Induced Regulatory T cells in Transplantation Tolerance

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Ye Chen

Sir William Dunn School of Pathology

Green-Templeton College

ABSTRACT

Induced regulatory T cells (iTreg) play an important role in the induction of tolerance to self and non-self antigens. Harnessing their suppressive potential has therapeutic implications for the treatment of autoimmune conditions and transplant rejection. Although the role of TGFβ-conditioned iTreg in natural and therapeutic tolerance is indisputable, their mechanism of action as well as factors that influence their function and stability *in vivo* remain unclear. Here it is shown that TGFβ-conditioning of T cells in the absence of any Foxp3 expression is insufficient for conferring a suppressive phenotype *in vivo,* whilst Foxp3 expression is sufficient to enable naïve T cells to become suppressive both *in vitro* and *in vivo*. Graft antigen was found to enhance the number of iTreg-derived $Foxp3^+$ cells localising to the draining lymph nodes of recipients, and this was associated with histone modifications at the *Foxp3* locus that suggested a stabilisation or 'affirmation' of Foxp3 expression. Finally, iTreg were shown to 'out-compete' naïve T cells in forming clusters with dendritic cells. Activated inflammatory T cells could also 'out-compete' naïve T cells. However, unlike activated T cells, iTreg did not activate interacting DCs to the same extent, and this may potentially be a mechanism of their action *in vivo*.

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ABBREVIATIONS

4HT	4-hydroxytamoxifen
Ab	Antibody
APC	Antigen presenting cell
ATRA	all-trans retinoic acid
BmDC	Bone marrow-derived dendritic cells
CBA	Mouse strain — eg. CBA/Ca
CD	Cluster of differentiation
cFoxp3	Conditional Foxp3 retroviral construct
ChIP	Chromatin Immunoprecipitation
CTLA	Cytotoxic T cell lymphocyte antigen
DC	Dendritic cell
DLN	Draining lymph node
DNA	Deoxyribonucleic acid
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisable
FCS	Fetal calf serum
Foxp3	Forkhead box P3
GITR	Glucocorticoid-induced TNF-related receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H-2	Murine histocompatibility system 2
hCD2	human CD2
HLA	Human leukocyte antigen
HPRT	Hypoxanthinephophoribosyl transferase
hr/hrs	Hour/Hours
HSP	Heat shock protein
ICAM	Intracellular adhesion molecule
ICS	Intracellular cytokine staining
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
i.p.	Intraperitoneal
IPEX	Immune dysfunction polyendocrinopathy, enteropathy, X linked
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
i.v.	Intravenous
LFA-1	Lymphocyte function-associated 1
LN(s)	Lymph node(s)
mAb	Monoclonal antibody
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mHC	Minor histocompatibility complex antigen
mRNA	Messenger ribonucleic acid

NDLN	Non-draining lymph node(s)
NOD	Non-obese diabetic
NK	Natural killer cell
T_a	Activated T cell
T_n	Naïve T cell
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pMHC	Peptide-MHC complex
PMN	Polymorphonuclear cell
RAG	Recombinase-activating gene
ROS	Reactive oxygen species
RNA	Ribonucleic acid
STZ	Streptozotocin
T1D	Type 1 diabetes
TBAC	Tris-buffered ammonium chloride
TCR	T cell receptor
TGFβ	Tumour growth factor β
Th	T helper cell
TNF	Tumour necrosis factor
$T_{TGFβ\text{-}con}$	TGFβ-conditioned T cell
Treg(s)	Regulatory T cell(s)
cTreg	Conditional Treg(s) (4HT/Tamoxifen treated *cFoxp3*-transduced T cell)
iTreg	Induced Treg(s)
nTreg	Natural Treg
VDJ	Variable, Diversity and Joining gene segments
WT	Wildtype

CHAPTER 1 INTRODUCTION

1.1 Regulatory T cells – the key to achieving therapeutic tolerance

The immune system is a double-edged sword. On one hand, it protects the host from foreign organisms and eliminates mutated or diseased host cells. On the other, aberrant, excessive or unwanted immune responses can cause debilitating pathology. Failure of the host to subdue aberrant activation of the immune system against its own tissues (autoimmunity) can result in the development of pathological changes in almost all types of tissues including glia (multiple sclerosis), gut lining (inflammatory bowel disease), skin (psoriasis) or the pancreas (diabetes mellitus). The immune system can also mount an unwanted attack on foreign tissues, for example in the context of transplantation.

In the past, drugs used to treat autoimmune conditions and to counter graft rejection have led to a general and non-specific immunosuppression. The challenge has been to find effective therapies that would render the immune system 'tolerant' towards self or foreign tissues in a specific and long-term manner. The discovery of regulatory immune cells along with the powerful phenomena associated with their action such as infectious tolerance and linked suppression has boosted hopes of therapeutically achieving specific and long-term tolerance. A better understanding of the mechanisms of regulatory cell function can be harnessed as the basis of future therapeutic strategies against autoimmune conditions and transplant rejection.

In the non-pathological state, the immune system uses a myriad of mechanisms to promote self-tolerance. Tolerance mechanisms can be central or peripheral with the former referring

to the deletion of self-reactive T cell-progenitors in the thymus due to high affinity of their T cell receptors (TCR) for self-antigens presented in the thymic medulla. Peripheral mechanisms exist to modify or delete self-reactive T cells that have escaped thymic deletion. These include receptor editing, where lymphocytes replace self-reactive T cell or B cell receptors with nonreactive receptors; anergy, where the cell becomes functionally inert towards self-antigens; privilege, where the self-antigen is inaccessible to the self-reactive lymphocytes; raised activation threshold, where inhibitory receptors on the T cell surface prevent its own activation; and regulatory T cells.

Regulatory T cells (Tregs) are a specialised subset of $CD4^+$ T cells that mediate so-called 'dominant' tolerance because they actively suppress the activation and expansion of self-reactive and over-reactive lymphocytes (Sakaguchi 2005; Simpson 2008; Waldmann 2008). Tregs have also been shown to promote tolerance to non-self graft-antigens. In fact, the field of transplant biology played a significant role in the eventual identification of these cells.

$CD4^+$ T cells were first implicated in the induction of transplant tolerance from studies showing that $CD4^+$ cells isolated from rats which were tolerant to their heart transplants after cyclosporin A treatment could prevent rejection of a heart graft of the same strain by naïve T cells (Hall et al. 1985). Furthermore, these $CD4^+$ cells were not recent émigrés from the thymus, as 'suppressor' $CD4^+$ cells could also develop in thymectomised mice. It was Waldmann et al., who showed that allospecific tolerance to allografts induced by anti-CD4 and anti-CD8 co-receptor blockade was reliant on suppressive $CD4^+$ cells (Qin et al. 1993). $CD4^+$ cells from tolerant animals could then prevent rejection by naïve $CD4^+$ or $CD8^+$ cells. Importantly, it was established that naïve T cells that were exposed to tolerant $CD4^+$ T cells became tolerant themselves, and were able to suppress fresh naïve T cells. This powerful

phenomenon where a population of tolerant cells can pass on tolerant properties to naïve cells was termed *infectious tolerance* (Qin et al. 1993; Waldmann 2008).

Groups working around the same time on the phenomenon of self-tolerance were also coming to the conclusion that a population of $CD4^+$ T cells was important for the immunoregulation of self-reactive T cells. The thymus was first identified as key for maintaining self-tolerance. Neonatal thymectomy around day 3 after birth could result in the development of systemic autoimmune damage (Kojima et al. 1976; Sakaguchi et al. 1982). The transfer of normal $CD4^+$ cells or $CD4^+$ thymocytes could prevent the development of autoimmunity, suggesting that $CD4^+$ cells, and perhaps a subset of them, were important for the maintenance of self-tolerance. Early search for markers distinguishing between self-reactive T cells and 'suppressor' T cells led to CD5 and the RB splice form of CD45 as two potential markers (Sakaguchi et al. 1985; Powrie et al. 1990). The transfer of $CD4^+$ cells depleted of $CD5^{high}$ or $CD45RB^{low}$ cells into lympho-deficient mice and rats led to the development of multi-organ inflammation and a wasting disease. The co-transfer of the eliminated population of cells prevented the development of this systemic autoimmunity (Sakaguchi et al. 1985).

Subsequently, a series of experiments led to the IL-2 receptor α chain (CD25) being put forward as a candidate marker for potential 'suppressor' $CD4^+$ cells (Sakaguchi et al. 1995). Inoculating $CD25^-$ splenic and lymph node-derived $CD4^+$ lymphocytes into T cell deficient nu/nu mice led to the development of multi-organ inflammation and circulating auto-antibodies. On the other hand, co-transfer of $CD25^+$ cells prevented the systemic autoimmunity. Furthermore, the authors showed that $CD25^+$ cells may play a role in dampening the immune response to allograft antigens. The co-transfer of $CD4^+$ cells

containing $CD25^+$ cells with $CD25^-$ cells into lympho-deficient animals led to slower rejection kinetics of allogeneic skin grafts than if $CD25^-$ cells were transferred alone. Shimizu et al. showed that $CD25^+$ cells can dampen tumour immunity (Shimizu et al. 1999). These immunosuppressive cells were termed "natural" regulatory T cells or 'nTreg' and CD25 has since become one of the best available surface marker for these cells. Subsequent studies of these cells led to the discovery of the transcription factor forkhead box P3 (Foxp3) as a 'master regulator' for the development and function of Tregs (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003).

Foxp3 is a member of the forkhead/winged-helix family of transcription factors. In the Scurfy mouse strain, the *Foxp3* gene, located on the X-chromosome, is mutated. These mice exhibit T cell hyper-expansion and activation leading to systemic inflammation (Brunkow et al. 2001). Mutation of the FOXP3 gene in humans causes a similar X-linked syndrome termed IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Gambineri et al. 2003). The pathological similarities between Scurfy/IPEX syndromes and the autoimmunity from Treg depletion studies led to work that identified Foxp3 as critical for the development of $CD4^+CD25^+$ Tregs. These studies revealed that in the resting state, Foxp3 is expressed by $CD4^+CD25^+$ peripheral T cells and $CD4^+CD25^+CD8^-$ thymocytes (Fontenot et al. 2003; Khattri et al. 2003). Foxp3 deficient mice were found to have few $CD4^+CD25^+$ T cells and develop scurfy-like systemic inflammation. These studies not only affirmed the importance of Foxp3 for the development of regulatory T cells, but also the critical role of these cells in the maintenance of peripheral self-tolerance.

Ontologically, $Foxp3^+$ Tregs generated centrally in the thymus are called 'natural' Tregs (nTreg). Thymic generation of nTreg involves high avidity interactions between self-peptide-

MHC complex on thymic cortical epithelial cells and T cell receptors on the developing thymocytes (Bensinger et al. 2001; Jordan et al. 2001; Apostolou et al. 2002). The co-stimulatory receptors CD28 and the cytokine IL-2 are also thought to be critical for nTreg lineage commitment (Schulze-Luehrmann et al. 2006; Ouyang et al. 2011). Recent studies have shown that Foxo proteins, downstream of the signaling intermediate Akt, directly regulate Foxp3 expression during nTreg development (Ouyang et al. 2010). Once generated, nTreg emigrate to the peripheral tissues, their self-antigen specificity allowing them to police self-reactive T cells that have escaped thymic negative selection. However, recent studies have shown that T cells with 'regulatory' properties can also arise in the periphery under various conditions. These include $Foxp3^-$ T cells in animals that had been rendered tolerant by co-receptor blockade (Graca et al. 2002), $Foxp3^+$ induced Tregs (iTreg), Tr1 cells (Groux et al. 1997) and a newly discovered subset of IL-35 secreting T cells (iTr35) (Collison et al. 2010).

The concept that 'suppressor' lymphocytes can be generated *de novo* in the periphery arose from experiments showing that therapeutically-induced transplant tolerance can be transferred to naïve cells which can in turn induce a new cohort of naïve cells to become tolerant to the graft antigen (infectious tolerance) (Qin et al. 1993). Interestingly, a subsequent study found that not only $CD4^+CD25^+$ cells, but $CD4^+CD25^-$ cells in mice that had been rendered tolerant to skin grafts by co-receptor blockade could prevent graft rejection by fresh splenocytes, although greater numbers of such $CD4^+CD25^-$ cells were required (Graca et al. 2002). In contrast, $CD4^+CD25^-$ cells from naïve mice could not prevent rejection by naïve splenocytes. In 2003, Chen et al. and other groups showed that Tregs can be induced *in vitro* by TCR activation in the presence of TGFβ (Chen et al. 2003; Fantini et al. 2004; Fu et al. 2004). Later, Cobbold et al. showed that when male-specific TCR transgenic mice devoid of nTreg were grafted with a male skin allograft under the cover of non-

depleting anti-CD4 antibodies, *de novo* Foxp3 expression and $CD4^+GITR^+$ cells were detected in the transplanted graft (Cobbold 2004).

At the same time, other groups demonstrated that the parenteral administration of subimmunogenic doses of cognate peptide either IV or subcutaneously to TCR-transgenic $CD4^+$ cells *in vivo* can also lead to the development of $CD4^+CD25^+$ cells that also express Foxp3 (Thorstenson et al. 2001; Apostolou et al. 2004; Verginis et al. 2008). Peptide antigen can also be coupled with anti-DEC 205 antibodies that target the peptide to dendritic cells in the T cell area of lymphoid tissues, which then instruct naïve T cells to become $Foxp3^+$ cells (Kretschmer et al. 2005). This study suggests that the peripheral generation of $Foxp3^+$ T cells from $Foxp3^-$ T cells is dependent on DCs, and importantly, dependent on TGFβ-signaling (see next sub-section). $CD103^+$ DCs in the gut-associated lymphoid tissues (GALT) have also been shown to mediate the generation of iTreg from naïve T cells (Coombes et al. 2007). The generation of iTreg in the gut is thought to be needed to dampen any immune response to gut commensal bacteria. Therefore, the disruption of this may lead to the development of inflammatory bowel disease.

Whilst $Foxp3^+$ nTreg and iTreg appear to be the dominant regulatory cells in a variety of contexts, there appears to be other $Foxp3^-$ regulatory type T cells that be induced from naïve T cells. These include Tr1 cells, which are $Foxp3^-$ $CD4^+$ cells that can be generated *in vitro* in the presence of IL-10 (Groux et al. 1997) or *in vivo* following the administration of oral antigen (Chen et al. 1994). These cells secrete IL-10 and TGFβ and can suppress the proliferation of naïve T cells *in vitro*. When adoptively transferred, they can also prevent the development of inflammatory colitis in lymphopenic mice. Recently, it was discovered that IL-35 secreted by activated T cells can promote the generation of a unique set of T cells

called iTr35 cells (Collison et al. 2010). These cells are Foxp3$^-$, secrete IL-35, and can suppress inflammation in a number of experimental models including experimental autoimmune encephalomyelitis and inflammatory bowel disease.

Regulatory T cells are a key member in the body's armoury of peripheral tolerance mechanisms. Their existence and critical importance for the regulation of auto-reactive T cells are beyond doubt. A deeper understanding of their development, mechanism of action and the division of labour of the various sub-classes of regulatory cells under both normal and pathological states will facilitate the development of specific and powerful therapeutics.

1.2 The role of Foxp3 in the function of Tregs

Early studies showed that Foxp3 deficiency resulted in a deficiency of nTreg and the development of a multi-organ inflammatory syndrome in both humans and mice (Brunkow et al. 2001; Gambineri et al. 2003). This suggested that Foxp3 was critical for the development of these cells. However, Foxp3 appears not only important for their development but also function. In recent times, the role played by Foxp3 in Treg function is being increasingly unravelled by molecular techniques, gene expression studies and retroviral transduction techniques.

Foxp3 is shown to interact with a number of transcription factors. It can bind factors such as nuclear-factor of activated T cells (NFAT) which is normally bound to AP-1 and NF-κB to promote the expression of genes such as IL-2 and IL-4. Foxp3 binding to NFAT turns the gene expression program of T cells from 'activation' to a 'suppressor' one, with downstream repression of IL-2 expression and upregulated expression of CD25 and CTLA-4 (Wu et al.

2006). Foxp3 can also physically bind to NF-κB to suppress its transcription of cytokines associated with T cell activation (Bettelli et al. 2005).

The importance of Foxp3 for Treg function was also shown in a study where the transgenic over-expression of Foxp3 in $CD4^+$ cells can lead to both $CD8^+$ and $CD4^+CD25^-$ cells adopting a regulatory phenotype *in vitro* (Khattri et al. 2003). Moreover, the ectopic expression of a *Foxp3* transgene in $CD4^+CD25^-$ T cells using retroviral transduction can confer suppressive properties *in vitro* (Hori et al. 2003). When these Foxp3-transduced cells were transferred *in vivo*, they could also inhibit the development of autoimmune colitis. Subsequently, a number of other groups have investigated the *in vivo* functional properties of Foxp3-transduced cells with mixed results (Jaeckel et al. 2005; Loser et al. 2005). In a non-lymphopenic mouse type 1 diabetes model, $1x10^6$ Foxp3-transduced polyclonal $CD4^+$ cells were ineffective in stopping established disease (Jaeckel et al. 2005). In a non-lymphopenic model of contact sensitivity, the injection of $2x10^6$ Foxp3-transduced polyclonal $CD4^+$ cells resulted in a partial suppression of allergic contact hypersensitivity (Loser et al. 2005). In another study, polyclonal Foxp3-transduced $CD4^+$ cells failed to prevent the development of collagen-induced arthritis (Andersen et al. 2008). Reasons for this include a possible low frequency of antigen specific cells within the transferred population or possibly altered homing to secondary lymphoid organs as a result of reduced expression of CD62L by transduced cells (Andersen et al. 2008).

Although Foxp3-transduced $CD4^+$ cells were effective in preventing minor-antigen mismatched skin graft rejection, they were shown to be most effective when they were TCR-specific for the graft antigen as polyclonal transduced cells were unable to prevent rejection

(Chai et al. 2005). The mixed efficacy of ectopic Foxp3-expressing cells raised the possibility that other factors may be important for Treg function.

In a study where the Foxp3 gene was deleted and replaced by a green fluorescent protein (GFP) gene in order to monitor transcriptional activity at the Foxp3 locus, the $GFP^{+}Foxp3^{null}$ cells surprisingly demonstrated some of the features of wildtype $Foxp3^{+}$ cells *in vitro* including anergy and intermediate expression of regulatory T cell markers CD25, CTLA-4 and GITR (Gavin et al. 2007). $GFP^{+}Foxp3^{null}$ also secreted very little IL-2 and other Th1 or Th2-type cytokines (IL-4, IFNγ, TNFα), suggesting that there may be Foxp3-independent repression of cytokine expression in these cells.

Sugimoto et al. compared the gene expression profiles of nTreg and Foxp3-transduced cells and showed that there were genes such as galectin and granzyme B that were transcribed in nTreg but not Foxp3-transduced cells (Sugimoto et al. 2006). This provided further evidence to the existence of Foxp3-independent processes in nTreg. In corroboration, Hill et al. performed a meta-analysis of gene expression profiles from nTreg, TGFβ-induced Tregs and Foxp3-transduced T cells and showed that Foxp3 expression only induced a fraction of the nTreg transcriptome (Hill et al. 2007). Other genes in the nTreg transcriptome were controlled by signals such as T cell activation through TCR, IL-2 and TGFβ. Of the genes that correlated with Foxp3 expression, there were some which were not directly induced by it, thus pointing to the existence of higher-order regulator(s). A recent paper suggested that Foxo transcription factors may be the higher order signal in both nTreg and TGFβ-induced Tregs as they can bind the *Foxp3* locus and control *Foxp3* promoter activity in these cells (Ouyang et al. 2010)

Hence, Foxp3 appears to be important and necessary for the functional phenotype of nTreg. However, other independent factors such as TGFβ, TCR activation and Foxo expression may also contribute to the regulatory phenotype. Indeed, it remains unclear if Foxp3 is *sine qua non* for the function of TGFβ-induced Tregs. Further investigation is required to dissect the relative importance of Foxp3 and factors such as TGFβ for the function of regulatory T cell subtypes.

1.3 TGFβ: role in therapeutic tolerance and the generation of iTreg

TGFβ has been shown to be important for the therapeutic induction of tolerance. This effect was later attributed to its ability to induce the generation of $Foxp3^+$ Tregs (iTreg) from $Foxp3^-$ cells in the periphery. However, a number of outstanding questions remain with regards to their behaviour *in vivo* including stability, mechanism and location of their action, and their contribution to the phenomenon of infectious tolerance.

TGFβ is a member of a family of regulatory cytokines with pleiotropic effects on a range of cell types in various settings including inflammation, wound healing, carcinogenesis, embryogenesis and immune regulation (Li et al. 2008). The predominant form TGFβ1 (others being TGFβ2 and TGFβ3) is secreted as a homodimer coupled with the latency-associated protein (LAP). TGFβ becomes active only when LAP undergoes proteolysis via a TGFβ activator produced by cells in various contexts. TGFβ activators can be integrins, reactive oxygen species or matrix metalloproteinases. TGFβ binding to its receptor – a tetrameric complex consisting of 2 TGFβ type I and two type II receptors, leads to the phosphorylation of transcription factors Smad2 and Smad3, which in turn translocate to the nucleus to complex with Smad4 and TIF1γ (Shi et al. 2003). TGFβ can also activate Smad-independent

pathways including PI3 kinase and mitogen-activated protein kinase pathways (Derynck et al. 2003).

Studies have shown that TGFβ is inextricably linked to peripheral tolerance. The deficiency of TGFβ1 leads to a systemic inflammatory state similar to that seen in Foxp3 deficiency, with hyperactivation of CD4 cells (Kulkarni et al. 1993). T cell-specific TGFβ receptor deficiency generates a neonatal inflammatory disease also similar to TGFβ1 deficiency or Foxp3 deficiency (Marie et al. 2006). This suggests that T lymphocytes are targets for TGFβ in the establishment of peripheral tolerance. Waldmann et al. demonstrated a role for TGFβ in therapeutically induced transplant tolerance by showing that T cell co-receptor blockade-induced transplant tolerance could be abrogated by anti-TGFβ blocking antibodies (Cobbold et al. 2004). Such therapeutically induced tolerance involved the *de novo* expression of Foxp3 and generation of $CD4^+GITR^+$ cells in the allograft, thus hinting at the link between TGFβ and the induction of Tregs in the periphery. Later, it was established that TGFβ-signalling specifically through T cells was critical for establishing therapeutic transplant tolerance (Daley et al. 2007).

Studies that examined the generation of iTreg in the periphery showed that this was dependent on unimpaired TGFβ signalling to the T cells (Kretschmer et al. 2005). $CD103^+$ DCs in the GALT also mediated the conversion of naïve T cells to $Foxp3^+$ iTreg in a TGFβ-dependent manner (Coombes et al. 2007). $Foxp3^+$ T cells could also be generated *in vitro* from TCR activation of $Foxp3^-$ cells in the presence of TGFβ (Chen et al. 2003; Fantini et al. 2004; Fu et al. 2004). IL-2 was also reported as being necessary for TGFβ-induced Foxp3 expression in naive $CD4^+$ cells (Davidson et al. 2007; Zheng et al. 2007). The molecular mechanisms for the induction of Foxp3 expression in the context of TGFβ-conditioning and

TCR stimulation are far from clear. It has been shown that Smad3 can bind to the Foxp3 enhancer (Tone et al. 2008). T cells that lack Smad3 show impaired Foxp3 induction. Interestingly, whilst TGFβ-induced Smad2/3 phosphorylation can be detected within minutes of TGFβ treatment, Foxp3 mRNA cannot be detected until 12-24 hrs after treatment (Chen et al. 2010). There are reports that all-trans retinoic acid (ATRA) can increase TGFβ-induced Foxp3 expression. ATRA may mediate its actions by enhancing Smad3 signaling (Xiao et al. 2008) or by relieving cytokine-mediated inhibition from a subset of $CD4^{+}CD44^{hi}$ cells (Hill et al. 2008). There are potentially other as yet unidentified transcription factors that may mediate Foxp3 induction via Smad3-independent mechanisms or by binding to the Foxp3 promoter.

The efficacy of *in vitro* generated iTreg in suppressing inflammation *in vitro* or *in vivo* has been variable. Indeed, $1x10^{7}$ antigen-specific TGFβ-conditioned T cells could prevent rejection of a male graft in a recipient whose T cells are specific for the HY antigen (Daley et al. 2007). TGFβ-induced Tregs could also inhibit the development of colitis when co-transferred with $CD4^{+}CD45RB^{hi}$ cells into a lymphopenic recipient. Whilst TGFβ-induced Tregs have been shown to be suppressive *in vitro* in a number of studies (Chen et al. 2003; Fantini et al. 2004), others have failed to demonstrate this capacity in their *in vitro* suppression assay (Hill et al. 2007). Most of these cells appear to lose Foxp3 expression after being re-stimulated with antigen in the absence of exogenous TGFβ (Floess et al. 2007). Aside from doubts about their suppressive potency, there are still questions regarding their stability in vivo, mechanism of action, whether they require Foxp3 for their function and also their contribution to infectious tolerance. Furthermore, there is a need for better assessment of the suppressive capacity of iTreg that can overcome the technical constraints and interpretive limitations associated with *in vitro* suppression assays.

1.4 Delineating the mechanism of Treg action using *in vitro* assays

It is far from clear how Tregs function *in vivo*. Since a good understanding of their mechanism of function can be harnessed for the generation of therapies, there is a need for continued investigation to dissect their mechanism of action. Whilst it is ultimately critical to understand their mechanism of function *in vivo*, a reductionist approach using *in vitro* assays is thought to offer valuable insight into their action *in vivo*. Nevertheless, it is becoming clear that findings from *in vitro* assays have limitations when translated to the *in vivo* scenario. In the culture dish, Tregs are not in their physiological environment which would contain a plethora of interacting factors such as stromal cells and as yet unidentified soluble factors (Waldmann et al. 2009). Indeed, their behavior and mechanism of action may differ depending on their location and their microenvironment, whether it is the lymphoid tissue or non-lymphoid tissue such as graft tissue. As a result, findings from both *in vitro* and *in vivo* studies may not correspond and indeed may be conflicting.

One *in vitro* assay used commonly is the cell-contact-based suppression assay. Much of currently accepted properties of Tregs come from such assays. However, the set-up of such assays has varied between laboratories, some using irradiated or mitomycin C-treated APCs for the activation of responder T cells, whilst others using plate-bound or soluble anti-CD3 and/or anti-CD28. In those groups that do not use APCs for responder T cell stimulation, any effect on APCS would not be measured. From such assays, some groups propose that Tregs function by inhibiting IL-2 production by responder T cells (Thornton et al. 1998), others suggest that Tregs compete with responder T cells for IL-2, resulting in apoptosis of the responder T cells (Pandiyan et al. 2007). However, the involvement of either of these mechanisms *in vivo* is still unclear.

The production of immunosuppressive cytokines by Tregs has also been proposed as a mechanism of Treg suppression. However, there is little evidence from *in vitro* studies that secreted IL-10 or TGFβ, both archetypal immunosuppressive cytokines, are involved in *in vitro* suppression of responder T cells (Thornton et al. 1998; Piccirillo et al. 2002). On the other hand, TGFβ has been shown to be essential for therapeutically-induced transplantation tolerance *in vivo* (Cobbold et al. 2004). Specifically, the loss of TGFβ-signaling in T cells as a result of expression of a dominant-negative receptor on T cells led to failure of coreceptor-blockade induced transplant tolerance (Daley et al. 2007). However, whether TGFβ in this setting is related to the loss of induction of iTreg or the failure of TGFβ signaling to alloreactive T cells remains to be investigated.

Another inconsistency between *in vitro* and *in vivo* observations of Treg behaviour include the finding that while Tregs appear to be anergic to TCR stimulation *in vitro*, they have been shown to expand in response to cognate antigen simulation *in vivo* (Walker et al. 2003).

In vitro suppression assays involving APCs as activators of responder T cells have also raised the possibility of the APC as a potential target of Treg function. nTreg constitutively express CTLA-4, and this has been shown to downregulate CD80 and CD86 expression on DCs, thus blocking costimulation (Onishi et al. 2008; Wing et al. 2008). The downregulation of CD80 and CD86 on DCs could not be shown when DCs were co-incubated with CTLA-$4^{-/-}$ Tregs. APCs such as DCs are critical for the initiation of the immune response (Banchereau et al. 2000). Therefore, modification of their costimulatory capacity may be a mechanism for Tregs to control the downstream immune response (Farquhar et al.).

One other possible mechanism whereby Tregs can influence DC function is through CD73, expressed on an overwhelming majority of TGFβ-induced Tregs (Frederico Regateiro, DPhil Thesis, 2009). CD73 is an ectoenzyme responsible for the conversion of AMP to the strongly immunosuppressive nucleoside adenosine (Thomson et al. 1990). Adenosine can act via the A2A receptor on T cells to prevent their activation and proliferation (Huang et al. 1997). Adenosine can also act on mature DCs to alter their cytokine expression profile from a Th1-polarising one to that of Th2. This involved reduced production of IL-12 and TNFα by the DCs and increased production of IL-10 (Panther et al. 2003). Hence, CD73-induced adenosine generation can be a potential mechanism of iTreg-mediated immunosuppression.

The ability of Tregs to modify APC function has also led to the development of assays that examine their physical interaction *in vitro.* Prior to this, observations of Treg-DC interaction were made *in vivo* using two-photon laser microscopy showing that the presence of nTreg prevents antigen-specific T cells from forming stable long interactions with antigen-loaded DCs (Tadokoro et al. 2006). Interestingly, polyclonal $CD4^+CD25^+$ Treg cells could also reduce stable interactions between antigen-specific T cells and DCs, albeit to a smaller degree (Tadokoro et al. 2006). Using the same technique of 2 photon laser scanning microscopy, Tang and colleagues showed that both $CD4^+CD25^-$ cells and nTreg can form stable interactions with antigen-loaded DCs *in vivo*. However, whereas $CD4^+CD25^-$ cell-DC interaction leads to proliferation and expansion of T cells, Treg-DC interaction led to reduced $CD4^+CD25^-$ cell priming (Tang et al. 2006). Both studies, however, did not examine the functional impact of the Treg-DC interaction on the DCs.

Onishi et al. examined Treg-DC interaction in a controlled *in vitro* setting using antigen-specific nTreg and naïve $CD4^+$ cells (Onishi et al. 2008). When fluorescently-labeled nTreg

were co-cultured with responder T cells (naïve T cells) labeled in a different colour in the presence of DCs, nTreg could out-compete responder T cells in forming aggregates around DCs, thus diminishing responder T cells' access to DCs. This process was antigen-dependent as aggregates did not form without antigen. In a separate functional assay, the authors showed that the incubation of nTreg with responder T cells and DCs led to reduced expression of CD80 and CD86 compared to DCs incubated with responder cells alone. This suggests that nTreg may function by preferentially interacting with DCs, thus physically displacing potential pro-inflammatory T cells, and functionally inhibiting the activation of DCs. What the relative contribution of the former and latter is on the overall 'suppression' of an inflammatory response is still unclear. Furthermore, the aforementioned studies have focused on nTreg and little is known about the interaction of iTreg or indeed other T cell subsets with DCs.

In summary, whilst *in vitro* assays have provided some insights into the mechanism of function of Tregs, they cannot replicate the complexity of the environment in which Tregs act *in vivo*. Hence, observations of Treg function *in vitro* may provide limited information and may indeed be misleading. This is especially true for suppression assays. Newer *in vitro* assays such as the aggregation assay reported by Onishi (Onishi et al. 2008) have their own limitations.

1.5 The stability and plasticity of induced Tregs

The stability of iTreg, whether generated *in vivo* in response to antigen or *in vitro* from TGFβ-conditioning and TCR activation, is still an area of ongoing research. A greater understanding of the factors that influence their stability *in vivo* will aid in the development of therapies

that can harness their inherent advantage over nTreg such as the potential to generate an unlimited number of antigen-specific Foxp3^{+} Tregs.

Studies have shown that the immediate cytokine environment can determine if the differentiation program of uncommitted naïve T cells will be that of regulatory T cell or an inflammatory cell. Whilst TGFβ alone can induce their differentiation into iTreg, TGFβ in combination with IL-6 leads to differentiation into Th17 cells (Bettelli et al. 2006). TGFβ in combination with IL-4 can also drive the differentiation of the IL-9 secreting Th9 cells (Dardalhon et al. 2008; Veldhoen et al. 2008). At least *in vitro*, iTreg themselves also appear to be able to re-differentiate into an inflammatory phenotype in the presence of IL-6 (Yang et al. 2008) or IL-23 (Osorio et al. 2008). Furthermore, Foxp3^{-} cells that were potentially derived from Foxp3^{+} iTreg appeared to have a memory-like phenotype and can secrete inflammatory cytokines (Zhou et al. 2009).

Gene expression studies have revealed distinct profiles for nTreg and *in vitro* generated Tregs (Hill et al. 2007; Haribhai et al. 2009). In the study of Haribhai and colleagues, *in vitro* TGFβ-conditioned cells were generated from Foxp3^{GFP} mice and Foxp3^{+} iTreg were sorted based on GFP expression (Haribhai et al. 2009). In the study of Hill and colleagues, unsorted TGFβ-conditioned cells were used for the gene expression study (however, the authors claim over 90% of TGFβ-conditioned CD4^{+} cells became Foxp3^{+}) (Hill et al. 2007). In both studies, iTreg differed from nTreg in that they displayed many genes which were related to TGFβ exposure, including genes for lymphotoxin α, aryl hydrocarbon receptor and CTLA-2b. Nevertheless, a number of genes were shared by nTreg and iTreg including CD103, CD25 and neuropilin-1. In the case of nTreg, chromatin immunoprecipitation studies have shown that the nTreg transcriptome contains genes directly bound by Foxp3 but also genes indirectly

modulated downstream (Zheng et al. 2007). In regard to Foxp3 expression, a significant percentage of iTreg appear to lose Foxp3 expression when transferred *in vivo* (Kretschmer et al. 2005; Haribhai et al. 2009). In contrast, >95% of nTreg retain Foxp3 expression 14 days after transfer into wildtype syngeneic recipients (Floess et al. 2007). Analysis of the *Foxp3* locus of nTreg and iTreg have shown that CpG motifs at the enhancer 2 region (also known as conserved non-coding sequence 2 or Treg-specific demethylated region) of the Foxp3 locus in iTreg is only partially demethylated, whereas this is fully demethylated in nTreg, implying greater phenotypic stability of nTreg (Floess et al. 2007; Lal et al. 2009).

To fully exploit the therapeutic potential of iTreg, a better understanding of the factors that influence their apparent instability is required. Such factors can be targeted to promote greater stability of these cells *in vivo*.

1.6 Thesis aims

Understanding how induced Tregs function and what factors influence their stability *in vivo* is vital for harnessing their powerful suppressive capacity for therapeutic tolerance induction. The advantage of iTreg over nTreg is the potential for the body to generate a large number of iTreg with a specificity determined by the inflammatory context.

Despite iTreg and nTreg sharing many properties, there are distinct differences in their genetic expression profiles and behaviour *in vivo*. As yet, it is unclear what role TGFβ has in producing final suppressive phenotype of iTreg. TGFβ-exposure alone induces the expression of a number of molecules independent of Foxp3 (Frederico Regateiro, DPhil Thesis, 2009). This includes CD73, which can mediate the production of the immunosuppressive molecule

adenosine. TGFβ can also directly act on $CD4^+$ and $CD8^+$ cells such that disturbed TGFβ signaling due to expression of a dominant-negative form of the TFG-β type II receptor on these cells results in their hyper-activation and differentiation (Gorelik et al. 2000). TGFβ can also act directly on DCs to hamper their maturation and activation with reduced class II MHC and CD86 expression, resulting in their providing lower levels of costimulation to T cells (Lu et al. 1997; Yamaguchi et al. 1997). Hence, TGFβ-exposure *in vitro* in the presence of DCs alone may confer a suppressive phenotype to the T cells. Alternatively, the end target of TGFβ-exposure in iTreg physiology may be Foxp3 expression. Here, the behaviour of TGFβ-conditioned iTreg in a skin transplantation system will be examined. Transplantation provides a good system to study the function of Tregs because the allograft provides a powerful and persistent source of antigenic stimulation. Furthermore, the degree, nature and temporal course of the immune response to the graft can be readily followed visually. The aims of the thesis are as follows.

Firstly, the role of TGFβ-exposure for the function of iTreg (defined as $Foxp3^+$ TGFβ-conditioned $CD4^+$ cells) was dissected by using a transgenic mouse that co-expresses a surface hCD2 molecule with Foxp3 in $CD4^+$ cells, thus allowing the purification of Foxp3-expressing cells (Komatsu et al. 2009).

The second aim was to understand the factors that impact on the stability of iTreg *in vivo* and are crucial to ensure their effective suppression in an inflammatory context and to avoid their 'conversion' to an inflammatory phenotype. Here, the role of antigen and its form of delivery in maintaining the stability of iTreg *in vivo* was investigated by means of graft transplantation and cognate peptide injected intravenously.

Thirdly, in comparison with TGFβ-conditioned T cells that do not express Foxp3, the suppressive capacity of T cells that express Foxp3 only in the transplantation context was also examined. Whilst this was examined in a previous study (Chai et al. 2005), here a system of conditional ectopic Foxp3 expression was used, thus allowing me to switch Foxp3 expression on or off on demand. Thus, I could interrogate if transduced-alloreactive T cells can be 'converted' into a regulatory phenotype at a later stage in the rejection response. The conditional system also allowed me to examine if Foxp3-transduced cells were still required for the maintenance of graft tolerance when the graft appeared to be well-healed.

Finally, the modification of DC activation has been shown to be an important mechanism of nTreg function (Onishi et al. 2008; Wing et al. 2008). This involved a down-modulation of DC surface CD80 and CD86. Furthermore, nTreg have been reported to aggregate around DCs in preference to naive T cells (Onishi et al. 2008). I asked whether similar mechanisms of preferential aggregation and functional de-activation might operate for TGFβ-conditioned induced Tregs and ectopic Foxp3 expressing T cells or if indeed, the competition assay merely reflects antigen-experience of the clustering cells.

CHAPTER 2 MATERIALS AND METHODS

2.1 Experimental animals

Mice were bred and maintained under specific pathogen-free conditions at the Sir William Dunn School of Pathology (University of Oxford, Oxford, UK). B6.hCD2.KI mice were made in collaboration with Shohei Hori. Briefly, a targeting construct encoding a human CD2-human CD52 fusion protein along with an internal ribosomal entry site was inserted into the 3' untranslated region of the *Foxp3* gene on the X-chromosome. All experimental procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 and received local ethical committee approval. All mice strains used are listed in **Table 2.1**.

Table 2.1

Mouse Strain	Abbreviation	Haplotype	TCR transgenic	Presence of nTregs	Reference
CBA/Ca	CBA	H-2^{k}+CBA minors	N o	Yes	
CBA/Ca.RAG-1$^{-/-}$	CBA.RAG$^{-/-}$	H-2^{k}+CBA minors	No	No	
C57B10.BR	B10.BR	H-2^{k}+B10 minors	No	Yes	
A1 (M).RAG-1$^{-/-}$	A1.RAG$^{-/-}$	H-2^{k}+CBA minors	Yes, recognise *DBY*-encoded peptide[1] presented by H-2E^{k}	No	(Zelenika et al. 1998)
A1(M).Rag-1$^{-/-}$ x CBA/Ca F1	A1.RAG$^{-/-}$ x CBA F1	H-2^{k}+CBA minors	Yes, as above	Yes	
C57BL/6	B6	H-2^{b}+B6 minors	No	Yes	
C57BL/6.RAG-1	B6.RAG$^{-/-}$	H-2^{b}+B6 minors	No	No	
B6.SJL	B6.SJL	H-2^{b}+B6 minors+Ptprc^{a} allele encoding the lymphocyte surface antigen CD45.1 or Ly5.1	No	Yes	(Waterstrat et al. ; Shen et al. 1985)
Marilyn.Rag-1$^{-/-}$	Marilyn	H-2^{b}+B6 minors	Yes, recognise *DBY*-encoded peptide[2] presented by H-2A^{b}	No	(Lantz et al. 2000)
Marilyn.Rag-1$^{-/-}$ .Foxp3^{hCD2}	Marilyn.hCD2.KI	H-2^{b}+B6 minors	Yes, as above	No	
C57BL/6.Foxp3^{hCD2}	B6.hCD2.KI	H-2^{b}+B6 minors		Yes	(Komatsu et al. 2009)

Table 2.1 Experimental Animals

[1] HYE^{k} peptide: REEALHQFRSGRKPI
[2] HYA^{b} peptide: NAGFNSNRANSSRSS

2.2 Media and buffers

4-hydroxtamoxifen (Sigma-Aldrich, UK)
Working concentration: 20ng/ml (in culture medium)

2xHBS
H_2O
200mM NaCl
10mM KCl
1.5mM Na_2HPO_4
12mM Dextrose
50mM HEPES

AUTOMACS running buffer
PBS
0.5% w/v BSA
2mM EDTA

AUTOMACS rinsing buffer
PBS
2mM EDTA

Chromatin immunoprecipitation lysis buffer
50mM TrisCl
10mM EDTA
1% SDS
Protease inhibitors (EDTA-free, Roche)
1mM PMSF

Fluorescence activated cell sorting (FACS) wash buffer
PBS
0.5% w/v bovine serum albumin (BSA)

FACS fixing solution
PBS
0.5% w/v BSA
2% w/v paraformaldehyde

FACS saponin intracellular staining buffer
PBS
0.5% w/v saponin (Sigma-Aldrich, UK)
0.5% w/v BSA
5% v/v Heat inactivated-NRS
0.1% w/v NaN_3

18.5% formaldehyde

H_2O	4.8ml
Paraformaldehyde	0.92g

1N KOH 35µl
Heated in microwave to dissolve.

IMDM complete
IMDM (PAA Laboratories, Somerset)
10% v/v ultra low Ig foetal calf serum (Gibco)
5mg/ml gentamicin
50µM 2-mercaptoethanol

Linear acrylamide carrier
5mg acrylamide in 200ul TE
1ul 10%APS
1ul TEMED
Mixed together and allowed to set, precipitated with ethanol. The pellet was dried and resuspended in 500µl of distilled H2O (ie. 10µg/µl).
Used 10-20 µg per precipitation.

R10
RPMI-1640 (PAA Laboratories, Somerset)
10% v/v ultra low protein foetal calf serum (Gibco)
50ug/ml Penicillin-Streptomycin
2mM L-glutamine
50µM 2-mercaptoethanol

Radioimmunoprecipitation assay (RIPA) buffer
10mM TrisCl
140mM NaCl
1mM EDTA
0.5mM EGTA
1% TX100
0.1% SDS
0.1% Na deoxycholate
Protease inhibitors (added before use for "RIPA ChIP buffer"):
1mM PMSF, 20mM Na butyrate

Phosphate-buffered saline (PBS)
137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4
1.47 mM KH_2PO_4
Adjust to a final pH of 7.4

Tamoxifen (Sigma-Aldrich, UK)
Working concentration: 10mg/ml in 10% v/v ethanol/oil mix for in vivo i.p. injections.

Tris-buffered ammonium chloride (TBAC)
9 parts 0.15M NH_4Cl
1 part 0.17M Tris
pH adjusted to pH 7.2 with 4M HCl

2.3 Antibodies

Antibodies used in this thesis are listed in **Table 2.2**.

Table 2.2

Specificity	Conjugate	Clone	Isotype	Company
CD3e	PerCP	145-2C11	Armenian hamster IgG1, k	BD Pharmingen
CD4	FITC	H129.19	rat (LOU) IgG2a, k	BD Pharmingen
	PerCP	RM4-5	rat (DA) IgG2a, k	BD Pharmingen
	APC	RM4-5	rat (DA) IgG2a, k	BD Pharmingen
CD8a	PE	53-6.7	rat (LOU) IgG2a, k	BD Pharmingen
CD11c	PerCP-Cy5.5	HL3	Armenian hamster IgG1, λ2	BD Pharmingen
CD25	PE	PC61	rat (OFA) IgG1, λ	BD Pharmingen
CD39	PE	24DMS1	rat IgG2b, k	eBioscience
CD40	PE	1C10	rat IgG2a, k	SouthernBiotech
CD62L	APC	MEL-14	rat (F344) IgG2a, k	BD Pharmingen
CD73	PE	eBioTY/11.8	rat IgG1	eBioscience
CD80	APC	1G10	rat IgG2a, k	abcam
CD86	APC	GL1	rat (LOU) IgG2a, k	BD Pharmingen
CD103	PE	M290	rat (LOU) IgG2a, k	BD Pharmingen
CD152	PE	UC10-4F10-11	hamster IgG1, k	BD Pharmingen
F4/80	PE	CI:A3-1	rat IgG2b	Serotec
GITR	PE	YGITR765	IgG2b	Serotec
I-A/I-E	FITC	2G9	rat (DA) IgG2a, k	BD Pharmingen
human CD2 domain 2	-	YTH655	rat IgG2b mAb	SWDSOP[1]
PD-1	PE	J43	Armenian hamster IgG	eBioscience
Foxp3	FITC	FJK-16s	rat IgG2a, k	eBioscience
	PE	FJK-16s	rat IgG2a, k	eBioscience
	APC	FJK-16s	rat IgG2a, k	eBioscience

Table 2.2 Antibodies

[1] Sir William Dunn School of Pathology

2.4 Cytokines for cell culture and synthetic peptides

Lyophilised IL-2 and IL-7 were purchased from Peprotech (UK) and reconstituted in sterile water. Lyophilised recombinant human TGFβ-1 was purchased from R&D Systems and reconstituted in 4mM HCl with 1mg/ml BSA. Supernatants enriched for GM-CSF were harvested from a cultured X63 cell line transfected with cDNA constructs encoding murine GM-CSF (D. Gray, Edinburgh). Cytokine-enriched supernatants were a gift of E. Adams who quantified cytokine concentrations. The GM-CSF containing supernatant was used at 2% v/v, which was equivalent to 5ng/ml of GM-CSF. Synthetic peptides, HYE^{k} (REEALHQFRSGRKPI) (Scott et al. 2000) and HYA^{b} (NAGFNSNRANSSRSS) (Lantz et al. 2000), were re-constituted in PBS from lyophilized powder and both used at a maximum working concentration of 100nM.

2.5 Preparation of dendritic cells, T cells and T cell clones

2.5.1 Bone marrow dendritic cell preparation

Bone marrow-derived DCs (BmDC) were prepared using a protocol adapted from Inaba et al (Inaba et al. 1992). Briefly, femurs and tibias were dissected from CBA or B6 mice (6-10 week old). Connective tissue and muscle attached to the bones were removed. A 10ml syringe and 26 gauge needle (Terumo, Europe) were used to flush the bones with R10 medium to extrude the bone marrow. The cells were then passed through a 70µm cell strainer (Becton Dickinson, UK) to remove unwanted debris and tissues. The cells were then pelleted by centrifugation, and resuspended in 45ml R10 medium supplemented with 2% GM-CSF-containing supernatant from the X63-GM-CSF cell line. The cell suspension was divided into three 100mm diameter petri dishes (Corning, US) at 15ml per dish. The cells were cultured for 7 days, with removal of non-adherent cells and the addition of fresh medium on day 3. On day 6, BmDCs were ‘re-plated’ where loosely adherent cells were first removed by

pipetting over the surface of the dish, then pelleted by centrifugation, and finally resuspended in fresh R10 medium containing GM-CSF and plated onto fresh petri dishes. On day 7 of culture, BmDCs were harvested by gently pipetting over the surface of the petri dish and collecting the cell suspension. Thus, tenuously adhered BmDCs were collected whilst tightly adherent stromal cells were discarded. BmDCs were then frozen down at -80^{o}C in 5x10^{6} aliquots in 10% dimethylsulfoxide (Sigma-Aldrich, UK). Depending on experimental needs, BmDCs were treated before freezing with 25µg/ml Mitomycin C (Sigma-Aldrich, UK) for 30min at 37^{o}C.

2.5.2 CD4^{+} T cell preparation

CBA CD4^{+} cells were isolated by negative selection using the AutoMACS separator as per the manufacturer's instructions (Miltenyi Biotec, UK). Spleens were taken from mice and a single-cell suspension was obtained by passing the splenocytes through a 70µm cell strainer. Splenocytes were then resuspended in MACS buffer (PBS 0.5% BSA 2mM EDTA) and incubated with biotin-antibody cocktail (antibodies against CD8a, CD11b, CD11c, CD19, CD45R, CD49b, CD105, Anti-MHC class II, and Ter-119) for 10 min at 4°C. Subsequently, more MACS buffer was added, and cells were incubated with anti-biotin microbeads for 15 min at 4°C. Cells were then washed in MACS buffer and resuspended in 500µl of MACS buffer. CD4^{+} T cells were sorted by magnetic depletion on the AutoMACS (program 'Deplete').

2.5.3 *In vitro* generation of induced Tregs

Splenocytes from A1.RAG$^{-/-}$ or Marilyn mice were treated with TBAC for 30 seconds before washing in R10 medium. 5x10^{5} splenocytes were then resuspended per well in a 24-well plate (Corning, US) along with 1x10^{5} mitomycin-treated CBA female or B6 female BmDCs.

HYE^{k} or HYA^{b} peptide was added at 100nM. For TGFβ-conditioned iTreg cultures, recombinant human TGFβ was also added at 2ng/ml. Control cell cultures received peptide only. In some cases, all-trans retinoic acid (ATRA) (Sigma-Aldrich, UK) was added at 10nM ('ATRA-treated' cultures). On day 7, lymphocytes from the cultures were harvested by Ficoll-Hypaque (1.083g/ml; Sigma-Aldrich) separation. The harvested cells were then used *in vitro* or resuspended in 1% PBS/BSA for adoptive transfer into mice. The percentage of Foxp3 expression was assessed using flow cytometry and was typically 20-40% for A1-RAG$^{-/-}$ TGFβ-conditioned cultures, and 50-90% for Marilyn.RAG$^{-/-}$ TGFβ-conditioned cultures i.e. 50-90% of TGFβ-conditioned cultures were Foxp3^{+} iTregs.

2.5.4 Preparation of polarised T cell clones

Dr. Elizabeth Adams generated the Th1 (R2.2), Th2 (R2.4), Tr1 (Tr1D1) and Th9 (3E7) polarised cell clones. The Th1 and Th2 clones were established by culturing spleen cells from a female A1.RAG$^{-/-}$ mouse which had rejected two grafts. The cells (5x10^{5}/well) were polarised by three cycles of weekly stimulation with male 5x10^{6} spleen cells in the presence of IL-2 (50U/ml) for Th1 and IL-4 (200U/ml) for Th2. Viable cells were then cloned at limiting dilution in 96 well plates with APCs (2x10^{5} male CBA MMC-treated spleen cells), the appropriate cytokine, expanded into 24 well plates and maintained by fortnightly stimulation of 2x10^{5} clone cells with 5x10^{6} male MMC-treated spleen cells and the appropriate cytokine.

Tr1D1 clone was generated from splenocytes from naïve female A1.RAG$^{-/-}$ mice, stimulated in recombinant mIL-10 (R&D Systems, 10ng/ml) for 3 weeks before cloning (Groux et al. 1997; Zelenika et al. 2001). The clone was maintained by culturing 2x10^{5} cloned Tr1D1 cells with 5x10^{6} male splenocytes in 24 well plates in medium containing IL-2 and IL-4, each at 20U/ml.

The 3E7 Th9 clone was generated by culturing $CD4^+$ cells from naïve female A1.$RAG^{-/-}$ spleens with female CBA spleen cells in 100nM HYE^k peptide, 1ng/ml rTGFβ, 10ng/mIL-4 for one week. Viable cells were recovered and restimulated as previously. After three cycles of weekly activation, cells were cloned in at limiting dilution in 96 well plates with $2x10^5$ female A1.$RAG^{-/-}$ splenocytes, 100nM of HYE^k peptide, 20U/ml IL-2, 10ng/ml IL-4 and TGFβ. Cells that secreted IL-9 were expanded.

All clone cell populations were maintained by seeding $2x10^5$ cells with $5x10^6$ CBA MMC-treated male spleen cells and the appropriate cytokines and used after 14 days when viable cells were collected by separation over Hypaque gradients.

2.6 Generation of 'conditional' Tregs

2.6.1 Retroviral vectors

Retroviral vectors except pCL-ECO (**Table 2.3**) were kindly provided by Kristian Andersen and Alexander Betz (MRC Laboratory of Molecular Biology, Cambridge). The conditional Foxp3 construct ('057' or '*cFoxp3*^{tgGFP}') was made by fusing Foxp3 cDNA with ERT2, a modified oestrogen binding domain, at its C-terminal. A green-fluorescent protein (GFP) gene was cloned in-frame with Foxp3 after the first five codons from the 5'-end in order to produce an ERT2-F(gfp)oxp3 fusion protein that can be visualised in the cell. These were then cloned into a Moloney Murine Leukemia Virus (MMLV)-backbone, a circular plasmid that also contained a GPI-linked and IRES-driven rat CD8a (rCD8) gene. The empty control vector contained only the GFP and rCD8 segments, but no Foxp3. The packaging vector, pCL-ECO, was purchased from Addgene® (Cambridge, MA) (**Figure 2.1**).

