 8+ peripheral T cells responding to staphylococcal enterotoxin B in vivo." European Journal of Immunology **21**(8): 1963-1966.

Mahnke, K., Y. Qian, et al. (2003). "Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells." Blood **101**(12): 4862-4869.

Mandapathil, M., B. Hilldorfer, et al. "Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells." J Biol Chem **285**(10): 7176-7186.

Marie, J. C., J. J. Letterio, et al. (2005). "TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells." J Exp Med **201**(7): 1061-1067.

Marshall, S. E., S. P. Cobbold, et al. (1996). "Tolerance and suppression in a primed immune system." Transplantation **62**(11): 1614-1621.

Mason, P. D., C. M. Robinson, et al. (1996). "Detection of donor-specific hyporesponsiveness following late failure of human renal allografts." Kidney Int **50**(3): 1019-1025.

Medawar, P. B. (1948). "Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye." Br J Exp Pathol **29**(1): 58-69.

Medzhitov, R. (2008). "Origin and physiological roles of inflammation." Nature **454**(7203): 428-435.

Mellor, A. L. and D. H. Munn (2008). "Creating immune privilege: active local suppression that benefits friends, but protects foes." Nature Reviews Immunology **8**(1): 74-80.

Menges, M., S. Rossner, et al. (2002). "Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity." J Exp Med **195**(1): 15-21.

Miyajima, M., C. M. Chase, et al. (2011). "Early acceptance of renal allografts in mice is dependent on foxp3(+) cells." Am J Pathol **178**(4): 1635-1645.

Mottet, C., H. H. Uhlig, et al. (2003). "Cutting edge: Cure of colitis by CD4(+) CD25(+) regulatory T cells." Journal of Immunology **170**(8): 3939-3943.

Ng, W. F., R. J. Baker, et al. (2001). "The role of T-cell anergy in the maintenance of donor-specific hyporesponsiveness in renal transplant recipients." Transplant Proc **33**(1-2): 154-155.

Nishimura, E., T. Sakihama, et al. (2004). "Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+CD25+CD4+ regulatory T cells." International Immunology **16**(8): 1189-1201.

Noelle, R. J., L. Marshall, et al. (1992). "Role of Contact and Soluble Factors in the Growth and Differentiation of B-Cells by Helper T-Cells." Mechanisms of Lymphocyte Activation and Immune Regulation Iv **323**: 131-138

204.

Onishi, Y., Z. Fehervari, et al. (2008). "Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation." Proc Natl Acad Sci U S A **105**(29): 10113-10118.

Pandiyan, P., L. Zheng, et al. (2007). "CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells." Nat Immunol **8**(12): 1353-1362.

Park, H., Z. Li, et al. (2005). "A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17." Nature Immunology **6**(11): 1133-1141.

Paust, S., L. Lu, et al. (2004). "Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease." Proc Natl Acad Sci U S A **101**(28): 10398-10403.

Peters, J. H., F. W. Preijers, et al. (2008). "Clinical Grade Treg: GMP Isolation, Improvement of Purity by CD127(pos) Depletion, Treg Expansion, and Treg Cryopreservation." Plos One **3**(9): -.

Polansky, J. K., K. Kretschmer, et al. (2008). "DNA methylation controls Foxp3 gene expression." European Journal of Immunology **38**(6): 1654-1663.

Proietto, A. I., S. van Dommelen, et al. (2008). "Dendritic cells in the thymus contribute to T-regulatory cell induction." Proc Natl Acad Sci U S A **105**(50): 19869-19874.

Qin, S., S. P. Cobbold, et al. (1993). ""Infectious" transplantation tolerance." Science **259**(5097): 974-977.

Qin, S. X., S. Cobbold, et al. (1987). "Cdj Monoclonal-Antibody Pairs for Immunosuppression and Tolerance Induction." European Journal of Immunology **17**(8): 1159-1165.

Qin, S. X., S. P. Cobbold, et al. (1993). "Infectious Transplantation Tolerance." Science **259**(5097): 974-977.