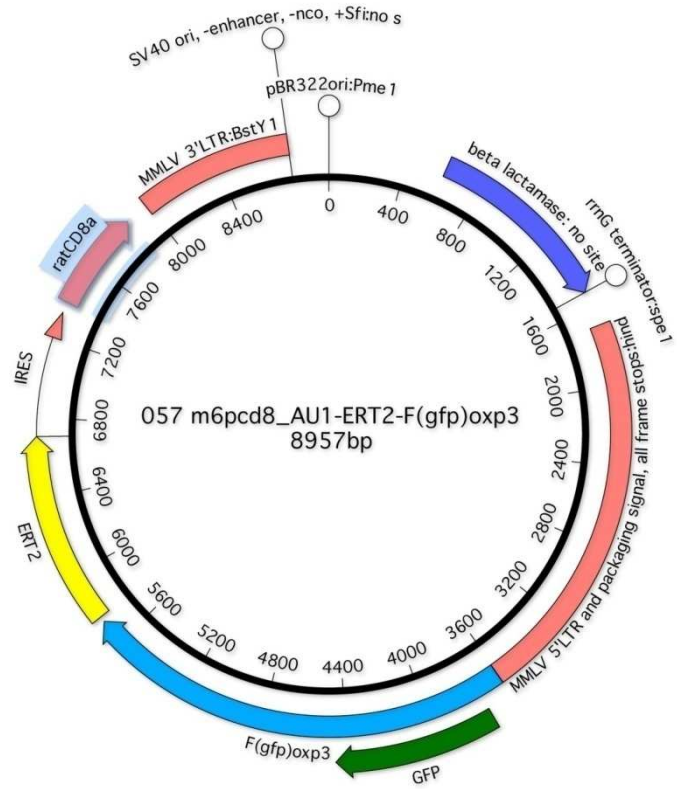
Table 2.3

Retroviral Construct	Abbreviated Linear Notation
cFoxp3^{tgGFP}	F(gfp)oxp3-ERT2-rCD8
cFoxp3^{GFP}	Foxp3-ERT2-GFP
cFoxp3^{rCD8}	Foxp3-ERT2-rCD8
empty vector	GFP-ERT2-rCD8
pCL-ECO	N/A

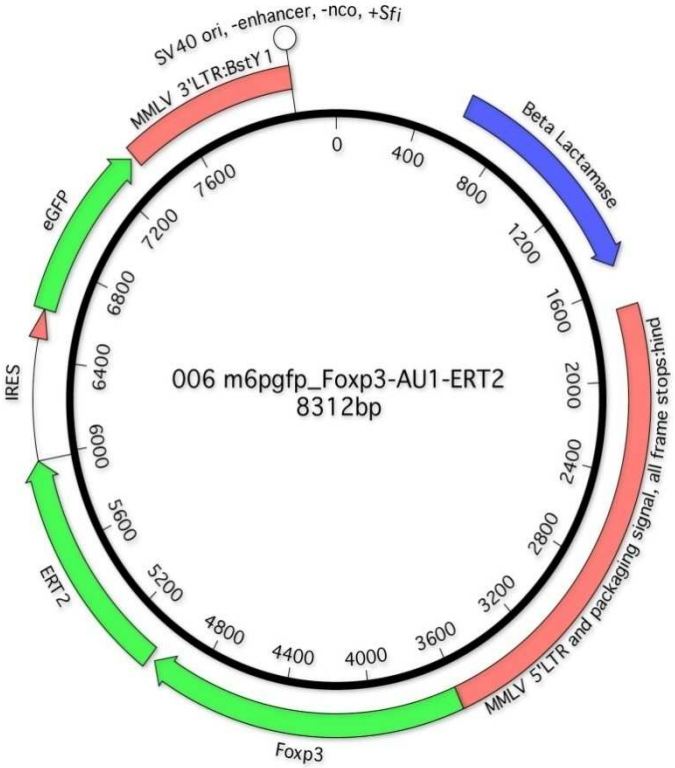
Table 2.3 Retroviral Constructs

Figure 2.1

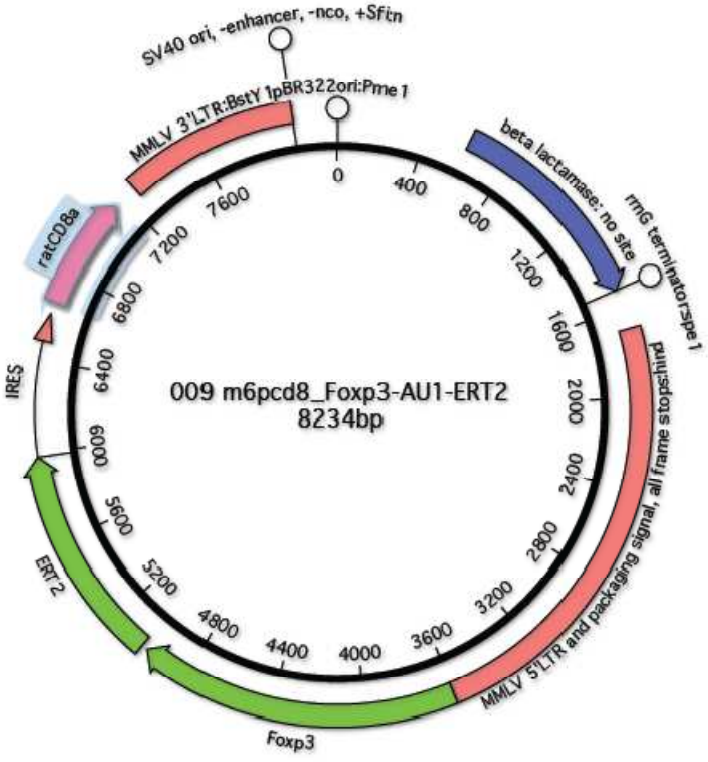
***cFoxp3*^{tgGFP} construct**



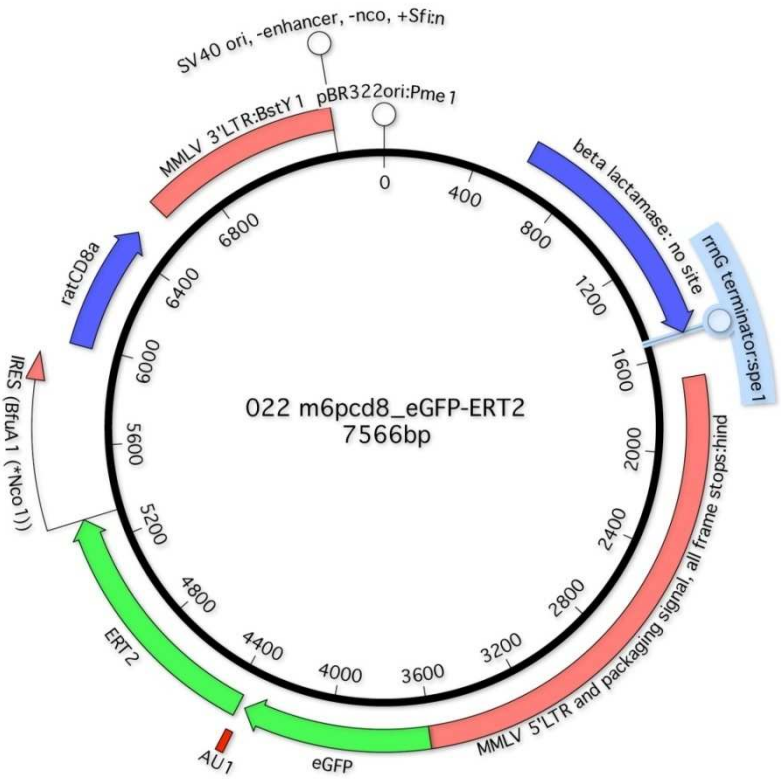
***cFoxp3*^{GFP} construct**



***cFoxp3*^{rCD8} construct**



Empty vector construct



pCL-ECO

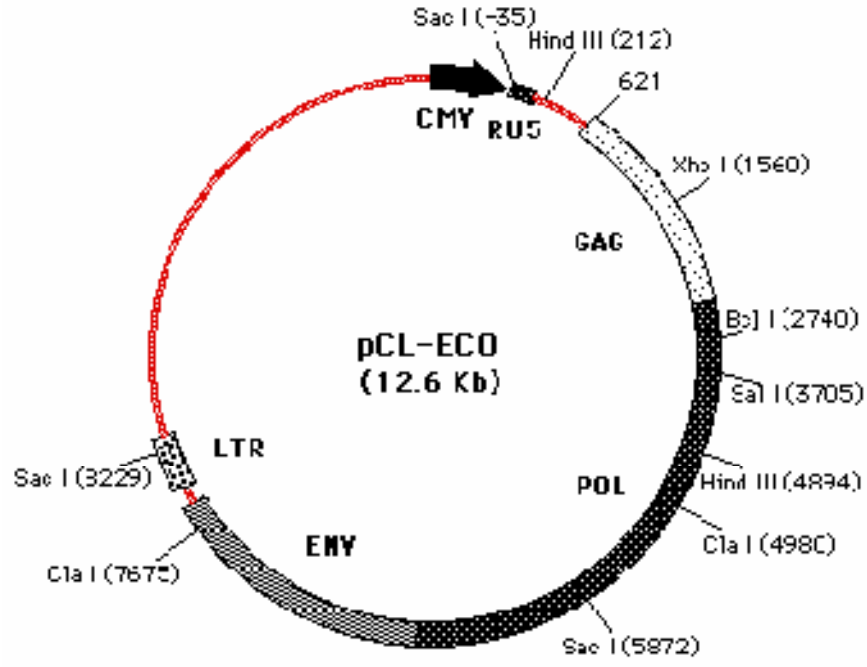


Image courtesy of Imgenex® (San Diego, CA)

Figure 2.1 Plasmid Maps

2.6.2 Preparation of plasmid DNA

Plasmid DNA was prepared using the QIAGEN Plasmid Maxi Kit (QIAGEN, HILDEN). A single colony of E. coli containing the target plasmid was cultured in 500ml of Luria-Bertani broth supplemented with ampicillin (100μg/ml) for 16hrs with vigorous shaking. Bacterial cells were harvested by centrifugation at 6000g and resuspended in Tris.Cl-EDTA (P1 buffer). Bacterial cells were then lysed with NaOH/SDS (P2 buffer). The lysate was neutralised with potassium acetate which causes the precipitation of cellular debris, chromosomal DNA and denatured proteins. The precipitate was removed and the clear lysate containing plasmid DNA was passed over an anion-exchange resin column (QIAGEN-TIP) which selectively bound the plasmid DNA. The column-bound DNA was washed with isopropanol/NaCl solution (Buffer QC) to remove contaminants. Plasmid DNA was eluted, washed with 70% ethanol, air-dried, and resuspended in sterile deionised H_2O.

2.6.3 Production of viral supernatant

The day before transfection, a confluent plate of HEK 293eT cells were split at 1 in 3 and plated in 6 well plates (Corning, US) at 2ml per well. Approximately 16 hours later (the following day), transfection mix was prepared (**Table 2.4**). In preparing the transfection mix, water was first added, followed by $CaCl_2$, then the plasmid DNA. A co-transfection was performed with an equal amount of pCL-ECO packaging plasmid (Imgenex, San Diego, CA) and a retroviral vector (**Figure 2.1**). Whilst vortexing the transfection mix on a low setting, 100μl 2x HBS was added drop-wise to achieve a total volume of 200μl. After waiting 30 seconds, the transfection mix (200μl) was added drop-wise to a single well in the 6 well plates. 6 hours following transfection, the medium in the well was replaced with fresh medium. A further 24hrs later, the viral supernatant was harvested and filtered through a 0.45μm filter. Protamine sulphate (600μg/ml) was added to the viral supernatant.

Table 2.4

Transfection mix	Volume
$CaCl_2$	12µl
pCL-ECO	2µg
Transfer vector	2µg
H_2O	Up to 100µl

Table 2.4 293eT Transfection Soup

2.6.4 Transduction of CD4+ T cells

AUTOMACS sorted $CD4^+$ cells were cultured at $2x10^5$ per well in a 96-well plate containing plate-bound anti-CD3 (KT3, 4µg/ml). The cells were activated for 24hr and then resuspended in a 1:2 mixture of viral supernatant and complete medium (IMDM + 10% v/v FCS + 10µm β-mercaptoethanol) supplemented with 5ng/ml of recombinant mIL-2 and 5ng/ml of recombinant mIL-7. This was followed by centrifugation at *500g* for 2hr at 32°C. Cells were analysed 40hr later (**Figure 2.2**).

Figure 2.2

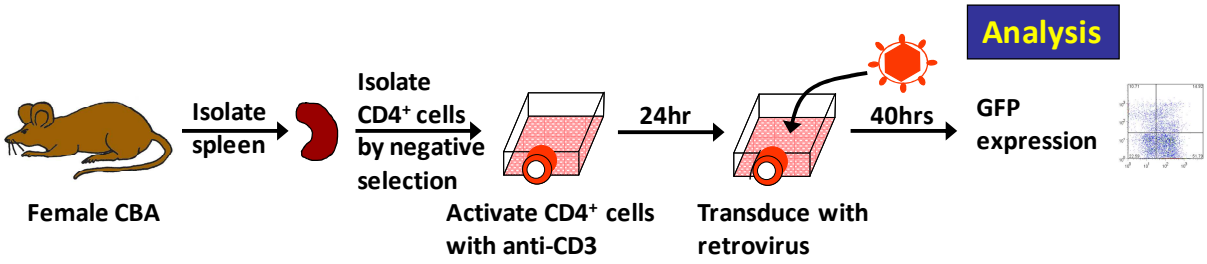


Figure 2.2 Transduction of $CD4^+$ cells

2.7 Fluorescence activated cell sorting (FACS)

2.7.1 Four-colour surface cytometry

Tissues were processed to produce single-cell suspensions. Spleens and lymph nodes were passed through a 70µm cell strainer and then treated with TBAC for 2 min at room temperature. For blood samples, water lysis was performed for 10 seconds. Surface staining was performed in 96-well plates with each well containing 2-5x10^5 cells. Cells were washed twice in FACS wash buffer and then incubated for 10min at room temperature with 10µg/ml anti-FcR block (2.4G2). Cells were then incubated with primary antibody (see Table 2.2) for 30min in the dark at 4^{o}C. This was followed by incubation with a secondary antibody or streptavidin conjugate for biotinylated primary antibodies. Between each step, cells were washed twice in FACS wash buffer. Finally, cells in each well were resuspended in 200µl FACS fixing solution. Analysis was performed on a FACSCalibur (Becton Dickinson, New Jersey) with dual laser excitation (488nm and 633nm). Data acquisition was performed with inter-channel compensation using CellQuest version 3.1 software (Becton Dickinson, NJ) and data analysis with FlowJo 7.2.4 software (Treestar Inc., Oregon).

2.7.2 Four-colour intracellular cytometry

After performing surface staining, cells were washed in PBS and resuspended in 200µl 2% v/v formaldehyde/PBS for 15min at room temperature. The cells were washed in FACS saponin buffer once before incubation with the staining antibody in FACS saponin buffer. Following incubation, cells were washed twice with FACS saponin buffer, once with FACS wash buffer and fixed with FACS fixing solution. For staining of Foxp3, the Foxp3-staining kit (eBioscience, CA) was used. Briefly, surface staining was first performed followed by washing in FACS wash buffer. Cells were then resuspended in fixation & permeabilization buffer for 30min at 4^{o}C.

Following this, cells were washed in permeabilization buffer and incubated with PE or APC-conjugated anti-Foxp3 for 30min at 4^{o}C. Then, cells were washed further in permeabilization buffer and finally resuspended in FACS wash buffer.

2.7.3 Mo-Flo FACS sorting of cells

Flow cytometry sorting was performed by Nigel Rust at the Sorting Facility at the Sir William Dunn School of Pathology (University of Oxford) using a MoFlo sorter (Dako Cytomation, Glostrup) was used. This instrument has 3 lasers emitting at the wavelengths of 488nm, 633nm and 405nm.

2.8 Dendritic cell and T cell co-clustering assay

2.8.1 Clustering assay

The clustering assay was based on that described by Onishi et al. (Onishi et al. 2008). Naïve T cells (Tn), iTregs, nTregs and T cell clones were labeled with membrane linker dyes PKH-67 (green dye) or PKH-26 (red dye) (Sigma-Aldrich, UK) according to the manufacturer's instructions. Labeling was performed in 15ml polypropylene tubes. Briefly, cells were washed and resuspended in serum-free medium to remove any proteins. They were then spun down and resuspended in Diluent C. Immediately, an equal volume of membrane linker dye diluted in Diluent C was added to obtain a final cell concentration of 5x10^{6} cells per ml of total reaction volume and dye concentration of 1µM. The cells were incubated for 2-3min. An equal volume of 1% PBS/BSA was added to stop the reaction. This was followed by washing the cells three times in complete medium. Cells were counted and competing T cell populations were co-cultured along with unlabeled bmDCs in 96well round-bottomed plates. A total number of 60,000 T cells consisting of various ratios of competing T cell populations

were cultured with 6000 bmDCs in a total volume of 200µl in each well. Cognate peptide was also added at a concentration of 10nM. 24hrs later, contents of each well were gently transferred into a well of an 8-well chamber slide (Lab-Tek, NY) and cell clusters were allowed to settle to the bottom of the well before examining under a confocal microscope.

2.8.2 Confocal microscopy

Confocal microscopy was performed on a Zeiss LSM 510 META laser scanning microscope (Carl Zeiss MicroImaging GmbH, Göttingen) using a x20 objective. The chamber slide was housed in a heated chamber adjusted to 4% CO2. Quantitative analysis of cell clusters were carried out using ImageJ software (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA) and LSM Image Browser software version 4.2 (Carl Zeiss MicroImaging GmbH, Göttingen).

2.9 Proliferation and suppression assays

Effector cells were Marilyn $CD4^+$ cells enriched by negative selection using AutoMACS (see **section 2.5.2**). Suppressor cells were Marilyn $CD4^+$ cells that had been transduced with retroviral vectors for 40hrs and then sorted on CD4 and GFP expression. BmDCs were obtained from the bone marrow of female B6 mice (see **section 2.5.1**). Suppressor cells and DCs were treated by mitomycin C (25µg/ml) for 30min at 37^oC and washed three times before being added to the assay. Each well of a 96 well plate contained 10^4 DCs, $2x10^4$ suppressors and $2.5x10^4$ effectors. HYA^b peptide was added as a dilution series (100nm, 10nm, 1nm, 0). Cultures were incubated for 72hrs. To measure proliferation, 3H-thymidine (GE Healthcare, UK) was added (0.5µCi/well) for the last 18hrs. The count of the incorporated isotope was then measured by a scintillation counter.

2.10 Molecular biology techniques

2.10.1 Chromatin immunoprecipitation (CHIP)

The chromatin immunoprecipitation assay was adapted from Dahl et al. (Dahl et al. 2008). Briefly, Dynabeads protein G (Invitrogen, UK) were washed twice with RIPA buffer with magnet. In a 1.5ml eppendorf tube (one per immunoprecipitation), 90µl RIPA buffer, 10µl washed dynabeads and 2.5µg of antibody were added. Antibodies used were anti-H3K4me3 (17-614, Millipore), anti-H3K27me3 (17-622, Millipore), anti-histone III (ab1791, Abcam) and control Immunoglobulin (Jackson Immunoresearch, UK). Dynabead-antibody mixes were rotated to ensure adequate mixing for 2hr at 4^{o}C. In the mean time, 1000-50,000 cells resuspended in PBS were fixed in 29µl of 18.5% formaldehyde for 8 minutes at room temperature to cross-link histones and DNA. This was then quenched with 71µl of 1M glycine stock for 5 minutes. Cells were then washed twice in cold PBS with vortexing and spinning at 500g for 10min. Chromatin was then sheared by sonicating (Sanyo MSE Soniprep 150 sonicator) 4 times for 10 seconds, placing the tubes on ice for 30 seconds in between each pulse. Chromatin was then collected by adding RIPA CHIP buffer to the sample, spinning at 14,000rpm and then collecting the supernatant. 100µl of chromatin was added to the dynabead-antibody complex and placed on a rotator at 4^{o}C overnight. 100µl of chromatin was kept aside as INPUT chromatin to which 10µl linear acrylamide carrier and 250µl 100% ethanol were added. This was spun at 14,000rpm and further washed with ethanol. Purification of the INPUT chromatin was then performed with 40µl 10% CHELEX-100. After rotation, CHIP samples were washed with 100µl ice cold RIPA buffer 3x5 minutes and with 100µl TE 1x5min with rotation at 4^{o}C. 40µl 10% CHELEX-100 was added and INPUT and CHIP samples were then boiled for 10 minutes and cooled to room temperature. 1µg proteinase K was added to the samples which were then allowed to incubate for 30 minutes at 55^{o}C. The samples were boiled again for 10 minutes and centrifuged for 10 seconds. 30µl

of supernatant (containing the DNA) was transferred to a new tube, and realtime PCR was performed.

2.10.2 Quantitative real-time PCR

Real-time quantitative PCR was carried out using SYBR Green Master Mix (Applied Biosystems) on an ABI 7500 Fast Real Time PCR System (Applied Biosystems) with SDS Software ver 1.3.1. Cycle conditions were 95^{o}C 10min followed by 50 cycles [95^{o}C 15s, 60^{o}C 60s]. Three biological replicates were performed as technical triplicates in a 96 well format. Prime pairs were purchased from Eurofins MWG Operon and used at a concentration of 0.02-0.25µM. Primer pairs used are shown below:

Foxp3 Promoter forward	5' – TTCCCATTCACATGGCAGGCTTCA – 3'
Foxp3 Promoter reverse	3' – TGAGATAACAGGGCTCATGAGAAACCACA – 3'
Foxp3 Enhancer 1 forward	5' – ATAGAAGACATACACCACCACGGCG – 3'
Foxp3 Enhancer 1 reverse	5' – TAAAGGAGACTGGAAGCCAACATGGG – 3'
Foxp3 Enhancer 2 forward	5' – GTTGTGACAACAGGGCCCAG – 3'
Foxp3 Enhancer 2 reverse	5' – CACTGTCTGTTGGGGCGTTC – 3'

In data analysis, the relative quantity for an immunoprecipitated target (eg. H3K4me3) was calculated with the formula:

$$Relative\ quantity\ (RQ) = 2^{Ct(control\ Ig) - Ct(target)}$$

Ct values were designated as the threshold cycle number for the amplification of DNA.

2.11 *In vivo* procedures

2.11.1 General anaesthesia

Mice were anaesthetised using 15mg/ml Ketaset (Ketamine) (Fort Dodge Animal Health) and 1mg/ml Rompun (Xylazine) (Bayer AG, Leverkusen) diluted in sterile filtered PBS and injected subcutaneously at 120µl/20g body weight using a BD Micro-Fine insulin syringe (BD Medical). Analgesia was given using Buprenorphine.

2.11.2 Skin grafting

All procedures were conducted in accordance with the U.K. Home Office Animals (Scientific Procedures) Act of 1986. Skin grafting was performed using a modified technique as described by Billingham *et al* (Billingham et al. 1953). The lateral thoracic wall of an anesthetised recipient mouse was shaven and a graft bed was prepared. Full thickness donor tail-skin of $1cm^2$ was placed in the bed and secured in place using paraffin gauze (Smith & Nephew, UK) and then cotton gauze. A layer of plaster of Paris (Gypsona, Smith & Nephew, UK) was then applied. An autoclip was used to attach the top edge of the cast to the skin at the back of the neck. On day 7, casts and gauze were removed and the skin examined daily for signs of rejection. Rejection was defined as total necrosis of the graft.

2.11.3 Adoptive transfer of $CD4^+$ cells

$CD4^+$ cells isolated from spleens by magnetic bead (**section 2.5.2**) or Mo-Flo (**section 2.7.3**) sorting were counted and then resuspended in PBS at the desired cell density. Mice were gently warmed using an infra-red lamp to enable vasodilation. 0.2ml of the cell suspension was injected intravenously into the lateral tail vein of mice.

2.11.4 Peptide injection

HYA^b peptide was diluted to the appropriate concentration (1, 10, or 100μg/ml) in sterile PBS and injected in a volume of 0.2ml intravenously into mice.

2.12 Statistical analysis

Statistical analysis was performed using Graphpad Prism software version 4.0. For cell clustering studies involving multiple test groups, ANOVA analysis was used followed by Bonferonni or Dunnett's posttest. Student's t test with two-tailed analysis was used for binary data sets. For skin transplant survival curves, *Log-rank Mantel-Cox test* was used for comparison between survival curves. *p* values less than 0.05 were considered significant.

CHAPTER 3 Factors influencing the function and stability of regulatory T cells induced by TGFβ

Introduction

Induced regulatory T cells (iTreg) have been shown to play an important role in the induction of tolerance *in vivo*. The induction of transplant tolerance using a regimen of co-receptor blockade antibodies was associated with *de novo* Foxp3 expression and the appearance of $CD4^+GITR^+$ cells in the transplanted graft (Cobbold et al. 2004). iTreg were also reported as necessary for the induction of tolerance in experimental colitis where iTreg and nTreg are thought to play a synergistic role in the induction of self-tolerance (Haribhai et al. 2009). Furthermore, the generation of iTreg in the gut is thought to be important in the maintenance of gastrointestinal homeostasis and tolerance to commensal organisms (Coombes et al. 2007). Despite their demonstrated suppressive ability, uncertainties remain regarding the importance of TGFβ and Foxp3 for their function, their stability *in vivo* and their mechanism of action.

The relative importance of TGFβ and Foxp3 expression for the function of iTreg have not been clarified. Whilst Foxp3 is important for the function of nTreg and is required to prevent autoimmunity, existing evidence regarding the role of iTreg in therapeutic tolerance remains circumstantial. Whilst TGFβ is important for both natural and therapeutically induced tolerance (Daley et al. 2007) and has been shown to generate $Foxp3^+$ iTreg both *in vivo* and *in vitro* (Chen et al. 2003; Fantini et al. 2004; Kretschmer et al. 2005; Coombes et al. 2007), it remains unclear whether TGFβ itself confers suppressive properties to iTreg independent of Foxp3 induction. It has been shown that TGFβ-exposure can upregulate expression of

molecules in $CD4^+$ T cells independent of Foxp3 such as lymphotoxin α, CTLA-2b and CD73 [(Hill et al. 2007), F. Regateiro, DPhil Thesis 2009] and can directly subdue activation of T cells and DCs (Yamaguchi et al. 1997; Gorelik et al. 2000). However, given that TGFβ leads to the expression of a number of transcription factors that can bind to an intronic enhancer site at the *Foxp3* locus (Tone et al. 2008), TGFβ may merely serve the purpose of inducing Foxp3 expression in iTreg.

To determine if $Foxp3^-$ TGFβ-conditioned $CD4^+$ T cells also have suppressive capacity, I established a transplantation model where graft antigen was presented only on recipient-type APCs, and where any rejection operated only through the indirect pathway. A "knock-in" mouse where Foxp3 expression was artificially linked to the expression of a surface human CD2 (hCD2) was then used to allow sorting of both $Foxp3^+$ and $Foxp3^-$ populations following TGFβ-conditioning. Both $hCD2^+$ ($Foxp3^+$) and $hCD2^-$ ($Foxp3^-$) subsets of TGFβ-conditioned cells were adoptively transferred into lympho-deficient recipients along with TCR transgenic naïve $CD4^+$ cells. A graft was transplanted the following day. Whilst the suppressive capacity of the $hCD2^-$ cells was examined, the suppressive potency of the $hCD2^+$ cells was also tested by performing a titration of putative suppressor cells against naïve $CD4^+$ T cells.

Finally, a pressing issue regarding iTreg has been to determine how they can become 'stabilised' *in vivo*. It is crucial to understand this from a practical and therapeutic standpoint in order to maximize their suppressive capacity, and to avoid any 'conversion' to an undesirable pro-inflammatory phenotype (Zhou et al. 2009). Floess et al., showed that iTreg generated *in vitro* by TGFβ-conditioning rapidly lost Foxp3 expression *in vitro* following antigen re-stimulation (Floess et al. 2007). *In vitro* generated iTreg transferred *in vivo* into

syngeneic recipients also appeared to show reduced Foxp3 expression within days (Kretschmer et al. 2005). Other groups have shown that CpG motifs at intronic enhancer site 2 (also known as Treg-specific demethylated region or CNS2) of the *Foxp3* locus are only weakly demethylated in iTreg in comparison with strong demethylation in nTreg (Floess et al. 2007). This would undoubtedly contribute to the instability of Foxp3 expression in iTreg. I wished to examine the role, if any, of cognate antigen and/or the tissue microenvironment in stabilizing Foxp3 expression of iTreg *in vivo*.

I hypothesised that antigen delivered in the context of a tissue microenvironment (i.e. a transplanted graft) may contribute to the stabilization of iTreg phenotype as measured by Foxp3 expression. This was examined by measuring Foxp3 expression of male antigen-specific TGFβ-conditioned T cells exposed to a male graft *in vivo*. These were compared to other cells exposed to a female graft or no graft at all. Whether the microenvironment of the graft was important for the stabilization of the iTreg phenotype was further examined by eliminating this variable using intravenous injection of the cognate peptide. Finally, to examine whether antigen-specific Foxp3$^+$ iTreg display greater stability of Foxp3 expression *in vivo* in the presence of graft antigen, Foxp3$^+$ cells were isolated from various sites of graft recipients – draining lymph nodes (DLN), non-draining lymph nodes (NDLN), spleen and blood. These cells were then examined for epigenetic changes, namely the histone modifications at their Foxp3 locus using chromatin immunoprecipitation (CHIP). Histone modifications can affect chromatin structure rendering the DNA more or less 'open' for binding of transcription factors or DNA repair molecules (Martin et al. 2005). CHIP was performed on sorted Foxp3$^+$ iTreg from the recipients with antibodies against trimethylated lysine 4 of histone 3 (H3K4me3: associated with activation of gene transcription) and trimethylated lysine 27 of histone 3 (H3K4me27: associated with repression of transcription)

(Martin et al. 2005). PCR was carried out to amplify regions of interest in the *Foxp3* locus including the proximal promoter, intronic enhancer region 1 (CNS1) and enhancer region 2 (CNS2 or TSDR). The ratio of H3K4me3 to H3K27me3 at each of these regions may give an indication of the degree of chromatin remodeling and transcriptional activity at the *Foxp3* locus which portends the stability of Foxp3 expression. Previously, Floess et al. showed that the ratio of H3K4me3 to H3K27me3 was greatly increased at the *Foxp3* locus of nTreg compared to $CD4^{+}CD25^{-}$ cells (Floess et al. 2007). Hence, the H3K4me3/H3K27me3 ratio at these regions is of interest to gauge whether the stability of Foxp3 expression by iTreg is enhanced by exposure to graft antigens.

3.1 Male CBA.RAG$^{-/-}$ skin is rejected by female Marilyn mice

The induction of Treg in the periphery is important for the establishment of transplant tolerance. However, how iTreg can induce graft tolerance is still unclear. Whilst it is known that acute graft rejection involves direct allorecognition mediated by donor APC, followed by indirect allorecognition mediated by host APC, it remains unclear at what stage iTreg are involved. Previously, it had been shown that the phenomenon of linked suppression can operate via the indirect pathway of allorecognition (Wise et al. 1998). Therefore, I first asked if iTreg derived from *in vitro* TGFβ-conditioning can suppress rejection operating through the indirect pathway of allorecognition.

I exploited a simple TCR transgenic model system where male CBA.RAG$^{-/-}$ (haplotype H-2^{b}) tail skin was grafted onto female Marilyn recipients. If rejection was to occur, it would occur via the indirect pathway of MHC presentation since Marilyn TCR-transgenic CD4^{+} cells would be specific for the HYA^{b} peptide presented by H-2A^{b} – only present on host-derived APC. Therefore, the male DBY peptides from the CBA.RAG$^{-/-}$ graft would have been processed by the Marilyn recipients' own DC into the HYA^{b} peptide fragments.

Indeed, male CBA.RAG$^{-/-}$ grafts were promptly rejected by female Marilyn recipients (**Figure 3.1**). Female CBA.RAG$^{-/-}$ grafts were not rejected. Control male B6.RAG$^{-/-}$ grafts (haplotype H-2^{b}) were also rejected with similar kinetics to the CBA.RAG$^{-/-}$ male grafts. This suggests that direct allorecognition is not essential for rapid rejection of the CBA.RAG$^{-/-}$ male grafts.

Figure 3.1

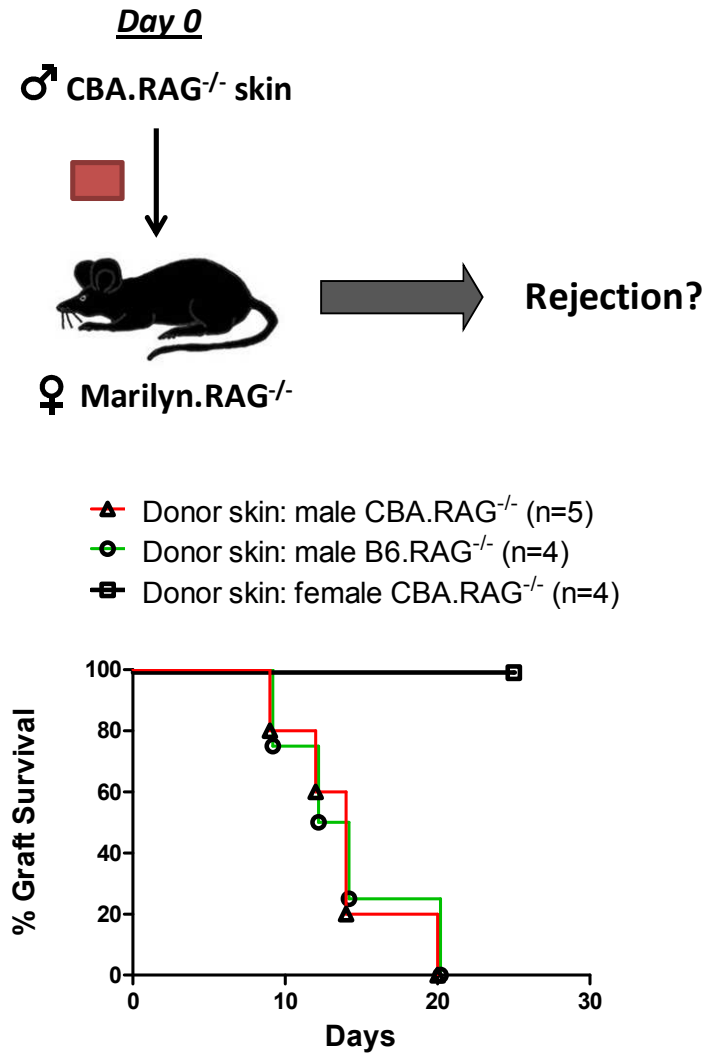


Figure 3.1 Female Marilyn recipients reject male CBA.RAG$^{-/-}$ skin grafts

Male CBA.RAG$^{-/-}$ tail skin was grafted onto female Marilyn recipients and the grafts were followed on a daily basis. Statistical analysis (Log-rank Mantel Cox):

	p value
male CBA.RAG$^{-/-}$ vs. female CBA.RAG$^{-/-}$	< 0.05
male B6.RAG$^{-/-}$ vs. female CBA.RAG$^{-/-}$	< 0.05
male CBA.RAG$^{-/-}$ vs. male B6.RAG$^{-/-}$	ns

3.2 As many as one million TGFβ-conditioned Marilyn CD4^{+} cells cannot prevent rejection of male CBA.RAG$^{-/-}$ skin by female Marilyn mice

To investigate if Treg induced via *in vitro* TGFβ-conditioning can suppress rejection via the indirect pathway, TGFβ-conditioned Marilyn CD4^{+} cells were transferred at various cell titrations (1x10^{6}, 5x10^{5} and 1x10^{5}) into Marilyn recipients grafted one day later with a male CBA.RAG$^{-/-}$ skin graft. However, as many as 1x10^{6} TGFβ-conditioned cells could not prevent rejection of the graft (**Figure 3.2**). This suggests that the strength of the rejection response in the recipient is greater than the capacity of 1x10^{6} iTreg to control it.

Figure 3.2

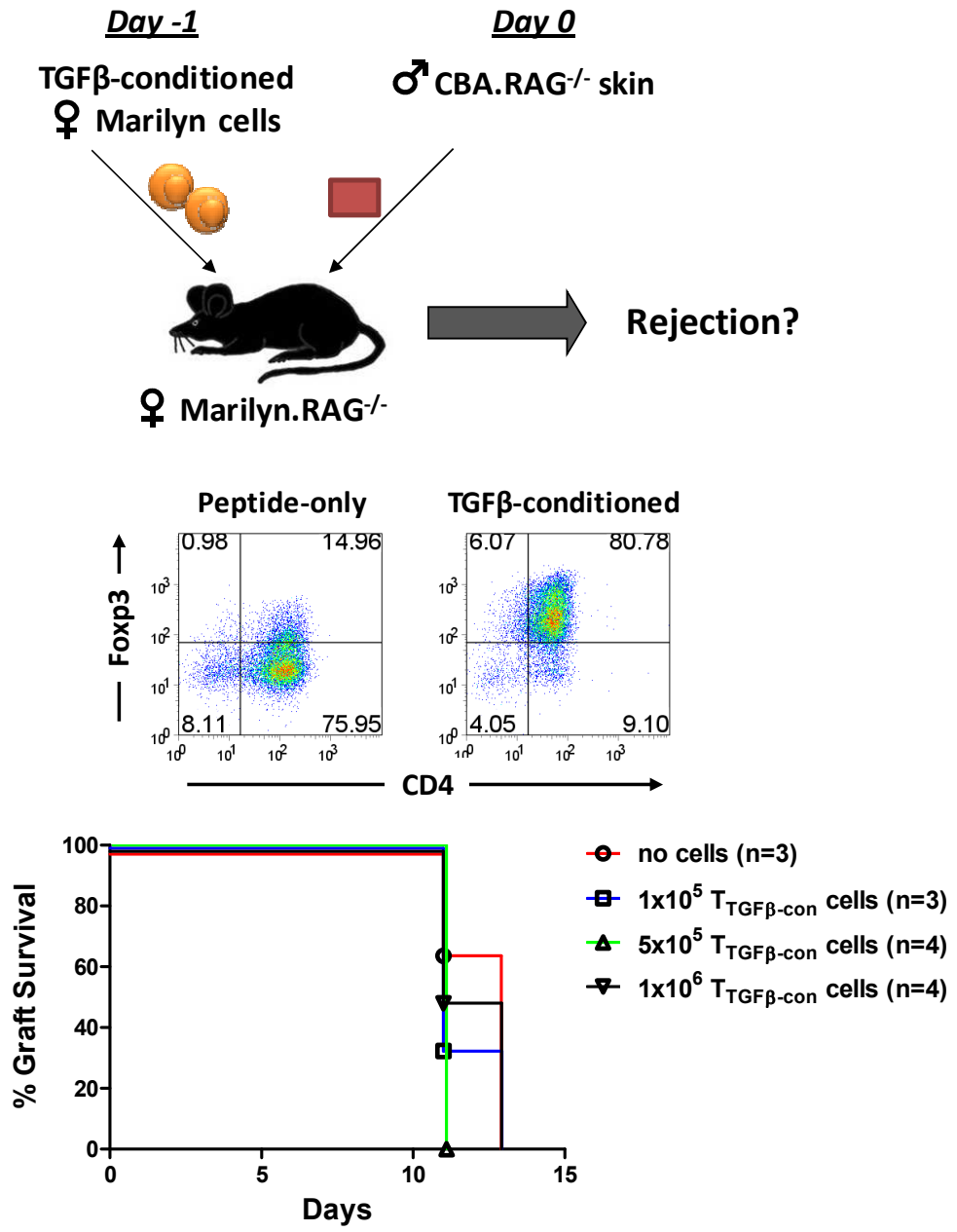


Figure 3.2 $1x10^6$ TGFβ-conditioned T cells fail to suppress male graft rejection by Marilyn recipients

$1x10^5$, $5x10^5$ or $1x10^6$ TGFβ-conditioned $CD4^+$ T cells ($T_{TGFβ\text{-}con}$) were adoptively transferred (i.v.) into Marilyn mice. The following day, male CBA.$RAG^{-/-}$ skin grafts were transplanted. Graft survival was followed on a daily basis. The FACS plot shows that 80% of the cells following TGFβ-conditioning were $CD4^+Foxp3^+$. Statistical analysis (Log-rank Mantel Cox): no statistically significant differences between the groups in terms of rejection kinetics.

3.3 TGFβ-conditioned Marilyn CD4+ cells can prevent male CBA.RAG$^{-/-}$ skin rejection by adoptively transferred naïve Marilyn CD4+ T cells

The overwhelming strength of the rejection response by male-antigen specific CD4$^+$ T cells could account for the inability of 1x10^6 TGFβ-conditioned CD4$^+$ T cells to prevent male graft rejection in Marilyn recipients. Therefore, I examined the ability of iTreg to suppress by limiting the number of effector T cells in an adoptive transfer into RAG$^{-/-}$ recipients, which lack any endogenous CD4$^+$ T cells.

Various numbers of female Marilyn CD4$^+$ T cells were transferred into female B6.RAG$^{-/-}$ recipients to determine the minimal dose capable of rejecting male skin graft. **Figure 3.3A** shows that as few as 2500 Marilyn CD4$^+$ T cells transferred one day before the transplant of a male CBA.RAG$^{-/-}$ skin graft could reject the graft. 1x10^4 Marilyn CD4$^+$ T cells, which would guarantee rejection of the CBA.RAG$^{-/-}$ male skin graft, were co-transferred with 1x10^6 TGFβ-conditioned Marilyn CD4$^+$ T cells (i.e. a hundred fold excess). When transferred alone, TGFβ-conditioned CD4$^+$ T cells did not reject the male graft. When co-transferred with naïve CD4$^+$ T cells, they were able to suppress graft rejection by naïve T cells (**Figure 3.3B**).

Figure 3.3A

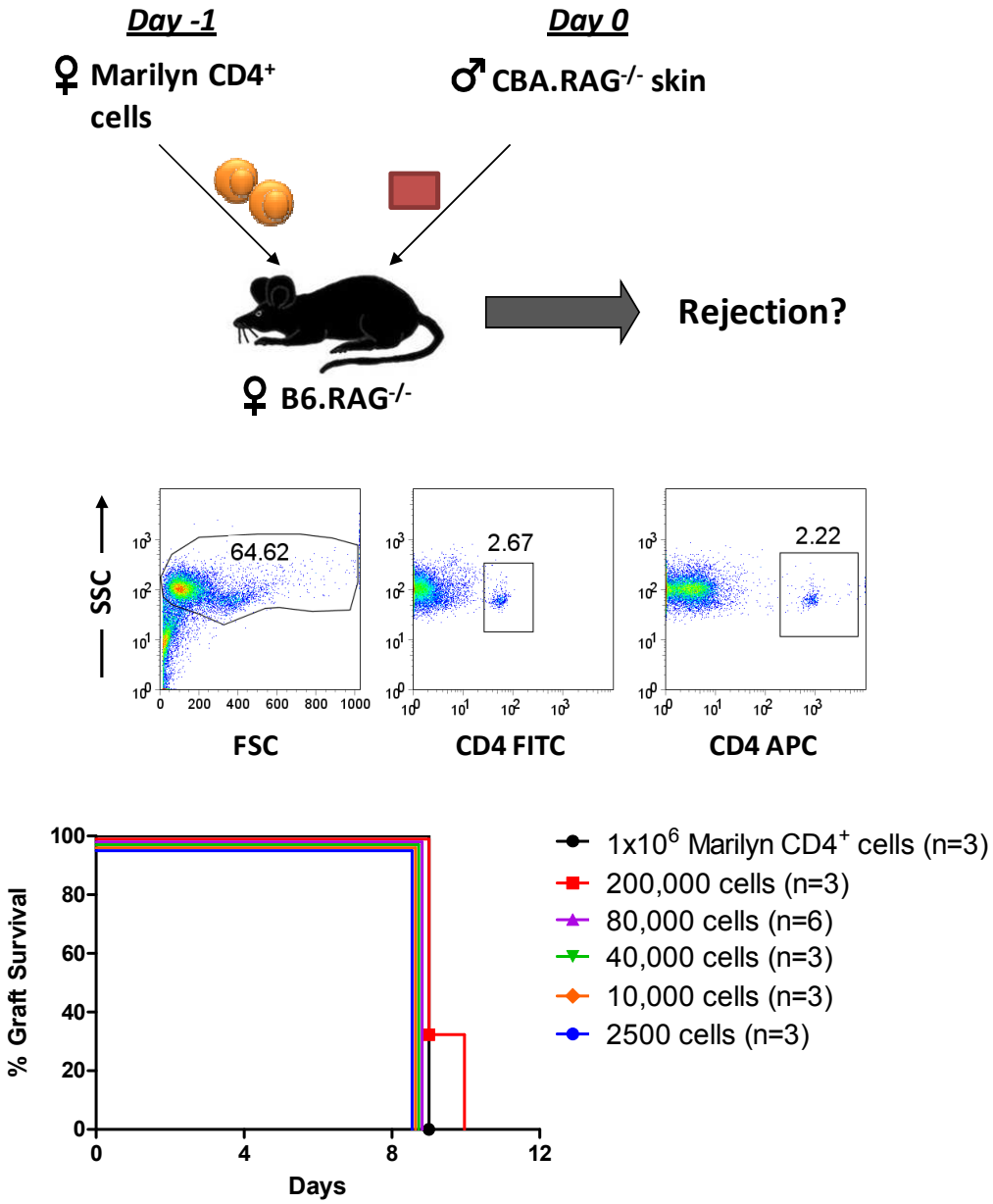


Figure 3.3A Very few Marilyn CD4$^+$ cells are needed to reject a male graft in a B6.RAG$^{-/-}$ recipient

FACS analysis was performed to determine the proportion of CD4$^+$ cells in Marilyn spleen. Splenocytes were stained with both anti-CD4 FITC and anti-CD4 APC antibodies, and an average of the two was deemed as the CD4$^+$ percentage (2.45%). Whole splenocytes containing the required number of CD4$^+$ cells were then transferred into B6.RAG$^{-/-}$ recipients. Various numbers of CD4$^+$ cells were transferred. Mice were grafted with a male CBA.RAG$^{-/-}$ graft on the following day. Grafts were followed on a daily basis. Difference in rejection kinetics between the groups was not statistically significant.

Figure 3.3B

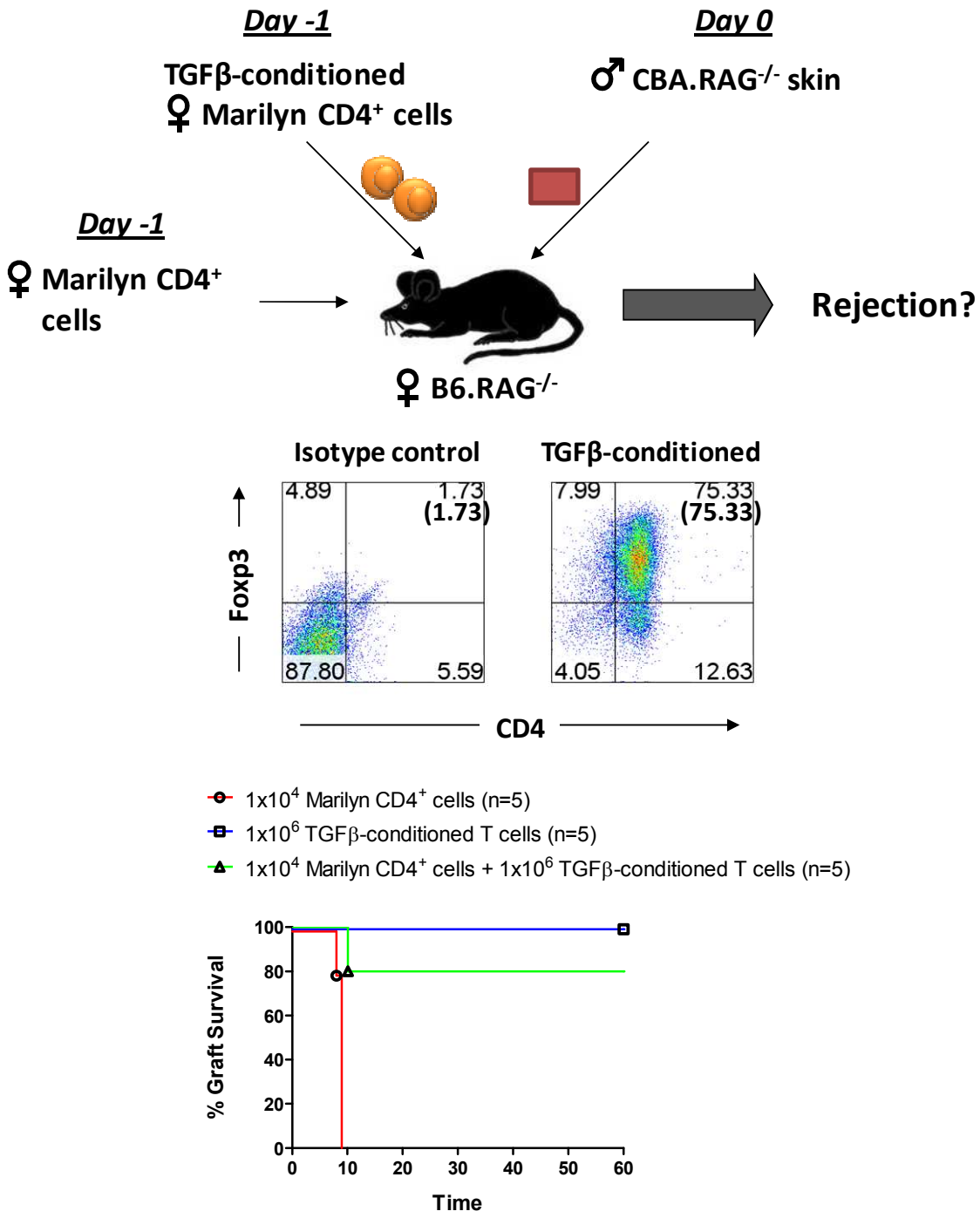


Figure 3.3B 1x10^6 TGFβ-conditioned T cells can suppress rejection of a male graft by 1x10^4 naïve CD4$^+$ T cells in a lympho-deficient recipient

1x10^6 TGFβ-conditioned Marilyn CD4$^+$ T cells (75% Foxp3$^+$) were co-transferred with 1x10^4 Marilyn CD4$^+$ T cells. Flow cytometry was performed to examine the Foxp3 expression of TGFβ-conditioned cells (75% of cells were CD4$^+$Foxp3$^+$). A male CBA.RAG$^{-/-}$ graft was transplanted the following day. Statistical analysis (Log-rank Mantel Cox):

	p value
Marilyn CD4$^+$cells vs TGFβ-conditioned T cells	< 0.01
Marilyn CD4$^+$cells vs Marilyn CD4$^+$cells + TGFβ-conditioned T cells	< 0.01

3.4 Within the TGFβ-conditioned Marilyn CD4$^+$ cells, Foxp3$^+$ cells cannot reject and are suppressive, whilst Foxp3$^-$ T cells retain the capacity to reject

To investigate if TGFβ-conditioning in the absence of Foxp3 expression can render cells 'suppressive' *in vivo*, Marilyn.hCD2.KI mice were used (Komatsu et al. 2009). These have a *hCD2* transgene knocked into the *Foxp3* locus on the X-chromosome, allowing for the co-expression of Foxp3 and surface hCD2 on the same cell. This enabled the flow cytometric sorting of TGFβ-conditioned CD4$^+$ T cells from these mice into hCD2$^+$ (Foxp3$^+$) and hCD2$^-$ (Foxp3$^-$) populations.

Previously, unsorted TGFβ-conditioned CD4$^+$ T cells were shown to be 'non-inflammatory' *in vivo* (**Figure 3.3B**). These unsorted populations always have a certain proportion of cells that remain Foxp3$^-$, despite the latter showing the expression of molecules regulated by TGFβ, such as CD103 and CD73. To investigate if TGFβ itself can induce regulatory capacity independently of Foxp3 expression and if Foxp3$^-$ TGFβ-conditioned CD4$^+$ T cells are also non-inflammatory *in vivo*, 1x10^6 hCD2$^-$ (Foxp3$^-$) TGFβ-conditioned cells were transferred into B6.RAG$^{-/-}$ recipients followed by a male graft. 1x10^6 unsorted or 1x10^6 hCD2$^+$ cells were also transferred in other groups. As expected, 1x10^6 unsorted TGFβ-conditioned cells did not reject a male graft. However, when anti-hCD2 ablative antibody was co-injected with unsorted cells, the grafts were rejected (**Figure 3.4A**). This suggests that the Foxp3$^+$ subpopulation of the injected cells was important for preventing rejection of the graft. Consistent with this, 1x10^6 hCD2$^-$ injected together with anti-hCD2$^-$ antibody (to ablate any Foxp3$^+$ contaminants) led to the rejection of the graft. This suggests that TGFβ-conditioning, independent of Foxp3 expression, was insufficient for keeping the cells 'non-inflammatory'. On the other hand, 1x10^6 hCD2$^+$ (Foxp3$^+$) cells did not reject the male graft.

To examine if $hCD2^+$ cells were not merely non-inflammatory, but could actively suppress rejection, the recipient mice underwent immunological challenge 30 days later with $1x10^5$ Marilyn $CD4^+$ T cells and a fresh male graft transplanted on the contralateral flank (**Figure 3.4B**). Recipients that had previously received unsorted or $hCD2^+$ TGFβ-conditioned cells resisted the challenge, and the 2^{nd} fresh male graft was not rejected. Recipients that had received $hCD2^-$ cells, in contrast, rejected the fresh male graft.

Figure 3.4A

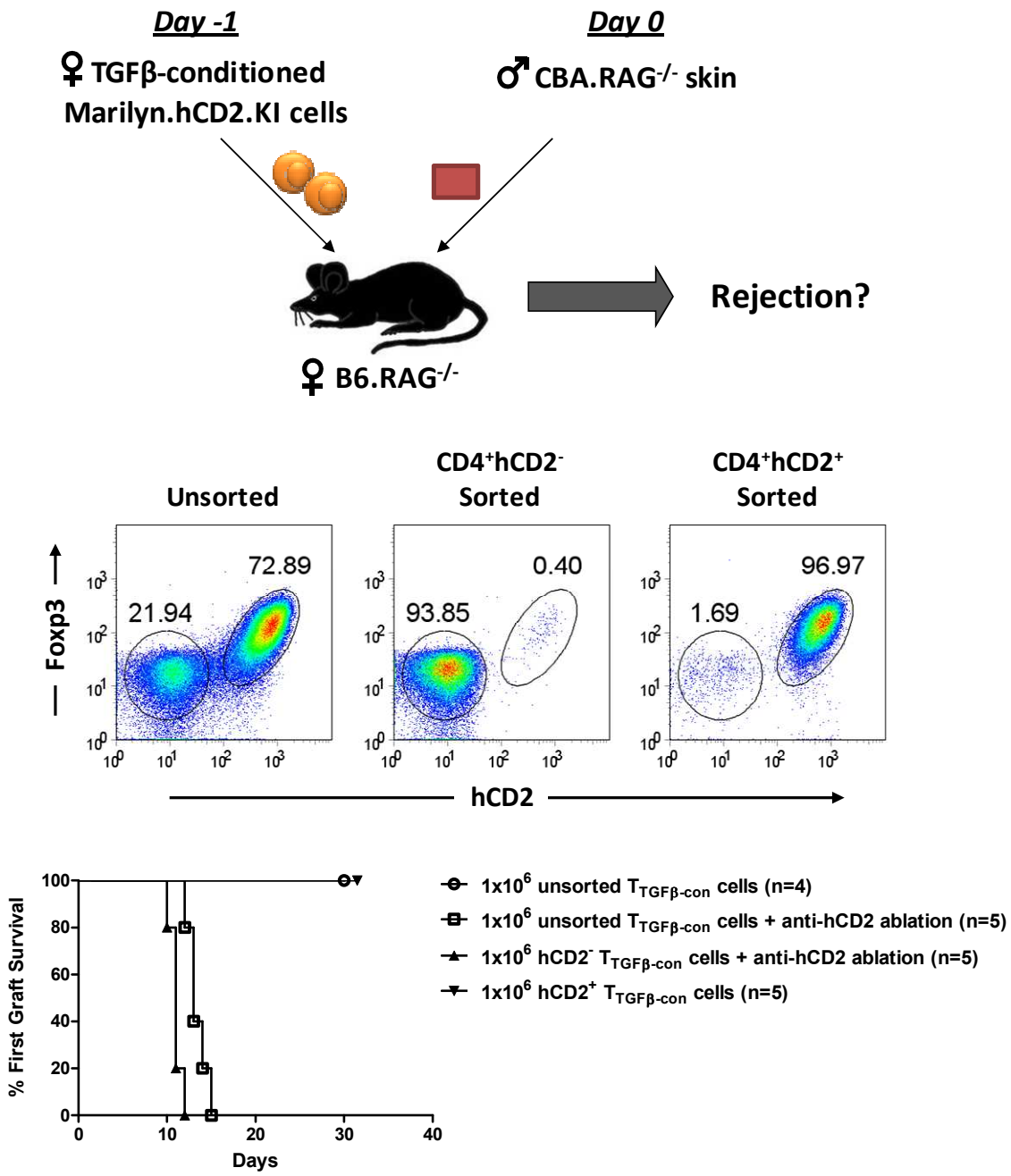


Figure 3.4A Sorted hCD2$^-$ TGFβ-conditioned T cells retain the ability to reject grafts whereas hCD2$^+$ T cells cannot reject

TGFβ-conditioned CD4$^+$ T cells (from Marilyn.hCD2.KI mice) were sorted into hCD2$^+$ and hCD2$^-$ subsets by Mo-flo and injected intravenously into B6.RAG$^{-/-}$ recipients followed by grafting of male CBA.RAG$^{-/-}$ tail skin. Sorted CD4$^+$hCD2$^+$ cells were over 96% hCD2$^+$Foxp3$^+$ (see FACS plot). Anti-hCD2 ablative antibody (1 mg YTH655, 1mg) was co-injected with unsorted and hCD2$^-$ TGFβ-conditioned cells. Statistical analysis (Log-rank Mantel Cox):

	p value
1x10^6 hCD2$^-$ cells (+Ab ablation) vs 1x10^6 unsorted cells	< 0.01
1x10^6 hCD2$^-$ cells (+Ab ablation) vs 1x10^6 unsorted cells (+Ab ablation)	< 0.01
1x10^6 hCD2$^-$ cells (+Ab ablation) vs 1x10^6 hCD2$^+$ cells	< 0.01

Figure 3.4B

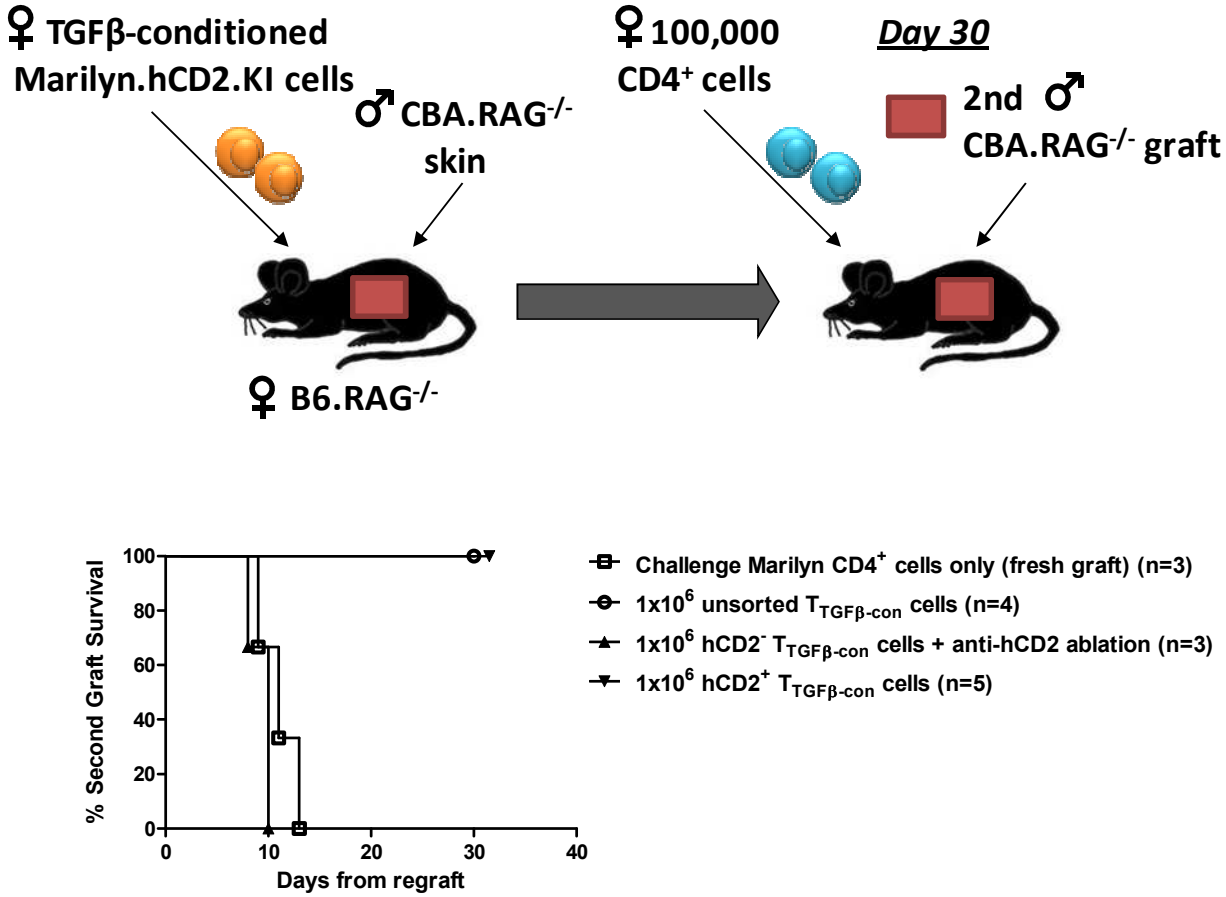


Figure 3.4B Sorted hCD2$^-$ TGFβ-conditioned CD4$^+$ T cells, unlike hCD2$^+$ cells, cannot resist immunological challenge by naïve CD4$^+$ T cells

30 days following the first skin transplant, 100,000 Marilyn CD4$^+$ cells were injected i.v. and a fresh male CBA.RAG$^{-/-}$ skin graft was transplanted on the contralateral flank of the recipients. Statistical analysis (Log-rank Mantel Cox):

	p value
1x10^6 hCD2$^+$ cells vs Marilyn CD4$^+$ cells	< 0.01
1x10^6 hCD2$^+$ cells vs 1x10^6 hCD2$^-$ cells (+Ab ablation)	< 0.01
1x10^6 hCD2$^+$ cells vs 1x10^6 unsorted cells	ns

3.5 Sorted hCD2$^+$ TGFβ-conditioned Marilyn T cells can prevent rejection mediated by naïve CD4$^+$ cells

Having established that Foxp3$^-$ TGFβ-conditioned cells are not suppressive *in vivo* and that Foxp3$^+$ TGFβ-conditioned cells can resist an immunological challenge mounted by naïve CD4$^+$ T cells, I next sought to investigate the capacity of Foxp3$^+$ TGFβ-conditioned cells to suppress a rejection response when co-transferred with naïve CD4$^+$ T cells.

When co-transferred with naïve Marilyn CD4$^+$ cells at a ratio of 5:1 (suppressor cell: naïve cell), hCD2$^+$ (Foxp3$^+$) TGFβ-conditioned cells demonstrated modest suppression with 50% of the male grafts remaining non-rejected (**Figure 3.5**). Suppression was not seen at 2:1 or 1:1 ratios.

Figure 3.5

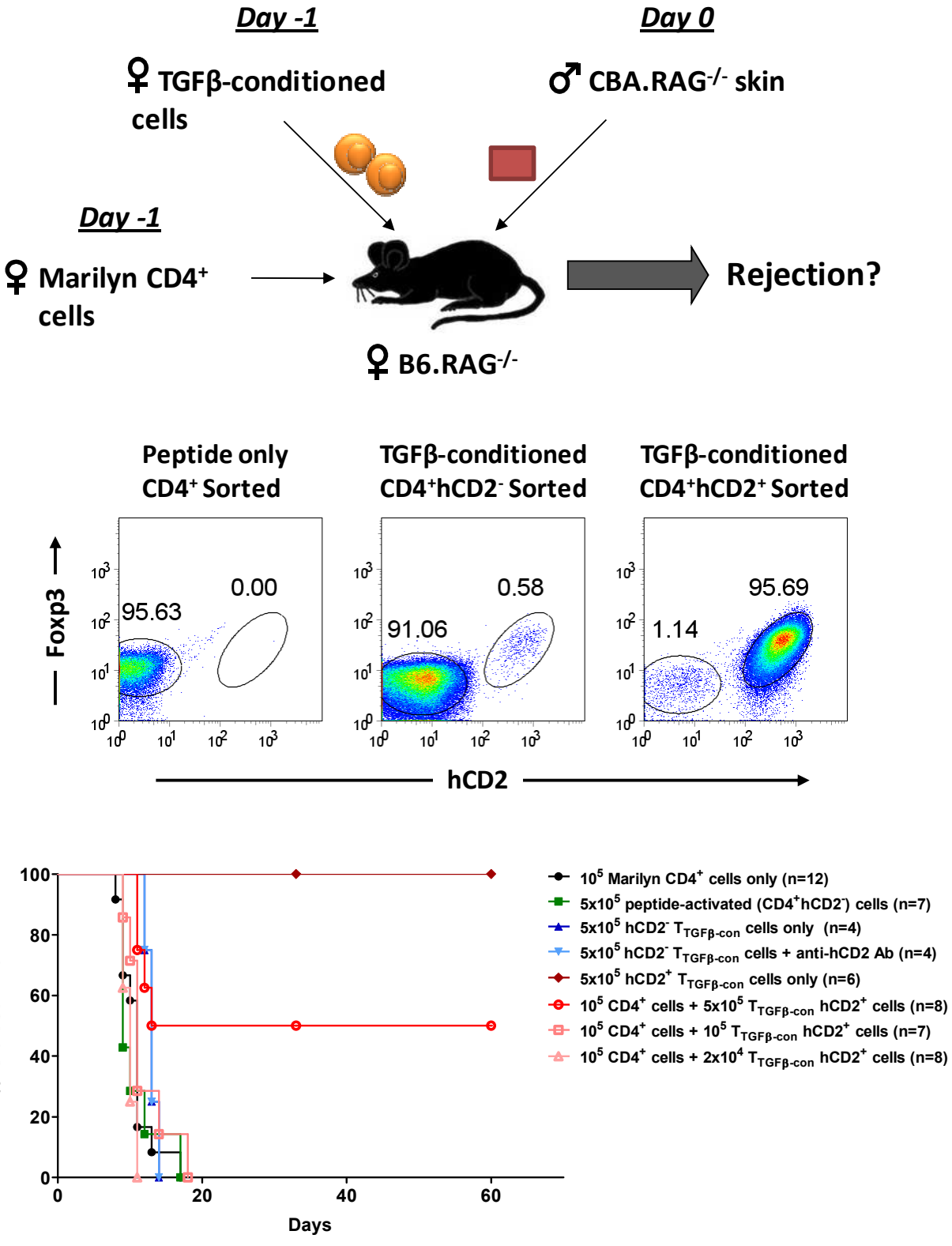


Figure 3.5 hCD2$^+$ TGFβ-conditioned CD4$^+$ T cells show modest suppression of graft rejection at 5:1 (suppressor : naïve cell) ratio

5x10^5 TGFβ-conditioned CD4$^+$ T cells ($T_{TGFβ\text{-}con}$) (from Marilyn.hCD2.KI mice) were sorted into hCD2$^+$ and hCD2$^-$ subsets by Mo-flo and injected i.v. along with naïve Marilyn CD4$^+$ cells into

B6.RAG$^{-/-}$ recipients at varying ratios (5:1, 1:1, 2:1). Sorted CD4^{+}hCD2^{+} cells were over 95% hCD2^{+}Foxp3^{+} (see FACS plot). On the next day, a male CBA.RAG$^{-/-}$ skin graft was transplanted. Results from two separate experiments were pooled. Statistical analysis (Log-rank Mantel Cox):

	p value
10^5 Marilyn CD4^{+} cells only vs 10^5 CD4^{+} cells + 5x10^5 $T_{TGFβ\text{-}con}$ hCD2^{+} cells	< 0.01
5x10^5 hCD2^{+} $T_{TGFβ\text{-}con}$ cells only vs 10^5 CD4^{+} cells + 5x10^5 $T_{TGFβ\text{-}con}$ hCD2^{+} cells	≤ 0.05
10^5 Marilyn CD4^{+} cells only vs 10^5 CD4^{+} cells + 10^5 $T_{TGFβ\text{-}con}$ hCD2^{+} cells	< 0.05
10^5 Marilyn CD4^{+} cells only vs 10^5 CD4^{+} cells + 2x10^5 $T_{TGFβ\text{-}con}$ hCD2^{+} cells	< 0.001

3.6 Antigen exposure enables a significant proportion of TGFβ-conditioned cells to retain Foxp3 expression

In the previous section, it was shown that Foxp3 expression was essential for the suppressive capacity of TGFβ-conditioned cells *in vivo*. This led to the question: can the Foxp3 expression of TGFβ-conditioned T cells remain stable *in vivo*, and what factors can influence the stability of that Foxp3 expression. I hypothesized that antigen and/or the graft microenvironment may play a role. To address this, I first investigated if TGFβ-conditioned T cells can maintain Foxp3 expression after an extended period *in vivo* in the absence of a skin graft.

TGFβ-conditioned cells were transferred into B6.RAG$^{-/-}$ recipients and after 30 days, the recipients were transplanted with a male CBA.RAG$^{-/-}$ graft. Surprisingly, these recipients did not reject their graft (**Figure 3.6**). In the control group, where recipients received a graft on day 0 as well as day 30, the grafts were also not rejected. This suggests that TGFβ-conditioned cells can remain functionally non-aggressive after an extended period in the absence of their cognate antigen. Whether this functional stability was related to the maintenance of Foxp3 expression was assessed by FACS analysis of draining LNs (DLN), non-draining LNs (NDLN), spleen and blood. In this study, some recipients received a delayed graft at day 30, some were grafted on day 0 and day 30, and some remained ungrafted for the duration of the experiment (**Figure 3.7A**). Other recipients were transplanted with a female graft on day 0 and a male graft on day 30 to interrogate whether a syngeneic graft would affect Foxp3 expression. Some recipient groups received TGFβ-conditioned cells which were treated with ATRA (10nM) as ATRA is thought to affect the peripheral generation of Tregs (Coombes et al. 2007; Mucida et al. 2007).

On day 60, the recipients were sacrificed and their DLN, NDLN, spleen and blood were analysed by FACS. **Figure 3.7B** shows that in the groups where recipients had received 1 or 2 grafts, a significantly greater proportion of $CD3^+CD4^+$ cells in the DLN expressed Foxp3 than recipients that were ungrafted (TGFβ ungrafted vs TGFβ male graft d30, $p<0.001$; TGFβ ungrafted vs TGFβ male graft d0, 30, $p<0.001$). ATRA-treatment of TGFβ-conditioned cells did not seem to influence the degree of Foxp3 expression (statistically non-significant), unlike the presence or absence of grafts. There was also a statistically significant increase in the percentage of Foxp3 expression of $CD3^+CD4^+$ cells when comparing recipients that received TGFβ-conditioned T cells and one male graft (day 30) with recipients that received 2 male grafts (day 0 & 30) in the DLN ($p<0.05$).

Because there were significantly more $CD3^+CD4^+$ cells in the DLN of grafted versus ungrafted recipients ($p<0.001$), there were also numerically more $Foxp3^+$ cells in the DLNs of those recipients as a percentage of total gated live cells (TGFβ ungrafted vs. TGFβ male graft d30, $p<0.01$; TGFβ ungrafted vs. TGFβ male graft d0, 30, $p<0.01$) (**Figure 3.7B**). Interestingly, there was no difference in Foxp3 expression of $CD3^+CD4^+$ cells between recipients that received a delayed male graft on day 30 or those that were grafted with a female graft and then a male graft. This suggests that the grafting process itself was insufficient to influence Foxp3 expression of the TGFβ-conditioned T cells.

To investigate this further, the experiment was repeated with a group of recipients receiving 2 female grafts (**Figure 3.8A**). If the grafting process itself was unimportant for maintaining Foxp3 expression, these recipients would be expected to show little difference in Foxp3 expression to recipients receiving no grafts. Indeed, these recipients showed a paucity of $CD4^+$ cells in the DLN, similar to ungrafted recipients (**Figure 3.8B**). In all the groups (1, 2 or

no grafts), the percentage of $CD4^+$ cells expressing Foxp3 was not significantly different. Nevertheless, there were significantly more $Foxp3^+$ cells amongst all live cells isolated from the DLN of recipients that received 1 or 2 male grafts compared to the ungrafted recipients (1 male graft vs. ungrafted, $p<0.01$; 2 male grafts vs. ungrafted, $p<0.01$) or recipients grafted with female grafts only (1 male graft vs. female grafts, $p<0.01$; 2 male grafts vs. female grafts, $p<0.001$) (**Figure 3.8B**). These results suggest that antigen in the context of a transplanted graft, but not the process of grafting on its own, is important for the maintenance of Foxp3 expression by TGFβ-conditioned cells.

Figure 3.6

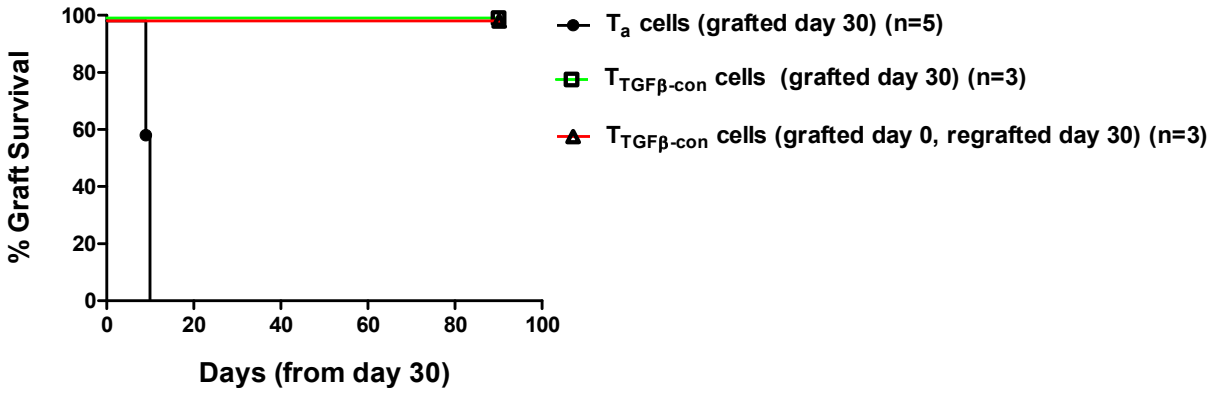


Figure 3.6 TGFβ-conditioned male-specific T cells remain non-aggressive and do not reject a male graft after being in vivo for 30 days

Marilyn TGFβ-conditioned $CD4^+$ T cells ($T_{TGFβ\text{-}con}$) or peptide-activated T cells (T_a) were transferred into female B6.$RAG^{-/-}$ recipients. Peptide-activated T cells were T cells activated in the presence of bmDCs and cognate peptide (HYA^b) but without TGFβ added. 30 days later, a male CBA.$RAG^{-/-}$ graft was transplanted and graft survival was followed daily. Control group recipients were grafted at day 0 and re-grafted at day 30. Log-rank (Mantel-Cox) analysis:

	p value
T_a cells vs. $T_{TGFβ\text{-}con}$ cells (grafted d30)	< 0.05
T_a cells vs. $T_{TGFβ\text{-}con}$ (grafted d0, 30)	< 0.05

Figure 3.7A

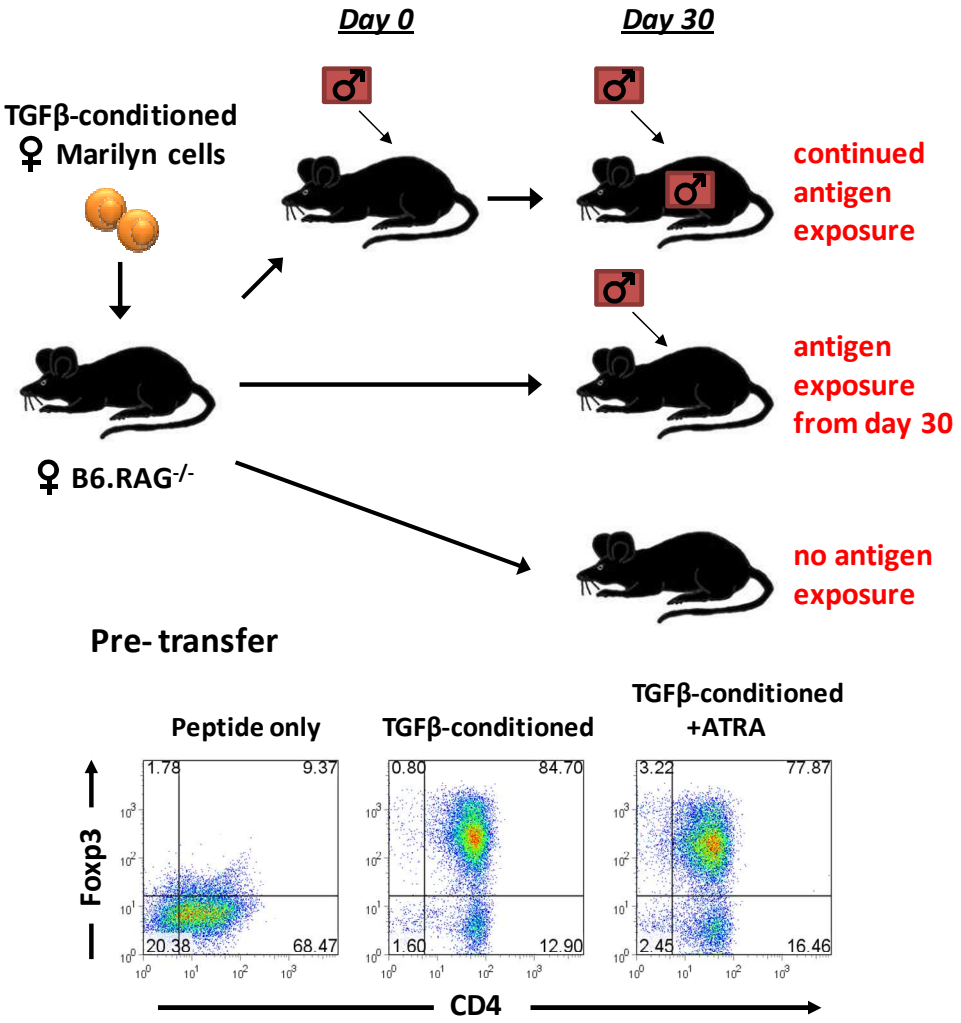
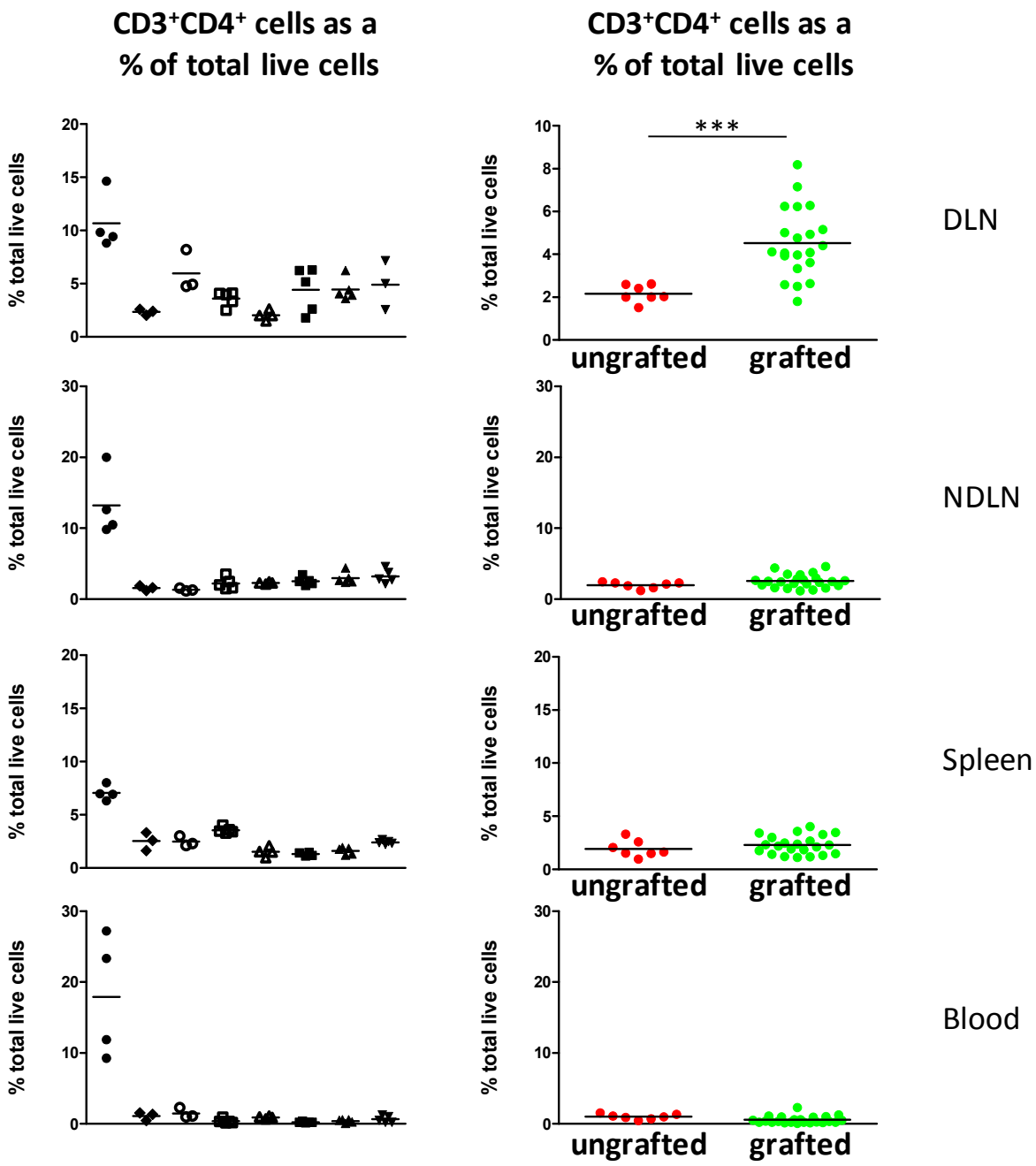


Figure 3.7A Experimental design and FACS profiles of cells transferred into recipient mice

Marilyn TGFβ-conditioned $CD4^+$ T cells were transferred into female $B6.RAG^{-/-}$ mice. A separate group of TGFβ-conditioned cells also received ATRA in vitro (10nM). The FACS profiles show the Foxp3 expression of cells pre-transfer. Some recipients were grafted with a male $CBA.RAG^{-/-}$ graft on day 0 and another graft on day 30, thus constantly exposing the cells to antigen. Other recipients were grafted on day 30. Some recipients received no graft at all. On day 60, the mice were sacrificed.

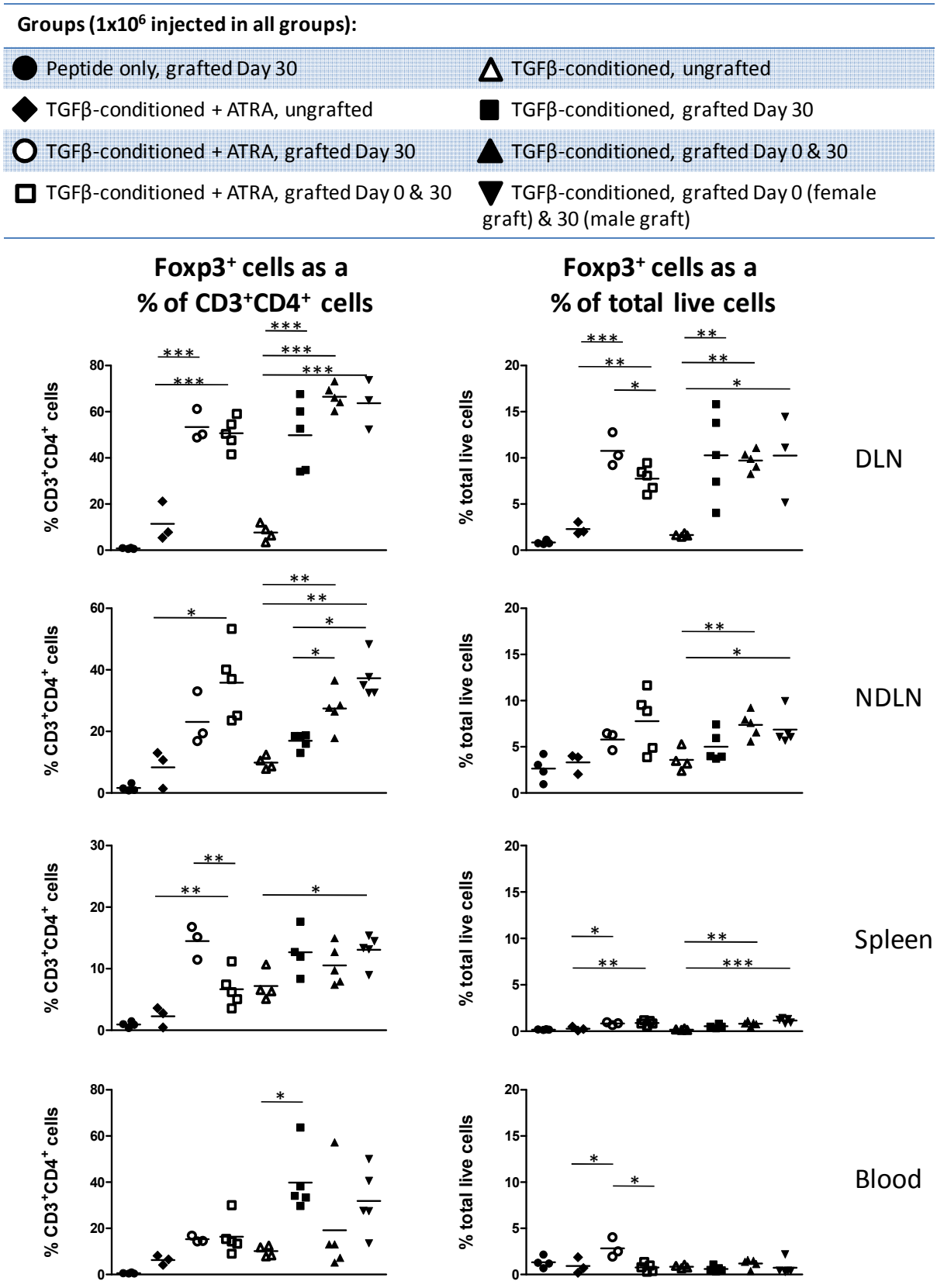
Figure 3.7B

Groups (1x10^6 injected in all groups):	
● Peptide only, grafted Day 30	△ TGFβ-conditioned, ungrafted
◆ TGFβ-conditioned + ATRA, ungrafted	■ TGFβ-conditioned, grafted Day 30
○ TGFβ-conditioned + ATRA, grafted Day 30	▲ TGFβ-conditioned, grafted Day 0 & 30
□ TGFβ-conditioned + ATRA, grafted Day 0 & 30	▼ TGFβ-conditioned, grafted Day 0 (female graft) & 30 (male graft)



** p=0.01-0.05* *** p=0.001-0.01* **** p<0.001*

Figure 3.7B (cont'd)



** p=0.01-0.05* *** p=0.001-0.01* **** p<0.001*

Figure 3.7B Higher proportion and number of Foxp3$^+$ cells amongst CD4$^+$ cells in the DLN of grafted recipients compared to ungrafted recipients

On day 60, the mice were sacrificed and their DLN, NDLN, spleen and blood were analysed by FACS. A gate was made around live cells on forward and side scatter. Further gating was performed on $CD3^+CD4^+$ cells and $Foxp3^+$ cells.

Statistical analyses (student's unpaired t test or one-way ANOVA with Dunnett's Multiple Comparison Test):

DLN ($CD3^+CD4^+$ cells as a % of total live cells)[1]	*p* value
ungrafted (combined) vs. grafted (combined)	<0.001

NDLN ($CD3^+CD4^+$ cells as a % of total live cells)	*p* value
ungrafted (combined) vs. grafted (combined)	ns

Spleen ($CD3^+CD4^+$ cells as a % of total live cells)	*p* value
ungrafted (combined) vs. grafted (combined)	ns

Blood ($CD3^+CD4^+$ cells as a % of total live cells)	*p* value
ungrafted (combined) vs. grafted (combined)	<0.05

DLN ($Foxp3^+$ cells as a % of $CD3^+CD4^+$ cells)[2]	*p* value
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d30)	<0.001
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d0, 30)	<0.001
TGFβ (ungrafted) vs. TGFβ (male graft d30)	<0.001
TGFβ (ungrafted) vs. TGFβ (male graft d0, 30)	<0.001
TGFβ (ungrafted) vs. TGFβ (female d0, male d30)	<0.001
TGFβ+ATRA (male graft d30) vs. TGFβ+ATRA (male graft d0, 30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (male graft d30)	<0.05
TGFβ (male graft d0, 30) vs. TGFβ (female d0, male d30)	ns

NDLN ($Foxp3^+$ cells as a % of $CD3^+CD4^+$ cells)	*p* value
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d30)	ns
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d0, 30)	<0.05
TGFβ (ungrafted) vs. TGFβ (male graft d30)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 30)	<0.001
TGFβ (ungrafted) vs. TGFβ (female d0, male d30)	<0.001
TGFβ+ATRA (male graft d30) vs. TGFβ+ATRA (male graft d0, 30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (male graft d30)	<0.05
TGFβ (male graft d0, 30) vs. TGFβ (female d0, male d30)	<0.05

Spleen ($Foxp3^+$ cells as a % of $CD3^+CD4^+$ cells)	*p* value
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d30)	<0.001
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d0, 30)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d30)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 30)	ns
TGFβ (ungrafted) vs. TGFβ (female d0, male d30)	<0.05
TGFβ+ATRA (male graft d30) vs. TGFβ+ATRA (male graft d0, 30)	<0.01
TGFβ (male graft d0, 30) vs. TGFβ (male graft d30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (female d0, male d30)	ns

Blood ($Foxp3^+$ cells as a % of $CD3^+CD4^+$ cells)	*p* value
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d30)	ns
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d0, 30)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d30)	<0.05
TGFβ (ungrafted) vs. TGFβ (male graft d0, 30)	ns

[1] Student's unpaired t test

[2] One-way ANOVA with Dunnett's Multiple Comparison Test

TGFβ (ungrafted) vs. TGFβ (female d0, male d30)	ns
TGFβ+ATRA (male graft d30) vs. TGFβ+ATRA (male graft d0, 30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (male graft d30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (female d0, male d30)	ns

DLN (Foxp3$^+$ cells as a % of total live cells)	*p* value
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d30)	<0.001
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d0, 30)	<0.01
TGFβ (ungrafted) vs. TGFβ (male graft d30)	<0.01
TGFβ (ungrafted) vs. TGFβ (male graft d0, 30)	<0.01
TGFβ (ungrafted) vs. TGFβ (female d0, male d30)	<0.05
TGFβ+ATRA (male graft d30) vs. TGFβ+ATRA (male graft d0, 30)	<0.05
TGFβ (male graft d0, 30) vs. TGFβ (male graft d30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (female d0, male d30)	ns

NDLN (Foxp3$^+$ cells as a % of total live cells)	*p* value
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d30)	ns
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d0, 30)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d30)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 30)	<0.01
TGFβ (ungrafted) vs. TGFβ (female d0, male d30)	<0.05
TGFβ+ATRA (male graft d30) vs. TGFβ+ATRA (male graft d0, 30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (male graft d30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (female d0, male d30)	ns

Spleen (Foxp3$^+$ cells as a % of total live cells)	*p* value
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d30)	<0.05
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d0, 30)	<0.01
TGFβ (ungrafted) vs. TGFβ (male graft d30)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 30)	<0.01
TGFβ (ungrafted) vs. TGFβ (female d0, male d30)	<0.001
TGFβ+ATRA (male graft d30) vs. TGFβ+ATRA (male graft d0, 30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (male graft d30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (female d0, male d30)	ns

Blood (Foxp3$^+$ cells as a % of total live cells)	*p* value
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d30)	<0.05
TGFβ+ATRA (ungrafted) vs. TGFβ+ATRA (male graft d0, 30)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d30)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 30)	ns
TGFβ (ungrafted) vs. TGFβ (female d0, male d30)	ns
TGFβ+ATRA (male graft d30) vs. TGFβ+ATRA (male graft d0, 30)	<0.05
TGFβ (male graft d0, 30) vs. TGFβ (male graft d30)	ns
TGFβ (male graft d0, 30) vs. TGFβ (female d0, male d30)	ns

Figure 3.8A

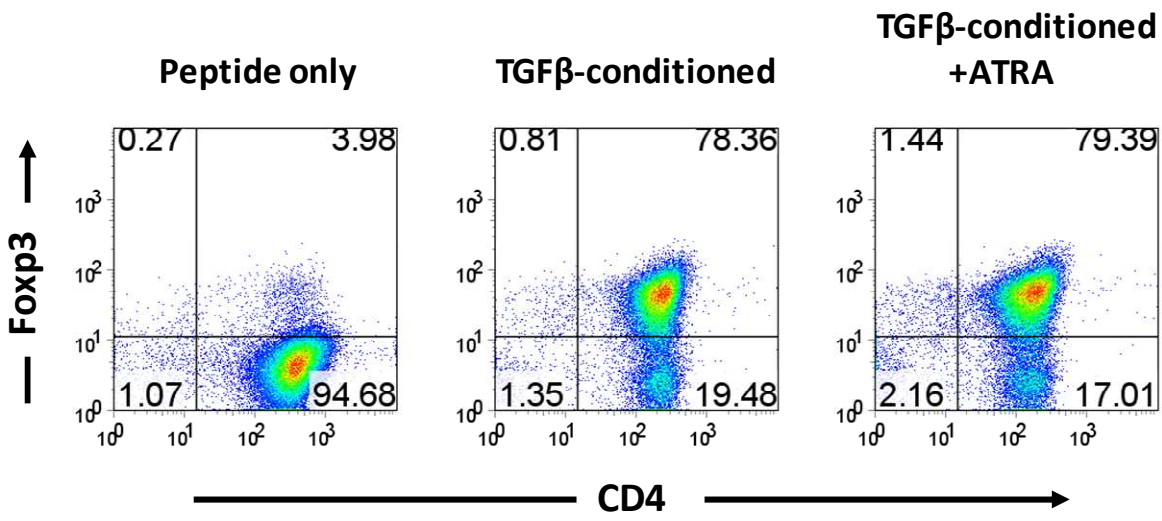
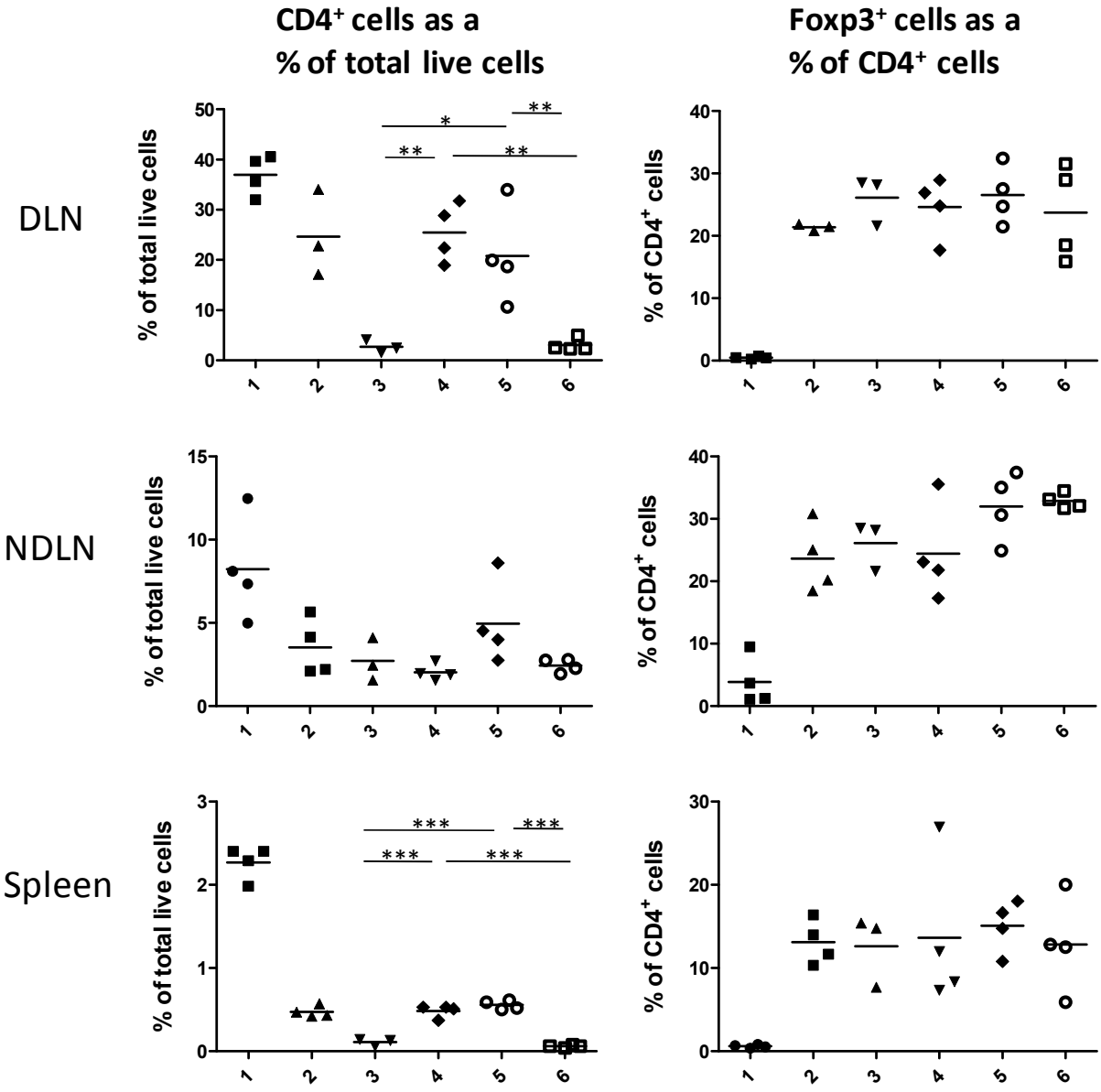


Figure 3.8A FACS profiles of cells pre-transfer

The profiles show the Foxp3 expression of TGFβ-conditioned $CD4^+$ T cells generated from Marilyn.hCD2.KI mice. Some TGFβ-conditioned cells also received ATRA in vitro (10nM).

Figure 3.8B

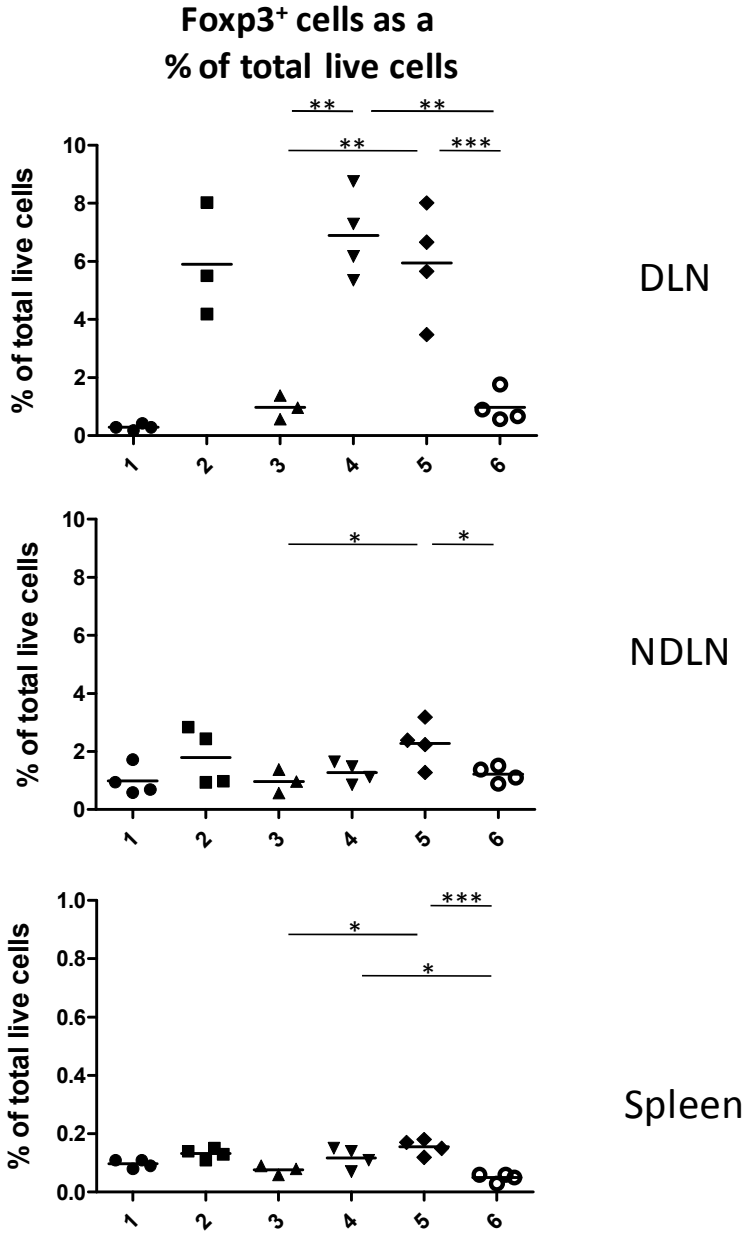
Groups (1x10^6 injected in all groups):	
1. Peptide only, grafted Day 23	4. TGFβ-conditioned, grafted Day 23
2. TGFβ-conditioned + ATRA, grafted Day 0 & 23	5. TGFβ-conditioned, grafted Day 0 & 23
3. TGFβ-conditioned, ungrafted	6. TGFβ-conditioned, female skin grafted Day 0 & 23



** p=0.01-0.05* *** p=0.001-0.01* **** p<0.001*

Figure 3.8B (cont'd)

Groups (1x10^6 injected in all groups):	
1. Peptide only, grafted Day 23	4. TGFβ-conditioned, grafted Day 23
2. TGFβ-conditioned + ATRA, grafted Day 0 & 23	5. TGFβ-conditioned, grafted Day 0 & 23
3. TGFβ-conditioned, ungrafted	6. TGFβ-conditioned, female skin grafted Day 0 & 23



** p=0.01-0.05* *** p=0.001-0.01* **** p<0.001*

Figure 3.8B Graft antigen helps maintain a high number of Foxp3$^+$ cells in the DLN of recipient mice

TGFβ-conditioned (with or without ATRA) $CD4^+$ T cells were transferred into female B6.$RAG^{-/-}$ mice. Some recipients were grafted with a male CBA.$RAG^{-/-}$ graft on day 0 and another graft on day 23. Other recipients were grafted on day 23. Some recipients received no graft at all. One group received a female CBA.$RAG^{-/-}$ graft on both day 0 and day 23. On day 46, the mice were sacrificed and their DLN, NDLN, spleen and blood were analysed by FACS. A gate was made around live cells. Further gating was performed on $CD4^+$ cells and $Foxp3^+$ cells.

Statistical analyses (one-way ANOVA with Dunnett's Multiple Comparison Test):

DLN ($CD4^+$ cells as a % of total live cells)	*p* value
TGFβ (ungrafted) vs. TGFβ (male graft d23)	<0.01
TGFβ (ungrafted) vs. TGFβ (male graft d0, 23)	<0.05
TGFβ+ATRA (male graft d0, 23) vs. TGFβ (male graft d0, 23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d23)	<0.01
TGFβ (female graft d0, 23) vs. TGFβ (male graft d0, 23)	<0.01

NDLN ($CD4^+$ cells as a % of total live cells)	*p* value
TGFβ (ungrafted) vs. TGFβ (male graft d23)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 23)	ns
TGFβ+ATRA (male graft d0, 23) vs. TGFβ (male graft d0, 23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d0, 23)	ns

Spleen ($CD4^+$ cells as a % of total live cells)	*p* value
TGFβ (ungrafted) vs. TGFβ (male graft d23)	<0.001
TGFβ (ungrafted) vs. TGFβ (male graft d0, 23)	<0.001
TGFβ+ATRA (male graft d0, 23) vs. TGFβ (male graft d0, 23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d23)	<0.001
TGFβ (female graft d0, 23) vs. TGFβ (male graft d0, 23)	<0.001

DLN ($Foxp3^+$ cells as a % of $CD4^+$ cells)	*p* value
TGFβ (ungrafted) vs. TGFβ (male graft d23)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 23)	ns
TGFβ+ATRA (male graft d0, 23) vs. TGFβ (male graft d0, 23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d0, 23)	ns

NDLN ($Foxp3^+$ cells as a % of $CD4^+$ cells)	*p* value
TGFβ (ungrafted) vs. TGFβ (male graft d23)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 23)	ns
TGFβ+ATRA (male graft d0, 23) vs. TGFβ (male graft d0, 23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d0, 23)	ns

Spleen ($Foxp3^+$ cells as a % of $CD4^+$ cells)	*p* value
TGFβ (ungrafted) vs. TGFβ (male graft d23)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 23)	ns
TGFβ+ATRA (male graft d0, 23) vs. TGFβ (male graft d0, 23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d0, 23)	ns

DLN ($Foxp3^+$ cells as a % of total live cells)	*p* value
TGFβ (ungrafted) vs. TGFβ (male graft d23)	<0.01
TGFβ (ungrafted) vs. TGFβ (male graft d0, 23)	<0.01
TGFβ+ATRA (male graft d0, 23) vs. TGFβ (male graft d0, 23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d23)	<0.01
TGFβ (female graft d0, 23) vs. TGFβ (male graft d0, 23)	<0.001

NDLN ($Foxp3^+$ cells as a % of total live cells)	*p* value
TGFβ (ungrafted) vs. TGFβ (male graft d23)	ns

TGFβ (ungrafted) vs. TGFβ (male graft d0, 23)	<0.05
TGFβ+ATRA (male graft d0, 23) vs. TGFβ (male graft d0, 23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d23)	ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d0, 23)	<0.05

Spleen (Foxp3$^+$ cells as a % of total live cells)	*p* value
TGFβ (ungrafted) vs. TGFβ (male graft d23)	ns
TGFβ (ungrafted) vs. TGFβ (male graft d0, 23)	<0.05
TGFβ+ATRA (male graft d0, 23) vs. TGFβ (male graft d0, 23)	<ns
TGFβ (female graft d0, 23) vs. TGFβ (male graft d23)	<0.05
TGFβ (female graft d0, 23) vs. TGFβ (male graft d0, 23)	<0.001

3.7 Can cognate antigen given intravenously stabilise TGFβ-conditioned T cells?

The previous section showed that cognate antigen provided in the context of a skin graft enables some of the TGFβ-conditioned cells to maintain Foxp3 expression and that the grafting process itself was insufficient for maintaining Foxp3 expression. This observation raised the possibility that antigen alone might be able to maintain Foxp3 expression. Previous work has shown that intravenous, oral or subcutaneous cognate peptide delivery into TCR-transgenic $RAG^{-/-}$ mice that lack any nTreg can result in the generation of $CD25^+$ cells that show Foxp3 expression and suppressive properties *in vitro* (Thorstenson et al. 2001; Apostolou et al. 2004; Dahlberg et al. 2007). Whilst these studies have examined the *generation* of Treg *in vivo*, they have not investigated whether cognate antigen delivery can *maintain* the expression of Foxp3 by iTreg. Therefore, I set out to answer this question.

TGFβ-conditioned $CD4^+$ T cells (86% $Foxp3^+$) were derived from Marilyn mice (**Figure 3.9A**), and injected into wildtype B6.SJL congenic female mice whose cells can be distinguished from the former by their expression of surface CD45.1 (Marilyn cells express CD45.2). HYA^b peptide was injected i.v. at 2μg or 20μg dosages every 3 days, and the peripheral lymph nodes (inguinal and axillary) and spleen from these mice were collected at 10 days after the onset of injection. Mice that received a high dose of peptide (20μg) showed a significantly higher proportion of $CD45.2^+$ cells amongst $CD4^+$ cells in both peripheral LNs (p<0.001) and spleens (p<0.001) of these mice (**Figure 3.9B**). A gate placed around live cells also showed significantly higher percentages of $CD4^+CD45.2^+$ cells in the spleen (p<0.001) and LN (p<0.05) of the 20μg peptide dose recipients. In these recipients, $CD45.2^+$ cells also accounted for a higher percentage of the total number of $Foxp3^+$ cells (spleen: p<0.001, LN: p<0.01), despite the Foxp3 percentage of total $CD4^+$ cells remaining similar whether or not peptide was

injected. Hence, there was an increase in $Foxp3^+CD45.2^+$ cells at the expense of $Foxp3^+CD45.1^+$ cells. However, the percentage of $CD45.2^+$ cells that were $Foxp3^+$ appeared to be lower in the 20μg peptide dose group in both spleen and lymph nodes (spleen, LN: $p<0.001$). Overall, these results suggest that high peptide dosage (20μg) had led to an expansion of both $Foxp3^+$ and $Foxp3^-$ TGFβ-conditioned $CD4^+$ cells at the expense of the native $CD45.1^+$ cells. The expansion of $Foxp3^-CD45.2^+$ cells might have been greater than that of $Foxp3^+CD45.2^+$ cells, thus resulting in a drop in the percentage of $Foxp3^+$ cells of total $CD4^+CD45.2^+$ cells.