Read, S. (2006). "Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo." J. Immunol. **177**: 4376-4383.

Read, S., V. Malmstrom, et al. (2000). "Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation." J Exp Med **192**(2): 295-302.

Rebello, P. R. U. B., G. Hale, et al. (1999). "Anti-globulin responses to rat and humanized CAMPATH-1 monoclonal antibody used to treat transplant rejection." Transplantation **68**(9): 1417-1420.

Robb, R. J. and W. C. Greene (1987). "Internalization of interleukin 2 is mediated by the beta chain of the high-affinity interleukin 2 receptor." J Exp Med **165**(4): 1201-1206.

Rocha, B. and H. von Boehmer (1991). "Peripheral selection of the T cell repertoire." Science **251**(4998): 1225-1228.

Saito, M., T. Iwawaki, et al. (2001). "Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice." Nat Biotechnol **19**(8): 746-750.

Sakaguchi, S., N. Sakaguchi, et al. (1995). "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor [alpha]-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases." J. Immunol. **155**: 1151-1164.

Scheuplein, F., N. Schwarz, et al. (2009). "NAD(+) and ATP Released from Injured Cells Induce P2X(7)-Dependent Shedding of CD62L and Externalization of Phosphatidylserine by Murine T Cells." Journal of Immunology **182**(5): 2898-2908.

Schmidt, E. M., C. J. Wang, et al. (2009). "CTLA-4 Controls Regulatory T Cell Peripheral Homeostasis and Is Required for Suppression of Pancreatic Islet Autoimmunity." Journal of Immunology **182**(1): 274-282.

Schneider, H., E. Valk, et al. (2005). "CTLA-4 up-regulation of lymphocyte function-associated antigen 1 adhesion and clustering as an alternate basis for coreceptor function." Proc Natl Acad Sci U S A **102**(36): 12861-12866.

Schwartz, R. H. (1990). "A cell culture model for T lymphocyte clonal anergy." Science **248**(4961): 1349-1356.

Schwartz, R. H. (2003). "T cell anergy." Annual Review of Immunology **21**: 305-334.

Scully, R., S. X. Qin, et al. (1994). "Mechanisms in Cd4 Antibody-Mediated Transplantation Tolerance - Kinetics of Induction, Antigen Dependency and Role of Regulatory T-Cells." European Journal of Immunology **24**(10): 2383-2392.

Semiletova, N. V., X. D. Shen, et al. (2010). "Intensity of transplant chronic rejection correlates with level of graft-infiltrating regulatory cells." Journal of Heart and Lung Transplantation **29**(3): 335-341.

Shull, M. M., I. Ormsby, et al. (1992). "Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease." Nature **359**(6397): 693-699.

Siegmund, K., M. Feuerer, et al. (2005). "Migration matters: regulatory T-cell compartmentalization determines suppressive activity in vivo." Blood **106**(9): 3097-3104.

Sloan-Lancaster, J., B. D. Evavold, et al. (1993). "Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells." Nature **363**(6425): 156-159.

Strasser, A., A. W. Harris, et al. (1991). "bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship." Cell **67**(5): 889-899.

Strauss, L., T. L. Whiteside, et al. (2007). "Selective survival of naturally occurring human CD4(+)CD25(+)Foxp3(+) regulatory T cells cultured with rapamycin." Journal of Immunology **178**(1): 320-329.

Streilein, J. W. (2003). "Ocular immune privilege: the eye takes a dim but practical view of immunity and inflammation." Journal of Leukocyte Biology **74**(2): 179-185.

Streilein, J. W., G. B. Toews, et al. (1979). "Corneal allografts fail to express Ia antigens." Nature **282**(5736): 326-327.

Takahashi, T., T. Tagami, et al. (2000). "Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4." J Exp Med **192**(2): 303-310.

Tang, Q. Z., K. J. Henriksen, et al. (2004). "In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes." Journal of Experimental Medicine **199**(11): 1455-1465.

Tarbell, K. V., S. Yamazaki, et al. (2004). "CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes." J Exp Med **199**(11): 1467-1477.