To investigate this possibility, $1x10^6$ $Foxp3^+$ TGFβ-conditioned cells derived from Marilyn.hCD2.KI mice and sorted by Mo-flo based on CD4 and hCD2 expression were transferred into B6.SJL mice (**Figure 3.10A**). By excluding $Foxp3^-$ cells, it was expected that the percentage of Foxp3-expressing cells within the $CD4^+CD45.2^+$ cells at 10 days would solely reflect $Foxp3^+$ cells' response to the intravenous peptide, thus eliminating any response of $Foxp3^-$ cells which would obfuscate interpretation. $CD45.2^+$ cells as a percentage of $CD4^+$ cells was greater in the peripheral LNs ($p<0.01$) and spleens ($p<0.001$) of mice that received high dose (20μg) peptide compared to when no peptide was administered (**Figure 3.10B**). However, this was at a much lower level (~0.3% and 0.1% in the spleen and LN respectively) compared to when unsorted cells were transferred (~2% and 1% in the spleen and LN respectively), lending support to the hypothesis of expansion of $Foxp3^-$ cells when unsorted cells were transferred. Again, the percentage of $Foxp3^+$ cells within the total $CD4^+CD45.2^+$ cells was lower in the mice that received 20μg dose peptide compared to no peptide (spleen, LN: $p<0.01$). Whilst it may seem that high peptide dosage led to a drop in Foxp3 expression of transferred $CD4^+CD45.2^+$ cells, this result may still be due to the possible expansion of 'contaminating' $hCD2^-$ cells. However, despite the lower percentage of Foxp3 in

the 20µg dose group, there were still detectable numbers of $Foxp3^+$ T cells in the spleen and LNs in these mice.

From the previous two experiments, one can conclude that high peptide dose (20µg) delivered intravenously over 10 days can lead to increased numbers of $CD4^+CD45.2^+$ cells. This is a result of an expansion of both $Foxp3^-$ and $Foxp3^+$ cells but with the $Foxp3^-$ cells predominating, thus leading to a drop in the percentage Foxp3 expression of $CD45.2^+$ cells.

Next, the mode of antigen-exposure was changed from intravenous peptide to a male graft ($B6.RAG^{-/-}$) to investigate whether the graft microenvironment would aid in maintaining Foxp3 expression of transferred $hCD2^+$ TGFβ-conditioned iTreg. Female grafts were also transplanted onto control recipients (**Figure 3.11A**). In those recipients transplanted with a male graft, a significantly greater percentage of gated $CD4^+$ cells from the DLN appeared to be $CD45.2^+$ cells ($p<0.001$) and $CD45.2^+Foxp3^+$cells ($p<0.001$) (**Figure 3.11B**). The statistical p value of <0.001 applies to comparison to either ungrafted or female graft recipients. This suggests an expansion of transferred $Foxp3^+$ cells in response to their cognate peptide. There was also a significantly lower percentage of $CD4^+CD45.2^+$ cells in the DLN of male graft recipients expressing Foxp3 compared to female graft recipients ($p<0.05$). This drop is similar to high peptide dose (20µg) intravenous peptide delivery. However, the drop is to ~40% compared to less than 20% for intravenous peptide delivery (**Figure 3.10B**). To explain this result, it is possible that the male graft environment provided a different and localised peptide dose to the intravenous peptide dose. Alternatively, the tissue microenvironment might have provided cues which helped maintain the survival and/or Foxp3 expression of these cells. It is also possible that in the intravenous peptide delivery experiments, cells might have migrated to organs other than the ones which were analysed.

Together, the data would suggest that antigen in the context of a graft helps maintain survival and/or Foxp3 expression of TGFβ-conditioned Foxp3$^+$ cells. In the presence of cognate peptide delivered in the context of a graft, there were more transferred CD45.2$^+$ cells despite lower Foxp3 expression as a percentage of CD45.2$^+$ cells. Hence, numerically, there was still more Foxp3$^+$ iTreg in those mice that received a male graft. Moreover, it would appear that in a conventional mouse, Foxp3 expression is homeostatically controlled such that an increase in the number of iTreg as a result of exposure to their cognate peptide leads to a corresponding decrease in regulatory T cells of other antigen-specificities.

Figure 3.9A

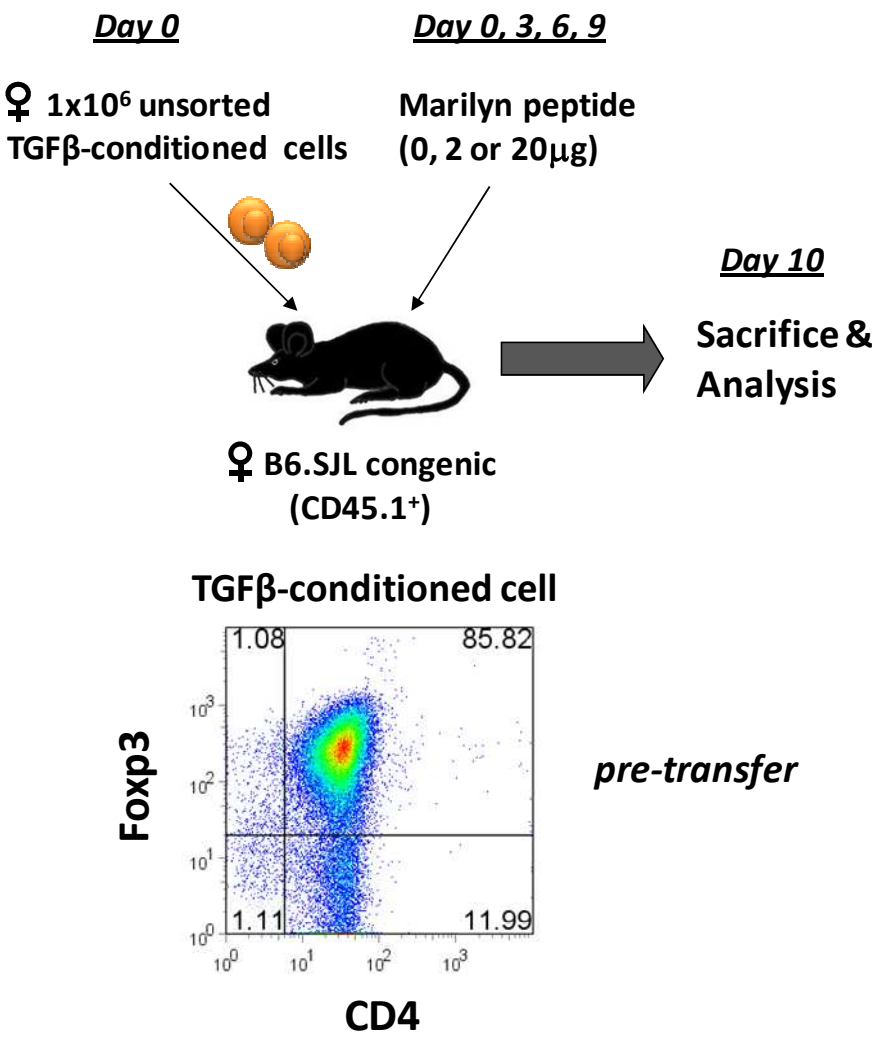
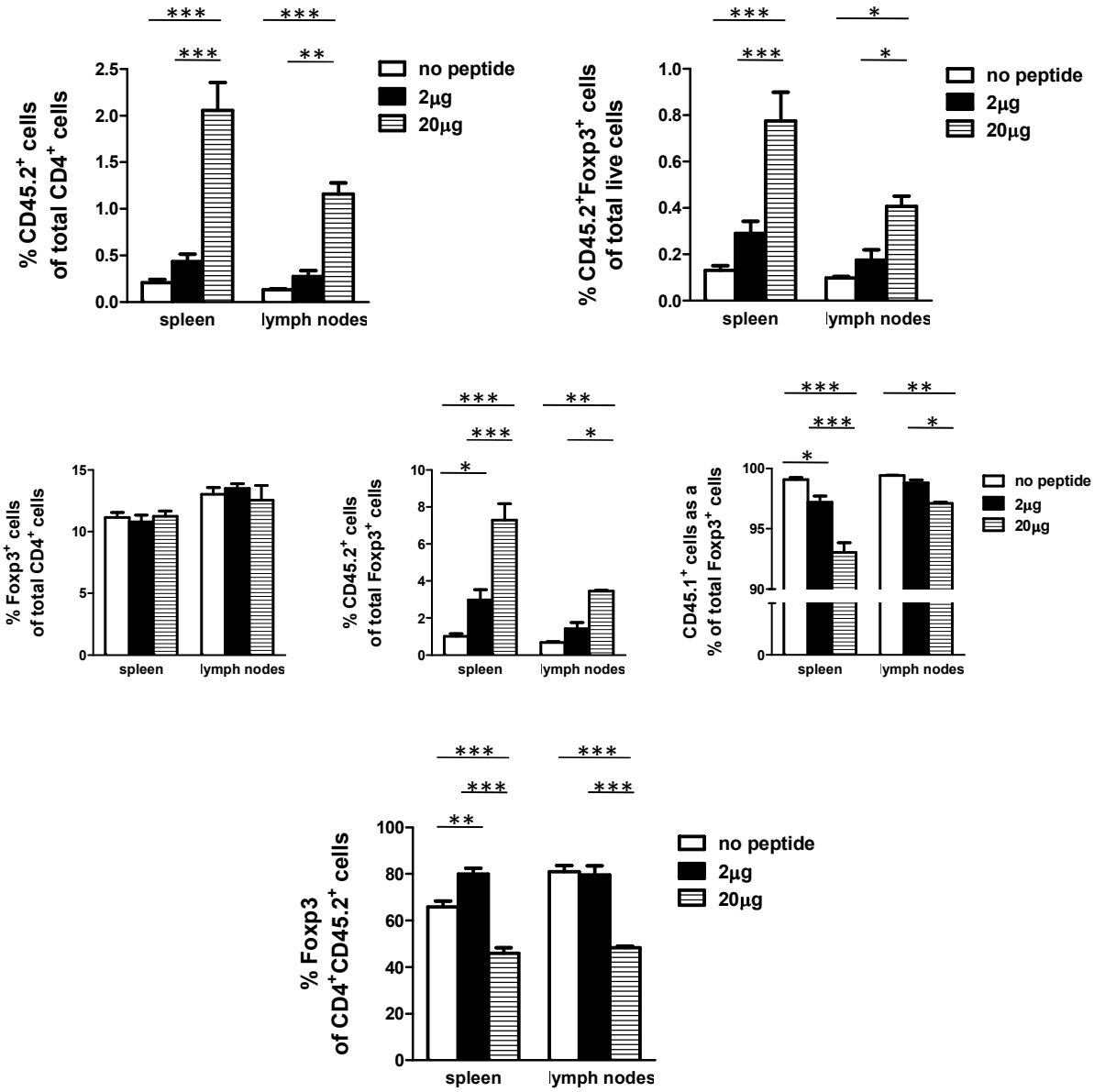


Figure 3.9A Experimental design and FACS profile of TGFβ-conditioned CD4⁺ T cells pre-transfer

1x10^6 unsorted TGFβ-conditioned CD4$^+$ T cells derived from Marilyn mice were transferred into B6.SJL congenic mice. The FACS plot shows that 85% of TGFβ-conditioned cells were CD4$^+$Foxp3$^+$. HYA^b peptide was injected at various doses (0μg/PBS only, 2μg, 20μg) every 3 days. The peripheral lymph nodes and spleens of the B6.SJL mice were harvested at day 10 and analysed by FACS.

Figure 3.9B



** p=0.01-0.05* *** p=0.001-0.01* **** p<0.001*

Figure 3.9B Higher numbers of transferred Foxp3+ TGFβ-conditioned CD4+ cells are found in wildtype hosts that have received a high dose of intravenous peptide

Cells from the peripheral lymph nodes (axillary and inguinal) and spleens were stained with antibodies against CD45.1, CD45.2, CD4 and Foxp3. A gate was placed around live cells.

Statistical analysis (two-way ANOVA with Bonferroni post-test):

% CD45.2 cells of total CD4+ cells

no peptide vs 2mcg

Spleen	ns
lymph nodes	ns

no peptide vs 20mcg

spleen	$p<0.001$
lymph nodes	$p<0.001$
2mcg vs 20mcg	
spleen	$p<0.001$
lymph nodes	$p<0.01$

% CD45.2$^+$Foxp3$^+$ cells of total livecells

no peptide vs 2mcg	
spleen	ns
lymph nodes	ns
no peptide vs 20mcg	
spleen	$p<0.001$
lymph nodes	$p<0.05$
2mcg vs 20mcg	
spleen	$p<0.001$
lymph nodes	$p<0.05$

% Foxp3$^+$ cells of total CD4$^+$ cells

no peptide vs 2mcg	
spleen	ns
lymph nodes	ns
no peptide vs 20mcg	
spleen	ns
lymph nodes	ns
2mcg vs 20mcg	
spleen	ns
lymph nodes	ns

% CD45.2$^+$ cells of total Foxp3$^+$ cells

no peptide vs 2mcg	
spleen	$p<0.05$
lymph nodes	ns
no peptide vs 20mcg	
spleen	$p<0.001$
lymph nodes	$p<0.01$
2mcg vs 20mcg	
spleen	$p<0.001$
lymph nodes	$p<0.05$

% CD45.1$^+$ cells of total Foxp3$^+$ cells

no peptide vs 2mcg	
Spleen	$p<0.05$
lymph nodes	ns
no peptide vs 20mcg	
spleen	$p<0.001$
lymph nodes	$p<0.01$

2mcg vs 20mcg	
Spleen	$p<0.001$
lymph nodes	$p<0.05$

% Foxp3$^+$ cells of CD4$^+$CD45.2$^+$ cells

no peptide vs 2mcg	
Spleen	$p<0.01$
lymph nodes	ns
no peptide vs 20mcg	
spleen	$p<0.001$
lymph nodes	$p<0.001$
2mcg vs 20mcg	
Spleen	$p<0.001$
lymph nodes	$p<0.001$

Figure 3.10A

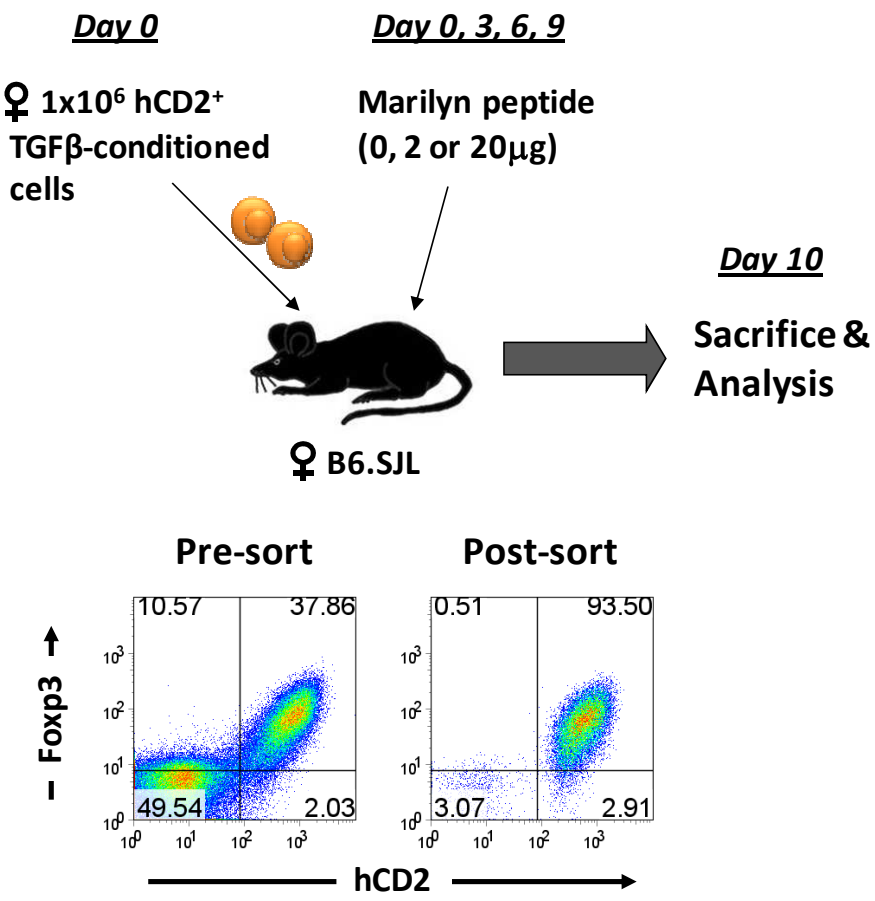
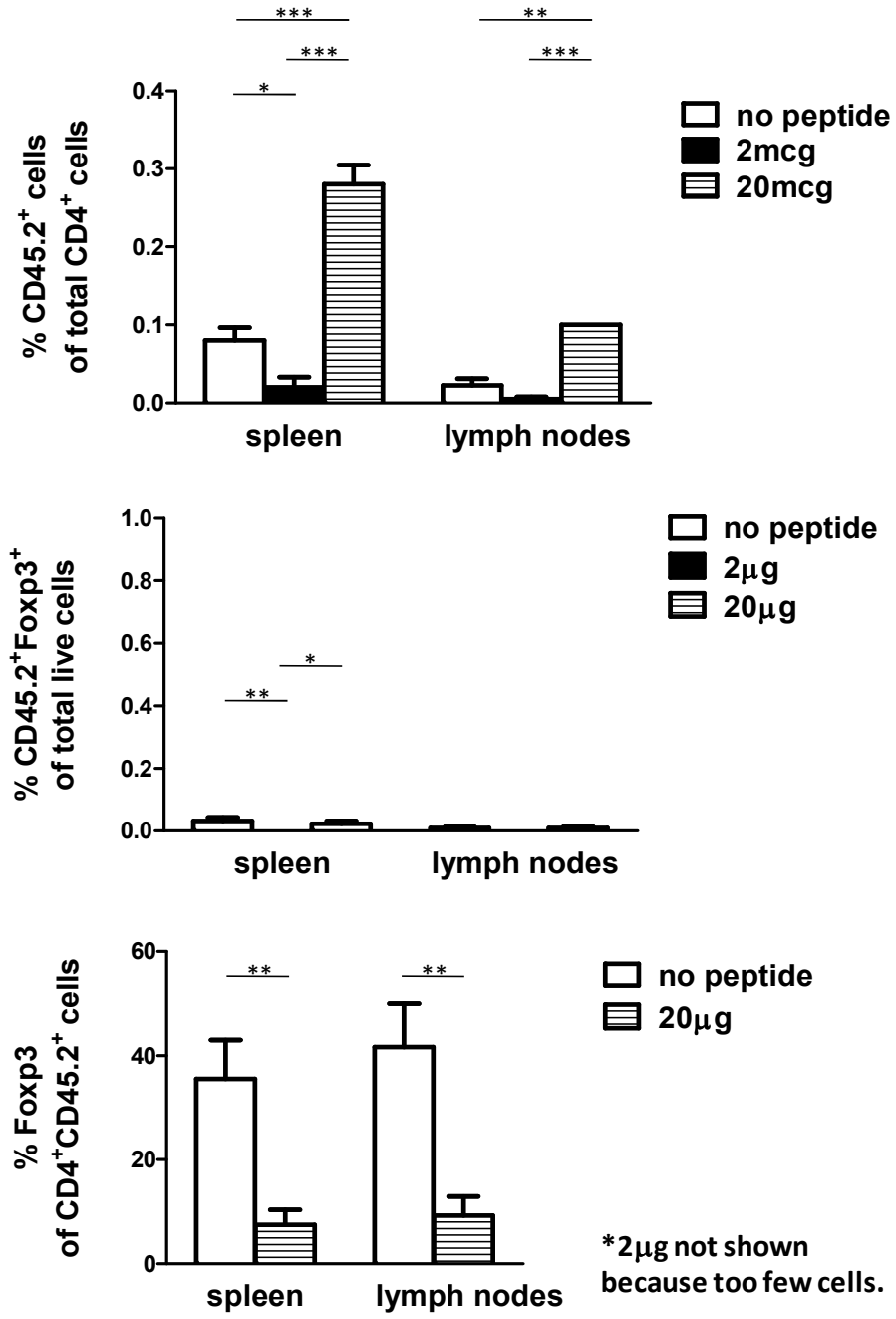


Figure 3.10A Experimental design and FACS profile of sorted TGFβ-conditioned T cells pre-transfer

$CD4^+hCD2^+$ cells were Mo-flo sorted from TGFβ-conditioned $CD4^+$ cells generated from Marilyn.hCD2.KI mice. $1x10^6$ $CD4^+hCD2^+$ cells were transferred into B6.SJL congenic mice. HYA^b peptide (dissolved in PBS) was injected at various dosages (0μg/PBS only, 2μg, 20μg) every 3 days. The peripheral lymph nodes and spleens of the B6.SJL mice were harvested at day 10 and analysed by FACS.

Figure 3.10B



** p=0.01-0.05* *** p=0.001-0.01* **** p<0.001*

Figure 3.10B Higher numbers of $CD4^+CD45.2^+$ cells found in recipients that have received a high dose of intravenous peptide

Cells from the peripheral lymph nodes (bilateral axillary and inguinal) and spleens were stained with antibodies against CD45.1, CD45.2, CD4 and Foxp3. A gate was placed around live cells. Statistical analysis was performed with two-way ANOVA with Bonferroni post-test. Percentage Foxp3 expression of $CD4^+CD45.2^+$ cells for 2µg is not shown due to too few cells.

Statistical analysis (two-way ANOVA with Bonferroni post-test):

% CD45.2$^+$ cells of total CD4$^+$ cells

no peptide vs 2mcg	
spleen	$p<0.05$
lymph nodes	Ns
no peptide vs 20mcg	
spleen	$p<0.001$
lymph nodes	$p<0.01$
2mcg vs 20mcg	
spleen	$p<0.001$
lymph nodes	$p<0.001$

% CD45.2$^+$Foxp3$^+$ cells of total livecells

no peptide vs 2mcg	
spleen	$p<0.01$
lymph nodes	ns
no peptide vs 20mcg	
spleen	ns
lymph nodes	ns
2mcg vs 20mcg	
spleen	$p<0.05$
lymph nodes	ns

% Foxp3$^+$ cells of CD4$^+$CD45.2$^+$ cells

no peptide vs 20mcg	
spleen	$p<0.01$
lymph nodes	$p<0.01$

Figure 3.11A

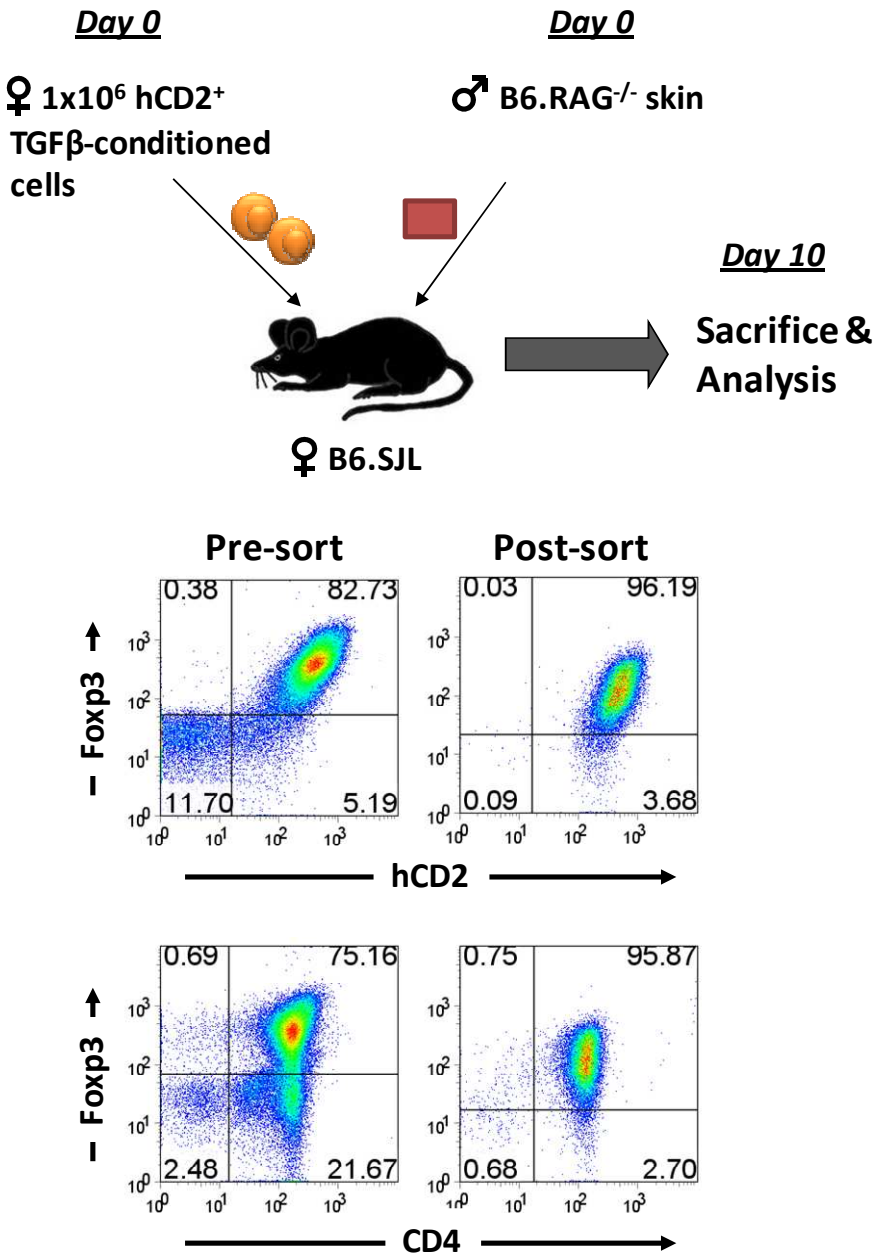
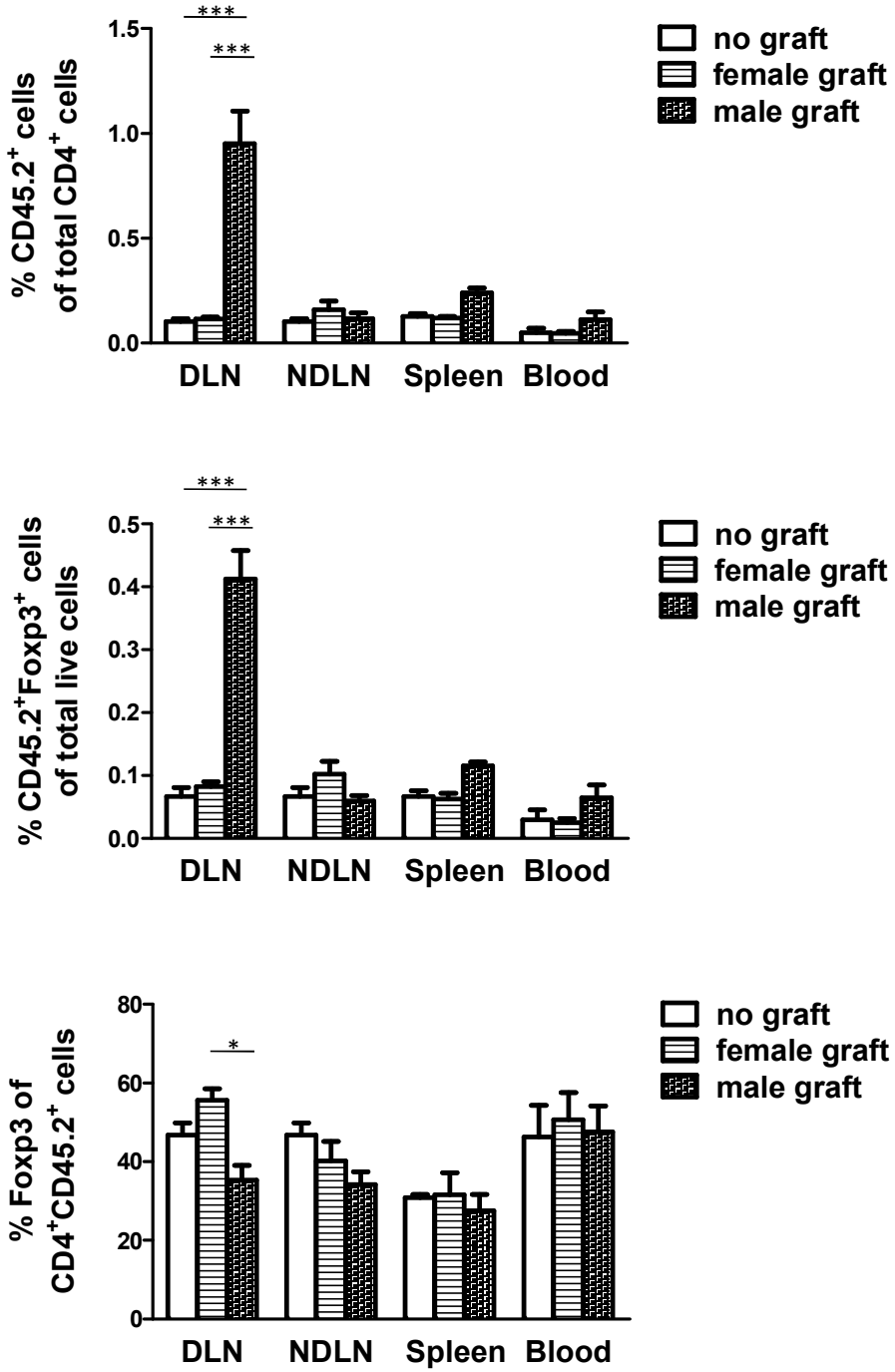


Figure 3.11A Experimental design and pre-transfer FACS profile of sorted Foxp3⁺ TGFβ-conditioned CD4⁺ T cells

1x10^6 Mo-flo sorted CD4$^+$hCD2$^+$ TGFβ-conditioned CD4$^+$ T cells derived from Marilyn.hCD2.KI mice were transferred into B6.SJL congenic mice. The FACS plot shows that over 95% of sorted CD4$^+$hCD2$^+$ cells expressed Foxp3. On the following day, recipients were transplanted with a male graft or a female graft or were left ungrafted. The B6.SJL mice were harvested at day 10 and analysed by FACS.

Figure 3.11B



** p=0.01-0.05* *** p=0.001-0.01* **** p<0.001*

Figure 3.11B Male graft transplantation leads to higher numbers of male-specific TGFβ-conditioned Foxp3$^+$ cells in the draining lymph node

Cells from the peripheral lymph nodes (axillary and inguinal) and spleen were stained with antibodies against CD45.1, CD45.2, CD4 and Foxp3. A gate was placed around live cells.

Statistical analysis (two-way ANOVA with Bonferroni post-test):

% CD45.2$^+$ cells of total CD4$^+$ cells
no graft vs. female graft

Draining lymph node	ns
Non-draining lymph nodes	ns
Spleen	ns
Blood	ns
no graft vs. male graft	
Draining lymph node	$p<0.001$
Non-draining lymph nodes	ns
Spleen	ns
Blood	ns
female graft vs. male graft	
Draining lymph node	$p<0.001$
Non-draining lymph nodes	ns
Spleen	ns
Blood	ns
% CD45.2$^+$Foxp3$^+$ cells of total live cells	
no graft vs. female graft	
Draining lymph node	ns
Non-draining lymph nodes	ns
Spleen	ns
Blood	ns
no graft vs. male graft	
Draining lymph node	$p<0.001$
Non-draining lymph nodes	ns
Spleen	ns
Blood	ns
female graft vs. male graft	
Draining lymph node	$p<0.001$
Non-draining lymph nodes	ns
Spleen	ns
Blood	ns
% Foxp3$^+$ cells of CD4$^+$CD45.2$^+$ cells	
no graft vs. female graft	
Draining lymph node	ns
Non-draining lymph nodes	ns
Spleen	ns
Blood	ns
no graft vs. male graft	
Draining lymph node	ns
Non-draining lymph nodes	ns
Spleen	ns
Blood	ns
female graft vs. male graft	
Draining lymph node	$p<0.05$
Non-draining lymph nodes	ns
Spleen	ns
Blood	ns

3.8 Permissive epigenetic modifications are found at the Foxp3 locus of cells isolated from draining lymph nodes of mice exposed to antigen

In the previous sections, I showed that male-specific Foxp3$^+$ iTreg generated from *in vitro* TGFβ-conditioning responded to a transplanted male graft *in vivo* by expanding and/or surviving. This led to an overall higher number of Foxp3$^+$ cells isolated from the DLN of male graft recipients compared to female graft recipients (**Figure 3.8B** and **3.11B**). I aimed to examine if this increase in number was also associated with an increase in the stability of Foxp3 expression by examining histone methylation at various key regions of the *Foxp3* locus. Some studies have examined epigenetic modifications at the Foxp3 locus of iTreg immediately following TGFβ-conditioning *in vitro* (Floess et al. 2007; Polansky et al. 2008). However, no reports have examined whether epigenetic changes can occur in response to factors such as graft antigen exposure following the *in vivo* transfer of TGFβ-conditioned iTreg.

1x10^6 Mo-flo sorted Foxp3$^+$ TGFβ-conditioned CD4$^+$ cells (from Marilyn.hCD2.KI mice) were injected into RAG$^{-/-}$ recipients. This was followed by the transplantation of a male graft. Control recipients received female graft or no grafts at all (**Figure 3.12A**). On day 21 post-transplant, the recipients were sacrificed, and cells isolated from the DLN, NDLN, spleen and blood were examined by FACS for the expression Foxp3. Pooled DLN and spleen cells from each group ('male graft', 'female graft', 'no graft') were sorted by Mo-flo into CD4$^+$hCD2$^+$ and CD4$^+$hCD2$^-$ populations. These were immunoprecipitated with antibodies against histone 3 lysine 4 trimethylation (H3K4me3) or histone 3 lysine 27 trimethylation (H3K4me27). Immunoprecipitated gene fragments were then amplified using PCR using primers specific for proximal promoter, intronic enhancer region 1 and enhancer region 2 of the *Foxp3* locus (**Figure 3.12C**). For control, CHIP was also performed on pre-transfer CD4$^+$hCD2$^+$ cells as well as the CD4$^+$hCD2$^-$ subpopulation.

The FACS analysis of tissue cells from graft recipients showed a significantly greater expansion of $CD3^+CD4^+$ in the DLN of male graft recipients compared to female graft or ungrafted recipients (**Figure 3.12B**). There was no difference in the NDLN, spleen or blood, suggesting that DLN was the site of expansion for the transferred cells. The greater percentage of $CD3^+CD4^+$ cells correlated with a higher percentage of $Foxp3^+$ cells as a percentage of live cells. This was seen in the DLN and spleen of the male graft recipients and is consistent with previous data (**Section 3.6**). In the DLN of 'male graft' group Foxp3 as a percentage of $CD3^+CD4^+$ was significantly higher than 'female group', although interestingly, not significantly higher than the 'no graft' group. On the other hand, in the spleen and blood of male graft recipients, significantly fewer $CD3^+CD4^+$ cells showed Foxp3 expression. This might be due to 'loss' of Foxp3 expression by $CD4^+hCD2^+$ cells or might be due to an expansion of 'contaminating' $CD4^+hCD2^-$ that were also transferred. Hence, the data suggests that antigen from a male graft boosts the number of $Foxp3^+$ iTreg following their transfer *in vivo*, and may help maintain their Foxp3 expression in the DLN. The epigenetic changes (that I will now describe) seen at the Foxp3 locus of male graft recipients suggest that their maintained Foxp3 expression might be secondary to a more 'open' conformation conducive to transcription.

The 5' proximal promoter, enhancer region 1 (also known as conserved non-coding region 1), and enhancer region 2 (also known as conserved non-coding region 2, or Treg Specific Demethylated Region) of the Foxp3 locus were examined for histone methylations associated with a more or less 'accessible' chromatin conformation (**Figure 3.12C**). These regions were selected as previous studies have shown that these are all key conserved non-coding sequences of the *Foxp3* gene. Furthermore, enhancer region 2 is a strongly demethylated region in nTreg, and DNA demethylation is frequently linked to histone

modifications (Dobosy et al. 2001). Specifically, both trimethylation of lysine 4 (H3K4me3) and trimethylation of lysine 27 (H3K4me27) on histone 3 were examined. Whilst the former is associated with active DNA transcription, the latter is associated with gene silencing (Martin et al. 2005). Hence, the ratio of H3K4me4/H3K4me27 gives an indication of the likelihood of Foxp3 transcription, with a higher ratio suggesting a greater likelihood for transcription.

In the proximal promoter region, the H3K4me3/H3K27me3 ratio was not statistically different between pre-transfer $CD4^+hCD2^+$ ($Foxp3^+$) and $CD4^+hCD2^-$ ($Foxp3^-$) cells (**Figure 3.12D**), suggesting that the TGFβ-conditioning does not confer more transcriptionally-permissive histone 3 trimethylations to the $Foxp3^+$ subset. However, in the spleens of male graft and ungrafted recipients, $CD4^+hCD2^+$ cells appeared to have a higher H3K4me3/H3K27me3 ratio than $CD4^+hCD2^-$ cells. This suggests that *in vivo*, $Foxp3^+$ cells had a more transcriptionally-permissive chromatin conformation at the proximal promoter than $Foxp3^-$ cells regardless of the presence of graft antigen.

At the intronic enhancer 1 region, pre-transfer $CD4^+hCD2^+$ cells displayed a significantly higher H3K4me3/H3K27me3 ratio than $CD4^+hCD2^-$ cells, suggesting that TGFβ-conditioning renders this region conducive to gene transcription (**Figure 3.12D**). This was also true for the sorted $CD4^+hCD2^+$ cells compared to sorted $CD4^+hCD2^-$ cells from 'male graft', 'female graft' and 'no graft' groups, reaching statistical significance in the spleens. In the 'male graft' group, $CD4^+hCD2^+$ cells in the spleen had a significantly higher H3K4me3/H3K27me3 ratio than pre-transfer $CD4^+hCD2^+$ cells, suggesting a more transcriptionally-permissive chromatin structure in the presence of antigen once the cells were transferred *in vivo*. There was no significant difference between the pre-transfer $CD4^+hCD2^+$ H3K4me3/H3K27me3 ratio and

that of 'female graft' and 'no graft' groups. 'Male graft' splenic $CD4^+hCD2^+$ cells also had a higher H3K4me3/H3K27me3 ratio than 'no graft' splenic $CD4^+hCD2^+$ cells. There was no difference in the H3K4me3/H3K27me3 ratio between cells from 'female graft' or 'no graft' groups in the spleen or DLN. Again, this suggests that $Foxp3^+$ cells in the presence of a male graft have a more 'open' Foxp3 locus for gene expression than in the presence of a female graft or no graft.

Similar to enhancer region 1, enhancer region 2 showed a higher H3K4me3/H3K27me3 ratio in pre-transfer $CD4^+hCD2^+$ cells than $CD4^+hCD2^-$ cells (**Figure 3.12D**). Sorted $CD4^+hCD2^+$ cells from the spleens of 'female graft' and 'male graft' groups also showed a higher H3K4me3/H3K27me3 ratio than sorted $CD4^+hCD2^-$ cells. This suggests that $CD4^+hCD2^+$ cells had a more transcriptionally-permissive enhancer 2 region than $CD4^+hCD2^-$ cells. The H3K4me3/H3K27me3 ratio was significantly higher in the 'male graft' $CD4^+hCD2^+$ cells from the spleen than pre-transfer $CD4^+hCD2^+$ cells at enhancer region 2. Hence, a male graft appeared to 'affirm' Foxp3 expression of $Foxp3^+$ iTreg following their transfer *in vivo*. $CD4^+hCD2^+$ cells sorted from 'female graft' spleens did not show a significantly higher H3K4me3/H3K27me3 ratio compared to pre-transfer suggesting that the male antigen is important for this 'affirmation' of Foxp3 expression at this region of the *Foxp3* locus. There was also a significantly higher H3K4me3/H3K27me3 ratio found for $CD4^+hCD2^+$ cells sorted from 'male graft' spleens than 'female graft' spleens, suggesting that cognate antigen, rather than the grafting process, plays a role in generating greater transcriptional-permissiveness at the enhancer 2 region. 'Male graft' spleen $CD4^+hCD2^+$ cells also appeared to have a higher H3K4me3/H3K27me3 ratio than 'no graft' spleen cells, although this was not statistically significant (p=0.06).

Figure 3.12A

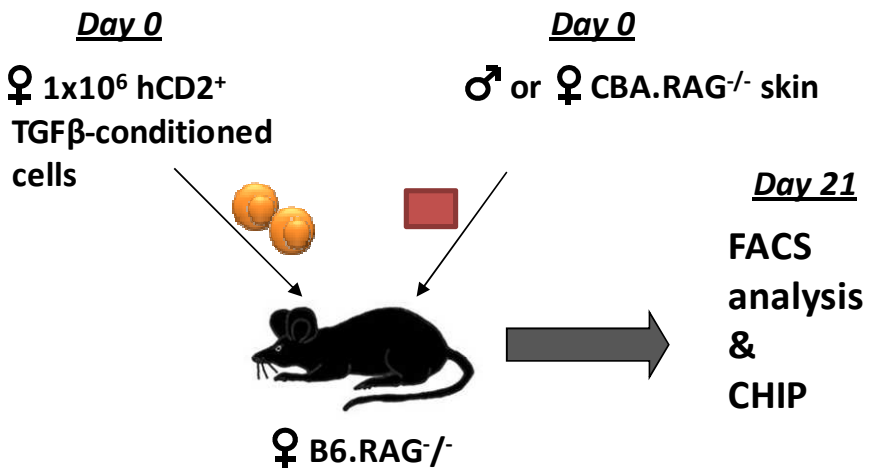
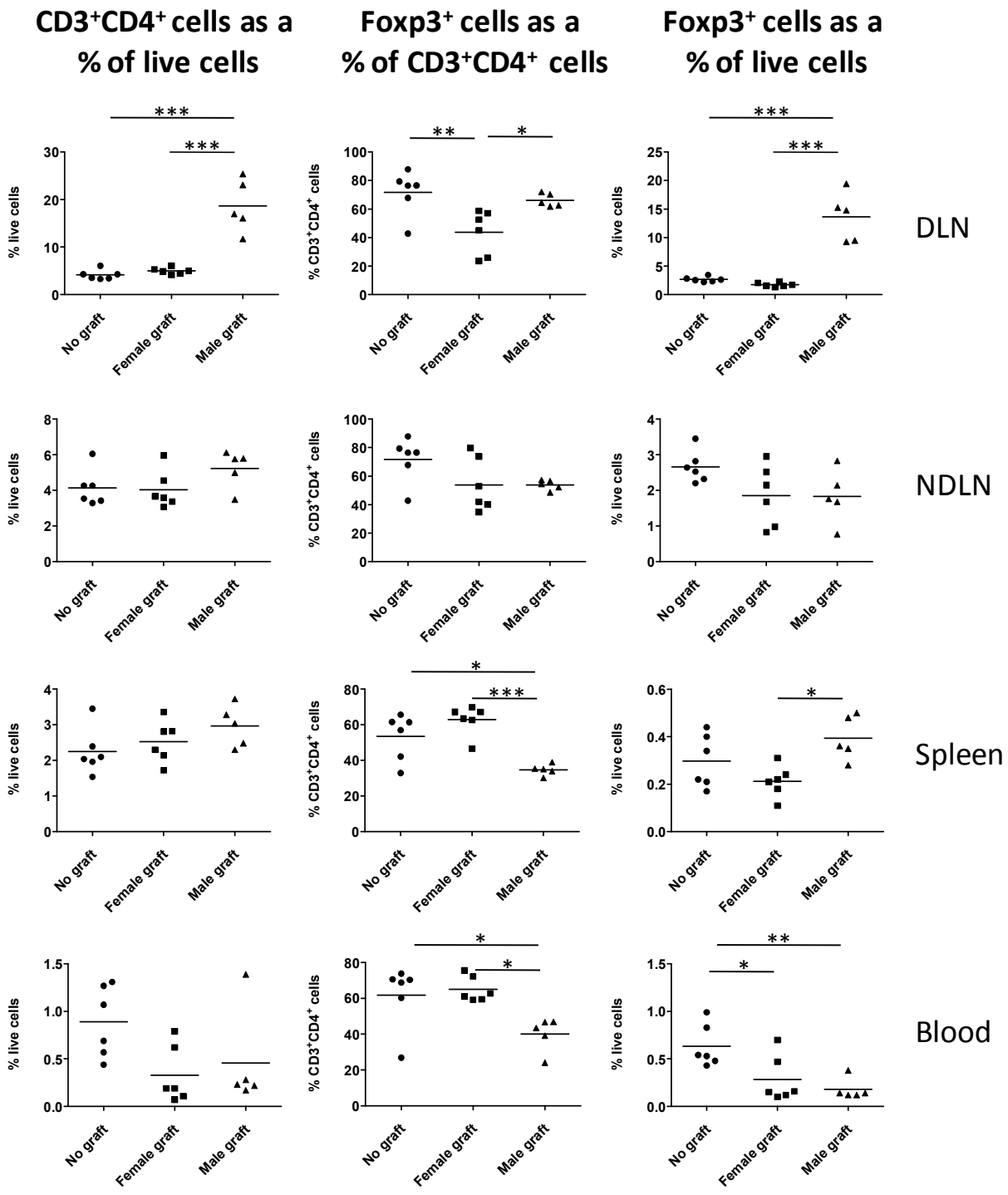


Figure 3.12A Experimental design

$1x10^6$ Mo-flo sorted $CD4^+hCD2^+$ TGFβ-conditioned $CD4^+$ cells derived from Marilyn.hCD2.KI mice were transferred into $B6.RAG^{-/-}$ mice. On the following day, some recipients were transplanted with a male graft, others with a female graft or were left ungrafted. The DLNs, NDLNs, spleen and blood of recipient mice were harvested at day 21 for FACS analysis and CHIP.

Figure 3.12B



** p=0.01-0.05* *** p=0.001-0.01* **** p<0.001*

Figure 3.12B FACS analysis of lymphoid tissues and blood from recipients

FACS analysis was performed on cells from harvested tissues. Cells were then stained with antibodies against CD3, CD4 and Foxp3. A gate was placed around live cells. Statistical analysis was performed using one-way ANOVA with Bonferonni post-test.

$CD3^+CD4^+$ cells as a % of live cells

DLN	*p* value
No graft vs Female graft	ns
No graft vs Male graft	<0.001
Female graft vs Male graft	<0.001

NDLN	*p* value
No graft vs Female graft	ns
No graft vs Male graft	ns
Female graft vs Male graft	ns

Spleen	*p* value
No graft vs Female graft	ns
No graft vs Male graft	ns
Female graft vs Male graft	ns

Blood	*p* value
No graft vs Female graft	ns
No graft vs Male graft	ns
Female graft vs Male graft	ns

$Foxp3^+$ cells as a % of $CD3^+CD4^+$ cells

DLN	*p* value
No graft vs Female graft	<0.01
No graft vs Male graft	ns
Female graft vs Male graft	<0.05

NDLN	*p* value
No graft vs Female graft	ns
No graft vs Male graft	ns
Female graft vs Male graft	ns

Spleen	*p* value
No graft vs Female graft	ns
No graft vs Male graft	<0.05
Female graft vs Male graft	<0.001

Blood	*p* value
No graft vs Female graft	ns
No graft vs Male graft	<0.05
Female graft vs Male graft	<0.05

$Foxp3^+$ cells as a % of live cells

DLN	*p* value
No graft vs Female graft	ns
No graft vs Male graft	<0.001
Female graft vs Male graft	<0.001

NDLN	*p* value
No graft vs Female graft	ns
No graft vs Male graft	ns
Female graft vs Male graft	ns

Spleen	*p* value
No graft vs Female graft	ns
No graft vs Male graft	ns

Female graft vs Male graft	<0.05

Blood	*p* value
No graft vs Female graft	<0.05
No graft vs Male graft	<0.01
Female graft vs Male graft	ns

Figure 3.12C

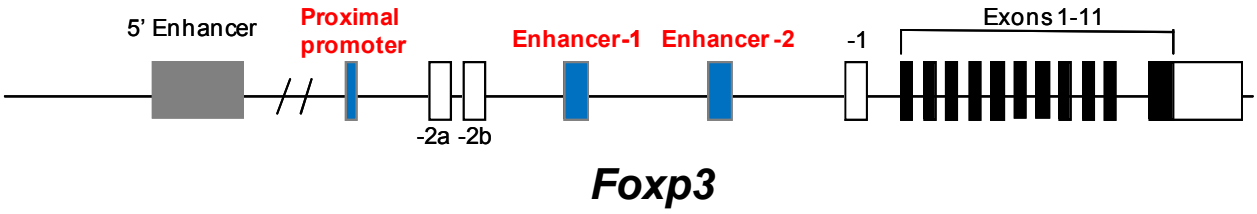
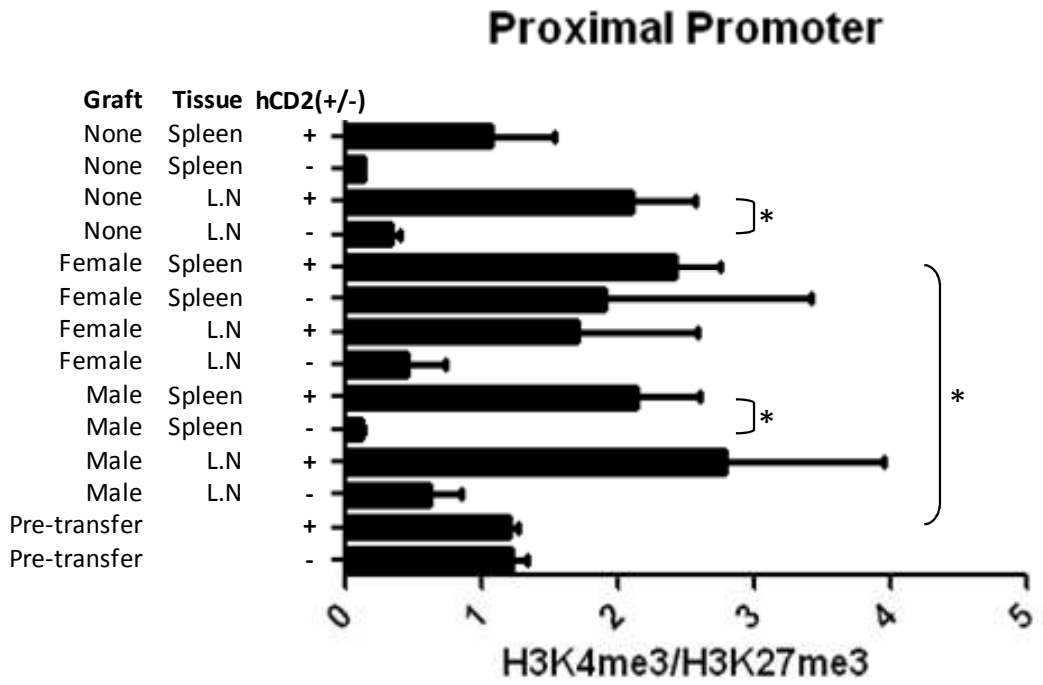
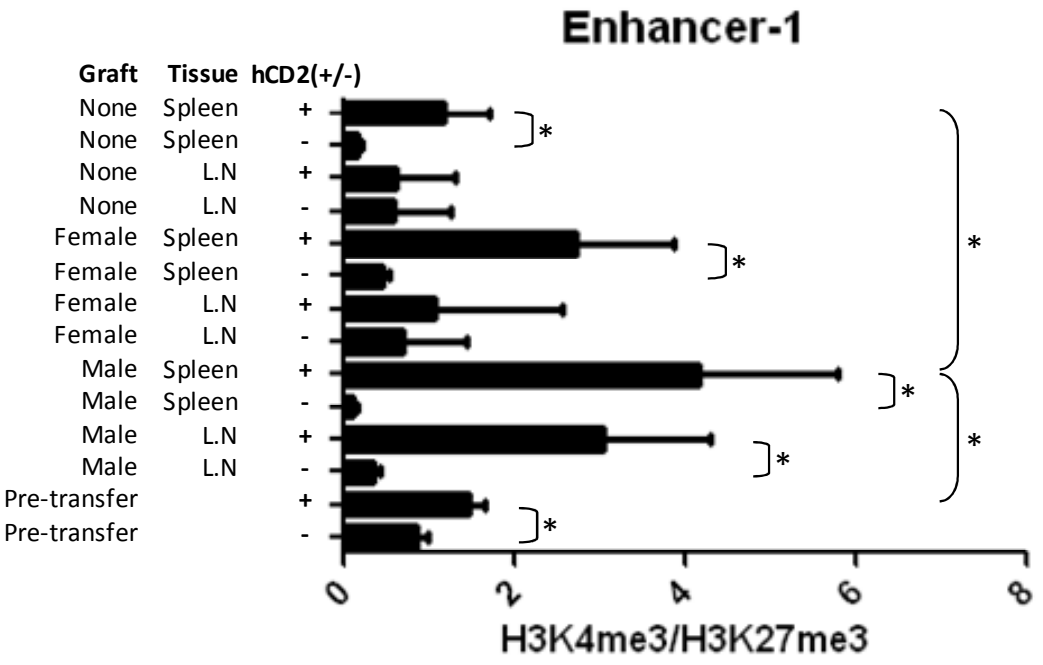


Figure 3.12C Diagrammatic representation of the Foxp3 gene locus

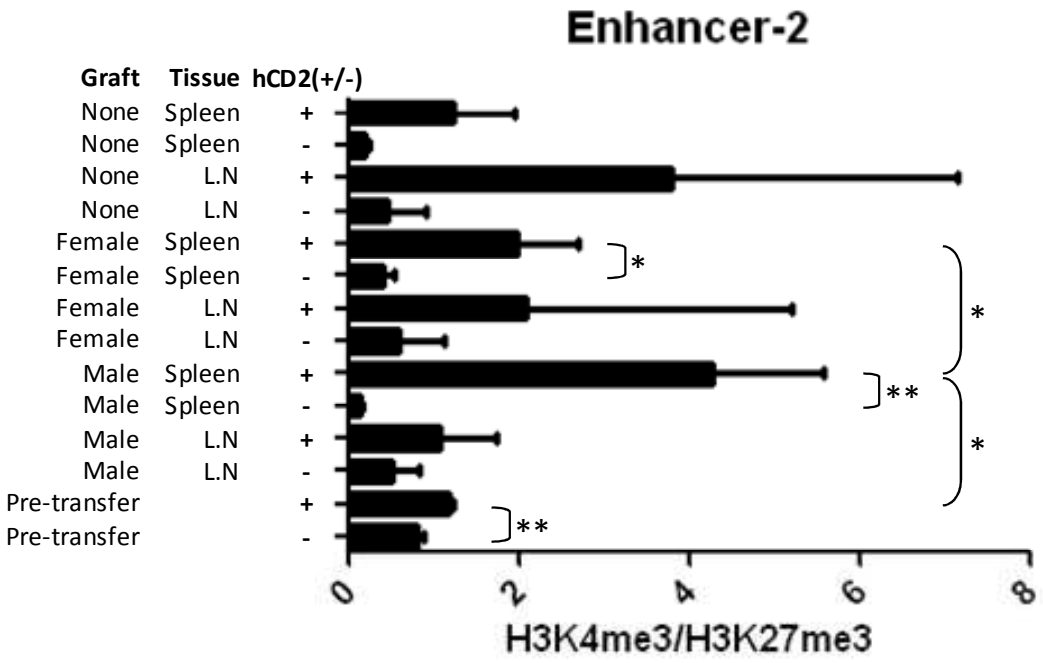
Figure 3.12D





** p=0.01-0.05* *** p=0.001-0.01* **** p<0.001*

Figure 3.12D (cont'd)



** p=0.01-0.05 ** p=0.001-0.01 *** p<0.001*

Figure 3.12D Graft antigen stabilises Foxp3 expression of in vitro TGFβ-conditioned iTreg

CHIP was performed on pre-transfer cytometrically-sorted $CD4^+hCD2^+$ cells and $CD4^+hCD2^-$ cells. On day 21 post-transplant, the recipients in all groups were sacrificed and $CD4^+hCD2^+$ and $CD4^+hCD2^-$ cells were sorted from pooled spleen and DLNs cells from all three groups: 'no graft', 'female graft' and 'male graft'. The following cell yields were obtained post-sorting: 'no graft' LN ($hCD4^+hCD2^-$ 130,000; $CD4^+hCD2^+$: 3598), 'no graft' spleen ($CD4^+hCD2^-$: 450,000, $CD4^+hCD2^+$: 22,000), 'female graft' LN ($CD4^+hCD2^-$: 10,000; $CD4^+hCD2^+$: 1079), 'female graft' spleen ($CD4^+hCD2^-$: 290,000; $CD4^+hCD2^+$: 18,000), 'male graft' LN ($CD4^+hCD2^-$: 25,000; $CD4^+hCD2^+$: 32,000), 'male graft' spleen ($CD4^+hCD2^-$: 370,000; $CD4^+hCD2^+$: 37,000). CHIP was performed using anti-H3K4me3, anti-H3K27me3, anti-histone III, and isotype control antibodies. PCR was performed using primers specific for proximal promoter, enhancer region 1 and enhancer region 2 of the *Foxp3* gene. Technical triplicates were performed. Statistical analysis was performed using unpaired two-tailed student's t test for each comparison:

Proximal Promoter Region

$hCD2^+$ vs. $hCD2^-$ (proximal promoter)	*p* value
Pre-transfer $hCD2^+$ vs. pre-transfer $hCD2^-$	*ns*
No graft spleen $hCD2^+$ vs. no graft spleen $hCD2^-$	*ns*
No graft LN $hCD2^+$ vs. no graft LN $hCD2^-$	*<0.05*
Female graft spleen hCD2+ vs. female graft spleen $hCD2^-$	*ns*
Female graft LN $hCD2^+$ vs. female graft LN $hCD2^-$	*ns*
Male graft spleen hCD2+ vs. male graft spleen $hCD2^-$	*<0.05*
Male graft LN $hCD2^+$ vs. male graft LN $hCD2^-$	*ns*

Pre-transfer vs. all groups (proximal promoter)	
Pre-transfer hCD2$^+$ vs. male spleen hCD2$^+$	*ns*
Pre-transfer hCD2$^+$ vs. male LN hCD2$^+$	*ns*
Pre-transfer hCD2$^+$ vs. female spleen hCD2$^+$	*<0.05*
Pre-transfer hCD2$^+$ vs. female LN hCD2$^+$	*ns*
Pre-transfer hCD2$^+$ vs. no graft spleen hCD2$^+$	*ns*
Pre-transfer hCD2+ vs. no graft LN hCD2$^+$	*ns*
Male graft vs. no graft (proximal promoter)	
Male graft spleen hCD2$^+$ vs. no graft spleen hCD2$^+$	*ns*
Male graft LN hCD2$^+$ vs. no graft LN hCD2$^+$	*ns*
Male graft vs. female graft (proximal promoter)	
Male graft spleen hCD2$^+$ vs. female graft spleen hCD2$^+$	*ns*
Male graft LN hCD2$^+$ vs. female graft LN hCD2$^+$	*ns*
Female graft vs. no graft (proximal promoter)	
Female graft spleen hCD2$^+$ vs. no graft spleen hCD2$^+$	*ns*
Female graft LN hCD2$^+$ vs. no graft LN hCD2$^+$	*ns*
Enhancer 1 Region	
hCD2$^+$ vs. hCD2$^-$ (Enhancer 1)	*p* value
Pre-transfer hCD2$^+$ vs. pre-transfer hCD2$^-$	*<0.05*
No graft spleen hCD2$^+$ vs. no graft spleen hCD2$^-$	*<0.05*
No graft LN hCD2$^+$ vs. no graft LN hCD2$^-$	*ns*
Female graft spleen hCD2+ vs. female graft spleen hCD2$^-$	*<0.05*
Female graft LN hCD2$^+$ vs. female graft LN hCD2$^-$	*ns*
Male graft spleen hCD2+ vs. male graft spleen hCD2$^-$	*<0.05*
Male graft LN hCD2$^+$ vs. male graft LN hCD2$^-$	*<0.05*
Pre-transfer vs. all groups (Enhancer 1)	
Pre-transfer hCD2$^+$ vs. male spleen hCD2$^+$	*<0.05*
Pre-transfer hCD2$^+$ vs. male LN hCD2$^+$	*ns*
Pre-transfer hCD2$^+$ vs. female spleen hCD2$^+$	*ns*
Pre-transfer hCD2$^+$ vs. female LN hCD2$^+$	*ns*
Pre-transfer hCD2$^+$ vs. no graft spleen hCD2$^+$	*ns*
Pre-transfer hCD2+ vs. no graft LN hCD2$^+$	*ns*
Male graft vs. no graft (Enhancer 1)	
Male graft spleen hCD2$^+$ vs. no graft spleen hCD2$^+$	*<0.05*
Male graft LN hCD2$^+$ vs. no graft LN hCD2$^+$	*ns*
Male graft vs. female graft (Enhancer 1)	
Male graft spleen hCD2$^+$ vs. female graft spleen hCD2$^+$	*ns*
Male graft LN hCD2$^+$ vs. female graft LN hCD2$^+$	*ns*
Female graft vs. no graft (Enhancer 1)	
Female graft spleen hCD2$^+$ vs. no graft spleen hCD2$^+$	*ns*

Female graft LN hCD2$^+$ vs. no graft LN hCD2$^+$	*ns*

Enhancer 2 Region

hCD2$^+$ vs. hCD2$^-$ (Enhancer 2)	*p* value
Pre-transfer hCD2$^+$ vs. pre-transfer hCD2$^-$	*<0.01*
No graft spleen hCD2$^+$ vs. no graft spleen hCD2$^-$	*ns*
No graft LN hCD2$^+$ vs. no graft LN hCD2$^-$	*ns*
Female graft spleen hCD2+ vs. female graft spleen hCD2$^-$	*<0.05*
Female graft LN hCD2$^+$ vs. female graft LN hCD2$^-$	*ns*
Male graft spleen hCD2+ vs. male graft spleen hCD2$^-$	*<0.01*
Male graft LN hCD2$^+$ vs. male graft LN hCD2$^-$	*ns*

Pre-transfer vs. all groups (Enhancer 2)	
Pre-transfer hCD2$^+$ vs. male spleen hCD2$^+$	*<0.05*
Pre-transfer hCD2$^+$ vs. male LN hCD2$^+$	*ns*
Pre-transfer hCD2$^+$ vs. female spleen hCD2$^+$	*ns*
Pre-transfer hCD2$^+$ vs. female LN hCD2$^+$	*ns*
Pre-transfer hCD2$^+$ vs. no graft spleen hCD2$^+$	*ns*
Pre-transfer hCD2+ vs. no graft LN hCD2$^+$	*ns*

Male graft vs. no graft (Enhancer 2)	
Male graft spleen hCD2$^+$ vs. no graft spleen hCD2$^+$	*ns*
Male graft LN hCD2$^+$ vs. no graft LN hCD2$^+$	*ns*

Male graft vs. female graft (Enhancer 2)	
Male graft spleen hCD2$^+$ vs. female graft spleen hCD2$^+$	*=0.05*
Male graft LN hCD2$^+$ vs. female graft LN hCD2$^+$	*ns*

Female graft vs. no graft (Enhancer 2)	
Female graft spleen hCD2$^+$ vs. no graft spleen hCD2$^+$	*ns*
Female graft LN hCD2$^+$ vs. no graft LN hCD2$^+$	*ns*

Discussion

In this chapter, I set out to investigate if TGFβ-conditioning alone can confer a suppressive phenotype to $CD4^+$ cells *in vivo* independent of Foxp3 expression. A mouse strain was used whose $CD4^+$ T cells co-expressed Foxp3 and surface hCD2 allowing for the separation of $Foxp3^+$ and $Foxp3^-$ populations following TGFβ-conditioning *in vitro*. Following transfer *in vivo*, I showed that $1x10^6$ male-specific $Foxp3^-$ TGFβ-conditioned T cells rejected a male graft. This suggests that TGFβ-conditioning was insufficient for conferring a 'non-inflammatory' phenotype to the T cells *in vivo*. Furthermore, an immunological challenge with $1x10^5$ naïve $CD4^+$ cells together with transplantation of a fresh graft resulted in rejection of the graft. Hence, $Foxp3^-$ TGFβ-conditioned T cells did not show any suppressive capacity. The data is consistent with the possibility that the major suppressive impact of TGFβ-conditioning for iTreg is to induce Foxp3 expression. However, this does not rule out that TGFβ may contribute to the suppressive capacity of iTreg in other ways. Indeed, the data also does not exclude the possibility that $Foxp3^-$ cells exposed to TGFβ may play a role in combination with the suppressive actions of $Foxp3^+$ iTreg *in vivo*.

On the other hand, $Foxp3^+$ TGFβ-conditioned T cells showed no rejection of the graft and could suppress an immunological challenge 30 days later with naïve $CD4^+$ cells. When co-injected with naïve $CD4^+$ cells at a 5:1 (iTreg:naïve cell) ratio, $Foxp3^+$ iTreg could prevent rejection in 50% of recipients. The demonstration of the suppressive capacity of $Foxp3^+$ iTreg is consistent with other reports of their suppressive function *in vivo* (Fantini et al. 2006; Haribhai et al. 2009). Whereas here, hCD2 expression was used as a means of sorting for $Foxp3^+$ cells, in those studies, $Foxp3^+$ iTreg were enriched by either sorting $CD25^+$ cells or GFP^+ cells derived from $Foxp^{GFP}$ mice. In this study, the potency of $Foxp3^+$ iTreg was shown to be modest. However, this may be due to the use of a lympho-deficient system which

provided a convenient reductionist system but which might have led to less effective homeostatic proliferation of Foxp3$^+$ iTreg compared to naïve CD4$^+$ cells (Suffner et al. 2010). Moreover, the lack of nTreg and indeed other T cells in the recipients may possibly prevent iTreg from acting synergistically with other regulatory cell types in suppressing the alloimmune response (Haribhai et al. 2009). Nevertheless, given that Foxp3 is important for the suppressive phenotype of TGFβ-conditioned iTreg, but that TGFβ may also contribute to their suppressive function, a question that inevitably arises is would Foxp3 expression in a CD4$^+$ T cell alone without TGFβ exposure lead to a more or less potent suppressive phenotype. This will be examined in Chapter 4.

I also investigated the impact of cognate antigen on the stability of Foxp3 expression by iTreg *in vivo*. Cognate antigen delivered in the form of a transplanted graft was found to lead to enhanced expansion and/or survival of Foxp3$^+$ iTreg in the DLNs of the recipients. When unsorted male-specific TGFβ-conditioned T cells were transferred into RAG$^{-/-}$ recipients and followed by a male graft transplant (either the next day or even >20 days later), there was an increase in the number of CD4$^+$ cells found in the DLNs of the recipients compared to female graft or ungrafted recipients. Although there was a drop in the percentage of Foxp3 expression of the CD4$^+$ cells from pre-transfer levels, the overall number of Foxp3$^+$ cells isolated from the DLN of male graft recipients was significantly higher than those of female graft or ungrafted recipients. This suggests that Foxp3$^+$ iTreg when injected, encountered graft antigen presented by recipient APCs in DLNs. This led to enhanced survival/expansion, with higher numbers of both Foxp3$^+$ and Foxp3$^-$ cells. The Foxp3$^-$ cells might originate from pre-transfer Foxp3$^-$ cells or might be Foxp3$^+$ iTreg that have lost their Foxp3 expression. Interestingly, when sorted Foxp3$^+$ cells were transferred into a wild-type host along with intravenous injection of the cognate peptide, comparatively little expansion of transferred

Foxp3$^+$ cells was seen in the peripheral LNs or the spleen. Alternatively, if *unsorted* TGFβ-conditioned T cells were transferred or if cognate peptide delivery was changed to that of a male graft, these hosts displayed a significantly higher number of Foxp3$^+$ cells in their peripheral lymph nodes (former) or draining lymph nodes (latter). This could be due to the presence of better antigen experience, or survival signals provided by the microenvironment of the graft, or to the Foxp3$^-$ portion of TGFβ-conditioned T cells, such as IL-2. IL-2 is not only critical for the generation of Foxp3$^+$ iTreg *in vitro* (Davidson et al. 2007; Zheng et al. 2007), its presence is even necessary to prevent the conversion of nTreg into inflammatory effector cells in lymphopenic recipients (Duarte et al. 2009). Therefore, it is feasible that IL-2 secretion may contribute to the enhanced survival and expansion of Foxp3$^+$ cells. A future experiment could involve peptide antigen injection along with IL-2 injection to test if this could improve the survival of Foxp3$^+$ iTreg *in vivo*. Also, survival and/or expansion of Foxp3$^+$ iTreg could also be distinguished by using CFSE staining.

Previously, cognate peptide delivered intravenously was shown to help transferred TGFβ-conditioned cells to maintain a greater percentage of Foxp3 expression (Kretschmer et al. 2005). However, in that study, cognate peptide was delivered via a DC-targeting anti-DEC205 antibody and only one peptide dosage was examined. Moreover, only unsorted iTreg were transferred *in vivo*, thus introducing the possibility of preferential expansion of Foxp3$^-$ populations in response to peptide.

Graft antigen also appears to enhance the 'stability' of Foxp3 expression by iTreg. Epigenetic modification such as DNA demethylation or histone modifications can alter the transcriptional activity of genes and these changes at critical lineage-determining genes can often signal the stability and commitment of a given cell lineage. A number of groups

showed that nTreg are strongly demethylated at the *Foxp3* locus whilst TGFβ-induced Tregs show weak demethylation and $CD4^{+}CD25^{-}$ cell show little or no demethylation (Floess et al. 2007; Lal et al. 2009). However, it is unclear how epigenetic modifications at the iTreg *Foxp3* locus may change *in vivo* when they are exposed to antigen and tissue signals.

I showed that the ratio of permissive to inhibitory histone modifications found at the enhancer 1 or 2 regions of the *Foxp3* locus in $Foxp3^{+}$ cells isolated from the spleens of male graft recipients was higher than those from female graft or ungrafted recipients. Moreover, this ratio was also higher in these cells compared to pre-transfer $Foxp3^{+}$ cells. The implication of this are that graft antigen plays a role in rendering the *Foxp3* gene in $Foxp3^{+}$ iTreg more conducive to transcription, hence stabilising their Foxp3 expression and 'affirming' their regulatory phenotype *in vivo*. Previously, a group showed that *in vivo* generated Tregs displayed more permissive epigenetic modifications than *in vitro* generated Treg (Polansky et al. 2008). The current data suggests that rather than ranking iTreg into various categories of potency and stability, these properties are variable and context-dependent as seen when *in vitro* generated Treg are transferred *in vivo*. Future work would involve examining DNA methylation of the *Foxp3* locus in addition to histone modifications in the same experimental setup.

In summary, graft antigen exposure appears to lead to higher numbers of $Foxp3^{+}$ iTreg in DLN, but not elsewhere (ie. spleen, blood, NDLN). These cells appear to be able to affirm their Foxp3-determined suppressive phenotype, as they appear epigenetically geared to more effective Foxp3 transcription.

In continuation of the investigation of the relative contribution of Foxp3 and TGFβ for the function if iTreg, I next examine whether forced Foxp3 expression in non-regulatory T cells in the absence of TGFβ-conditioning can render the cells suppressive *in vitro* and *in vivo*, specifically in the setting of preventing graft rejection. The suppressive potency of such cells is compared with that of TGFβ-conditioned iTreg, and seen to be more potent.

CHAPTER 4 T cells ectopically expressing Foxp3 are suppressive both *in vitro* and *in vivo*

Introduction

In the previous chapter, TGFβ-conditioned but Foxp3 negative $CD4^+$ cells failed to show a 'suppressive' phenotype *in vivo*. Therefore, it remains to be clarified whether the sole outcome of TGFβ-conditioning of T cells is the induction of Foxp3. To examine if Foxp3 expression alone is sufficient for suppression of transplant rejection, a retroviral conditional expression system was exploited. Previously, ectopic expression of Foxp3 in $CD4^+CD25^-$ cells was shown to confer regulatory properties *in vitro*. However, reports are mixed regarding the suppressive functions of such cells, with some models reporting effectiveness (Chai et al. 2005; Loser et al. 2005), whilst others being less certain (Jaeckel et al. 2005; Andersen et al. 2008).

In the conditional system developed by Andersen et al. and used in the experiments described in this chapter (Andersen et al. 2008), Foxp3 expression can be induced *on demand* in naïve T cells by transducing them with a murine leukaemia virus-derived retrovirus carrying a conditional Foxp3 transgene (*cFoxp3*). The product of this transgene is the chimeric protein Foxp3-ERT2 (also referred to as cFoxp3 from this point on), where ERT2 is a modified ligand binding domain of the human estrogen receptor. A number of retroviral constructs have been made where the Foxp3-ERT2 protein is linked, either directly or via an internal ribosomal entry site (IRES), to a rat CD8 reporter molecule (rCD8) and/or green fluorescent protein (GFP) to facilitate the identification or isolation of transduced cells.

The key advantage of such a conditional system is that functional Foxp3 can be induced or 'switched off' on demand. In the context of transplantation this is important because the suppressive capacity of $Foxp3^+$ cells at various stages of graft rejection can, in principle, be examined. Moreover, because *cFoxp3*-transduced cells would remain potentially proinflammatory until tamoxifen administration, they would be expected to undergo normal clonal expansion in response to foreign graft antigens. This may allow an expanded population of graft-specific *cFoxp3*-transduced cells to emerge, thus forgoing the need for the transfer of antigen-specific *cFoxp3*-transduced cells as reported in other studies (Chai et al. 2005; Jaeckel et al. 2005). Andersen et al. (Andersen et al. 2008) have shown that, in an inflammatory murine model of arthritis, translocation of *cFoxp3* during the course of the evolution of arthritis can reverse the disease. In this chapter I investigated whether the same would be true of the graft rejection response. Moreover, stopping tamoxifen treatment might allow one to examine the importance of Treg in maintaining graft tolerance in a well-healed allograft. The *in vitro* functional effect of ectopic Foxp3 expression in the absence of TGFβ was also examined.

The aims of this chapter were:

1) To optimize transfection and transduction of retroviral constructs.

Numerous conditions were tested in order to optimize the transfection of 293eT cells and the transduction of $CD4^+$ cells. These include the volume of $CaCl_2$ used for $CaPO_4$ transfection and the duration of and volume of viral supernatant used for transduction.

2) To examine the phenotype and behavior of conditional Foxp3-transduced regulatory T cells (cTreg) *in vitro*

A number of groups have shown that ectopic Foxp3 expression can lead to the expression of Treg-like characteristics *in vitro* (Hori et al. 2003; Chai et al. 2005). This section aimed to further consolidate this data, and confirm whether conditional Foxp3 expression could similarly generate Treg characteristics. Specifically, these include: the expression of Treg surface markers, anergy, suppression of effector T cells, and the lack of secretion of cytokines. cTreg are defined as T cells transduced with the *cFoxp3* vector and treated with 4HT or tamoxifen thus allowing the cFoxp3 protein to become functional.

3) To investigate if cTreg are capable of suppressing graft rejection

The aim of the last section was to investigate the behaviour of cTreg *in vivo* – would these cells behave as proinflammatory cells or as non-inflammatory Treg-like cells in response to an allograft? cTreg behaviour *in vivo* was first investigated by inducing cFoxp3 translocation prior to transfer of cTreg, thus allowing cFoxp3 to become functional within the cell prior to grafting. Subsequently, the onset and frequency of tamoxifen-treatment were varied to examine the importance of the timing of cTreg action in the alloimmune response. The transplantation system used was initially multiple-minor-antigen mismatched skin grafts transplanted onto lymphodeficient mice, but was further extended to fully MHC-mismatched grafts and lymphoreplete mice. The extension to fully MHC-mismatched grafts allowed the behaviour of cTreg to be examined in the context of increased immunogenicity of the graft.

4.1 Optimisation of 293eT cell transfection and transduction of primary T cells

My first goal was to achieve optimal transfection of 293eT packaging cells with retroviral constructs and the highest transduction rate of primary T cells with the virus. Transfection of 293eT cells with the retroviral constructs was performed using $CaPO_4$ transfection according to published protocols [(Andersen et al. 2008), and see section **2.6.3**]. The volume of $CaCl_2$ used in the transfection mix proved key towards maximising the efficiency of transfection. Using the *cFoxp3*^{tgGFP} construct (**Figure 2.1**), various volumes of $CaCl_2$ were used in a total transfection mix volume of 200µl per well in a 6 well plate. I found that 12µl was the optimum volume generating the highest percentage transfection as measured by GFP expression – 46% (**Figure 4.1A**). *cFoxp3*^{tgGFP} virus generated from 293eT transfection using 12µl $CaCl_2$ produced a high rate of transduction of primary T cells ($CD4^+CD25^-$ cells sorted from a B6 spleen) – **Figure 4.1B**. Therefore, future transfections were performed using 12µl of $CaCl_2$ in the transfection mix. Viral supernatant was harvested 24hr following transfection.

An attempt was also made to optimise conditions for transduction. Replacing the $CD4^+$ cell transduction medium that contained viral supernatant with fresh medium 12-18hr after adding the virus did not lead to an increase in transduction efficiency. Likewise, adding 100µl of viral supernatant instead of 75µl in the transduction wells did not increase transduction efficiency. However, 24hr viral supernatant appeared to produce a greater transduction rate than the 48hr viral supernatant (**Figure 4.1B**).

Figure 4.1A

Transfection $CaCl_2$ volume (µl)	Transfection %
8	1
10	37
12	46
14	35
16	40

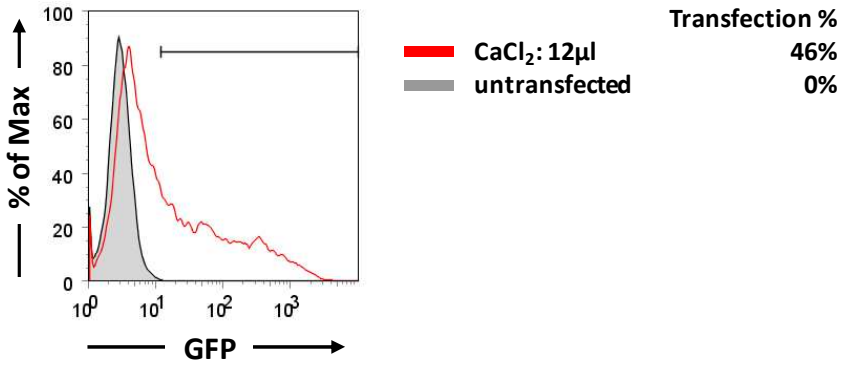


Figure 4.1A Optimising transfection of 293eT cells

$CaPO_4$ transfection of 293eT cells with the retroviral constructs was optimised (section 2.6.3). Various volumes of $CaCl_2$ were used in a total transfection mix volume of 200µl per well in a 6 well plate. Transfection efficiency was indicated by measuring GFP expression of the 293eT cells.

Figure 4.1B

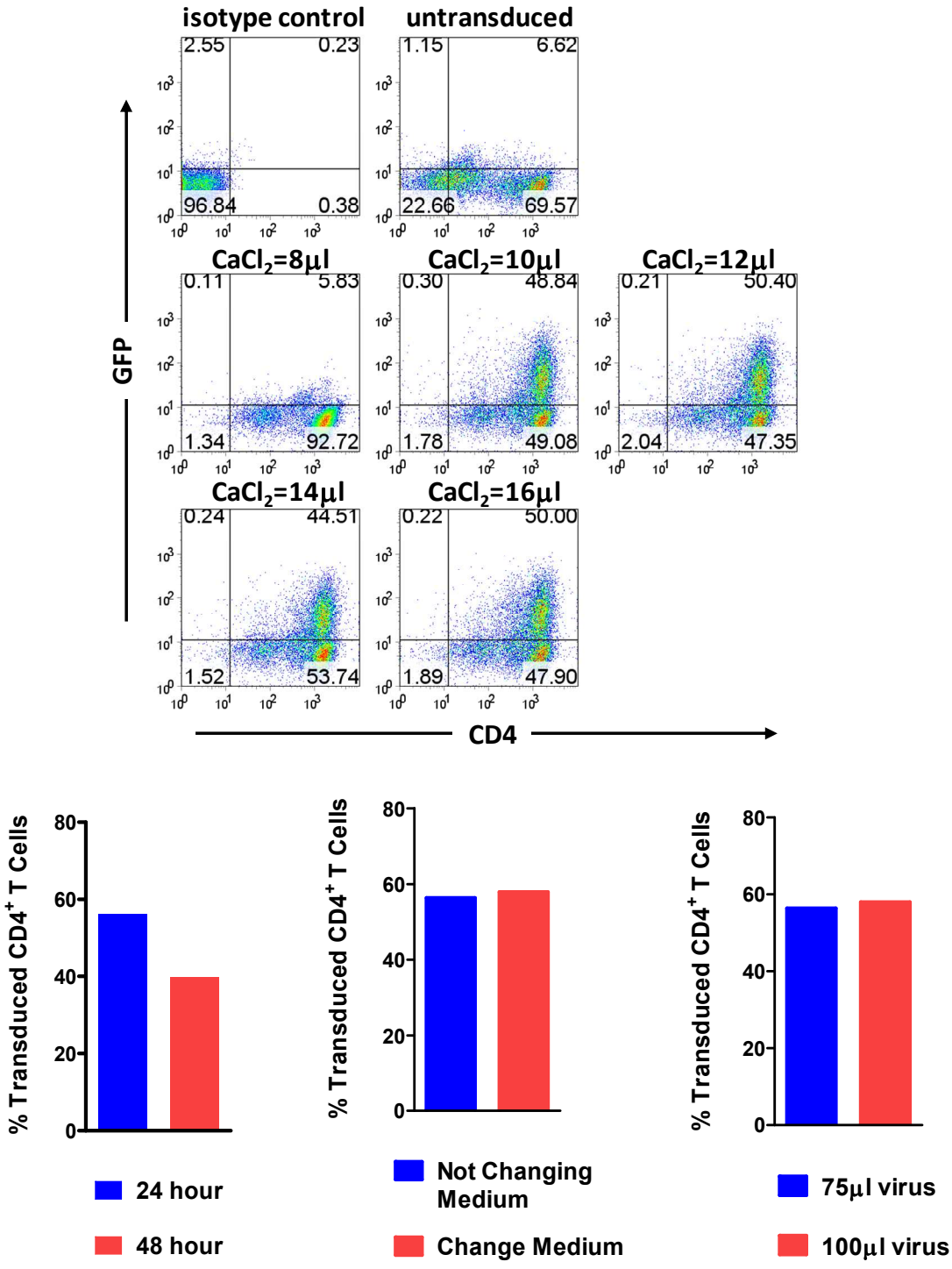
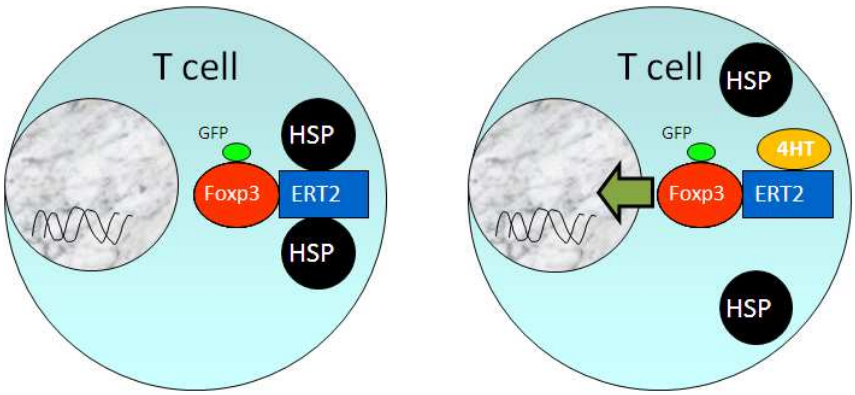


Figure 4.1B Optimising transduction of T cells

Transfection supernatant (*cFoxp3*^{tgGFP}) was added to MACS-sorted B6 CD4^{+}CD25^{-} cells in a 96 well plate. The transduction efficiencies using viral supernatants generated from various volumes of $CaCl_2$ are shown in the FACS plots. Using the supernatant generated from 12µl of $CaCl_2$, various conditions were tried to optimise transduction. Both 24hr and 48hr transfection supernatants were tried (left panel). In some wells, the medium was changed after 24hrs (centre panel), and also 100µl virus was added instead of 75µl (right panel). Transduction rate was indicated by GFP expression of the transduced cells.

4.2 The *cFoxp3* transgene product undergoes nuclear translocation upon addition of 4HT *in vitro*

The conditional expression system is based on the principle that the *cFoxp3* transgene product will only be functional when it is in the nucleus where it can behave as a transcription factor and have access to its multiple gene targets. The oestrogen binding domain (ERT2) of the chimeric *cFoxp3* product – Foxp3-ERT2, is a modified ligand binding domain of the human oestrogen receptor. This domain is bound to heat shock proteins in the cytoplasm, thus physically 'anchoring' the transgene product in the cytoplasm (Rossant et al. 1999). The binding of 4-hydroxytamoxifen (4HT), the active metabolite of tamoxifen, releases Foxp3-ERT2 from binding to heat shock proteins, allowing it to translocate to the nucleus (see below).



As Foxp3 is tagged to GFP in the case of the construct *cFoxp3*^{tgGFP}, the nuclear translocation of Foxp3-ERT2 can be visualised using confocal microscopy (**Figure 4.2A & B**). In that case, the green staining of the GFP-tagged transgene product could be seen as co-localising with blue staining of the nucleus (DRAQ-5 stain). To initiate nuclear translocation of the transgene product, cells were incubated in 4HT (50nM) at the outset of transduction. To assess the efficiency of nuclear translocation, visual quantitation was performed on three randomly selected views of either 4HT treated or untreated *cFoxp3*^{tgGFP}-transduced cells visualised

under x200 magnification (**Figure 4.2A & B**). With the addition of 4HT, the percentage of cells that have translocated at 40hrs from the onset of transduction was 70%, and this increased to over 90% at 64hrs (**Figure 4.2C**). However, there was a level of GFP-DRAQ-5 co-localisation seen in the 4HT untreated group of 10-15%.

Figure 4.2A

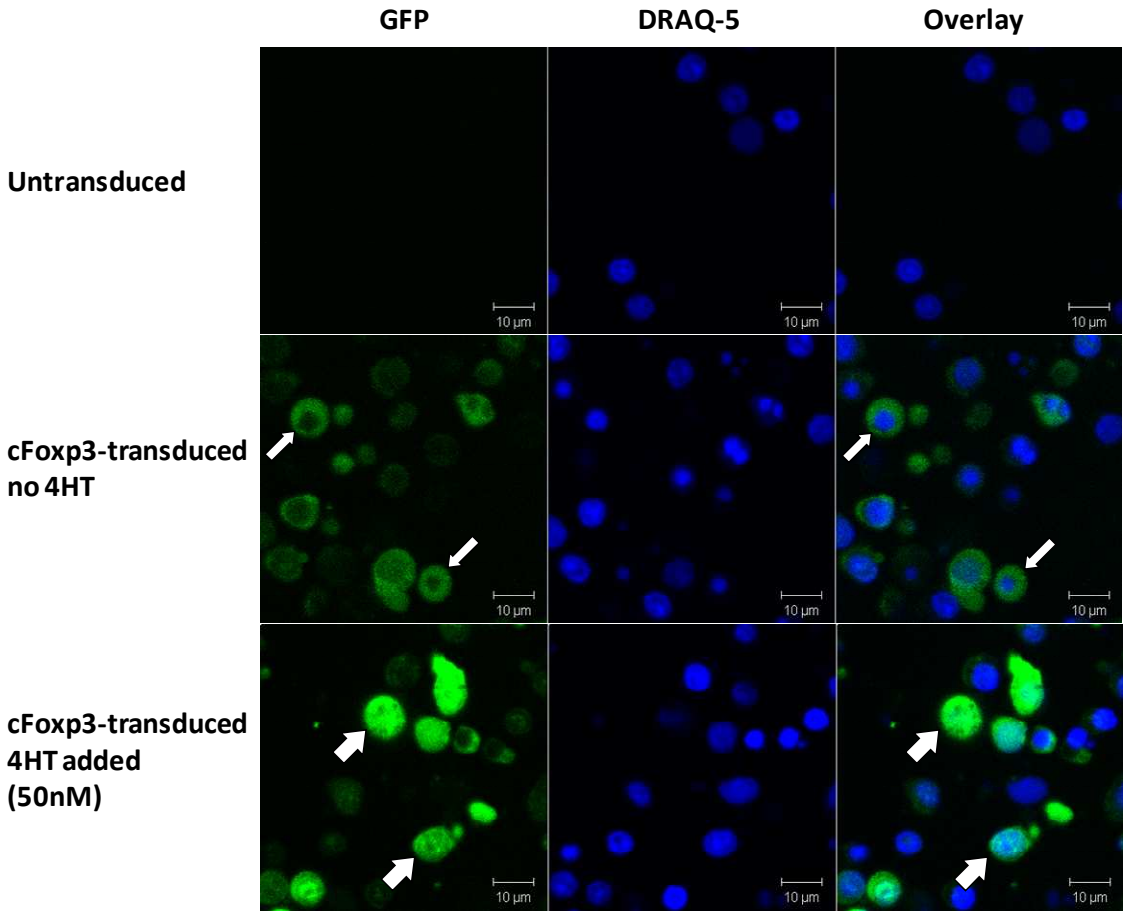


Figure 4.2A Nuclear translocation of cFoxp3 product at 40hrs from onset of transduction

$CD4^{+}CD25^{-}$ cells were purified from the spleen of a CBA mouse and transduced with *cFoxp3*^{tgGFP}. At 40hrs, GFP^{+} cells were sorted using flow cytometry and examined using confocal microscopy. The blue-coloured DRAQ-5 dye stains the nuclei of these cells. Thin arrows point to cells that do not show co-localisation of GFP and DRAQ-5 staining. Wide arrows show cells displaying co-localisation.

Figure 4.2B

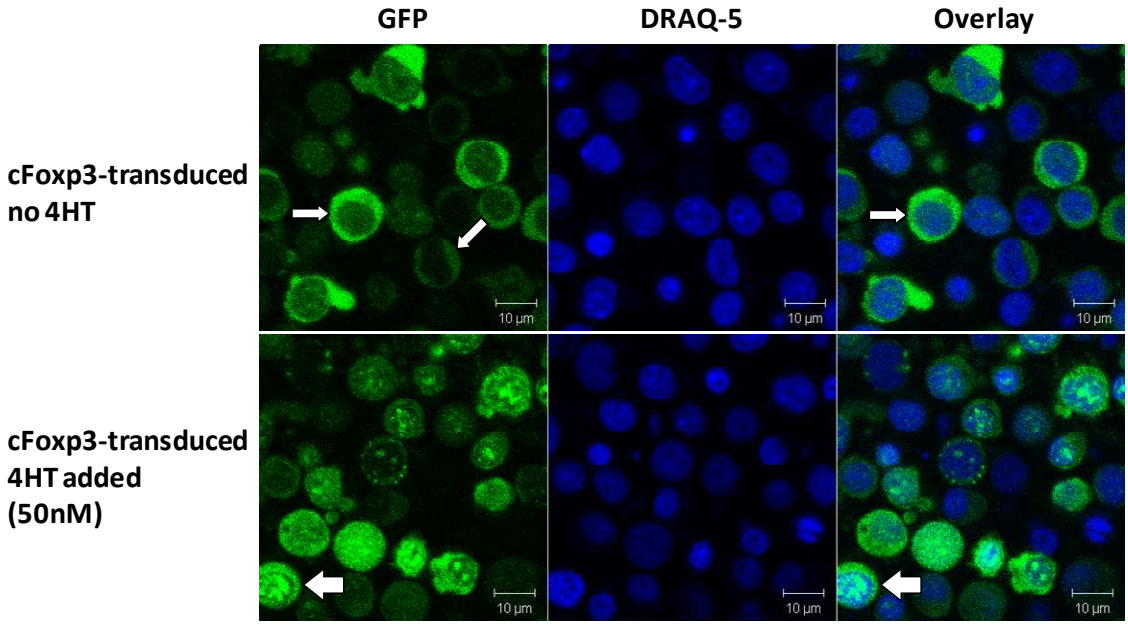


Figure 4.2B Nuclear translocation of cFoxp3 product at 64hrs from onset of transduction

Nuclear translocation at 64hrs from onset of transduction. Cells were prepared as in Figure 4.2A. Thin arrows point to cells not showing co-localisation of GFP and DRAQ-5 staining, while wide arrows point to those cells which are showing co-localisation.

Figure 4.2C

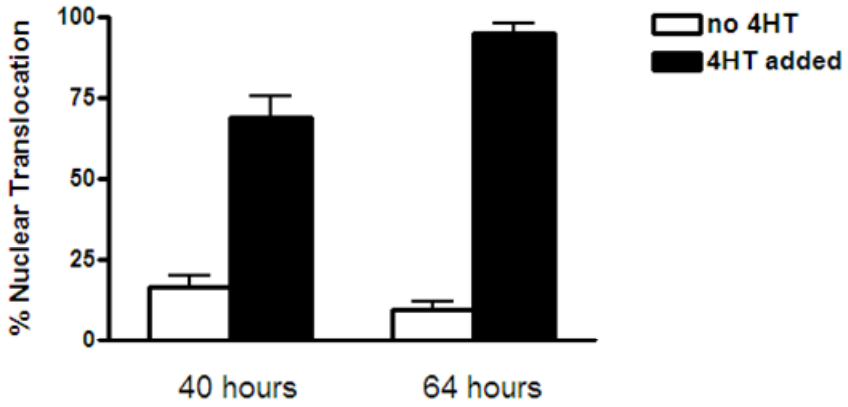


Figure 4.2C Quantification of nuclear translocation at 40hrs and 64hrs

Three random microscopy fields were selected and cells that showed co-localisation of GFP and DRAQ-5 stain were counted as having undergone nuclear translocation of the *cFoxp3*^{tgGFP} product. Each field that was examined contained at least 20 cells, with an average of 30 cells counted per field. The error bars represent standard error of the mean. Statistical significance (two-way ANOVA) comparing both timepoints and the two conditions (no 4HT versus 4HT added): $p<0.01$.

4.3 'Conditional' Treg (cTreg) express regulatory T cell markers

Both Hori et al. (Hori et al. 2003) and Chai et al. (Chai et al. 2005) have shown that T cells transduced with a non-conditional Foxp3 vector (Foxp3-MIGR1) expressed marginally higher-intensity staining for surface GITR, CD25, CD103 and also intracellular CTLA-4 compared to cells transduced with control vector. To assess whether T cells transduced with *cFoxp3* constructs also express markers characteristic of regulatory T cells and whether this was 'conditional' upon the addition of tamoxifen, $CD4^+CD25^-$ cells from CBA mice were transduced with *cFoxp3*^{tgGFP}and assessed for the expression of Foxp3, CD25, GITR, CTLA-4, CD62L, CD103 and CD73 at 40hr, 64hr and 88hr from the onset of transduction (when the viral supernatant is added to the transduction wells). At the 40hr timepoint, transduced cells were sorted based on CD4 and GFP expression and incubated for a further 24hrs (64hrs from onset of transduction) or 48hrs (88hrs from onset of transduction) in the presence of IL-2 and IL-7 (as during transduction). IL-2 and IL-7 were added as these are cytokines important for T cell proliferation and survival (Fry et al. 2002). At each timepoint, expression of regulatory T cell markers was assessed by FACS. These were also compared with sorted $CD4^+CD25^+$ nTreg from naive spleens (for CTLA-4 expression) and from TGFβ-conditioned T cells (for CD103 and CD73 expression) (**Figure 4.3A**).

Expression of Foxp3, CD25, GITR and intracellular CTLA-4 were high in cTreg (*cFoxp*^{tgGFP}-transduced cells with 4HT treatment) (**Figure 4.3B**). Foxp3 expression was also seen in *cFoxp*^{tgGFP}-transduced cells without 4HT treatment, however the levels were much lower than cTreg. This may be due to the nuclear location of the transgene product following 4HT induction providing it with protection from degradation in the cytoplasm by lysosomes or proteasomes (Fukuda et al. 2005). Expression of CD25 was found to be increased in cells transduced with the empty vector due to TCR stimulation by anti-CD3, which is required for

transduction. However, CD25 expression fell to lower levels at 60hr and 88hr in the empty-vector-transduced cells, but remained high in cTreg. A similar pattern was also found with GITR expression – whilst also high at 40hr in activated but untransduced or empty-vector-transduced cells, its expression dropped to lower levels at 64hr and 88hr. In contrast, cTreg maintained a high expression of GITR. cTreg also appeared to express increased levels of CTLA-4, and this was more marked at the later timepoints: 64hr and 88hr.

CD62L, also known as L-selectin or SELL, is a surface molecule expressed on leukocytes, and is required for their migration from high endothelial venules to secondary lymphoid organs (Fu et al. 2004). Whilst CD62L is highly expressed in naïve T cells, its expression is low in activated T cells. Non-conditional Foxp3-MIGR1 transduced cells express low levels of CD62L (Andersen et al. 2008). The expression of CD62L was found to be maintained at a high level (43% of cells) in cTreg compared to untransduced, empty-vector-transduced or *cFoxp3*^{tgGFP}-transduced cells without 4HT treatment (**Figure 4.3C**). This suggests that the expression of functional *cFoxp3* helps maintain CD62L expression, possibly enabling a high proportion of cTreg to home to secondary lymphoid organs.

In contrast, CD103, a surface integrin whose expression is induced by TGFβ (Kilshaw et al. 1990; Robinson et al. 2001) and CD73, an ecto-5'-nucleotidase up-regulated by TGFβ-induced iTreg (Frederico Regateiro, DPhil thesis 2009, University of Oxford), were not found to be highly expressed by cTreg (**Figure 4.3D**).

Figure 4.3A

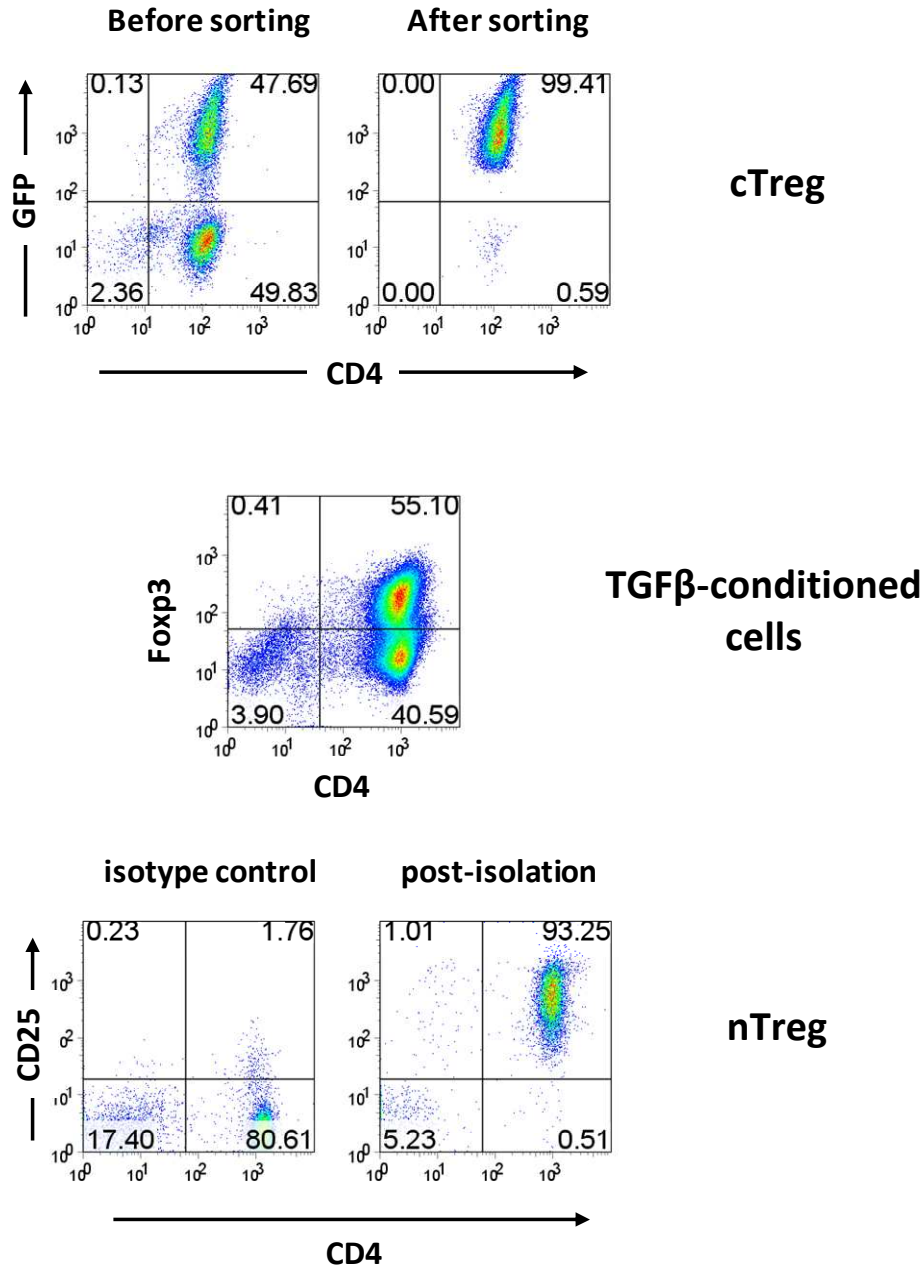


Figure 4.3A Pre-analysis: T cell populations

Upper panel: *cFoxp3*^{tgGFP}-transduced cells were sorted by Mo-flo based on GFP and CD4 expression. Middle panel: splenocytes from A1.RAG$^{-/-}$ mice were cultured for 6-7 days in the presence of cognate peptide and 2ng/ml TGFβ. Lower panel: nTreg were sorted using the Miltenyi Biotec CD4^{+}CD25^{+} isolation kit. The FACS plots are representative of typical isolations.

Figure 4.3B

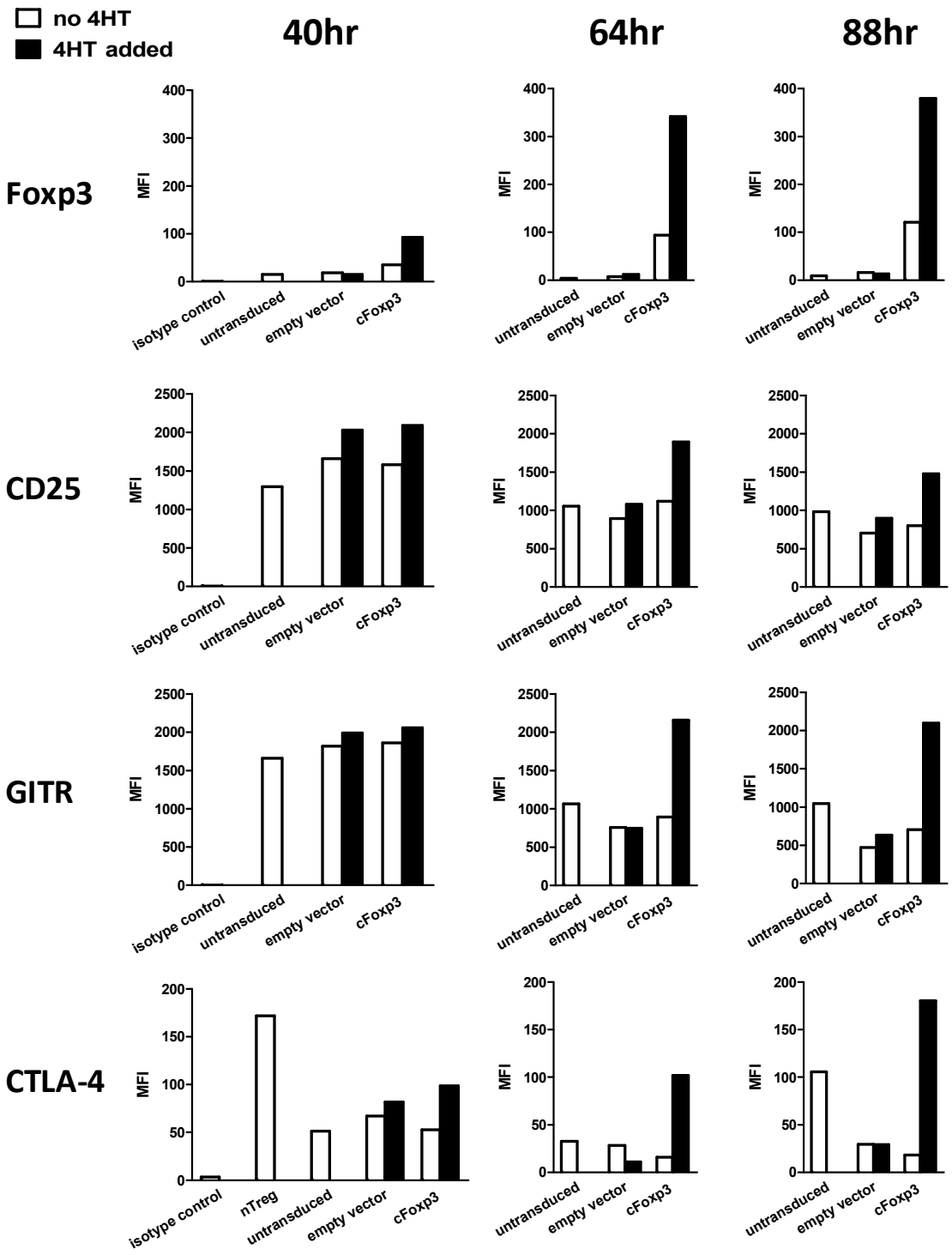


Figure 4.3B cTreg express molecules characteristic of regulatory T cells

The expression of Foxp3, CD25, GITR and CTLA-4 were assessed by FACS at 40hr, 64hr and 88hr following the onset of transduction. Transduced cells were gated on live $CD4^{+}GFP^{+}$ cells. nTreg were gated on live $CD4^{+}$ cells. Data shown are representative of three independent experiments.

Figure 4.3C

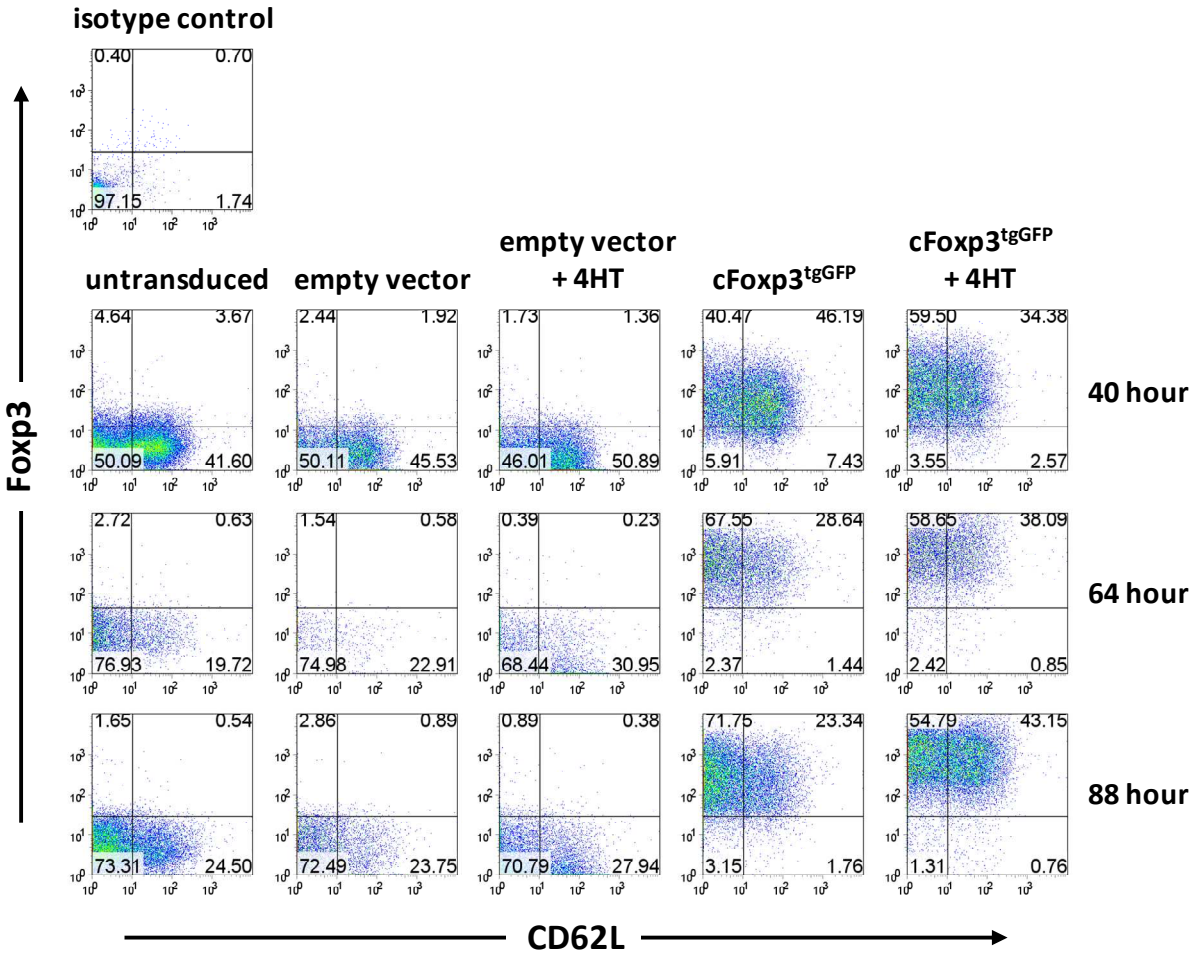


Figure 4.3C cTreg express CD62L

CD62L expression as assessed by FACS at 40hr, 64hr and 88hr following the onset of transduction. Transduced cells were gated on live $CD4^{+}GFP^{+}$ cells.