Taylor, A. W., J. W. Streilein, et al. (1992). "Identification of alpha-melanocyte stimulating hormone as a potential immunosuppressive factor in aqueous humor." Curr Eye Res **11**(12): 1199-1206.

Taylor, A. W., J. W. Streilein, et al. (1994). "Immunoreactive vasoactive intestinal peptide contributes to the immunosuppressive activity of normal aqueous humor." Journal of Immunology **153**(3): 1080-1086.

Thornton, A. M. and E. M. Shevach (1998). "CD4+CD25+ Immunoregulatory T Cells Suppress Polyclonal T Cell Activation In Vitro by Inhibiting Interleukin 2 Production." J. Exp. Med. **188**(2): 287-296.

Tone, Y., K. Furuuchi, et al. (2008). "Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer." Nat Immunol **9**(2): 194-202.

Trzonkowski, P., M. Bieniaszewska, et al. (2009). "First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells." Clinical Immunology **133**(1): 22-26.

Turley, D. M. and S. D. Miller (2007). "Peripheral tolerance induction using ethylenecarbodiimide-fixed APCs uses both direct and indirect mechanisms of antigen presentation for prevention of experimental autoimmune encephalomyelitis." Journal of Immunology **178**(4): 2212-2220.

Turnquist, H. R., G. Raimondi, et al. (2007). "Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance." Journal of Immunology **178**(11): 7018-7031.

Turnquist, H. R., G. Raimondi, et al. (2007). "Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance." J Immunol **178**(11): 7018-7031.

Uhlig, H. H., J. Coombes, et al. (2006). "Characterization of Foxp3(+)CD4(+)CD25(+) and IL-10-secreting CD4(+)CD25(+) T cells during cure of colitis." Journal of Immunology **177**(9): 5852-5860.

Utset, T. O., J. A. Auger, et al. (2002). "Modified anti-CD3 therapy in psoriatic arthritis: a phase I/II clinical trial." J Rheumatol **29**(9): 1907-1913.

Verginis, P., H. S. Li, et al. (2005). "Tolerogenic semimature dendritic cells suppress experimental autoimmune thyroiditis by activation of thyroglobulin-specific CD4+CD25+ T cells." Journal of Immunology **174**(11): 7433-7439.

Vieira, P. L., J. R. Christensen, et al. (2004). "IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells." Journal of Immunology **172**(10): 5986-5993.

Vukmanovic-Stejic, M., Y. Zhang, et al. (2006). "Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo." J Clin Invest **116**(9): 2423-2433.

Waldmann, H. and S. Cobbold (1998). "How do monoclonal antibodies induce tolerance? A role for infectious tolerance?" Annual Review of Immunology **16**: 619-644.

Waldmann, H., S. Cobbold, et al. (1987). "Monoclonal-Antibodies for the Depletion of Specific Subpopulations of Lymphocytes." Ciba Foundation Symposia **129**: 194-208.

Waldmann, H., S. P. Cobbold, et al. (1989). "Tolerance Induction in the Adult Using Monoclonal-Antibodies to Cd4, Cd8, and Cd11a (Lfa-1)." Cold Spring Harbor Symposia on Quantitative Biology **54**: 885-892.

Waldmann, H., A. Polliak, et al. (1984). "Elimination of graft-versus-host disease by in-vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1)." Lancet **2**(8401): 483-486.

Walker, L. S. and A. K. Abbas (2002). "The enemy within: keeping self-reactive T cells at bay in the periphery." Nature Reviews Immunology **2**(1): 11-19.

Wang, H. M., H. J. Kaplan, et al. (1987). "The distribution and ontogeny of MHC antigens in murine ocular tissue." Invest Ophthalmol Vis Sci **28**(8): 1383-1389.

Watanabe, N., Y. H. Wang, et al. (2005). "Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus." Nature **436**(7054): 1181-1185.

Waterhouse, P., J. M. Penninger, et al. (1995). "Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4." Science **270**(5238): 985-988.

Webb, S., C. Morris, et al. (1990). "Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity." Cell **63**(6): 1249-1256.

Weiner, H. L. (2001). "Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells." Immunological Reviews **182**: 207-214.

Wekerle, T., J. Kurtz, et al. (2002). "Mechanisms of transplant tolerance induction using costimulatory blockade." Current Opinion in Immunology **14**(5): 592-600.

Williams, L. M. and A. Y. Rudensky (2007). "Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3." Nat Immunol **8**(3): 277-284.

Wing, K., Y. Onishi, et al. (2008). "CTLA-4 control over Foxp3+ regulatory T cell function." Science **322**(5899): 271-275.

Wise, M. P., F. Bemelman, et al. (1998). "Linked suppression of skin graft rejection can operate through indirect recognition." J Immunol **161**(11): 5813-5816.

Wong, J., D. Mathis, et al. (2007). "TCR-based lineage tracing: no evidence for conversion of conventional into regulatory T cells in response to a natural self-antigen in pancreatic islets." J Exp Med **204**(9): 2039-2045.

Wood, K. J., A. Bushell, et al. (2011). "Immunologic unresponsiveness to alloantigen in vivo: a role for regulatory T cells." Immunological Reviews **241**(1): 119-132.

Wood, K. J. and S. Sakaguchi (2003). "Regulatory T cells in transplantation tolerance." Nature Reviews Immunology **3**(3): 199-210.

Xia, G. L., M. Shah, et al. (2009). "Prevention of allograft rejection by amplification of Foxp3(+)CD4(+)CD25(+) regulatory T cells." Translational Research **153**(2): 60-70.

Yamaizumi, M., E. Mekada, et al. (1978). "One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell." Cell **15**(1): 245-250.

Yan, Z., S. K. Garg, et al. (2009). "Extracellular redox modulation by regulatory T cells." Nature Chemical Biology **5**(10): 721-723.

Yanagi, Y., Y. Yoshikai, et al. (1984). "A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains." Nature **308**(5955): 145-149.

Yao, Z., Y. Kanno, et al. (2007). "Nonredundant roles for Stat5a/b in directly regulating Foxp3." Blood **109**(10): 4368-4375.

Yrlid, U. and M. J. Wick (2000). "Salmonella-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells." J Exp Med **191**(4): 613-624.

Zhang, L. I., D. R. Martin, et al. (1992). "Peripheral deletion of mature CD8+ antigen-specific T cells after in vivo exposure to male antigen." Journal of Immunology **148**(12): 3740-3745.

Zhang, N., B. Schroppel, et al. (2009). "Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response." Immunity **30**(3): 458-469.

Zhao, D. M., A. M. Thornton, et al. (2006). "Activated CD4+CD25+ T cells selectively kill B lymphocytes." Blood **107**(10): 3925-3932.

Zheng, L., R. Sharma, et al. (2007). "A novel role of IL-2 in organ-specific autoimmune inflammation beyond regulatory T cell checkpoint: both IL-2 knockout and Fas mutation prolong lifespan of Scurfy mice but by different mechanisms." J Immunol **179**(12): 8035-8041.

Zheng, Y., S. Josefowicz, et al. (2010). "Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate." Nature **463**(7282): 808-812.

Zhou, X., S. L. Bailey-Bucktrout, et al. (2009). "Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo." Nature Immunology **10**(9): 1000-1007.

Zinkernagel, R. M., G. N. Callahan, et al. (1978). "Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus." Nature **271**(5642): 251-253.

Zinkernagel, R. M. and P. C. Doherty (1974). "Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis." Nature **251**(5475): 547-548.

Zuo, T., L. Wang, et al. (2007). "FOXP3 Is an X-Linked Breast Cancer Suppressor Gene and an Important Repressor of the HER-2/ErbB2 Oncogene." Cell **129**(7): 1275-1286.

Zwar, T. D., S. Read, et al. (2006). "CD4+CD25+ regulatory T cells inhibit the antigen-dependent expansion of self-reactive T cells in vivo." J Immunol **176**(3): 1609-1617.