Figure 4.3D

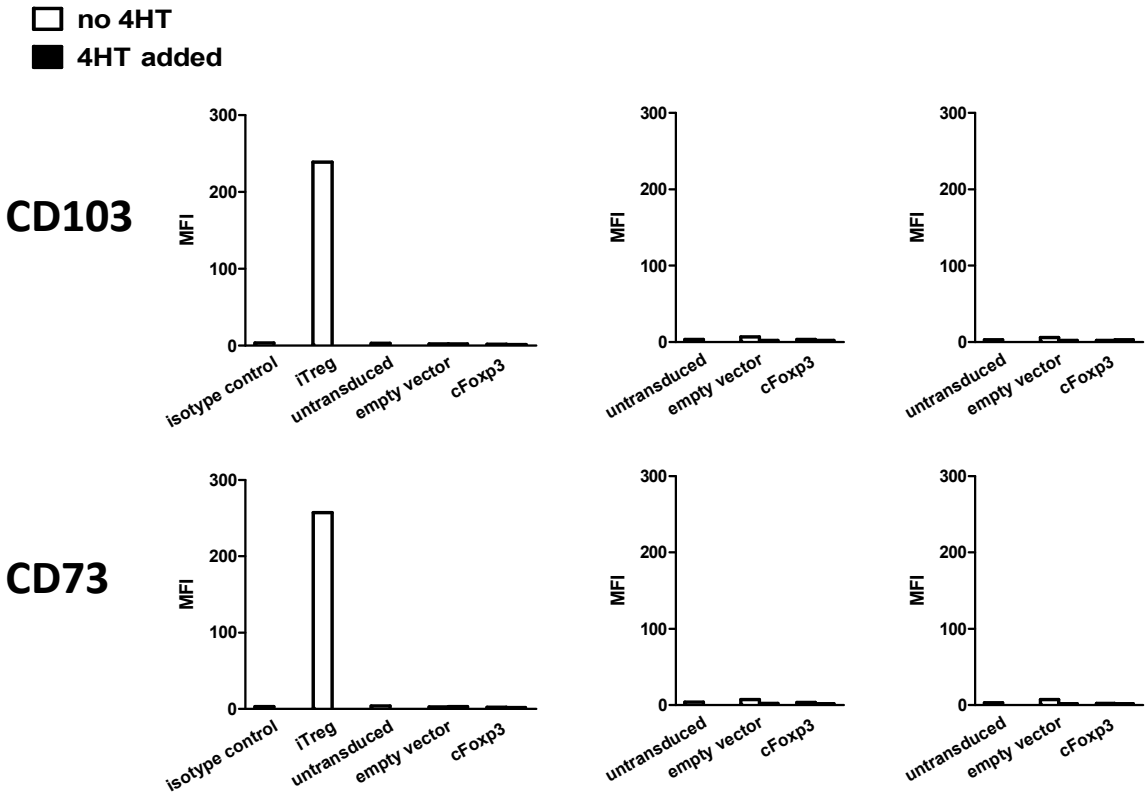


Figure 4.3D cTreg do not express CD103 or CD73

CD103 and CD73 expression as assessed by FACS at 40hr, 64 and 88hr following the onset of transduction. Transduced cells were gated on live $CD4^+GFP^+$ cells. TGFβ-conditioned cells were gated on live $CD4^+$ cells.

4.4 cTreg display anergy and can suppress proliferation of effector T cells *in vitro*

In vitro studies have shown that nTreg display a lack of response to antigen – referred to as *anergy* - and can suppress the proliferation of effector T cells (Sakaguchi 2005). This is thought to be contact-dependent. Chai et al. have shown that Foxp3-MIGR1-transduced T cells also show anergy and a suppressive capacity *in vitro* (Chai et al. 2005). To examine the proliferation and suppressive capacity of cTreg, $CD4^+$ cells from Marilyn mice were transduced with *cFoxp3*^{tgGFP} or the empty vector. Two technical replicates were performed for each sample, and the experiment was carried out twice. Representative data are shown.

cTreg derived from TCR-transgenic Marilyn $CD4^+$ cells displayed anergy *in vitro*, 66hrs after incubation with DCs and their cognate peptide **(Figure 4.4A)**. In contrast, T cells transduced with empty vector or with *cFoxp3*^{tgGFP} but without 4HT treatment proliferated normally. cTreg also showed marked suppression of effector T cell proliferation after 72hrs co-incubation with effector T cells (**Figure 4.4B**). Suppression was not seen when cFoxp3^{tgGFP}-transduced cells were not treated with 4HT.

Figure 4.4A

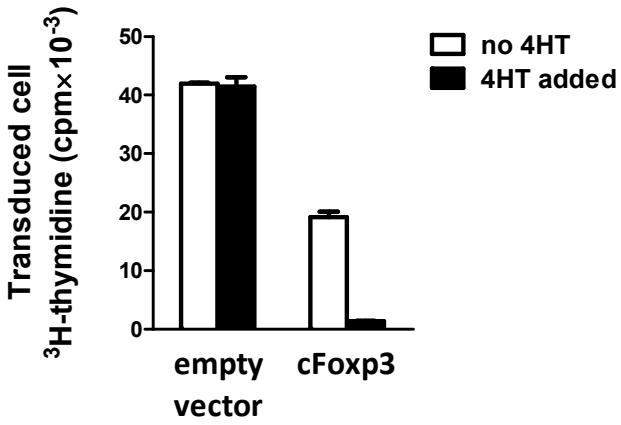


Figure 4.4A cTreg display anergy in vitro

Marilyn CD4$^+$ cells were transduced with *cFoxp3*^{tgGFP} or empty vector. 40hrs after transduction, cells were sorted on CD4 and GFP expression and incubated for a further 66hrs with mitomycin C–treated DCs and their cognate peptide (HYA^b: 100nM), with or without 4HT (50nM). Cell proliferation was assessed by 3H-thymidine incorporation. Statistical analysis performed using two-way ANOVA with Bonferonni posttest.

no 4HT vs 4HT added	*p* value
empty vector	ns
cFoxp3	<0.001

Figure 4.4B

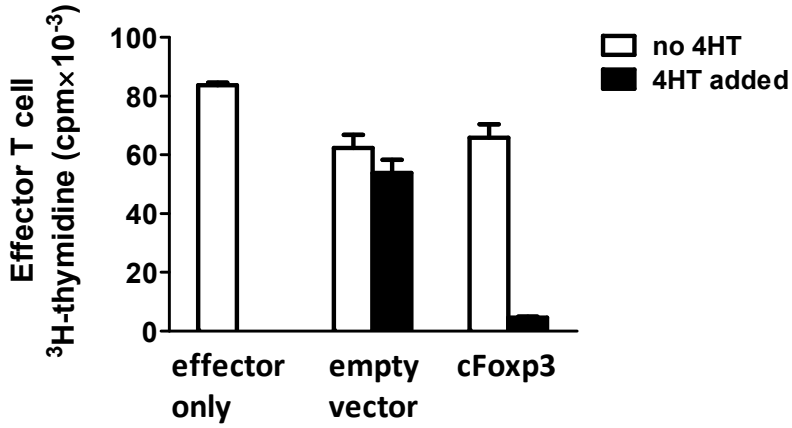


Figure 4.4B cTreg show suppression of effector cell proliferation in vitro

cFoxp3^{tgGFP} or empty-vector-transduced Marilyn CD4$^+$ cells (40hr transduction) were treated with mitomycin C and incubated with naïve Marilyn CD4$^+$ cells (effector cells) and DCs presenting their cognate peptide, with or without 4HT (50nM). Proliferation of effector cells was assessed 72hrs later. In each well of a 96 well plate contained 10^4 bmDCs, $2x10^4$ suppressors and $2.5x10^4$ effectors. Statistical analysis performed using two-way ANOVA with Bonferonni posttest.

no 4HT vs 4HT added	*p* value
effector only	N/A
empty vector	Ns
cFoxp3	<0.001

4.5 cTreg secrete low levels of cytokines

In vitro, nTreg do not secrete cytokines in response to activation (Chen et al. 2003). Foxp3 appears to negatively regulate the transcription of cytokines such as IL-2 (Marson et al. 2007; Zheng et al. 2007). To determine if conditional Foxp3 expression can result in a cytokine secretion profile similar to nTreg, $CD4^+CD25^-$ cells were isolated from spleens of B6 mice, and retrovirally transduced with *cFoxp3*^{tgGFP}. 40hrs after the transduction, cells were sorted upon their expression of GFP and CD4, and placed in fresh medium without any exogenous IL-2 or IL-7, in anti-CD3 coated plates for a further 48hrs. Cells were plated at $2x10^5$ per well in a 96-well plate. After 48hrs, supernatant was collected and cytokine secretion was assessed using Bio-Rad Multiplex Assay System. As controls, $Foxp3^+$ nTreg were sorted from B6.hCD2.KI mice based on hCD2 expression. These mice were described in the previous section.

cTreg (cFoxp^{tgGFP}-transduced + 4HT treatment) were found to secrete very low quantities of cytokines and chemokines, including cytokines such as IL-1, IFNγ, IL-4, IL-10 and TNFα (**Figure 4.5**). In contrast, cells transduced with empty vector or with *cFoxp3*^{tgGFP} but without 4HT treatment secreted significantly higher levels of cytokines and chemokines. This suggests that 4HT treatment itself, the process of retroviral transduction or T cells with untranslocated cFoxp3 did not lead to a reduction in cytokine output. IL-12, a cytokine not characteristically secreted by T cells, was also found to be elevated. This anomaly could indicate the presence of contaminating cells such as $CD4^+$ DCs. Hence, results must be interpreted in light of this.

As expected, nTreg secreted the lowest levels of all the 18 cytokines and chemokines that were tested, despite receiving the same anti-CD3 activation.

Figure 4.5

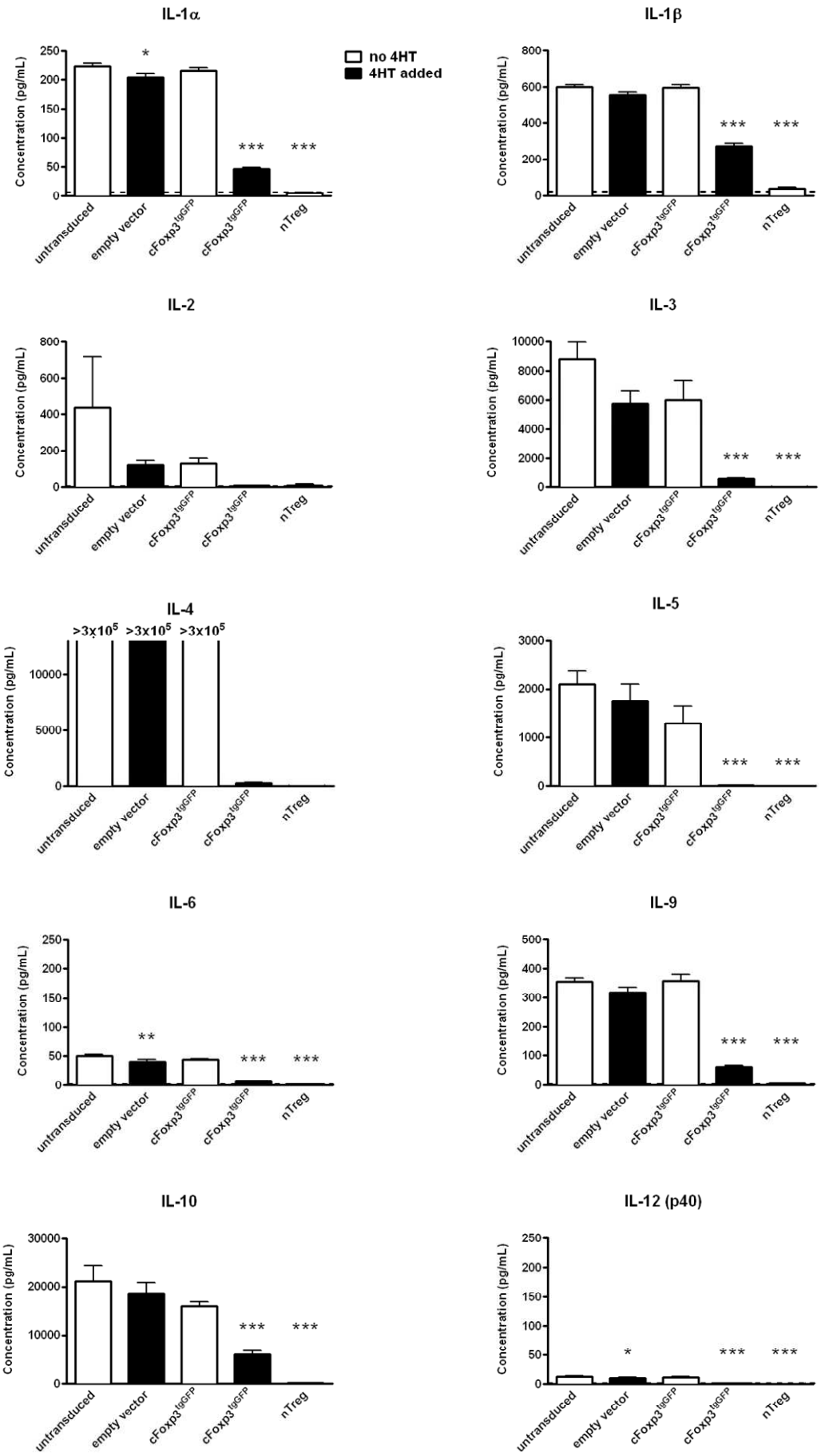

IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-9
IL-10
IL-12 (p40)
no 4HT
4HT added
Concentration (pg/mL)
>3x10^5
untransduced
empty vector
cFoxp3tgGFP
nTreg

Figure 4.5 (cont'd)

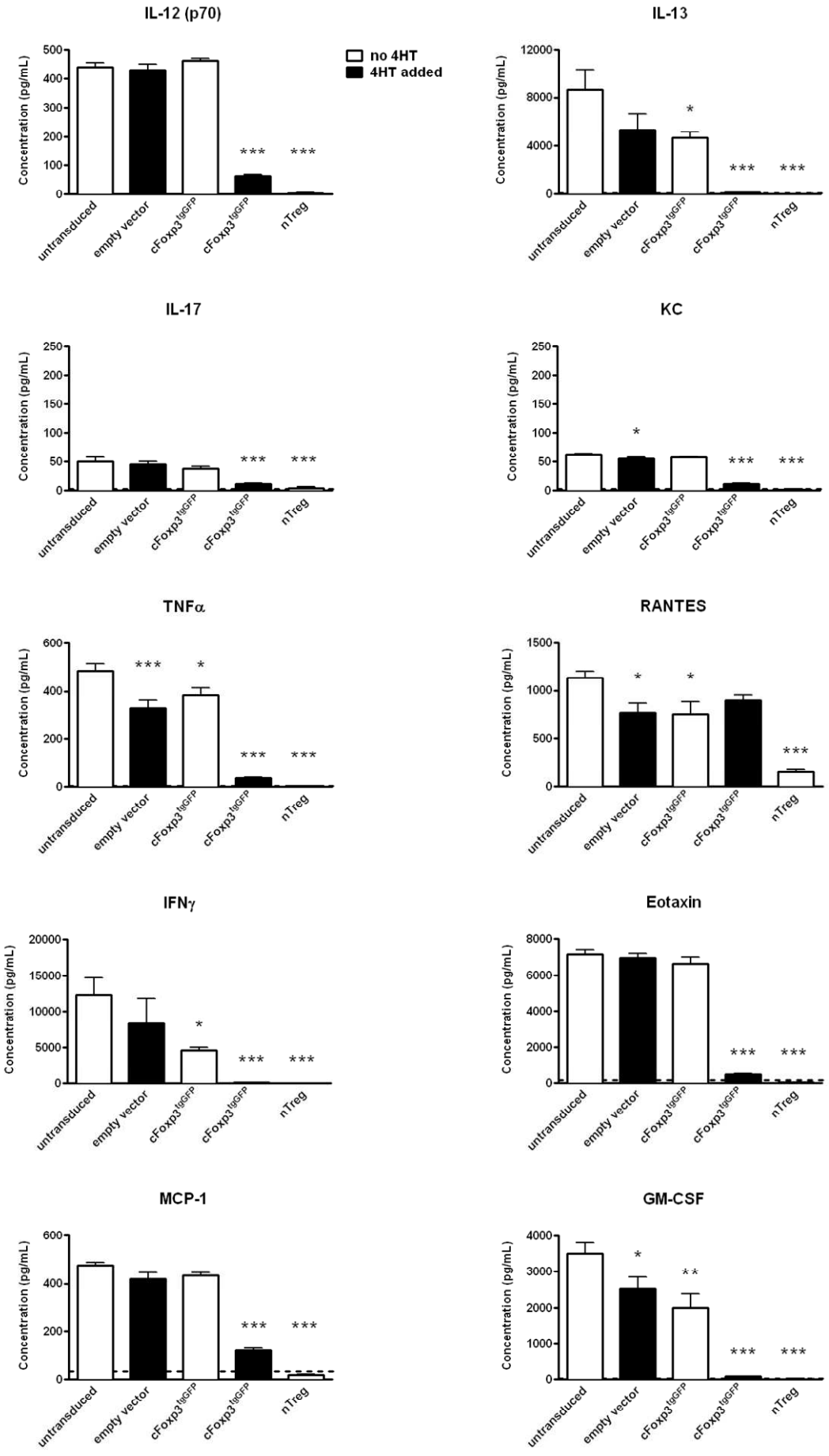


Figure 4.5 Cytokine expression profile of cTreg

$CD4^+CD25^-$ cells were purified from B6 spleen and transduced with *cFoxp3*^{tgGFP} or empty vector. For comparison, $Foxp3^+$ nTreg were isolated from B6.hCD2.KI mice based on hCD2 expression. 40hrs after the onset of transduction, transduced $CD4^+$ cells were Mo-flo sorted based on CD4 and GFP expression and $2x10^5$ cells/well were plated in fresh medium on anti-CD3 coated 96-well plates for a further 48hrs. Supernatant was then collected and analysis performed using the Bio-Plex Cytokine Multiplex Cytokine Assay. For statistical analysis, one-way ANOVA with Dunnett's comparison test was used (all groups compared to untransduced cells).

* p=0.01-0.05
** p=0.001-0.01
*** p<0.001

4.6 cTreg do not reject minor antigen-mismatched skin graft and can suppress graft rejection in lymphoreplete recipients

Previous studies have reported mixed findings regarding the effectiveness of ectopic Foxp3-expressing cells in behaving as suppressor cells *in vivo*. Whilst some studies suggest that these cells can suppress autoimmune dermatitis and graft rejection (Chai et al. 2005; Loser et al. 2005), others report their limited efficacy in the control of established diabetes or collagen-induced arthritis (Jaeckel et al. 2005; Andersen et al. 2008).

To demonstrate that this system of conditional ectopic Foxp3 expression can confer Treg-like properties *in vivo* similar to non-conditional systems, cFoxp3^{tgGFP}-transduced cells were treated with 4HT *in vitro* to induce cFoxp3-ERT translocation. Either 20,000 or 1x10^{6} 4HT-treated cFoxp3^{tgGFP}-transduced cells (i.e. cTreg) were transferred into lymphodeficient RAG$^{-/-}$ recipients followed by grafting with a male graft. Tamoxifen was administered i.p. on a daily basis (1mg per day) from the day of cell transfer (Day 0). The daily tamoxifen treatment ensured that the cTreg phenotype was actively maintained. In one group of recipients, cells were treated with *in vitro* 4HT, but recipients were not followed up with daily tamoxifen treatment. In another group of recipients, cells were not treated with 4HT, and recipients were not administered with tamoxifen (**Figure 4.6A**).

None of the recipients that received transduced cells pre-incubated *in vitro* with 4HT and then continued on daily tamoxifen rejected their grafts in the first 21 days post-transplantation (**Figure 4.6A**). Recipients that received *in vitro* 4HT treatment but no daily tamoxifen treatment rejected their grafts, suggesting that tamoxifen treatment after cell transfer is important for maintaining a 'non-inflammatory' phenotype. At day 21, when the grafts in the tamoxifen-treated group were fully healed with healthy hair growth present,

tamoxifen treatment was ceased. Five days following cessation of tamoxifen (day 26), the grafts started to show signs of rejection. Both the $1x10^6$ and 20,000 groups then rapidly rejected their grafts in the ensuing days. By Day 56, all animals except one in the $1x10^6$ group had completely rejected their grafts.

FACS analysis of the DLN, NDLN, spleen and blood on Day 56 showed that cells were present in all groups whether or not they received tamoxifen treatment (**Figure 4.6B**). However, recipients that received tamoxifen treatment showed a lower $CD4^+$ percentage of total live cells in the DLN and spleens. In these recipients, a greater percentage of $CD4^+$ cells also appeared to express CD25. Moreover, the percentage of $CD4^+$ cells expressing GFP, Foxp3 or CD62L did not appear to be higher than non-tamoxifen-treated recipients. However, at this stage, these recipients had been off tamoxifen treatment for more than 30 days.

Having shown that cTreg are non-inflammatory *in vivo*, I next investigated if these cells can suppress rejection. For this purpose, a TCR-transgenic system was exploited where *in vitro* 4HT-treated *cFoxp3*^{tgGFP}-transduced Marilyn $CD4^+$ cells were injected into Marilyn.hCD2.KI mice followed by transplantation of a male CBA.$RAG^{-/-}$ graft. Hence, both recipient $CD4^+$ cells and transferred cTreg would be targeting HYA^b peptide presented by recipient APCs. Control recipients only received tamoxifen treatment and rapidly rejected their grafts by day 11 post-transplant. In contrast, recipients that had been injected with $5x10^5$ cTreg did not reject their grafts (**Figure 4.7**). On day 27, when grafts in the group that had received cTreg appeared well-healed, tamoxifen administration was ceased. This led to the rejection of all the grafts within the next 1-2 weeks.

The prevention of graft rejection by cTreg builds upon the finding of Chai et al. in 2005, who had shown that $5x10^5$ ectopically expressing (non-conditional) Foxp3 cells could prevent rejection of syngeneic male skin in female (CBAxB6) F1 recipients (Chai et al. 2005). In preventing graft rejection in TCR-transgenic recipients, cTreg have shown themselves to be powerful mediators of suppression *in vivo*. Moreover, the rejection of grafts that follows cessation of tamoxifen treatment suggests that continued tamoxifen treatment may be necessary to maintain cTreg suppressive phenotype *in vivo*, and that the non-rejection of an allogeneic skin graft is a balancing act between rejection and suppression even at 27 days following grafting.

Figure 4.6A

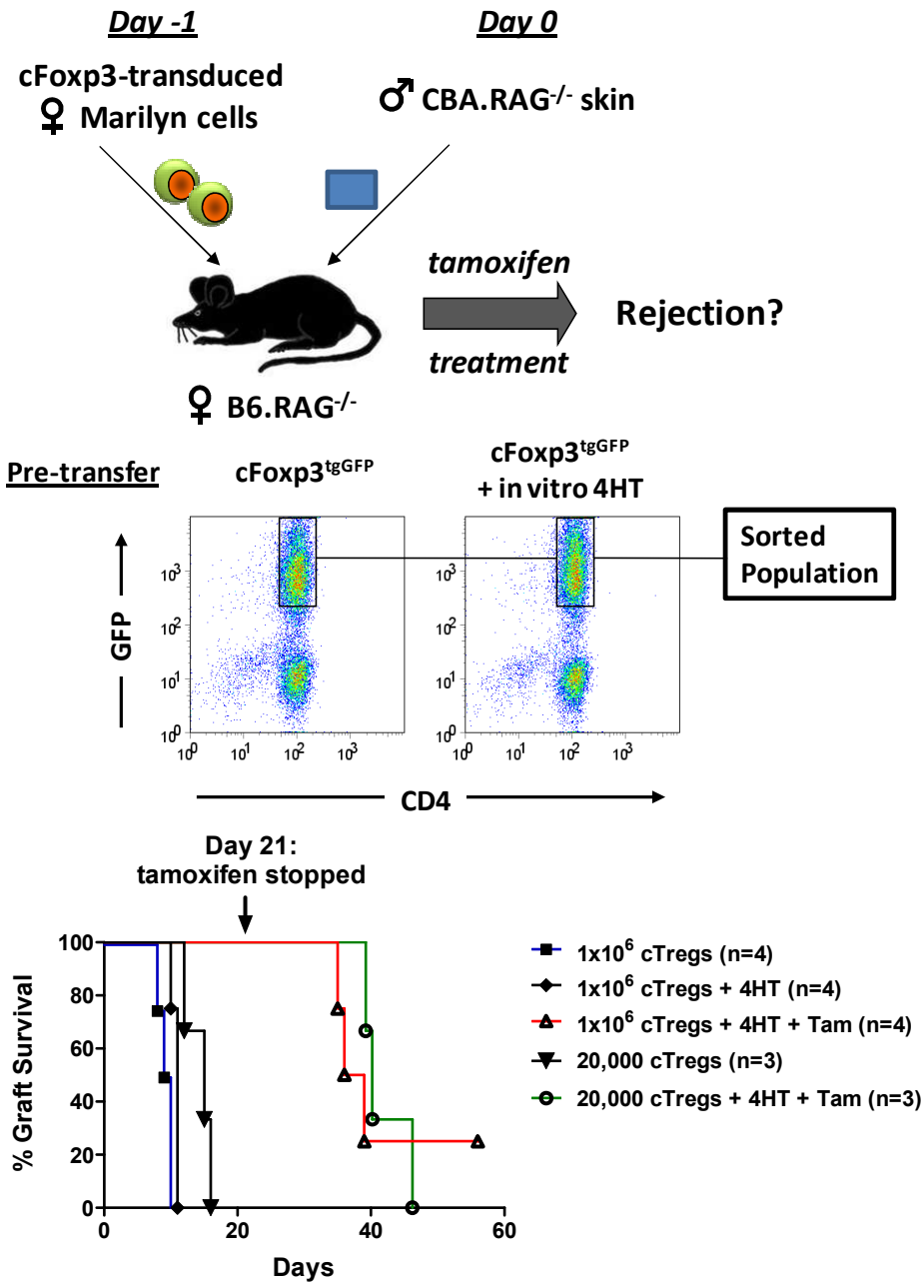


Figure 4.6A Lack of rejection of male skin grafts by male-specific cTreg is conditional upon daily tamoxifen administration

$CD4^+$ cells from spleens of Marilyn mice were transduced with *cFoxp3*^{tgGFP} and also received 4HT treatment. 20,000 or $1x10^6$ cells were transferred into B6.RAG$^{-/-}$ mice and these mice received daily tamoxifen. As controls, some recipients did not receive tamoxifen treatment. Graft survival was followed daily. On Day 21, tamoxifen treatment was ceased, and graft survival was followed until day 56. Statistical analysis (log-rank Mantel-Cox):

	p value
$1x10^6$ cFoxp3 vs. $1x10^6$ cFoxp3 + 4HT	< 0.05
$1x10^6$ cFoxp3 vs. $1x10^6$ cFoxp3 + 4HT + Tam	< 0.01
$1x10^6$ cFoxp3 + 4HT vs. $1x10^6$ cFoxp3 + 4HT + Tam	< 0.01
20,000 cFoxp3 vs. 20,000 cFoxp3 + 4HT + Tam	< 0.05
20,000 cFoxp3 + 4HT + Tam vs. $1x10^6$ cFoxp3 + 4HT + Tam	ns

Figure 4.6B

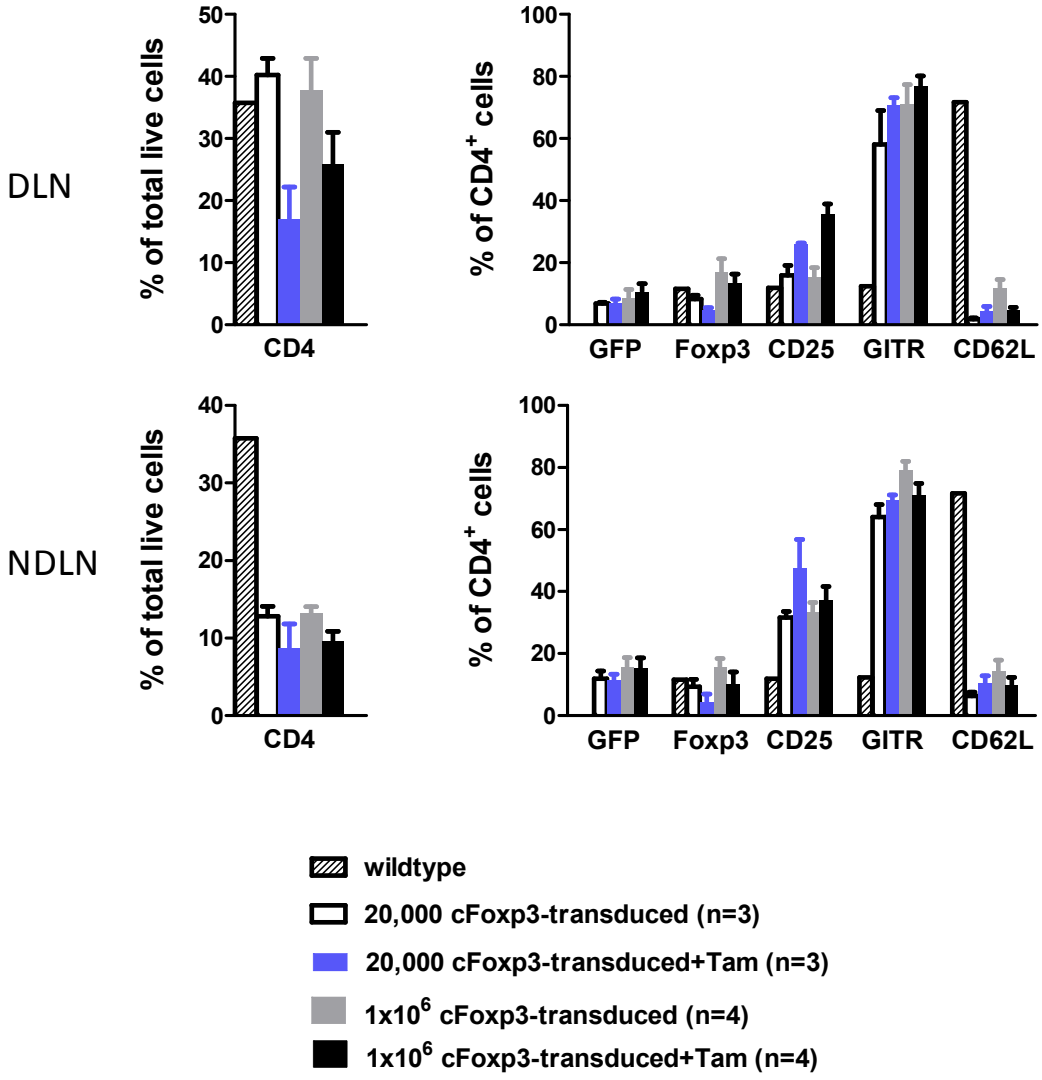

DLN
NDLN
% of total live cells
CD4
% of CD4+ cells
GFP
Foxp3
CD25
GITR
CD62L
wildtype
20,000 cFoxp3-transduced (n=3)
20,000 cFoxp3-transduced+Tam (n=3)
1x10^6 cFoxp3-transduced (n=4)
1x10^6 cFoxp3-transduced+Tam (n=4)

Figure 4.6B (cont'd)

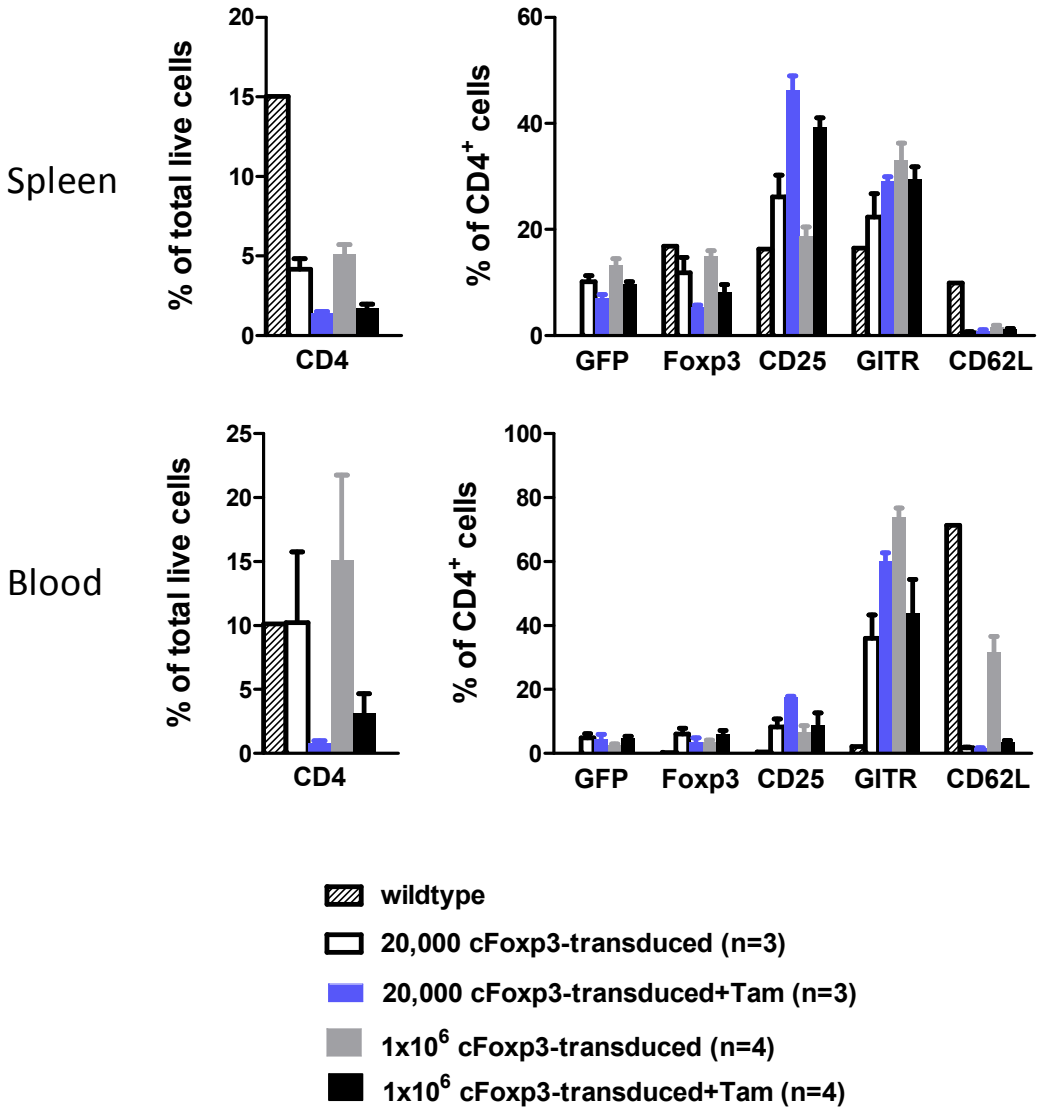


Figure 4.6B FACS analysis of the draining LN, non-draining LN, spleen and blood harvested from recipient mice on Day 56 post-transplantation

A FACS gate was drawn on live $CD4^+$ cells. $CD4^+$ cells from a single wildtype female B6 was also analysed by FACS for comparison. Statistical analysis (excluding the single wildtype reading) was performed using one-way ANOVA (CD4 as a % of total live cells, comparison between all the groups) and two-way ANOVA (expression of markers as a percentage of $CD4^+$ cells).

CD4 as a % of total live cells:	*p* value
DLN	<0.05
NDLN	ns
Spleen	<0.001
Blood	ns

Expression of markers as a percentage of CD4 cells:
DLN (*p* values shown)
20,000 cFoxp3-transduced vs 20,000 cFoxp3 transduced+Tam

GFP	ns
Foxp3	ns
CD25	ns
GITR	ns
CD62L	s

$1x10^6$ cFoxp3-transduced vs $1x10^6$ cFoxp3-transduced+Tam

GFP	ns
Foxp3	ns
CD25	<0.001
GITR	ns
CD62L	ns

NDLN (*p* values shown)

20,000 cFoxp3-transduced vs 20,000 cFoxp3-transduced+Tam

GFP	ns
Foxp3	ns
CD25	< 0.05
GITR	ns
CD62L	ns

$1x10^6$ cFoxp3-transduced vs $1x10^6$ cFoxp3-transduced+Tam

GFP	ns
Foxp3	ns
CD25	ns
GITR	ns
CD62L	ns

Spleen (*p* values shown)

20,000 cFoxp3-transduced vs 20,000 cFoxp3-transduced+Tam

GFP	ns
Foxp3	ns
CD25	<0.001
GITR	ns
CD62L	ns

$1x10^6$ cFoxp3-transduced vs $1x10^6$ cFoxp3-transduced+Tam

GFP	ns
Foxp3	<0.05
CD25	<0.001
GITR	ns
CD62L	ns

Blood (*p* values shown)

20,000 cFoxp3-transduced vs 20,000 cFoxp3-transduced+Tam

GFP	ns
Foxp3	ns
CD25	ns
GITR	<0.001
CD62L	ns

$1x10^6$ cFoxp3-transduced vs $1x10^6$ cFoxp3-transduced+Tam

GFP	ns
Foxp3	ns
CD25	ns
GITR	<0.001
CD62L	<0.001

Figure 4.7

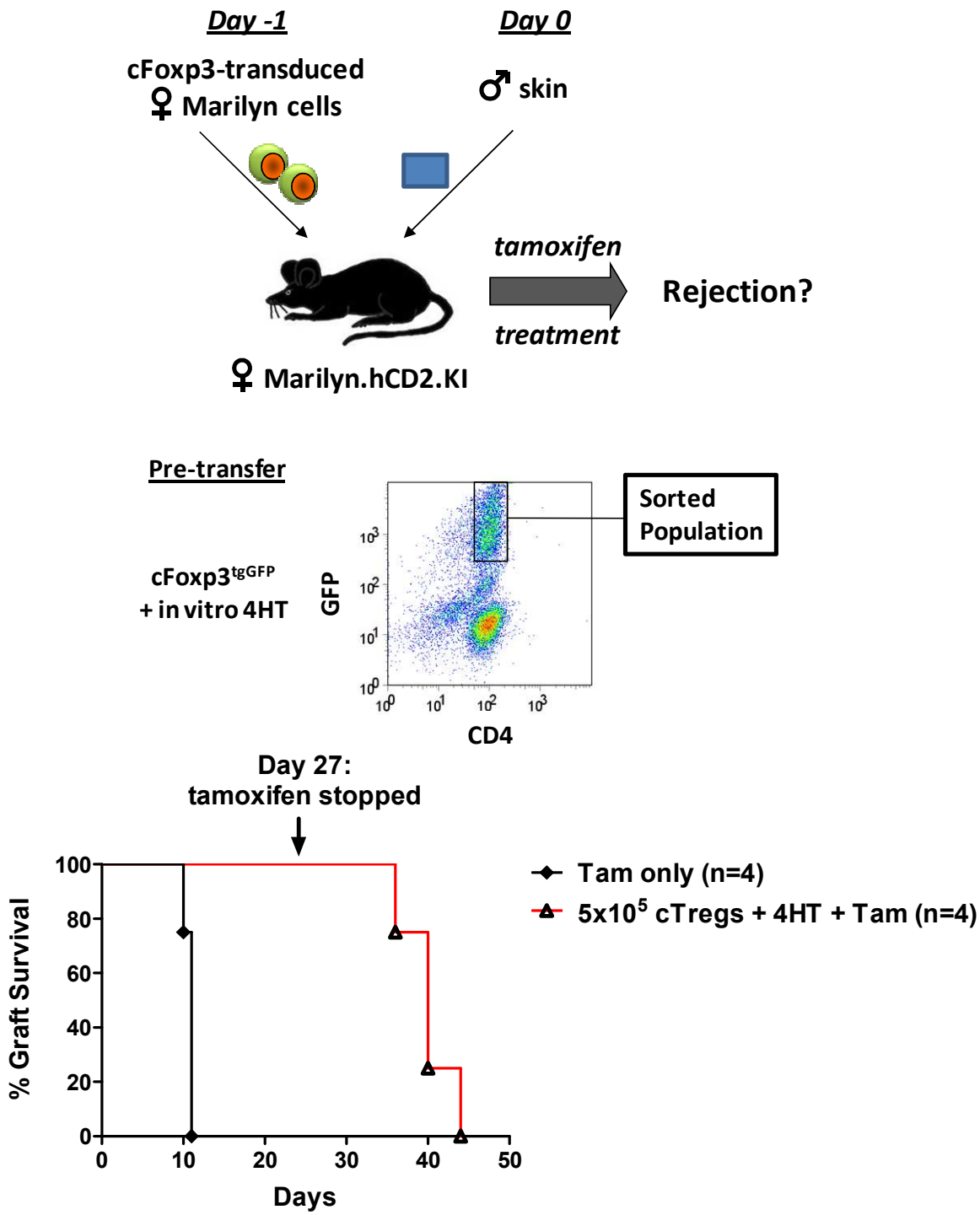


Figure 4.7 Male-specific cTreg can suppress rejection of male graft in a TCR-transgenic recipient

Mo-flo sorted *in vitro* 4HT-treated 5x10^5 cFoxp3^{tgGFP}-transduced cells were transferred into Marilyn.hCD2.KI mice followed by transplantation of a male CBA.RAG$^{-/-}$ graft the next day. Daily tamoxifen (1mg) was administered. Control recipients did not receive cells, but were treated with daily tamoxifen. Statistical significance (log-rank Mantel Cox): $p<0.05$.

4.7 Unsorted polyclonal cTreg remain able to reject a major-MHC mismatch graft in a lymphopenic recipient

Andersen et al. proposed that the conditional Foxp3 expression system enables control over timing of the 'conversion' into cTreg such that delayed tamoxifen administration can allow clonal expansion of transduced cells to occur (Andersen et al. 2008). Hence, unsorted polyclonal cTreg were shown to suppress arthritis development in a model of collagen-induced arthritis where tamoxifen treatment was initiated on day 15 post transfer of cells, 4 days before development of clinical signs in control untreated mice.

To investigate if delayed tamoxifen treatment could enable unsorted polyclonal cTreg to suppress graft rejection, a major-MHC mismatch skin transplantation system was established, where sex-matched donor B6 skin (haplotype H-2^b) was grafted onto a lymphodeficient CBA.RAG$^{-/-}$ recipient (haplotype H-2^k) following adoptive transfer of transduced CBA CD4$^+$ cells. A titration of cell numbers needed to lead to rejection was performed – from a minimum of $5x10^4$ up to $5x10^5$ cells. As few as $1x10^5$ *cFoxp3*^{rCD8}-transduced CBA CD4$^+$ cells can reject a major-mismatched graft in the absence of *in vitro* 4HT treatment or tamoxifen (**Figure 4.8A**). The transduction rate was 33%. The kinetics of rejection was most rapid at the highest number of cells – $5x10^5$.

In a second experiment, the highest number of cells – $5x10^5$ – was used to test whether delayed tamoxifen administration can 'convert' *cFoxp3*-transduced cells into cTreg *in vivo*. The proportion of transduced cells was 35%. Tamoxifen was administered from day 7 as this may allow *cFoxp3*-transduced cells to expand in response to graft antigen. However, all the groups, including empty vector-transduced, *cFoxp3*-transduced and *cFoxp3*-transduced with tamoxifen mice rejected their grafts within 20 days from the day of transplantation (**Figure**

4.8B). There are various non-mutually exclusive factors that might have contributed to this. Administering tamoxifen as late as day 7 might be too late to stop the rejection response as the inflammatory cascade might already be set in motion. The frequency of tamoxifen administration may also be inadequate (tamoxifen administered on day 7, 8, 15, 16). The number of cells – $5x10^5$, might also be too high, and skew the response towards rejection. The transduction rate (35%) may also be too low, again skewing the response towards rejection.

To address these possible factors, the experiment was repeated using fewer cells ($1x10^5$), injecting tamoxifen from the day of grafting (Day 0) and increasing the frequency of tamoxifen administration to every second day (**Figure 4.8C**). The transduction rate was around 25%. In one group, the *cFoxp3*^{GFP}-transduced cells were also treated with 4HT *in vitro*. However, all the groups still rejected their grafts. The low transduction rate of 25% might have account for this since non-transduced cells might have quantitatively exceeded the cTreg during the rejection response.

Figure 4.8A

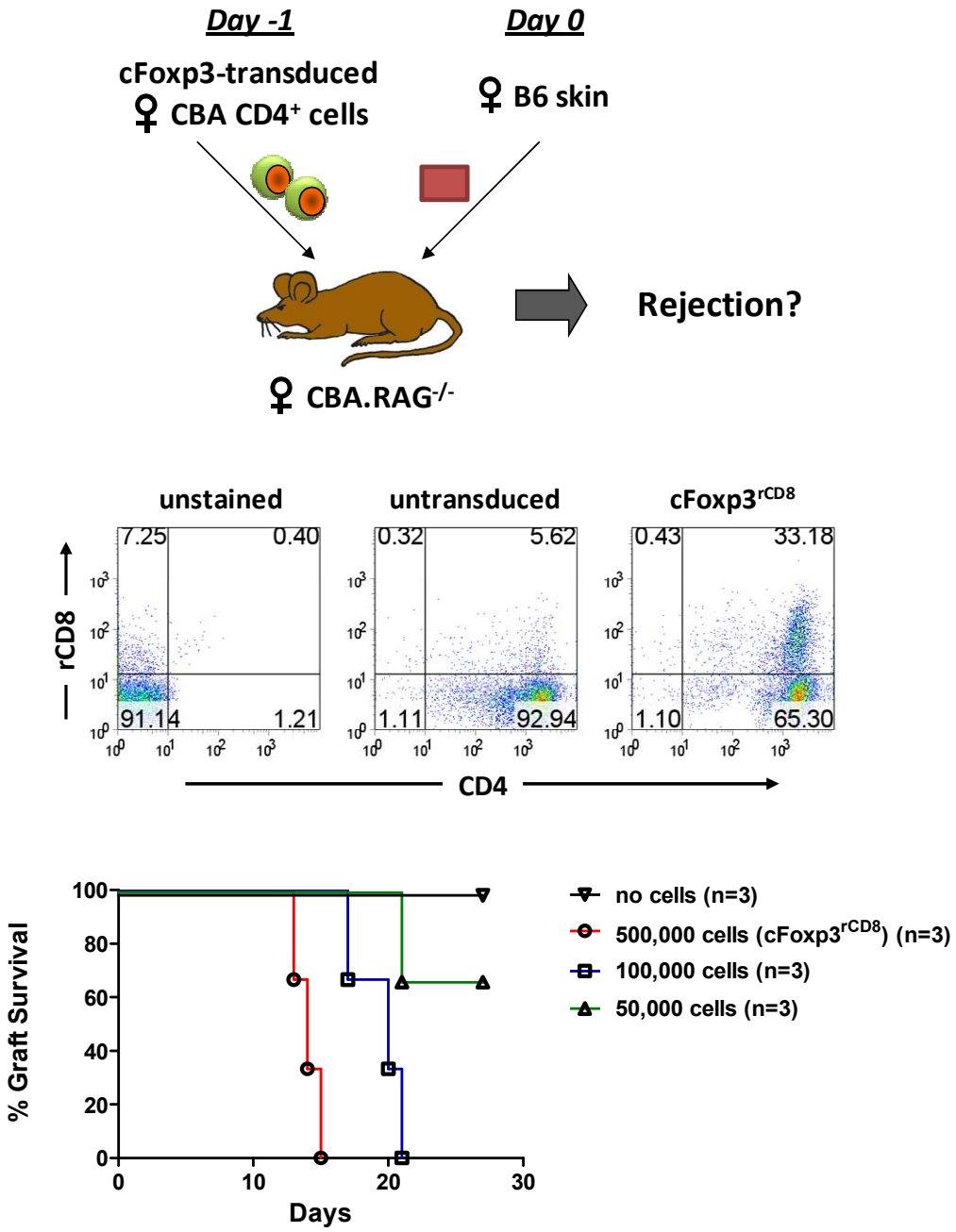


Figure 4.8A Titration of CBA CD4+ cells to reject B6 skin in a CBA.RAG-/- recipient.

cFoxp3^{rCD8}-transduced CD4^{+} cells (33% transduction rate) from female CBA spleens were transferred into CBA.RAG$^{-/-}$ mice and female B6 skin was grafted the following day. Tamoxifen was not administered. Statistical analysis (log-rank Mantel-Cox):

	p value
no cells vs. 500,000 cells	< 0.05
no cells vs. 100,000 cells	< 0.05
no cells vs. 50,000	ns

Figure 4.8B

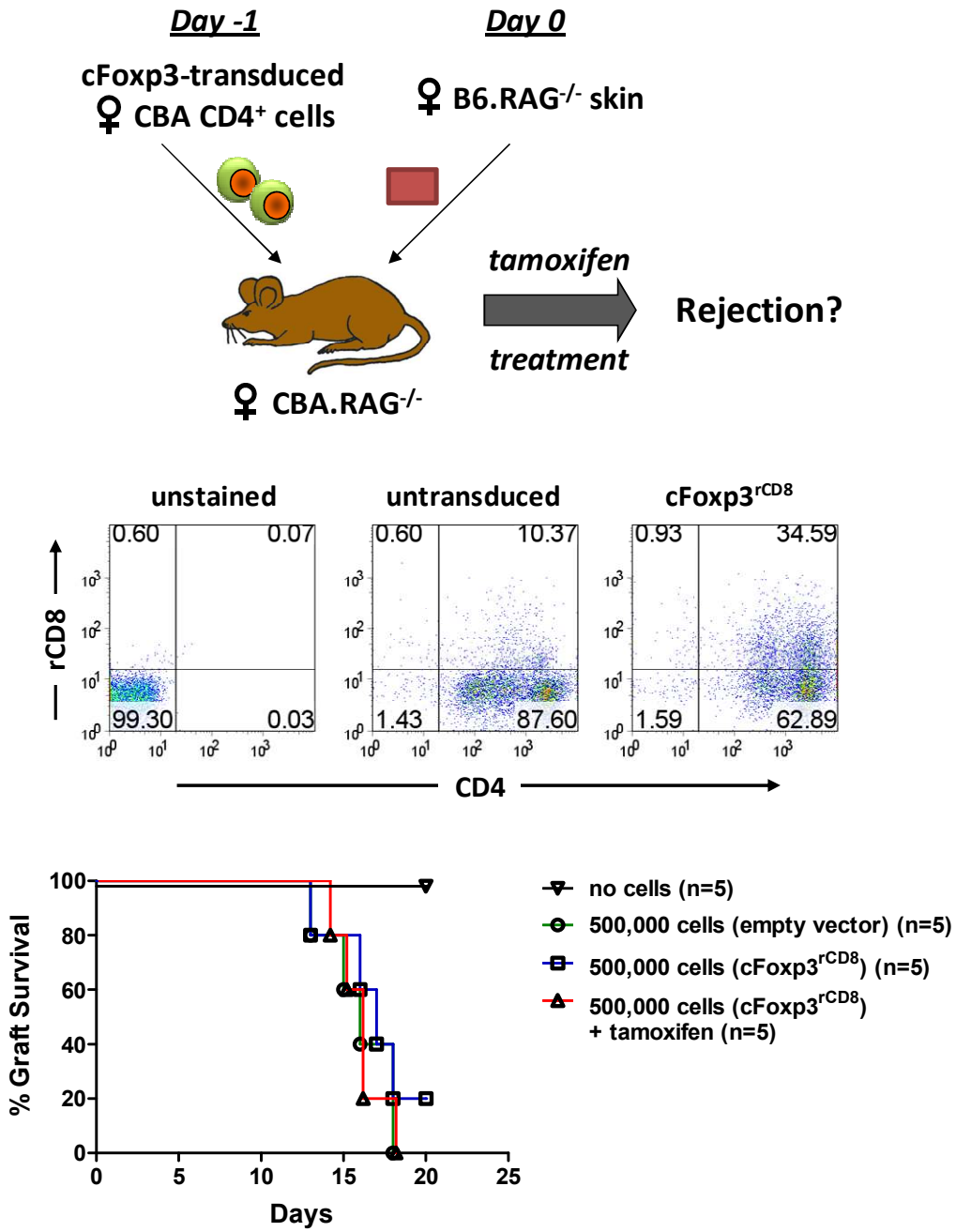


Figure 4.8B Unsorted cFoxp3-transduced polyclonal CD4+ cells reject a fully MHC mismatched graft in a lymphopenic recipient

5x10^5 unsorted *cFoxp3*^{rCD8}-transduced cells were adoptively transferred into CBA.RAG$^{-/-}$ mice and skin from B6.RAG$^{-/-}$ mice was transplanted the following day. The transduction rate was 35%. 1mg Tamoxifen was administered on day 7, 8, 15 and 16. Statistical analysis (log-rank Mantel-Cox):

	p value
no cells vs. 500,000 cells (empty vector)	<0.01
no cells vs. 500,000 cells (cFoxp3^{rCD8})	<0.05
no cells vs. 500,000 cells (cFoxp3^{rCD8} + tamoxifen)	<0.01

Figure 4.8C

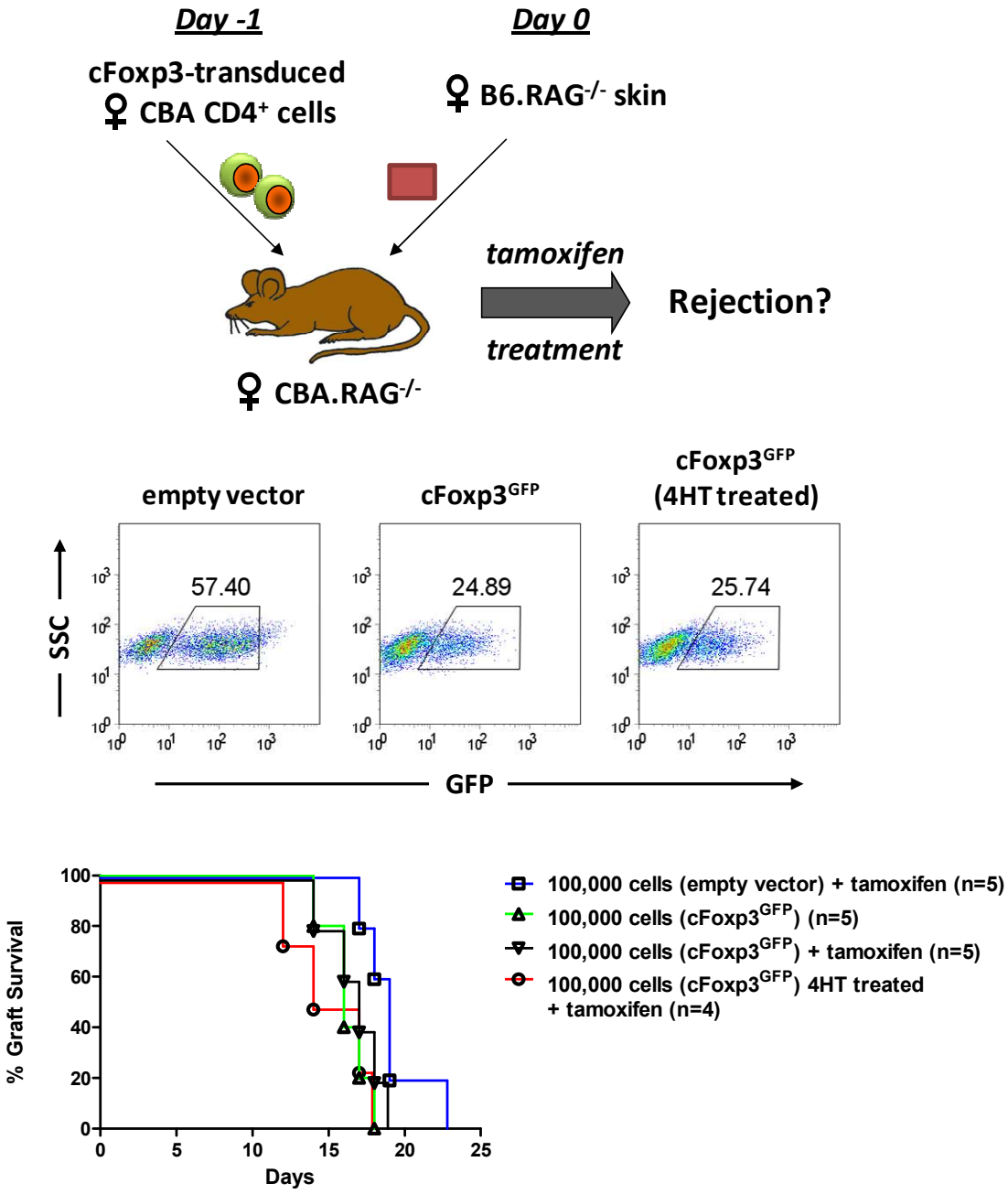


Figure 4.8C Unsorted cFoxp3-transduced polyclonal CD4+ cells reject a fully MHC mismatched graft in a lymphopenic recipient

Donor and recipient mice were as in B). 1x10^5 cells were transferred. Tamoxifen was given every second day starting from Day 0 (day of transplantation) at a dose of 1mg/day. The transduction rate was 25%. Statistical analysis was not performed as all groups rejected their grafts.

4.8 Polyclonal cTreg remain able to reject a major-mismatch skin graft in a semi-lymphoreplete recipient

In the previous section, cells were transferred into RAG$^{-/-}$ recipients which were then grafted. This allowed one to examine behaviour of the transferred cells *in vivo* without the complication of the recipient's own immune system. However, the issue of differential homeostatic expansion of *cFoxp3*-transduced and untransduced cells could be important since unsorted cell populations were transferred. To circumvent this issue, a system was set up where female CBA.RAG$^{-/-}$ skin was grafted onto Marilyn recipients. Marilyn recipients are semi-lymphoreplete because the only CD4^{+} cells in these mice are TCR transgenic and specific for a male antigen-derived peptide presented by H-2^{b}. Hence, they would not respond to the CBA.RAG$^{-/-}$ female graft and would not be reactive towards adoptively transferred B6 CD4^{+} cells. Transferred female B6 cells are of the same sex and haplotype as Marilyn mice, and would be expected to respond to the fully MHC-mismatched CBA.RAG$^{-/-}$ skin graft.

A titration showed that as few as 20,000 *cFoxp3*- transduced B6 CD4^{+} cells, given without tamoxifen administration, can reject a CBA.RAG$^{-/-}$graft in such a semi-replete system (**Figure 4.9A**). Therefore, 50,000 transduced cells were used for the next experiment. Assuming a 40% transduction efficiency, 20,000 cells would still behave as effectors in this system. Additionally, a low number of cells was expected to slow the kinetics of rejection, and therefore provide a window of opportunity for polyclonal cTreg to engage in 'suppression'.

Despite using 50,000 cells with a transduction rate of 38% (**Figure 4.9B**), and administering tamoxifen from Day 0 (day of transplant) at a frequency of every 2 days, the CBA.RAG$^{-/-}$ skin graft was still rejected by *cFoxp3*-transduced B6 CD4^{+} cells. The low number of mice was a

limitation of this particular experiment. Nevertheless, the results point out that even in this semi-lymphoreplete system, it is possible that the untransduced population quantitatively 'over-powered' the *cFoxp3*-transduced population.

Figure 4.9A

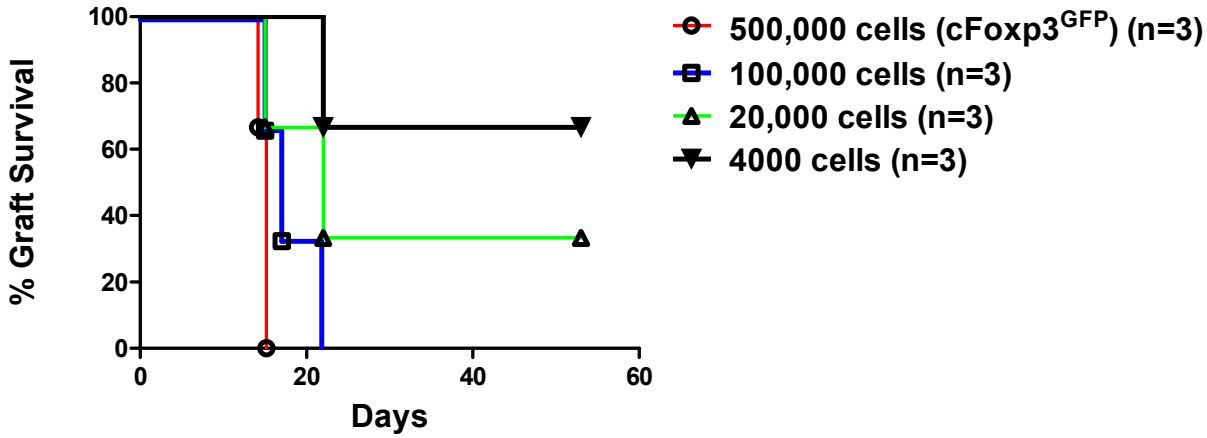


Figure 4.9A Titration of the number of cFoxp3^{GFP} transduced polyclonal cTreg required to reject a MHC mismatched graft in semi-lymphoreplete recipients

$CD4^+CD25^-$ cells were isolated from female B6 spleens and transduced with *cFoxp3*^{GFP}. 40hrs after transduction, 500,000, 100,000, 20,000 or 4000 cells were adoptively transferred into Marilyn mice which were then grafted with female CBA.RAG$^{-/-}$ skin. Tamoxifen was not administered. Statistical significance (Log-rank Mantel Cox):

	p value
4000 vs 500,000 cells	< 0.05
4000 vs 100,000 cells	< 0.05
4000 vs 20,000 cells	ns

Figure 4.9B

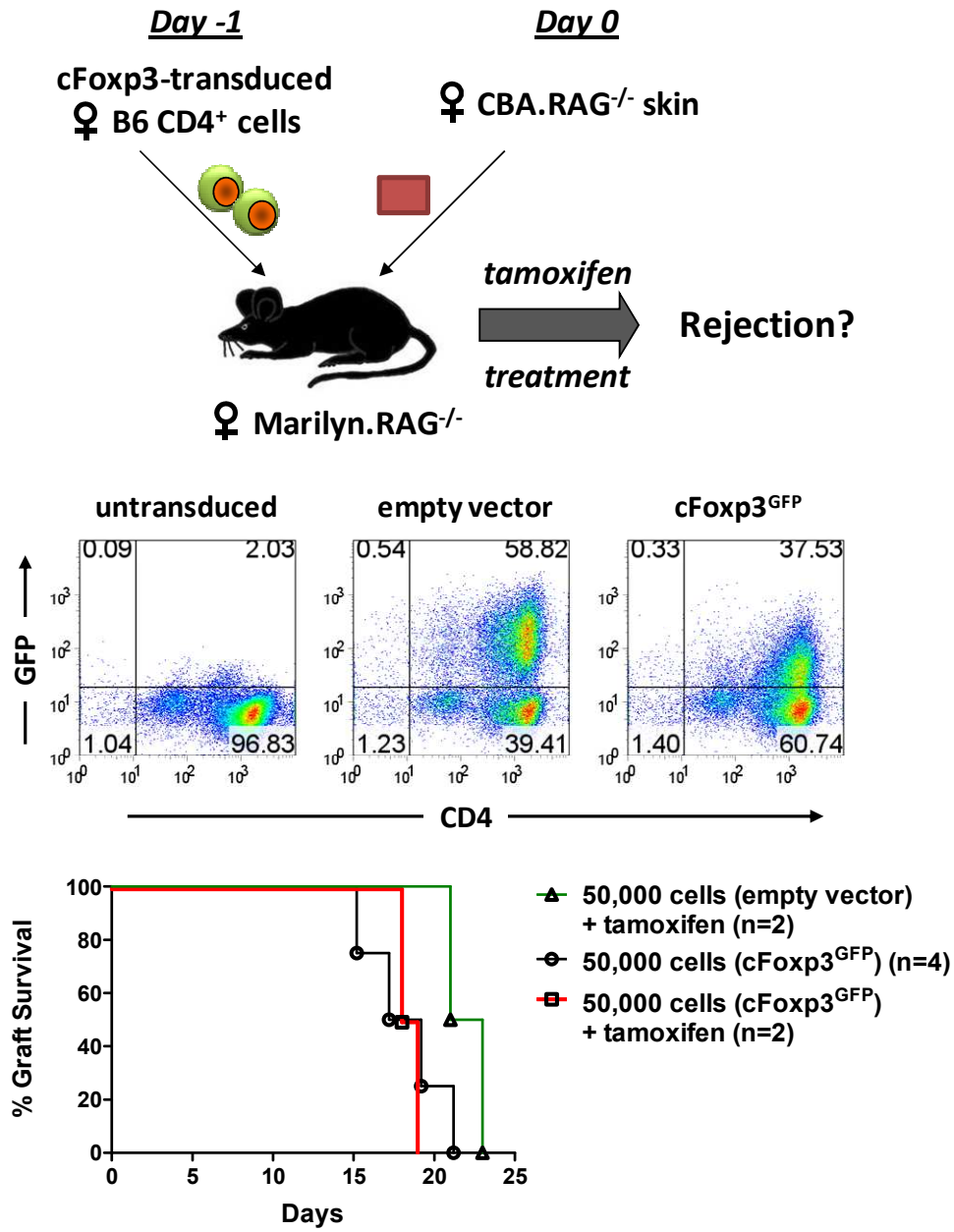


Figure 4.9B Polyclonal cTreg are still able to reject a fully MHC-mismatched graft in a semi-lymphopenic recipient

$CD4^+CD25^-$ cells were isolated from female B6 spleens and transduced with *cFoxp3*^{GFP}. 40hrs after transduction, 50,000 cells (transduction rate of 38%) were adoptively transferred into Marilyn mice which were then grafted with female CBA.$RAG^{-/-}$ skin. Tamoxifen was administered every second day, starting from the day of grafting (Day 0). Statistical significance (Log-rank Mantel Cox):

	p value
50,000 (empty vector) vs 50,000 (cFoxp3^{GFP}) + tam	ns
50,000 (cFoxp3^{GFP}) vs 50,000 (cFoxp3^{GFP}) + tam	ns

4.9 Polyclonal cTreg cannot prevent the rejection of grafts mismatched for multiple minor antigens in lymphoreplete recipients

The transduction rate of $CD4^+$ cells using *cFoxp3* vectors was consistently between 30% and 80%. This meant that between 20% and 70% of adoptively transferred cells were not transduced and could contribute to rejection. In section 4.1, Mo-flo sorted TCR-transgenic cTreg were shown to be non-inflammatory *in vivo*. Hence, transduced polyclonal cFoxp3-transduced cells were Mo-flo sorted in order to examine the behaviour of these purified cells *in vivo*. To further mimic the physiological state, these cells were transferred into a lymphoreplete host.

A multiple minor antigen-mismatched skin graft (B10.BR) was transplanted onto a sex-matched lymphoreplete recipient (CBA), and $1x10^6$ sorted *cFoxp3*^{tgGFP}-transduced cells were adoptively transferred to prevent rejection of the skin graft (**Figure 4.10**). Tamoxifen was administered every second day either from day 2 or day 5. However, sorted cTreg could not prevent rejection of multiple minor antigen-mismatched skin grafts. Possible reasons for this are that the tamoxifen administration regimen (delayed onset of administration and then every second day) may be inadequate for inducing or maintaining cTreg phenotype *in vivo*, or that the $1x10^6$ cTreg may lack the potency to stop the rejection by the wildtype recipient's own cells.

Figure 4.10

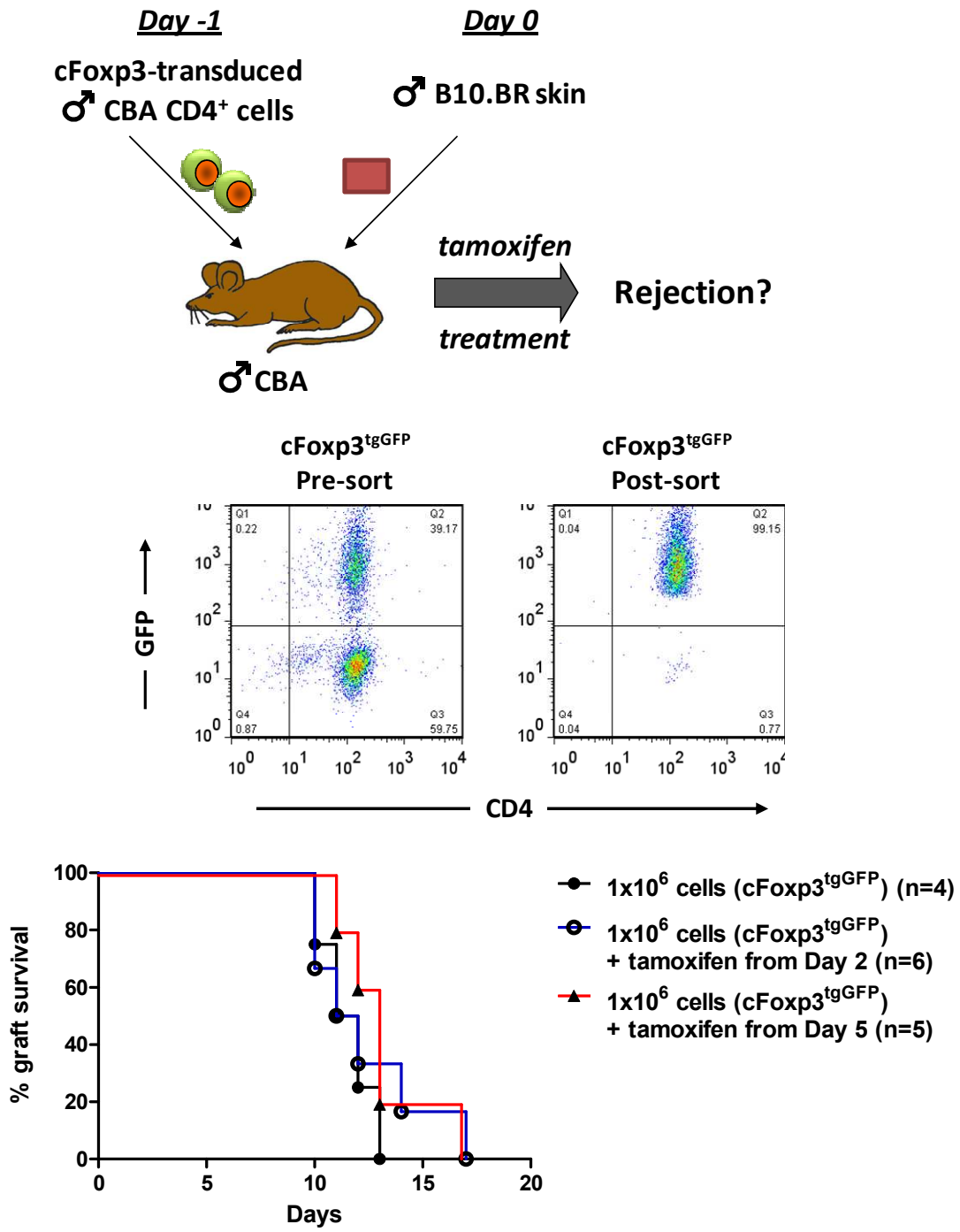


Figure 4.10 Polyclonal cTreg fail to suppress rejection of multiple minor antigen-mismatched grafts by lymphoreplete recipients

$CD4^+CD25^-$ cells were purified from male CBA mice spleens and transduced with *cFoxp3*^{tgGFP}. 40hrs after transduction, the cells were Mo-flo sorted based on CD4 and GFP expression. Post-sort purity of $CD4^+GFP^+$ cell was >99%. $1x10^6$ of the sorted cells were adoptively transferred into male CBA mice and a B10.BR skin graft was transplanted. Tamoxifen was administered every other day, starting from Day 2 or Day 5 post-transplantation. One group of recipients did not receive any tamoxifen. The difference in the kinetics of rejection between the groups was not statistically significant.

4.10 At low cell numbers, male antigen-specific cTreg do not reject a male skin graft in a lymphodeficient recipient

So far, polyclonal cTreg in combination with delayed tamoxifen administration had not shown Treg-like behaviour *in vivo*. On the other hand, TCR-transgenic cTreg with *in vitro* 4HT pre-treatment and daily tamoxifen administration starting from the day of transfer had been shown to be non-inflammatory when transferred into $RAG^{-/-}$ recipients and could suppress graft rejection in TCR-transgenic $RAG^{-/-}$ recipients. Hence, using TCR-transgenic cTreg and $RAG^{-/-}$ recipients, I set out to investigate if a lack of 4HT pre-treatment and delayed tamoxifen administration would still allow cFoxp3-transduced cells to display a Treg-phenotype *in vivo*.

Male antigen-specific $CD4^+$ cells from Marilyn mice were transduced with $cFoxp3^{tgGFP}$ and sorted on GFP and CD4 expression. Initially, 20,000 cells were transferred into $B6.RAG^{-/-}$ mice. A male graft was transplanted and followed by tamoxifen administration from day 7 to allow for antigen-specific expansion in the first 6 days.

Mice receiving 20,000 male-specific cTreg did not reject the male skin graft. However, mice injected with *cFoxp3*-transduced cells without tamoxifen treatment or empty vector-transduced cells rejected their grafts (**Figure 4.11**). On day 30 post-transplant, the mice were sacrificed, and DLN (ipsilateral axillary LN), NDLN (contralateral axillary LN), spleen and peripheral blood were examined for the presence of transferred cells (**Figure 4.12B**). The percentage of $CD4^+$ cells of total live cells is lowest in the cTreg (*cFoxp3* vector + Tam) group. This is the case in all the organs that were examined with the exception of blood. However, the cTreg group also had the highest proportion of $CD4^+$ cells expressing GFP, Foxp3, CD25 and CD62L. The pre-transfer expression of Foxp3 by cTreg is shown in **Figure 4.12A**.

The high percentage of cTreg expressing CD62L compared with tamoxifen untreated cells or empty-vector-transduced cells (**Figure 4.12B**) is similar to high percentage expression of CD62L *in vitro*. As CD62L is required to facilitate lymphocyte entry into secondary lymphoid organs, the relative high proportion of cTreg expressing CD62L in the DLN and NDLN suggest that they are capable of homing to these tissues. The intensity of Foxp3 staining of $CD4^{+}GFP^{+}$ cells also appeared to be highest in the cTreg group, consistent with the pattern seen *in vitro*, and suggests the nuclear translocation and consequent protection of the *cFoxp3* transgene product. All in all, the presence of cTreg in the lymph nodes and spleen suggests that cTreg are capable of homing to lymphoid organs.

The experiment was repeated, but in addition to transferring 20,000 cells, higher numbers of cells were also transferred – 100,000 and $1x10^6$ (**Figure 4.13**). Again, 20,000 cTreg did not reject the skin graft. However, the transfer of high cell numbers (100,000 cells or $1x10^6$ cells) resulted in graft rejection in the majority of the recipients. One possible reason that higher cell numbers rejected the graft was that the greater number of cells led to a rapid onset of rejection by unconverted *cFoxp3*-transduced cells, and so the administration of tamoxifen at Day 7 was too late for converted cTreg to reverse the process. To test this hypothesis, a second graft was transplanted, as converted cTreg should not be expected to reject the second graft.

Interestingly, the second graft transplanted in the higher cell number groups (100,000 and $1x10^6$) was still rejected, but with slower kinetics (**Figure 4.13**). The groups that received no tamoxifen treatment showed rapid rejection within 10 days. The difference in kinetics of rejection of the second and first grafts suggests that there were indeed more converted

cTreg at the time of the second transplant that had slowed the secondary rejection response by non-converted cells.

In summary, delayed tamoxifen administration still allows cFoxp3-transduced cells to 'convert' to cTreg. However, this may not be sufficient to stem the rejection response when there is a high number of 'unconverted' *cFoxp3*-transduced cells.

Figure 4.11

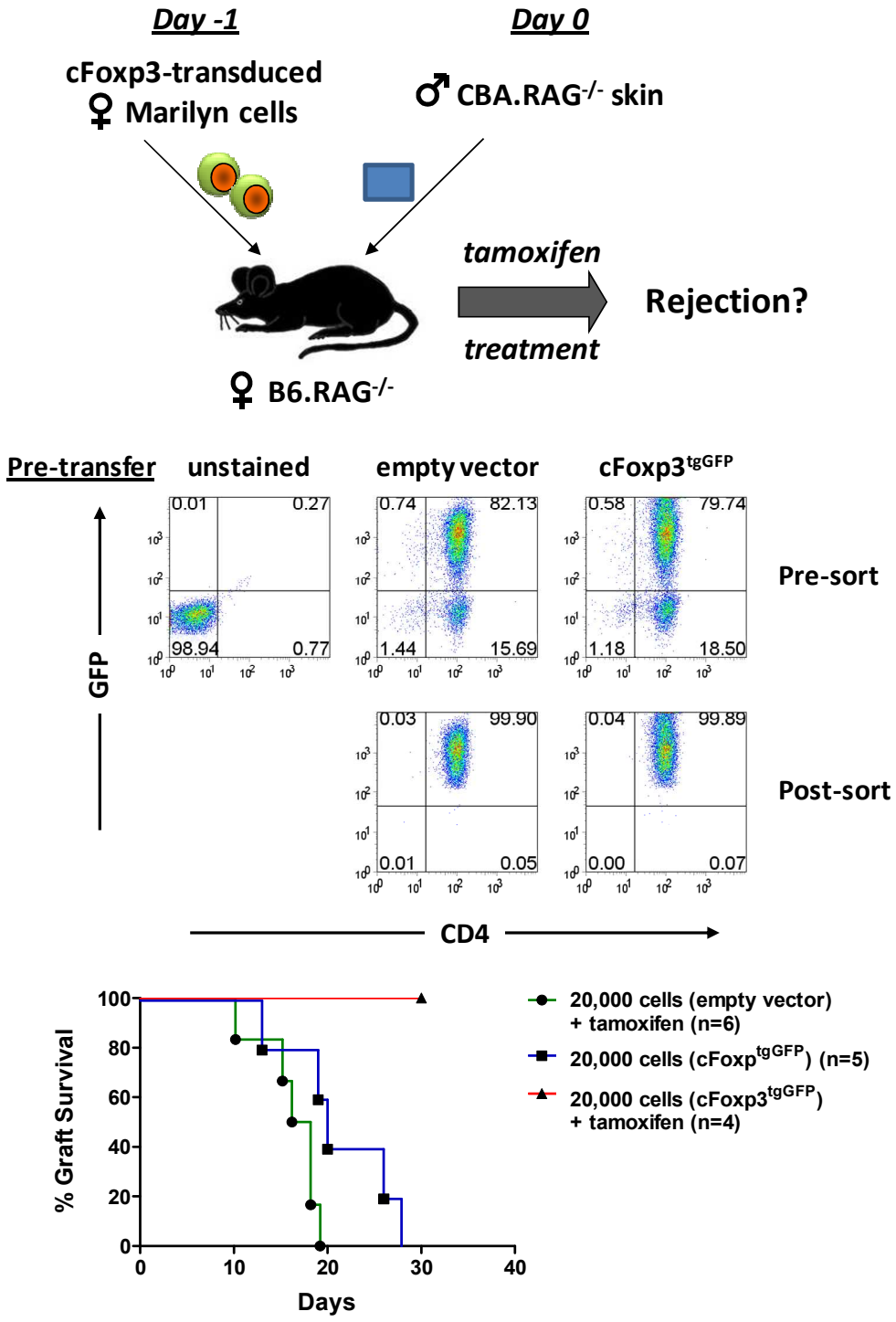


Figure 4.11 Low numbers of Mo-flo sorted TCR transgenic cTreg do not reject minor-antigen mismatched skin grafts

$CD4^+$ cells from Marilyn mice were transduced with *cFoxp3*^{tgGFP} for 40hrs. $CD4^+GFP^+$ cells were then sorted using Mo-Flo and injected IV into B6.$RAG^{-/-}$ mice. A male graft was transplanted the following day. Tamoxifen administration was given every second day starting from day 7 following transplantation. For controls, transplant recipients either did not receive tamoxifen treatment, or received empty-vector-transduced cells. Statistical significance (Log-rank Mantel Cox):

	p value
20,000 (empty vector) + tam vs 20,000 (cFoxp3^{tgGFP}) + tam	< 0.01
20,000 (cFoxp3^{tgGFP}) vs 20,000 (cFoxp3^{tgGFP}) + tam	< 0.01

Figure 4.12A

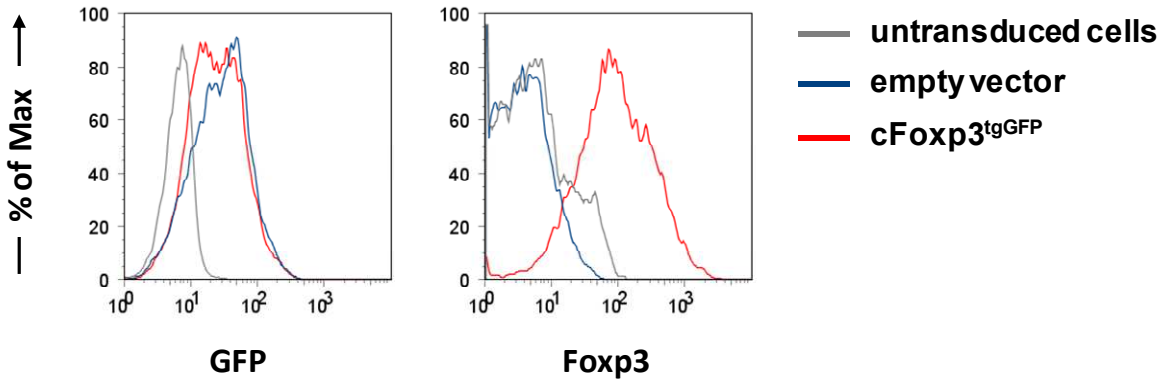


Figure 4.12A Pre-transfer expression of Foxp3 by transduced cells

Pre-transfer, the transduced Marilyn cells were sorted based on CD4 and GFP expression. Foxp3 expression of untransduced, empty-vector-transduced and *cFoxp3*-transduced cells is shown.

Figure 4.12B

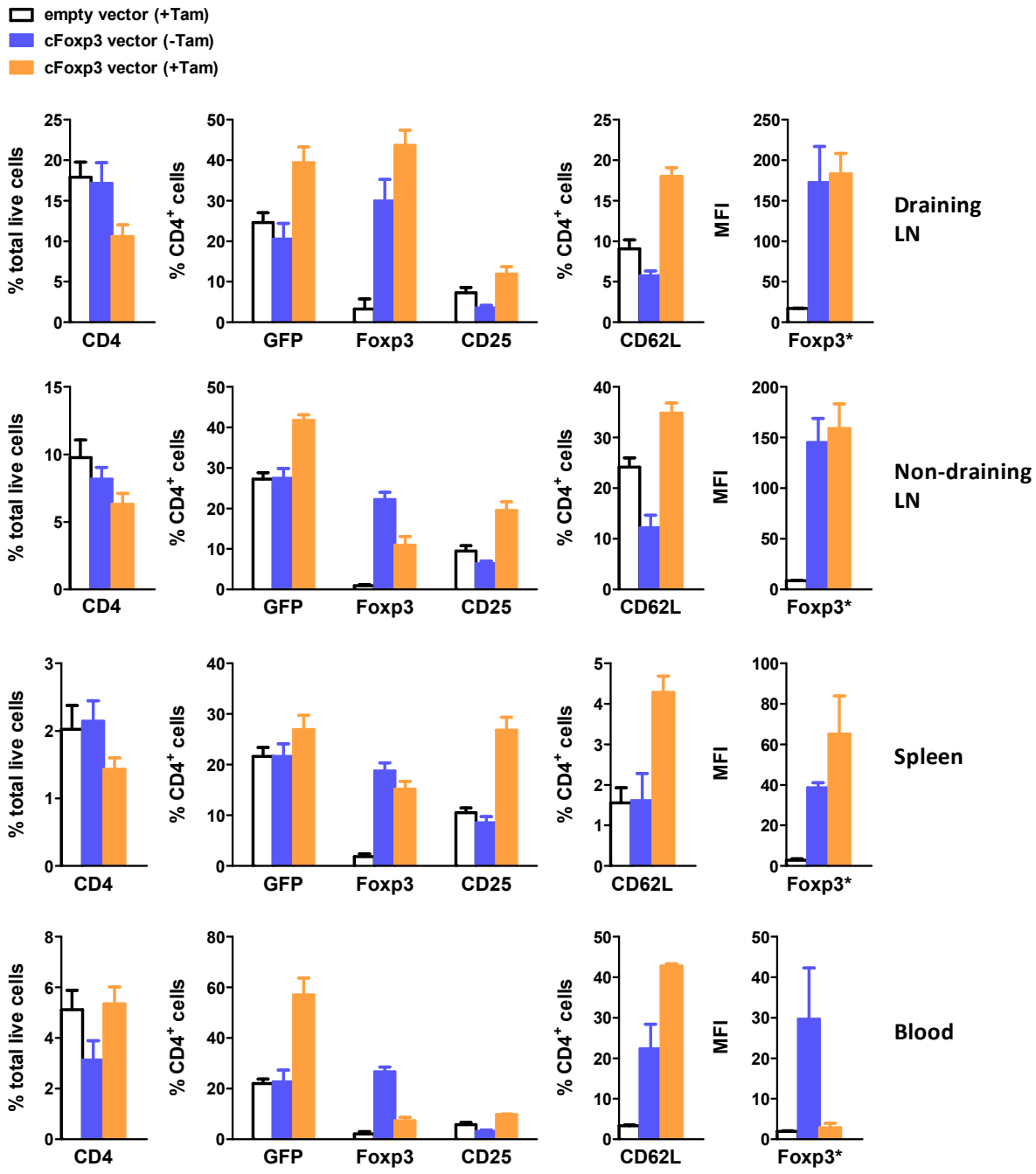


Figure 4.12B cTreg are found in secondary lymphoid organs of lymphopenic recipients

Cells from draining LN, non-draining LN, spleen and blood were obtained on Day 30 post-transplantation and stained with antibodies for CD4, Foxp3, CD25 and CD62L. A FACS gate was drawn on live $CD4^+$ cells. To examine the intensity of Foxp3 expression, gating was performed on live $CD4^+GFP^+$ cells. The median fluorescence intensity is shown with SEM.

Figure 4.13

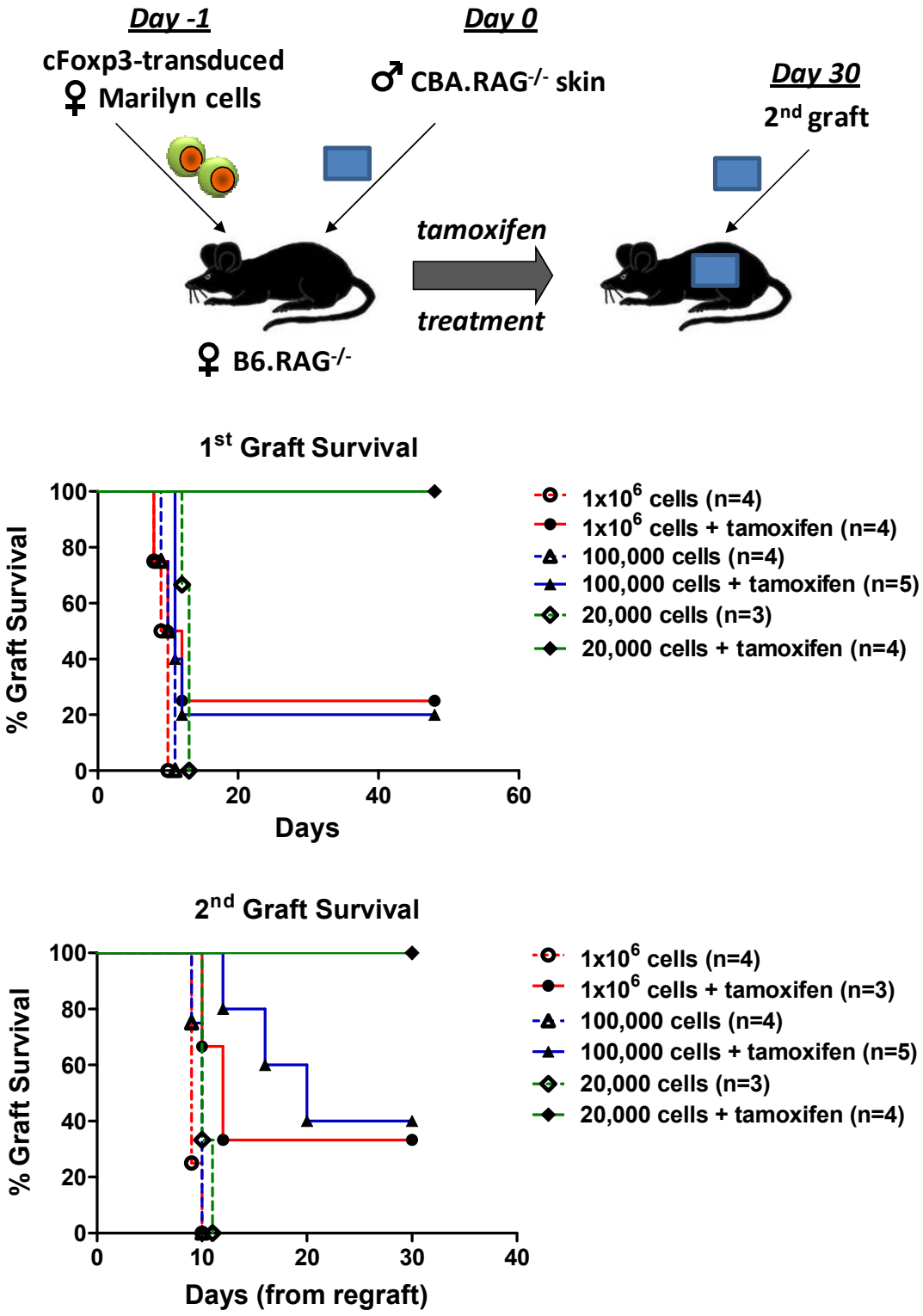


Figure 4.13 cTreg show slower rejection kinetics of a second graft

20,000, 100,000 or 1x10^6 *cFoxp3*^{tgGFP}-transduced Marilyn CD4$^+$ cells were transferred into B6.RAG$^{-/-}$ recipients followed by CBA.RAG$^{-/-}$ male skin grafts. Some recipients received tamoxifen every second day from day 7, whilst others did not receive any tamoxifen. On Day 30 post-transplant, a second male CBA.RAG$^{-/-}$ skin graft was transplanted on the opposite flank. Statistical significance (Log-rank Mantel Cox):

1st Graft Survival	*p* value
20,000 cells vs 20,000 cells + tamoxifen	<0.05
100,000 cells vs 100,000 cells + tamoxifen	ns
$1x10^6$ cells vs $1x10^6$ cells + tamoxifen	ns
20,000 cells + tam vs 100,000 cells + tam	<0.05
20,000 cells + tam vs $1x10^6$ cells + tam	<0.05

2nd Graft Survival	*p* value
20,000 cells vs 20,000 cells + tamoxifen	<0.01
100,000 cells vs 100,000 cells + tamoxifen	<0.01
$1x10^6$ cells vs $1x10^6$ cells + tamoxifen	<0.05
20,000 cells + tam vs 100,000 cells + tam	ns
20,000 cells + tam vs $1x10^6$ cells + tam	ns
100,000 cells + tam vs $1x10^6$ cells + tam	ns

Discussion

In this chapter, I showed that primary T cells transduced with a conditional Foxp3 construct and treated with tamoxifen can display regulatory properties both *in vitro* and *in vivo*. Although using a novel conditional Foxp3 construct, the data echo findings by other groups that have used a non-conditional Foxp3 construct (Hori et al. 2003; Chai et al. 2005; Loser et al. 2005) and is consistent with findings in a rheumatoid arthritis model by Andersen et al. using the same construct (Andersen et al. 2008). The initial basis for choosing this conditional approach was to ensure that potential effectors got into the target site (eg. lymphoid tissue), and only then translocating the cFoxp3. This approach appeared to work for resolution of a murine model of inflammatory arthritis. However, in the studies conducted with transplants, it appeared that there was little benefit in achieving cFoxp3 translocation once the rejection process had started. Rather, ectopic expression of Foxp3 was only effective where translocation had been achieved in advance of infusing cTreg. Therefore, the strength of a graft rejection response may mitigate against translocation of Foxp3 midstream in the graft rejection process.

After establishing optimal conditions for transfection and transduction, the theoretical basis of the conditional system was verified visually *in vitro* by examining *cFoxp3*-transduced cells using confocal microscopy. The cells showed an increasing percentage of nuclear translocation of the GFP-tagged *cFoxp3*-ERT2 protein in a time-dependent manner. At 40hrs after the onset of transduction, already a large proportion of cells (around 70%) showed nuclear translocation of *cFoxp3*-ERT2. This increased to >95% at 64hrs. To demonstrate that *cFoxp3*-ERT2 translocation led to a gain of function, cTreg expression of characteristic Treg surface markers were examined and compared to that of *cFoxp3*-transduced cells without tamoxifen, empty-vector-transduced cells, TGFβ-conditioned cells and nTreg. Indeed, cTreg

expressed even higher levels of CD25 and GITR notwithstanding the high expression by empty-vector-transduced cells as a result of anti-CD3 activation prior to transduction. cTreg also expressed high levels of intracellular CTLA-4, comparable to expression by nTreg. CTLA-4 is constitutively expressed by nTreg, and is thought to be essential for the contact-dependent suppression mediated by nTreg. The mechanism of CTLA-4 function may be by reducing expression of co-stimulatory molecules such as CD80 and CD86 on the surface of dendritic cells (Sansom et al. 2006; Wing et al. 2008). The expression of TGFβ-sensitive CD103 or CD73 levels were low in cTreg, whereas Chai et al. showed marginally greater expression of CD103 in Foxp3/MIGR1-transduced $CD4^+$ cells (Chai et al. 2005).

Interestingly, cTreg appeared to maintain expression of CD62L over time. More than 3 days following the onset of transduction, 43% of the transduced cells express CD62L compared to 23% of non-tamoxifen treated cells, 28% of empty-vector-transduced cells and 25% untransduced cells. CD62L is important for T cell traversal into secondary lymphoid organs via high endothelial venules. It is the receptor for ligands expressed on endothelial cells such as GlyCAM-1. Expressed in large amounts on naïve cells, CD62L is downregulated upon activation, and low expression is usually a feature of memory T cells. CD62L is also expressed on approximately 50-70% of nTreg in lymphoid organs (Lee et al. 2007). Those nTreg that express CD62L were shown to be more potent at suppression than the $CD62L^-$ subpopulation *in vitro*, although their suppressive capacity was not dependent on their CD62L expression (Fu et al. 2004). The expression of CD62L in 43% of cTreg after 88hrs of transduction suggests that cTreg retain ability to re-enter secondary lymphoid organs. In contrast, one study found that only 25% of non-conditional Foxp3-transduced cells expressed CD62L 48hrs following transduction (Andersen et al. 2008).

Functionally, cTreg displayed anergy *in vitro* and could completely suppress proliferation of effector cells at a ratio of 0.8 suppressor to 1 effector (20,000 suppressors + 25,000 effector cells in each well). This demonstrated that the *in vitro* regulatory potency of these cells is akin to that of nTreg (Khattri et al. 2003). Non-conditional *Foxp3*-transduced Treg have also been shown to display suppressive properties *in vitro* (Hori et al. 2003; Chai et al. 2005; Loser et al. 2005).

Previous studies have shown that ectopically expressed Foxp3-transduced cells produce high levels of IL-10 and low amounts of IL-2 compared to control-transduced cells (Hori et al. 2003; Loser et al. 2005). However, the multiplex study performed found that cytokine production of cTreg is reduced across the board, including IL-2, IL-10, IL-4, IFNγ and TNFα. This was comparable to low levels of cytokines produced by nTreg (Thornton et al. 1998). Only levels of RANTES, a chemokine responsible for recruiting a number of leukocytes to inflammatory sites, were similar between cTreg and control-transduced cells. The finding of low IL-10 production by cTreg is in contrast to another study which showed that non-conditional Foxp3-transduced cells produce higher levels of IL-10 (Loser et al. 2005). However, levels of IL-10 produced by cTreg were still higher than that of nTreg. IL-12p70, a cytokine not characteristically secreted by T cells was found to be elevated in the supernatant of the multiplex assay. This could be due to contaminating $CD4^+$ non-T cells such as DCs and macrophages. In a future repeat of the experiment, cells would be isolated based on CD3 and CD4 co-expression (rather than CD4 expression alone) before being plated for the cytokine assay.

The non-inflammatory and suppressive properties of cTreg *in vitro* ultimately translated to Treg-like behaviour in a transplantation setting. The timing and frequency of tamoxifen

treatment and the purity of transduced cells that were transferred proved to be important variables to establish an accurate assessment of cTreg behaviour. As it turned out, achieving cFoxp3 translocation prior to the initiation of the rejection response was critical for demonstrating the suppressive properties of cTreg. Using purified transduced cells that had been treated with 4HT *in vitro* and administering tamoxifen on a daily basis after transplantation of a minor antigen-mismatched skin graft, the grafted skin was not rejected even when as many as $1x10^6$ cells were adoptively transferred. The allograft underwent rejection only when tamoxifen administration was stopped after 21 days. This suggests that reversion to an inflammatory phenotype by stopping tamoxifen can lead to the rejection of an apparently well-healed graft. FACS analysis showed the presence of cells in the DLNs of tamoxifen-treated recipients, suggesting that the lack of initial rejection is not due to death or non-homing of cTreg, but the genuine non-inflammatory nature of cTreg. The presence of cTreg in the secondary lymphoid organs was also shown in recipients that had received as few as 20,000 *cFoxp3*-transduced cells. The suppressive potency of cTreg was demonstrated by their ability to stop graft rejection by TCR-transgenic $CD4^+$ cells. Whilst polyclonal cTreg were shown unable to prevent the rejection of major-MHC mismatched skin, in those experiments, polyclonal cells contained less than 50% *cFoxp3*-transduced cells, and tamoxifen treatment was either delayed or performed on a non-daily basis. Indeed, using monoclonal sorted cFoxp3-transduced cells pre-treated with 4HT may result in the non-rejection of a major-MHC mismatched graft. This may be attempted in a future experiment.

From these *in vivo* experiments, one can conclude that ectopic Foxp3 expression alone can confer regulatory properties to $CD4^+$ cells both *in vitro* and *in vivo*, and that functional $Foxp3^+$ cells are important for both inducing and maintaining the acceptance of a well-

healed skin graft. On this basis I can conclude that TGFβ-conditioned iTreg (Chapter 3) largely owe their suppressive properties to the Foxp3 that had been induced by TGFβ.

CHAPTER 5 Induced and conditional regulatory T cells out-compete naïve T cells in aggregation around dendritic cells

Introduction

Although regulatory T cells have been known for more than 17 years there are no universally accepted methods to study their function *in vitro*. The latter would be of great value in a reductionist approach to identify molecular mechanism for suppression. Having established that iTreg and cTreg could suppress rejection *in vivo*, I wished to set up an *in vitro* system whereby I could study and compare the suppressive mechanisms used by nTreg, cTreg and iTreg.

A well-characterised property of nTreg is that they suppress *in vitro* proliferation of other T cells in a contact-dependent manner. This is demonstrated in their inability to suppress across a semi-permeable membrane (Takahashi et al. 1998; Thornton et al. 1998). Amongst the plethora of possible contact-dependent mechanisms of suppression, modification of APC function is thought to be an important mechanism employed by nTreg. APCs that are modified may show reduced expression of co-stimulatory molecules (e.g. CD80 and CD86) or may increase production of immunosuppressive factors such as the enzyme indoleamine 2,3-dioxygenase (IDO) (Grohmann et al. 2002; Oderup et al. 2006). Since APCs such as DCs are critical for the initiation of the immune response (Banchereau et al. 2000), the modification of their activation status may be a powerful mechanism for controlling the nature of the downstream immune response (Farquhar et al.).

A recent report compared the *in vitro* aggregation of nTreg and effector/responder T cells around DCs by dye-labeling the T cell populations (Onishi et al. 2008). When nTreg were co-

cultured with responder T cells (naïve T cells) in the presence of DCs, nTreg were found to out-compete responder T cells in forming aggregates around DCs, thus preventing responder T cells from accessing the DCs. This process was antigen-dependent as aggregates did not form without antigen. Functionally, the nTreg incubation with responder T cells and DCs led to reduced expression of surface activation markers CD80 and CD86 compared to DCs incubated with responder cells alone (Onishi et al. 2008).

The physical interaction of nTreg with DCs has also been observed *in vivo*, and this is thought to be a mechanism for the prevention of effector T cell priming (Tadokoro et al. 2006; Tang et al. 2006).

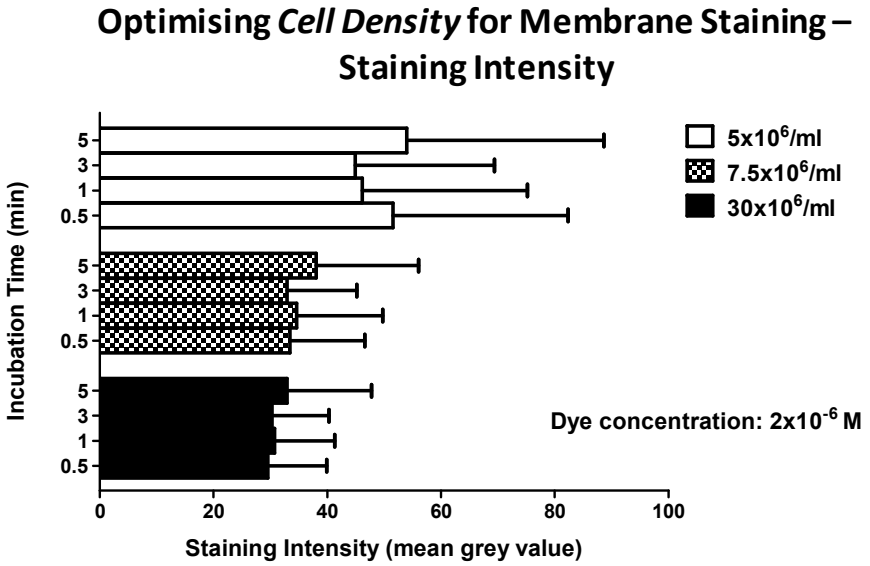
If competitive aggregation is an important part of nTreg function, it is possible that iTreg or cTreg may perform similarly, and as a consequence have a functional impact on DCs. However, to establish the validity of the aggregation assay as a measure of Treg function, I wished to investigate whether competitive aggregation is a sole property of regulatory type cells or shared by other T cell types. Using confocal microscopy and quantitative software tools, this chapter examines the *in vitro* aggregation of TGFβ-conditioned T cells (as representative of iTreg) and cTreg, comparing them with naïve and activated cells and also polarised T cell clones. Furthermore, the effect of TGFβ-conditioned T cells and cTreg on the upregulation of CD86 and CD40 on DCs is compared to that of pro-inflammatory polarised cell clones, with the expectation that they would not upregulate activation markers on DCs to the same extent as pro-inflammatory T cells (Onishi et al. 2008).

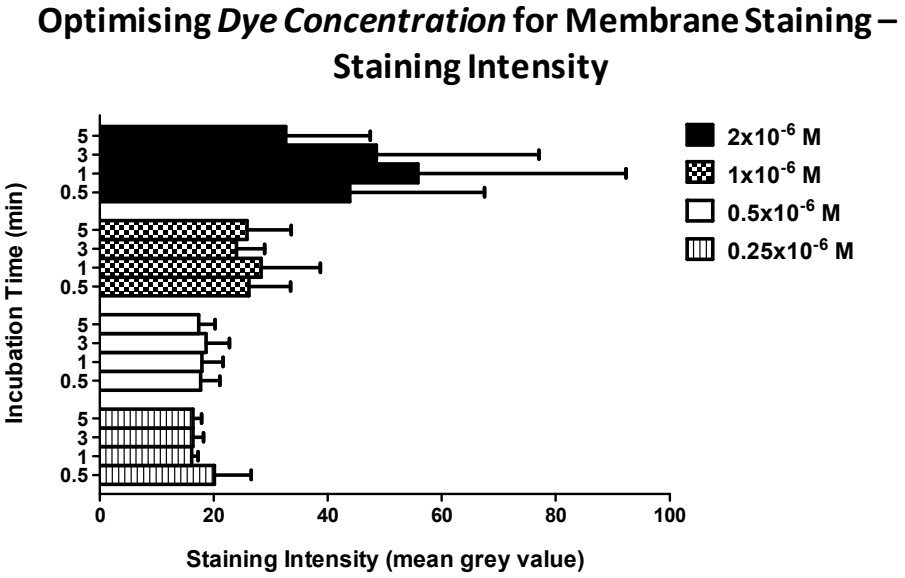
5.1 Optimising cell density and dye concentration for the T cell-dendritic cell aggregation assay

Whilst the competitiveness of nTreg in aggregating around DCs has been demonstrated (Onishi et al. 2008), it is unclear whether iTreg or cTreg share this mechanism of suppression. An *in vitro* assay was established to assess the aggregation competitiveness of iTreg and cTreg. This involved co-incubating different T cell populations with bmDCs. The T cell populations were pre-treated with either a green (PKH-67) or red (PKH-26) membrane dye prior to co-incubation. After 24hrs of co-incubation, the cultures were then examined using confocal microscopy. 3 random clusters were photographed in each culture condition, and a tally of the number of red and green cells in each cluster was made using ImageJ® .

First, the membrane dye treatment of cells needed to be optimised. To maximize intensity of membrane staining, a titration of cell density was performed. A cell density of $5x10^6$ cells/ml (200µl staining volume per $1x10^6$ cells) produced the highest staining intensity as measured by the mean grey values of each image taken (**Figure 5.1**). This titration of cell density was performed with the dye concentration fixed at $2x10^{-6}$M. Subsequently, the dye concentration was also titrated to maximize staining intensity and cell yield, as cell deaths resulted from the procedure and cell wash steps. Although a dye concentration of $2x10^{-6}$M produced the brightest stains, the cell yield was low (**Figure 5.1**). Hence, $1x10^{-6}$M was chosen to provide a compromise between staining intensity and cell yield. The duration of incubation of cells with the membrane dyes was also titrated from 30 seconds to 5 minutes, however it was found to have little effect on staining intensity or cell yield. A dye treatment time of 3 minutes was chosen. Hence for all subsequent aggregation assays, the following parameters were used: a cell density of $5x10^6$ cells/ml, dye concentration of $1x10^{-6}$M and 3 minutes of dye treatment.

Figure 5.1


Optimising *Cell Density* for Membrane Staining – Staining Intensity
Incubation Time (min)
5
3
1
0.5
5x10^6/ml
7.5x10^6/ml
30x10^6/ml
Dye concentration: 2x10^-6 M
0
20
40
60
80
100
Staining Intensity (mean grey value)


Optimising *Dye Concentration* for Membrane Staining – Staining Intensity
Incubation Time (min)
5
3
1
0.5
2x10^-6 M
1x10^-6 M
0.5x10^-6 M
0.25x10^-6 M
0
20
40
60
80
100
Staining Intensity (mean grey value)

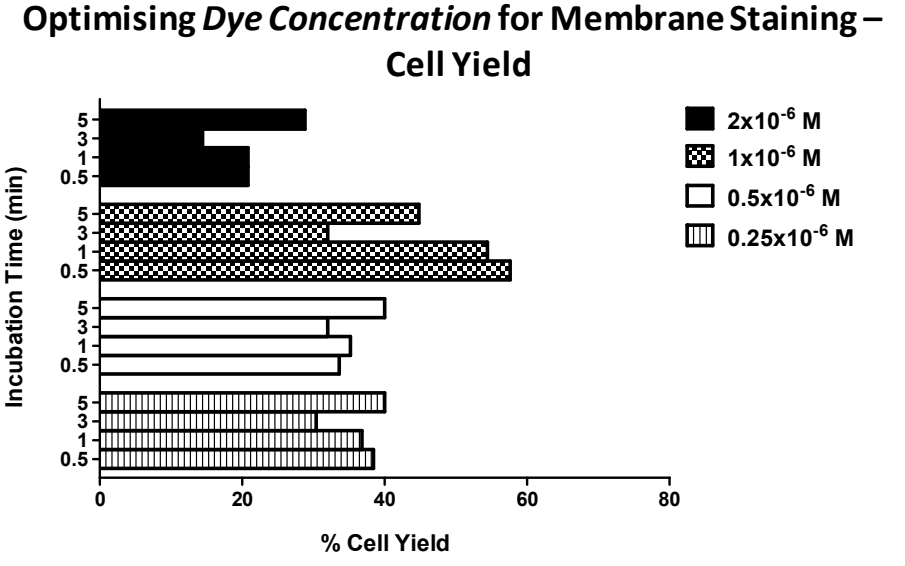

Optimising *Dye Concentration* for Membrane Staining – Cell Yield
Incubation Time (min)
5
3
1
0.5
2x10^-6 M
1x10^-6 M
0.5x10^-6 M
0.25x10^-6 M
0
20
40
60
80
% Cell Yield

Figure 5.1 Optimisation of protocol for membrane-staining – titrating cell density and dye concentration

A1.RAG$^{-/-}$ CD4^{+} cells were isolated from spleens and treated with the membrane dye PKH-67 (green). These were co-incubated with female CBA bmDCs and HYE^{k} peptide (100nM) at various cell densities and dye concentrations. Cultures were examined under a Zeiss LSM 510 META laser scanning microscope. Fields were photographed with the settings unchanged (laser intensity, image gain, pinhole). Staining intensity was assessed by examining a random microscopy field containing at least 50 cells. The mean grey value (staining intensity) of the field was calculated using Image J® software. The mean and standard deviation are shown for each condition. For the counting of cells to estimate cell yield, trypan blue stain was used.

5.2 nTreg out-compete naïve T cells in aggregating around dendritic cells

Onishi et al. showed that $CD4^+CD25^+$ nTreg can out-compete naïve T cells in aggregating around splenic DCs in an *in vitro* assay (Onishi et al. 2008). They used the OVA-specific DO11.10 TCR transgenic system ($H\text{-}2^d$), whereas here, the HY antigen-specific A1 system was used ($H\text{-}2^k$). Having optimised membrane-dye conditions, I first asked whether nTreg from the A1 system would also behave competitively in aggregation around DCs.

Using optimised membrane-dye conditions, nTreg and naïve T cells were sorted from ($A1.Rag^{-/-}$ x CBA) F1 mice based on CD4 and CD25 expression and incubated together at 1:1 ratio with bmDCs and HYE^k peptide (100nM) for 48hrs. IL-2, important for the function and maintenance of nTreg (Nelson 2004), was also added to the cultures at 5ng/ml. Naïve T cells were labeled with a green-membrane dye whilst nTreg were treated with the red dye. When the clusters were examined, nTreg showed a preferential aggregation around bmDCs compared to the naïve effector cells such that approximately 70% of cells in the clusters were nTreg (**Figure 5.2**). This result was consistent with that shown by Onishi et al. (Onishi et al. 2008).

Figure 5.2

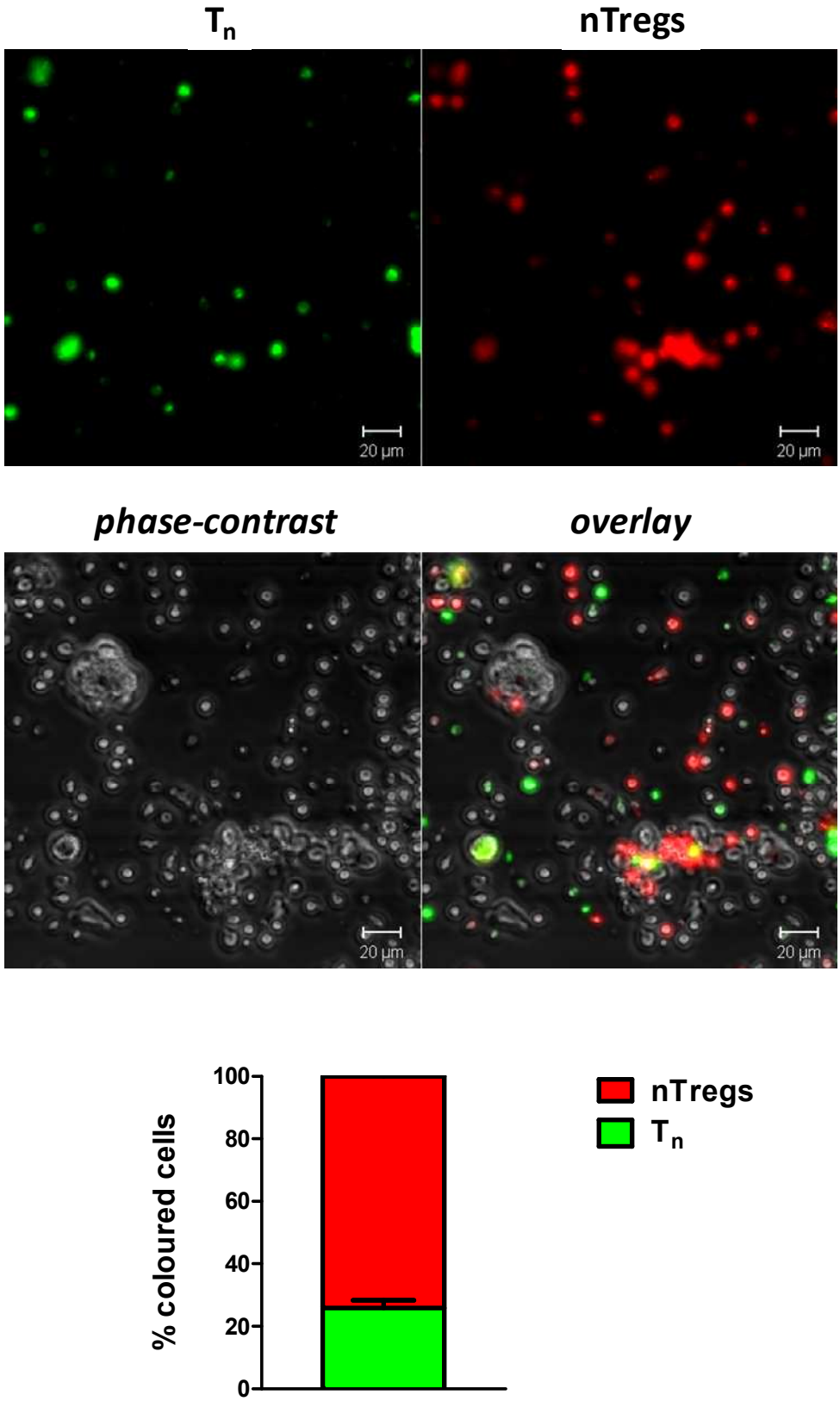


Figure 5.2 nTreg out-compete naïve T cells in T cell-DC clusters

nTreg ($CD4^+CD25^+$) and naïve T cells ($CD4^+CD25^-$) (T_n) were sorted using Mo-Flo from A1.$Rag^{-/-}$ x CBA F1 mice on CD4 and CD25 expression. They were then labeled with

membrane dyes (nTreg: red, T_n: green) and incubated together at 1:1 ratio with female bmDCs and HYE^k peptide (100nM) for 48hrs after membrane dye treatment. IL-2 was also added to the cultures at 5ng/ml. At 48hrs, cells were transferred to glass-bottomed chamber slides and visualized using a Zeiss LSM 510 META laser scanning microscope. 3 random clusters were photographed, and the stained cell populations in the clusters were quantified using ImageJ software®. The mean with SEM is shown. *p* value was <0.001 using unpaired student t test with two-tailed analysis.

5.3 TGFβ-conditioned T cells and activated T cells both out-compete naïve T cells

Having established that nTreg can out-compete naïve T cells, the next question was whether TGFβ-conditioned $CD4^+$ cells can also out-compete naïve $CD4^+$ T cells. Splenocytes were conditioned in TGFβ for 7 days in the presence of bmDCs and their cognate peptide as described in **Section 2.5.3**. $CD4^+$ cells were then enriched from the cultures using ficoll density gradients. On FACS analysis, the TGFβ-conditioned cells were 72% positive for Foxp3 expression (**Figure 5.3A**). Activated T cells (peptide-activated, but without TGFβ-conditioning) were also cultured with naïve T cells to assess clustering. For the aggregation assay, the different cell populations were incubated for 24hrs with bmDCs and peptide. As seen in **Figure 5.3B**, TGFβ-conditioned cells ($T_{TGFβ\text{-}con}$) were found to out-compete naïve T cells (T_n) at all T_n : $T_{TGFβ\text{-}con}$ cell ratios. Activated T cells also out-competed naïve T cells at all cell ratios. There was no statistically significant difference in the ability of TGFβ-conditioned T cells or peptide-activated T cells to out-compete naïve T cells in DC clustering.

Figure 5.3A

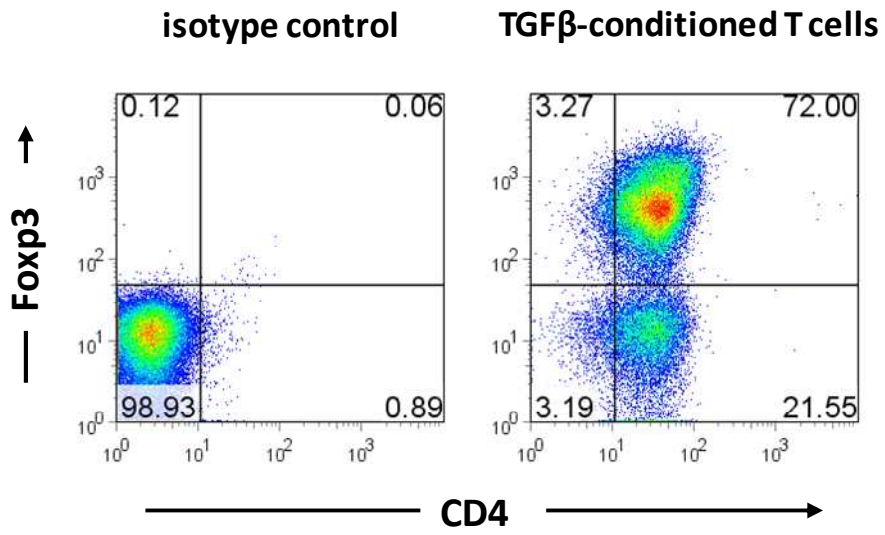


Figure 5.3A FACS profile of TGFβ-conditioned T cells used for the aggregation assay

TGFβ-conditioned T cells were derived by activating A1.RAG$^{-/-}$ splenic cells with female CBA bmDC in the presence of TGFβ (2ng/ml) and HYE^{k} peptide (100nM) for 7 days. The Foxp3 expression at this stage was 72% of total live cells after ficoll separation.

Figure 5.3B

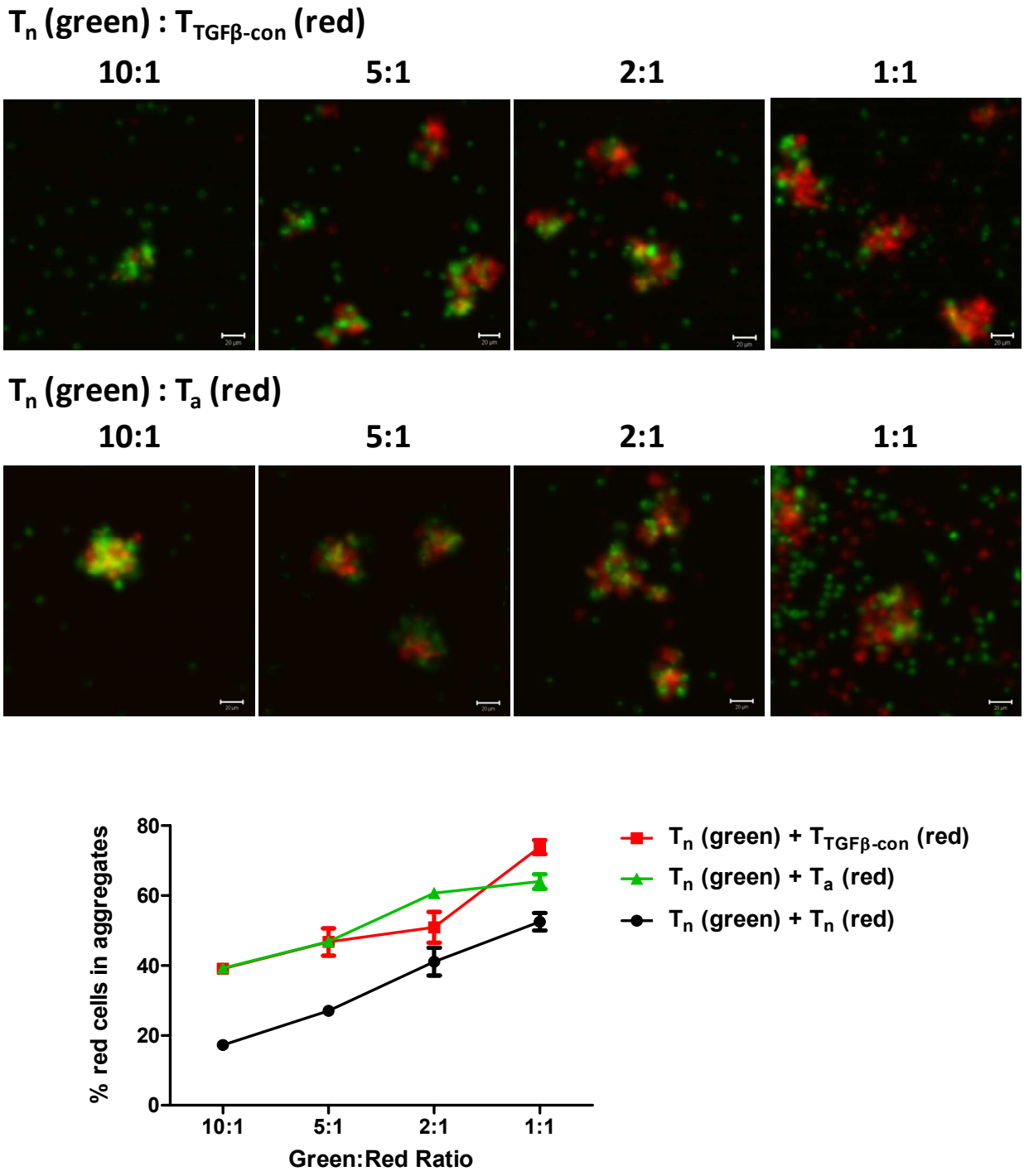


Figure 5.3B TGFβ-conditioned T cells out-compete naïve T cells in aggregating around DCs

TGFβ-conditioned T cells ($T_{TGFβ\text{-}con}$) can out-compete naïve T cells (T_n) in aggregating around DCs at various $T_{TGFβ\text{-}con}$: T_n cell ratios. Cell populations were membrane-stained with either PKH-67 (green) or PKH-26 (red) dyes and incubated for 24hrs, and then visualised by microscopy. Naïve T cells were also co-incubated with peptide-activated T cells (T_a) for comparison. Four random clusters were photographed, and the respective cell populations

in T cell-DC clusters were quantified. For statistical analysis, 2 way ANOVA with Bonferonni post-test was used.

Tn (green)+Tn (red) vs Tn (green)+iTreg (red)	*p* value
10:1	<0.001
5:1	<0.001
2:1	< 0.05
1:1	<0.001

Tn (green)+Tn (red) vs Tn (green)+Ta (red)	*p* value
10:1	<0.001
5:1	<0.001
2:1	<0.001
1:1	<0.01

Tn (green)+iTreg (red) vs Tn (green)+Ta (red)	*p* value
10:1	ns
5:1	ns
2:1	<0.05
1:1	< 0.05

5.4 Competitive aggregation of TGFβ-conditioned T cells and conditional regulatory T cells is not dependent on Foxp3 expression

Having shown that nTreg and TGFβ-conditioned T cells can out-compete naïve T cells around DCs, I next asked if this was also true for retrovirally transduced cTreg (cFoxp3-transduced T cells treated with 4HT *in vitro*). cTreg were derived from transducing $CD4^+$ T cells isolated from Marilyn.hCD2.KI mice and these were co-incubated with naïve $CD4^+$ cells together with B6 bmDCs and HYA^b peptide. 4HT was also added *in vitro* to ensure functional 'activation' of cFoxp3 (50nM). As controls, empty-vector-transduced $CD4^+$ cells with 4HT and $cFoxp3^{tgGFP}$-transduced $CD4^+$ cells without 4HT were used. The various cell populations were incubated with naïve $CD4^+$ cells at 2:1, 1:1 and 1:2 ratios (**Figure 5.4**). Whilst cTreg could out-compete naïve $CD4^+$ cells, both empty-vector-transduced cells and cFoxp3-transduced $CD4^+$ cells without 4HT treatment could also out-compete naïve T cells. There was no evidence that the aggregation potency of cTreg was vastly superior to that of control-transduced cells at various cell ratios. Hence, Foxp3 may not be essential for the competitive aggregation of cTreg.

To investigate whether Foxp3 was important for competitive aggregation of iTreg, $Foxp3^+$ and $Foxp3^-$ populations of TGF-conditioned T cells from Marilyn.hCD2.KI mice were purified based on their expression of hCD2 and CD4 (**Figure 5.5**). $hCD2^+$ ($Foxp3^+$) and $hCD2^-$ ($Foxp3^-$) cells were then co-incubated with naïve T cells. At both 10:1 and 1:1 ratios, there was no statistically significant difference in the aggregation potency of $hCD2^+$ and $hCD2^-$ cells compared to naïve T cells. This suggests that $Foxp3^+$ TGFβ-conditioned T cells are no better at out-competing naïve T cells than $Foxp3^-$ TGFβ-conditioned T cells (**Figure 5.5**).

Figure 5.4

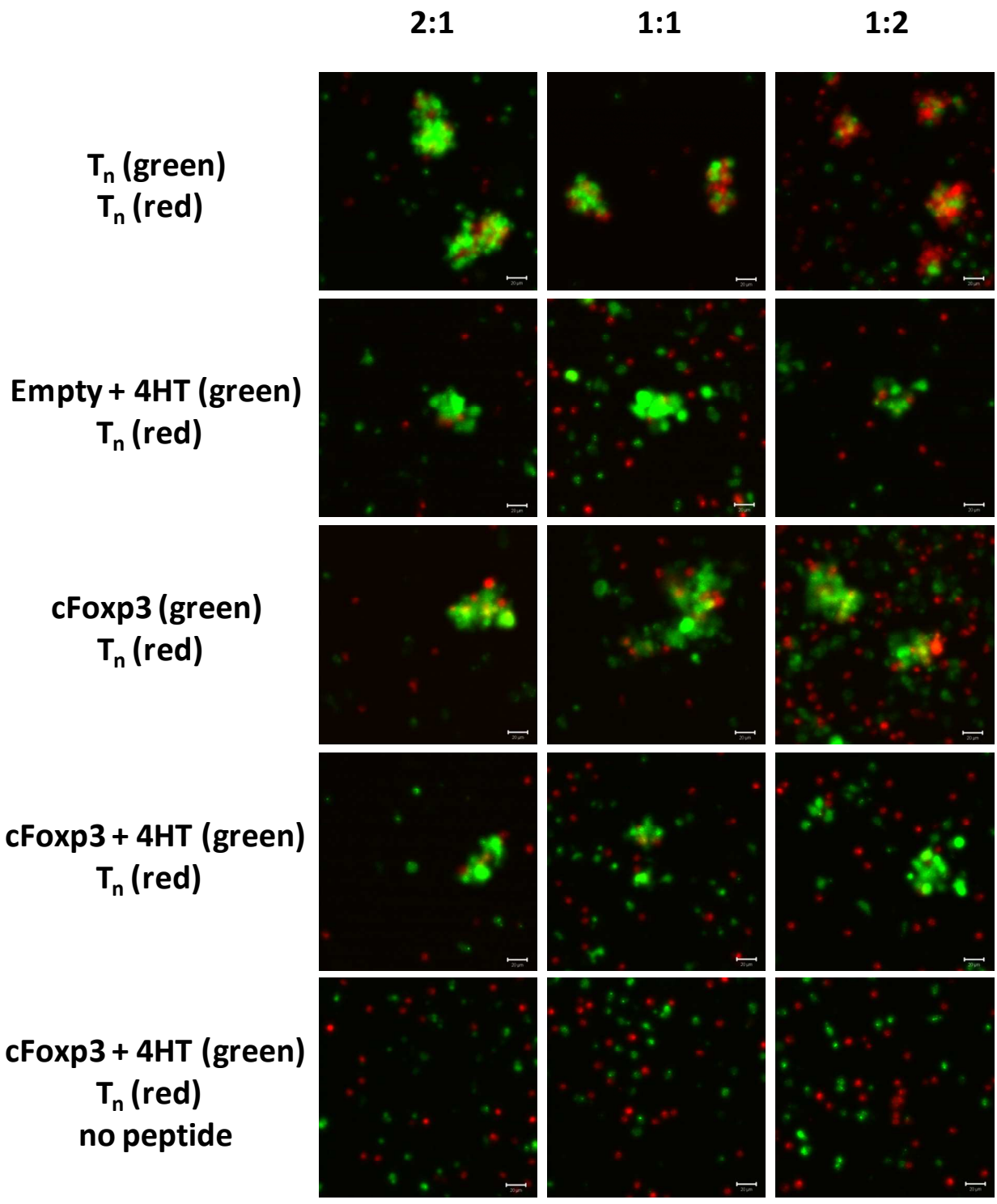

2:1
1:1
1:2
T_n (green)
T_n (red)
Empty + 4HT (green)
T_n (red)
cFoxp3 (green)
T_n (red)
cFoxp3 + 4HT (green)
T_n (red)
cFoxp3 + 4HT (green)
T_n (red)
no peptide

Figure 5.4 (cont'd)

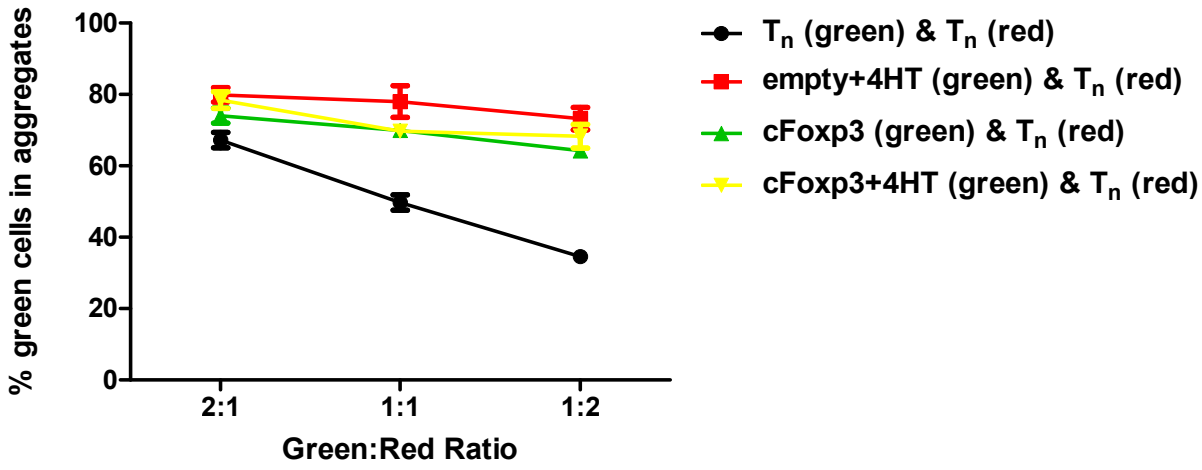


Figure 5.4 cTreg are not better at out-competing naïve T cells in aggregating around DCs than empty-vector-transduced T cells or 4HT-non-treated cFoxp3-transduced T cells

$CD4^+$ cells from Marilyn.hCD2.KI mice were transduced with $cFoxp3^{tgGFP}$ in the presence of 4HT (50nM) for 40hrs and then sorted by flow cytometry. After membrane dye treatment, $CD4^+GFP^+$ cells (cTreg) were then incubated with naïve sorted $CD4^+$ cells (T_n) in the presence of B6 female bmDCs and 4HT (50nM) at 2:1, 1:1 and 1:2 transduced cell : naïve $CD4^+$ ratios. T cell-DC clusters were then examined with confocal microscopy. For control, empty-vector-transduced T cells (treated with 4HT) and cFoxp3-transduced but 4HT-non-treated cells were also coincubated with naïve cells. 4 random clusters were quantified for each group at each cell ratio. Statistical analysis was performed using 2 way ANOVA with Bonferonni post-test.

Tn (green)+Tn (red) vs empty+4HT (green) & Tn (red)	*p* value
2:1	<0.01
1:1	<0.001
1:2	<0.001

Tn (green)+Tn (red) vs cFoxp3 (green) & Tn (red)	*p* value
2:1	ns
1:1	<0.001
1:2	<0.001

Tn (green)+Tn (red) vs cFoxp3+4HT (green) & Tn (red)	*p* value
2:1	<0.01
1:1	<0.001
1:2	<0.001

empty+4HT (green) & Tn (red) vs cFoxp3 (green) & Tn (red)	*p* value
2:1	ns
1:1	ns
1:2	< 0.05

empty+4HT (green) & Tn (red) vs cFoxp3+4HT (green) & Tn (red)	*p* value
2:1	ns
1:1	ns
1:2	ns

cFoxp3 (green) & Tn (red) vs cFoxp3+4HT (green) & Tn (red)	*p* value
2:1	ns
1:1	ns
1:2	ns

Figure 5.5

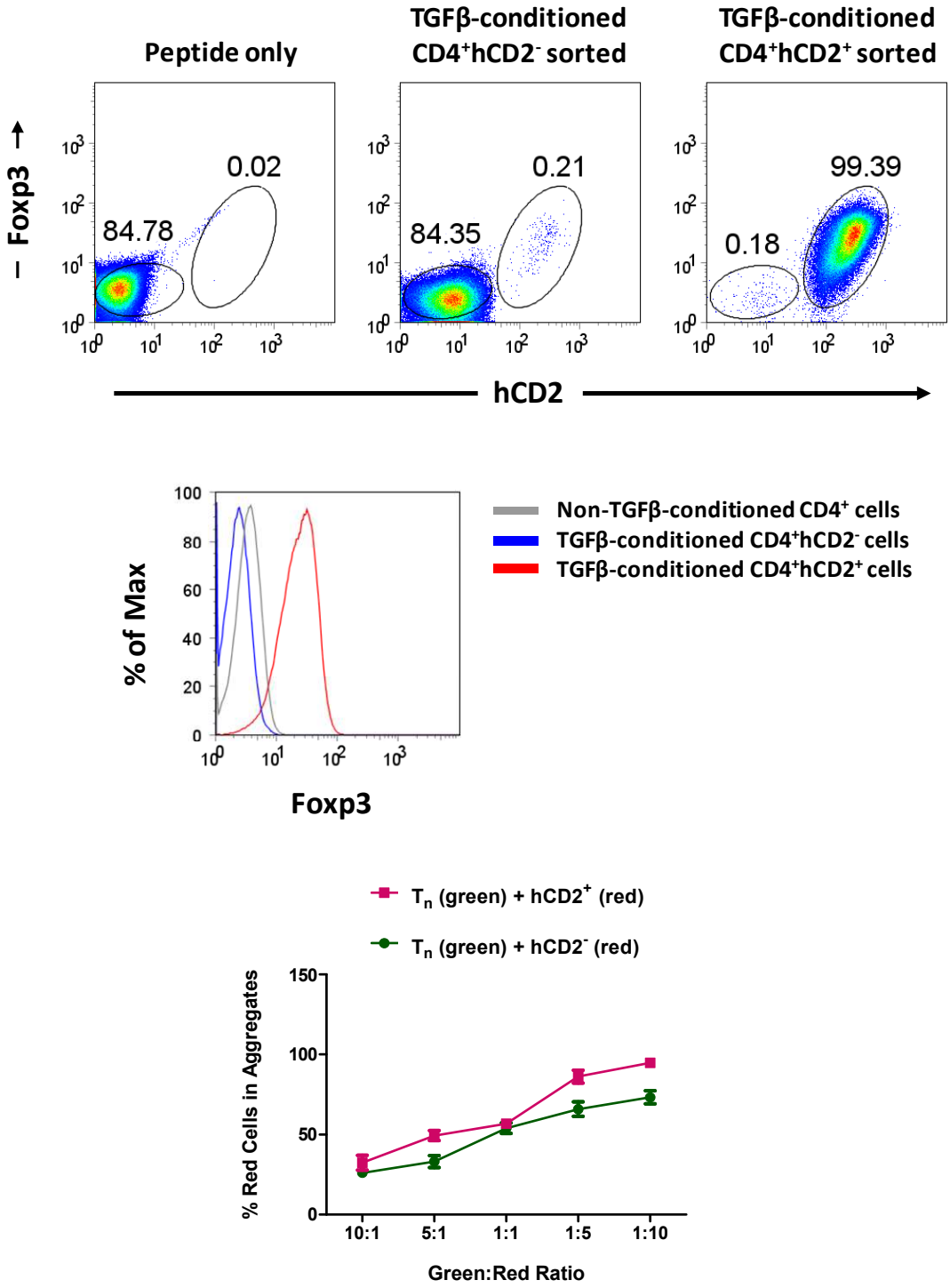


Figure 5.5 Foxp3$^+$ TGFβ-conditioned T cells (iTreg) are no better at outcompeting naïve T cells than Foxp3$^-$ TGFβ-conditioned T cells

TGFβ-conditioned T cells from Marilyn.hCD2.KI mice were sorted based on the expression of hCD2 and CD4. The Foxp3 expression of CD4$^+$hCD2$^-$ and CD4$^+$hCD2$^+$ cells are shown. The

hCD2$^-$ and hCD2$^+$ subsets of TGFβ-conditioned cells were then incubated with naïve T cells (T_n) in the presence of B6 female bmDCs for 24hrs. Quantitation was then conducted on the T-DC clusters. Statistical analysis was performed using 2 way ANOVA with Bonferonni post-test:

Tn (green) + hCD2$^-$ (red) vs Tn (green) + hCD2$^+$ (red)	*p* value
10:1	ns
5:1	< 0.05
1:1	ns
1:5	< 0.01
1:10	< 0.001

5.5 Polarised T cell clones also out-compete naïve T cells

Whilst nTreg, iTreg and cTreg all express Foxp3, they also share the common characteristic of being antigen-experienced. Prior antigen experience may explain the result that empty-vector-transduced $CD4^+$ cells and $Foxp3^-$ TGFβ-conditioned cells can out-compete naïve T cells in aggregation equally well compared to cTreg and iTreg. To confirm if antigen-experience is sufficient for competitive aggregation of T cells, polarised cell clones (Th1, Th2, Tr1, Th9) were incubated with naïve T cells (see **Section 2.5.4** on the generation of polarised cell clones). Both naïve $CD4^+$ T cells and polarised cell clones were established from A1.$RAG^{-/-}$ mice. In contrast to naïve T cells, polarised T cell clones had been activated for 14 days with the cognate peptide prior to the aggregation assay.

As seen in **Figure 5.6**, all polarized cell clones were found to out-compete naïve T cells (T_n). This was statistically significant at all cell ratios for Th9 and Th2 cell clones, and at 2:1 and 1:1 cell ratios for Th1 and Tr1 cell clones. At the 1:1 ratio, Th9, Tr1 and Th1 appeared to the most competitive at aggregating, with around 70% cells in the T-DC clusters being red (polarised cell clone). Hence, antigen-experienced pro-inflammatory and regulatory cell types all appear to out-compete naïve antigen inexperienced cells.

Figure 5.6

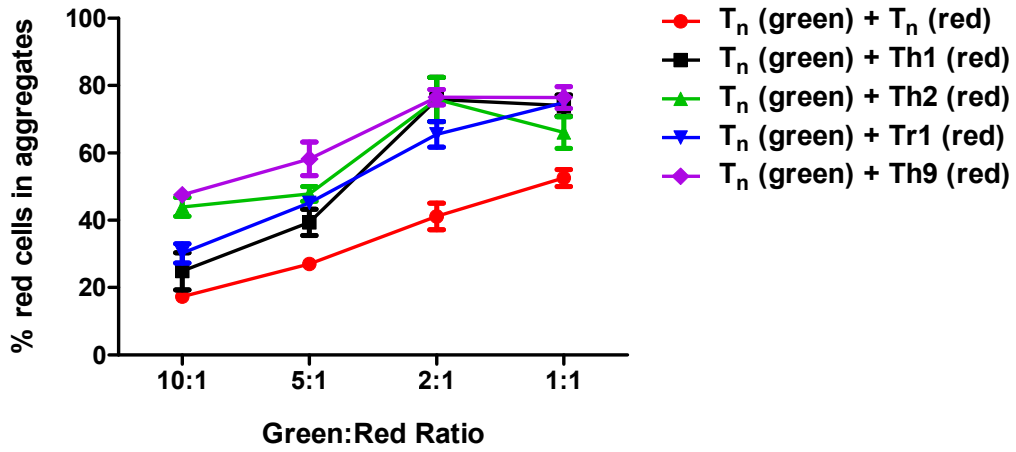


Figure 5.6 Polarised inflammatory cell clones out-compete naïve T cells in aggregating around DCs

Polarised Th1, Th2, Tr1 and Th9 cell clones were labelled with the red membrane dye PKH-26 and naïve $CD4^+$ T cells isolated from $A1.RAG^{-/-}$ splenic cells were labelled with the green membrane dye PKH-67. Cells were incubated with female bmDCs and HYE^k peptide (100nM) for 24hrs before being examined by confocal microscopy. Quantitation was performed by counting coloured aggregating cells in 4 random clusters. Statistical analysis was performed using 2 way ANOVA with Bonferonni post-test.

Tn (green)+Tn (red) vs Tn (green)+Th1 (red)	*p* value
10:1	ns
5:1	ns
2:1	<0.001
1:1	<0.001

Tn (green)+Tn (red) vs Tn (green)+Th2 (red)	*p* value
10:1	<0.001
5:1	<0.001
2:1	<0.001
1:1	< 0.05

Tn (green)+Tn (red) vs Tn (green)+Tr1 (red)	*p* value
10:1	ns
5:1	<0.01
2:1	<0.001
1:1	<0.001

Tn (green)+Tn (red) vs Tn (green)+Th9 (red)	*p* value
10:1	<0.001
5:1	<0.001
2:1	<0.001
1:1	<0.001

5.6 TGFβ-conditioned T cells and cTreg do not out-compete other activated cell populations in aggregation around DCs

Having established that both regulatory cells and pro-inflammatory polarised cell clones can out-compete naïve T cells, it may be that antigen-experience is more important for the ability of T cells to competitively cluster around dendritic cells. Onishi et al. attributed the competitive aggregation of nTreg to their LFA-1 expression, as LFA-1-deficient nTreg fail to out-compete naïve T cells in aggregation (Onishi et al. 2008). As LFA-1 is upregulated on T cell activation (Shiina et al. 2002), this would be consistent with the observation that antigen-experienced $CD4^+$ cells show superior competitive aggregation compared to naïve $CD4^+$ cells. Moreover, nTreg are thought to be more antigen-experienced than naïve T cells, due to their being selected via positive selection in the thymus (Apostolou et al. 2002). They may also experience self antigens in the periphery on a constant basis (Fisson et al. 2003).

I investigated whether there was a Treg-intrinsic superiority in competitive aggregation by comparing TGFβ-conditioned T cells or cTreg with activated or polarised cell clones in co-incubations. Sorted cTreg (*cFoxp3*-transduced $CD4^+$ cell, 4HT treated) were co-incubated with untransduced but anti-CD3 activated $CD4^+$ cells for 24hrs. Empty-vector-transduced cells and non-4HT-treated *cFoxp3*-transduced cells were also coincubated with untransduced activated cells for comparison. cTreg did not out-compete activated T cells more so than control-transduced cells (**Figure 5.7**).

To examine the aggregation competitiveness of TGFβ-conditioned T cells in comparison with other antigen-experienced cell types, the former were co-incubated with activated but non-polarised T cells and polarised cell clones at varying ratios of 10:1, 5:1 2:1, 1:1, 1:2, 1:5 and 1:10 for 24hrs. There was no statistically significant difference in the competitive

aggregation of TGFβ-conditioned T cells with activated T cells compared with dye-control TGFβ-conditioned T cell incubations (**Figure 5.8**). Furthermore, TGFβ-conditioned cells competed poorly in aggregation against the polarised cell clones Th1 and Tr1. (**Figure 5.8**).

Hence, whilst cTreg and TGFβ-conditioned T cells compete favourably against naïve T cells, they do not appear to have a competitive advantage in relation to other antigen-experienced cell populations.

Figure 5.7

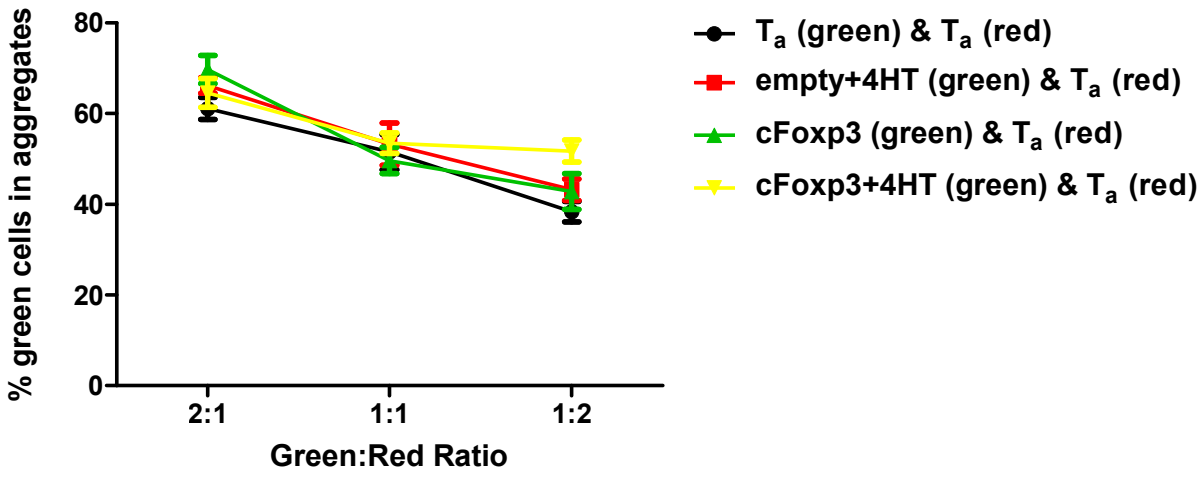


Figure 5.7 cTreg do not out-compete activated T cells in aggregation

$CD4^+$ cells from Marilyn.hCD2.KI mice were transduced with $cFoxp3^{tgGFP}$ in the presence of 4HT (50nM) for 40hrs and then sorted by flow cytometry. $CD4^+GFP^+$ cells (cTreg) were then incubated with anti-CD3 activated but untransduced $CD4^+$ cells (T_a) in the presence of B6 female bmDCs and 4HT (50nM) at 2:1, 1:1 and 1:2 cell ratios. T cell-DC clusters were then examined with confocal microscopy. For control, empty-vector-transduced T cells (treated with 4HT) and cFoxp3-transduced but 4HT-non-treated cells were also coincubated with untransduced cells. Statistical analysis was performed using 2 way ANOVA with Bonferonni post-test.

Ta (green)+Ta (red) vs empty+4HT (green) & Ta (red)	*p* value
2:1	ns
1:1	ns
1:2	ns

Ta (green)+Ta (red) vs cFoxp3 (green) & Ta (red)	*p* value
2:1	ns

1:1	ns
1:2	ns

Ta (green)+Ta (red) vs cFoxp3+4HT (green) & Ta (red)	*p* value
2:1	ns
1:1	ns
1:2	<0.01

empty+4HT (green) & Ta (red) vs cFoxp3 (green) & Ta (red)	*p* value
2:1	ns
1:1	ns
1:2	ns

empty+4HT (green) & Ta (red) vs cFoxp3+4HT (green) & Ta (red)	*p* value
2:1	ns
1:1	ns
1:2	ns

cFoxp3 (green) & Ta (red) vs cFoxp3+4HT (green) & Ta (red)	*p* value
2:1	ns
1:1	ns
1:2	ns

Figure 5.8

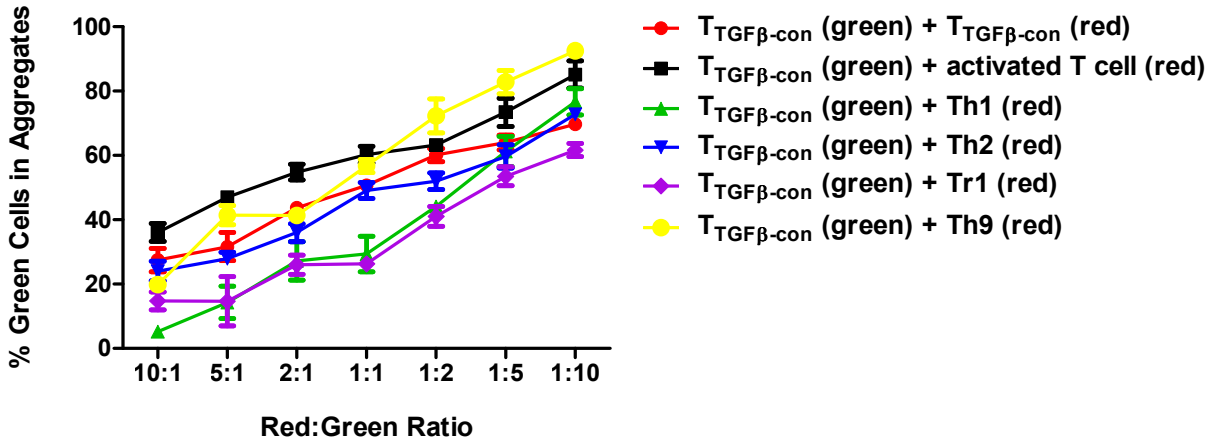


Figure 5.8 TGFβ-conditioned T cells do not out-compete antigen-experienced T cells

Polarised Th1, Th2, Tr1 and Th9 cell clones and 7-day peptide-activated A1.RAG$^{-/-}$ CD4^{+} cells were labelled with the red membrane dye (PKH-26). TGFβ-conditioned T cells ($T_{TGFβ\text{-}con}$) were labelled with the green membrane dye (PKH-67). The percentage of TGFβ-conditioned T cells expressing Foxp3 was 46%. Activated T cells were prepared in the same way as TGFβ-conditioned T cells, except TGFβ was not added to the culture. Statistical analysis was performed using 2 way ANOA with Bonferonni post-test.

$T_{TGFβ\text{-}con}$ (green)+$T_{TGFβ\text{-}con}$ (red) vs $T_{TGFβ\text{-}con}$ (green)+activated T cell (red)	*p* value

10:1	ns
5:1	<0.01
2:1	ns
1:1	ns
1:2	ns
1:5	ns
1:10	< 0.05

$T_{TGFβ\text{-}con}$ (green)+$T_{TGFβ\text{-}con}$ (red) vs $T_{TGFβ\text{-}con}$ (green)+Th1 (red)	*p* value
10:1	<0.001
5:1	<0.01
2:1	<0.01
1:1	<0.001
1:2	<0.05
1:5	ns
1:10	ns

$T_{TGFβ\text{-}con}$ (green)+$T_{TGFβ\text{-}con}$ (red) vs $T_{TGFβ\text{-}con}$ (green)+Th2 (red)	*p* value
10:1	ns
5:1	ns
2:1	ns
1:1	ns
1:2	ns
1:5	ns
1:10	ns

$T_{TGFβ\text{-}con}$ (green)+$T_{TGFβ\text{-}con}$ (red) vs $T_{TGFβ\text{-}con}$ (green)+Tr1 (red)	*p* value
10:1	<0.05
5:1	<0.01
2:1	<0.001
1:1	<0.001
1:2	<0.001
1:5	ns
1:10	ns

$T_{TGFβ\text{-}con}$ (green)+$T_{TGFβ\text{-}con}$ (red) vs $T_{TGFβ\text{-}con}$ (green)+Th9 (red)	*p* value
10:1	ns
5:1	ns
2:1	ns
1:1	ns
1:2	ns
1:5	<0.001
1:10	<0.001

5.7 TGFβ-conditioned T cells do not activate dendritic cells to the same degree as activated polarized T cells

Dendritic cells are important for both the induction of primary immune responses and the promotion of immunological tolerance (Farquhar et al. ; Banchereau et al. 2000). Upon encounter with T cells, DCs are activated via CD40-CD40L interaction, leading to the upregulation of CD80 and CD86 co-stimulatory molecules and release of cytokines. CD86 is one of the key molecules for the amplification of the T cell response (Caux et al. 1994). CD40 is also upregulated upon bmDC maturation and activation. To examine whether iTreg can modulate the expression of activation markers such as CD40 and CD86 on DCs, bmDCs were examined after 24hrs or 48hrs culture with TCR-transgenic TGFβ-conditioned T cells in the presence of cognate peptide (100nM). For comparison, naïve T cells and polarised T cell lines were also co-incubated with bmDCs.

BmDCs co-incubated with Th1 and Th9 polarised $CD4^+$ cells for 24hrs were found to express high levels of CD86 (MFI: 584 and 326 respectively), with much lower levels found on those co-incubated with Th2, Tr1, naive and TGFβ-conditioned T cells (MFI: 71, 63, 59 and 87) at 24hrs post-incubation (**Figure 5.9**). The same is also true at 48hrs. Similarly, at 24hrs, CD40 was expressed highly by bmDCs co-incubated with Th1 and Th9 polarised cell clones (MFI: 609 and 561), but lower levels were found on those co-incubated with TGFβ-conditioned cells, Th2, Tr1 and naive cells (MFI: 15, 188, 268, 20). A similar picture also emerged for the expression of class II MHC on bmDCs, with Th1 and Th9 polarised cells producing the highest levels of expression and lower levels produced by TGFβ-conditioned cells, Tr1 cells and naïve T cells.

Hence, antigen-experienced cells such as TGFβ-conditioned T cells and polarised $CD4^+$ clones, whilst effective in competitive aggregation compared with naïve T cells, are not equal in their ability to induce up-regulation of activation markers CD86, CD40 and class II MHC on bmDCs. TGFβ-conditioned T cells appear less effective at activating bmDCs than polarised inflammatory cell types such as Th1 and Th9 cells.

Figure 5.9

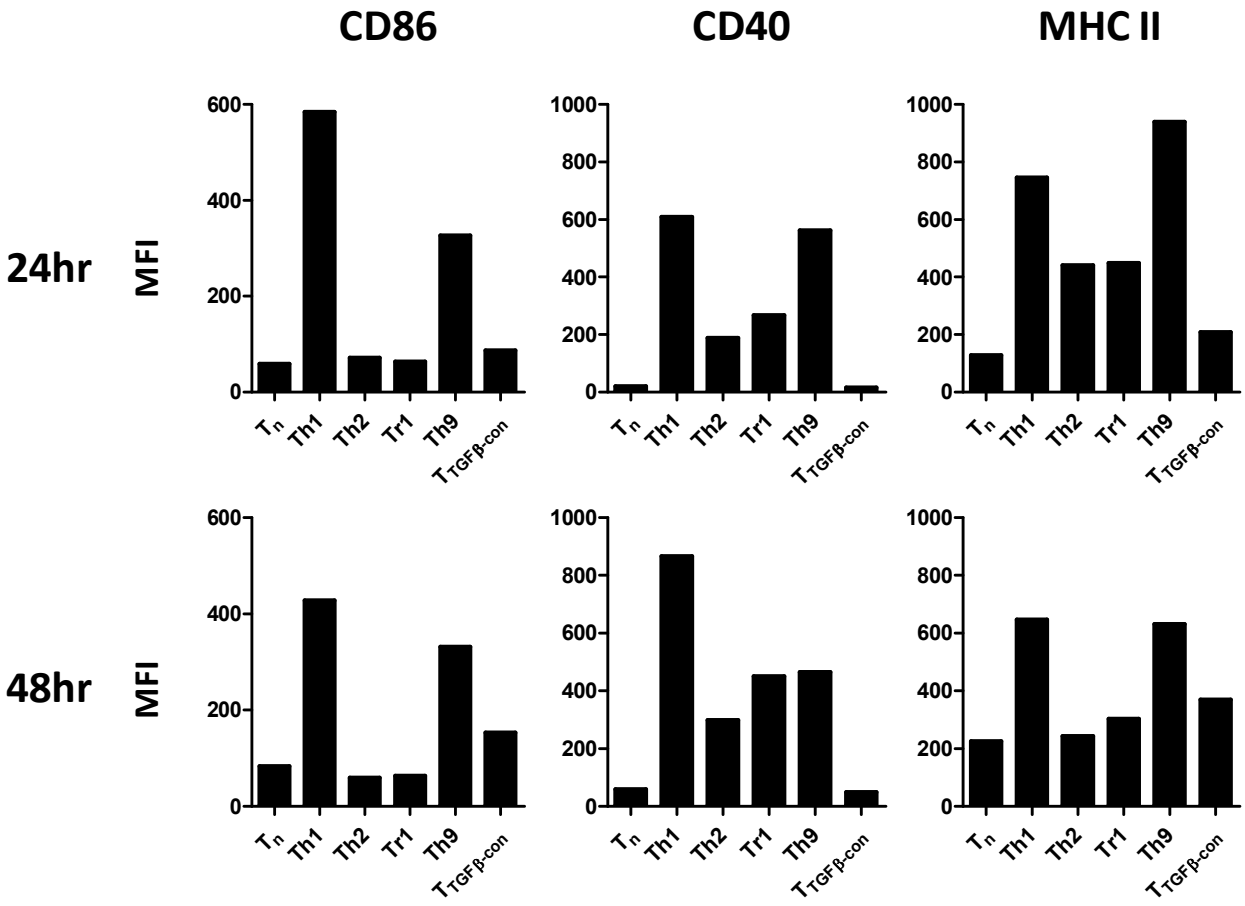


Figure 5.9 TGFβ-conditioned T cells do not activate dendritic cells to the same extent as pro-inflammatory polarised cell T clones

CBA female bmDCs were incubated with naïve T cells (T_n), TGFβ-conditioned T cells ($T_{TGFβ\text{-}con}$) and various polarised cell clones. DCs ($CD11c^+$) were examined by flow cytometry at 24hrs and 48hrs time points for CD86, CD40 and Class II MHC expression.

5.8 Attempts to determine if TGFβ-conditioned T cells influence the activation of dendritic cells by naïve T cells.

The previous section showed that TGFβ-conditioned T cells do not activate DCs to the same degree as polarised T cell clones such as Th1 or Th9 cells. Given that TGFβ-conditioned cells can out-compete naïve T cells in aggregating around DCs, I next investigated whether their co-incubation with naïve T cells would influence the activation of DCs compared to DCs incubated with naïve T cells alone. Because polarised inflammatory T cells could also out-compete naïve T cells in aggregation, these cells were also co-incubated with naïve T cells to investigate their combined effect on DC activation.

Unfortunately naïve T cells gave very little DC activation in this readout system. A 24hr co-incubation of TGFβ-conditioned cells with naïve T cells resulted in higher levels of CD86, CD40 and class II MHC expression on bmDCs at both 2:2 and 10:2 (T_n: $T_{TGF\text{-}con}$ T cell) ratios (MFI for 2:2 ratio: 215, 2485, 1105 respectively) compared to DCs incubated with T_n alone (MFI: 3, 152, 879) or those incubated with T_n and nTreg (MFI: 60, 512, 832). However, these expression levels were lower compared to bmDC co-incubated with inflammatory polarised T cell clones such as Th1 (MFI for 2:2 ratio: 470, 8385, 1473) (**Figure 5.10A**). The addition of the Tr1 clone was also non-stimulatory. Because naïve cells stimulated DC poorly, I could not, as I had wished, determine whether the regulatory populations were inhibitory to DC activation.

To investigate whether $Foxp3^+$ or $Foxp3^-$ subpopulations of TGFβ-conditioned T cells would modulate DCs differently, $hCD2^+$ ($Foxp3^+$) and $hCD2^-$ ($Foxp3^-$) subsets of TGFβ-conditioned T cells (derived from Marilyn.hCD2.KI mice) were co-incubated with peptide-only activated cells together with B6-derived bmDCs. When incubated alone with bmDCs, both $hCD2^+$ and

hCD2$^-$ cells appeared to upregulate CD86 and CD40 on bmDCs less effectively than peptide-activated T cells alone (**Figure 5.10B**). When co-incubated with peptide-activated T cells, both hCD2$^+$ and hCD2$^-$ cells did not further upregulate CD86 and CD40 expression on DCs compared to activated T cells alone. These results suggest that Foxp3$^+$ and Foxp3$^-$ TGFβ-conditioned T cells do not activate DCs to the same extent as peptide-activated T cells, and do not further contribute to the activation (or inhibition) of DCs by activated T cells when co-incubated with these cells. Taken together, TGFβ-conditioned T cells not only can physically displace naïve T cells from contact with DCs, but also only modestly activate the DCs themselves. Like 'civil servants', these physically competitive but functionally inert cells may contribute to the dampening of the immune response in an inflammatory context by limiting the capacity of naïve CD4$^+$ T cells to bind to DC (Waldmann et al. 1998).

Figure 5.10A

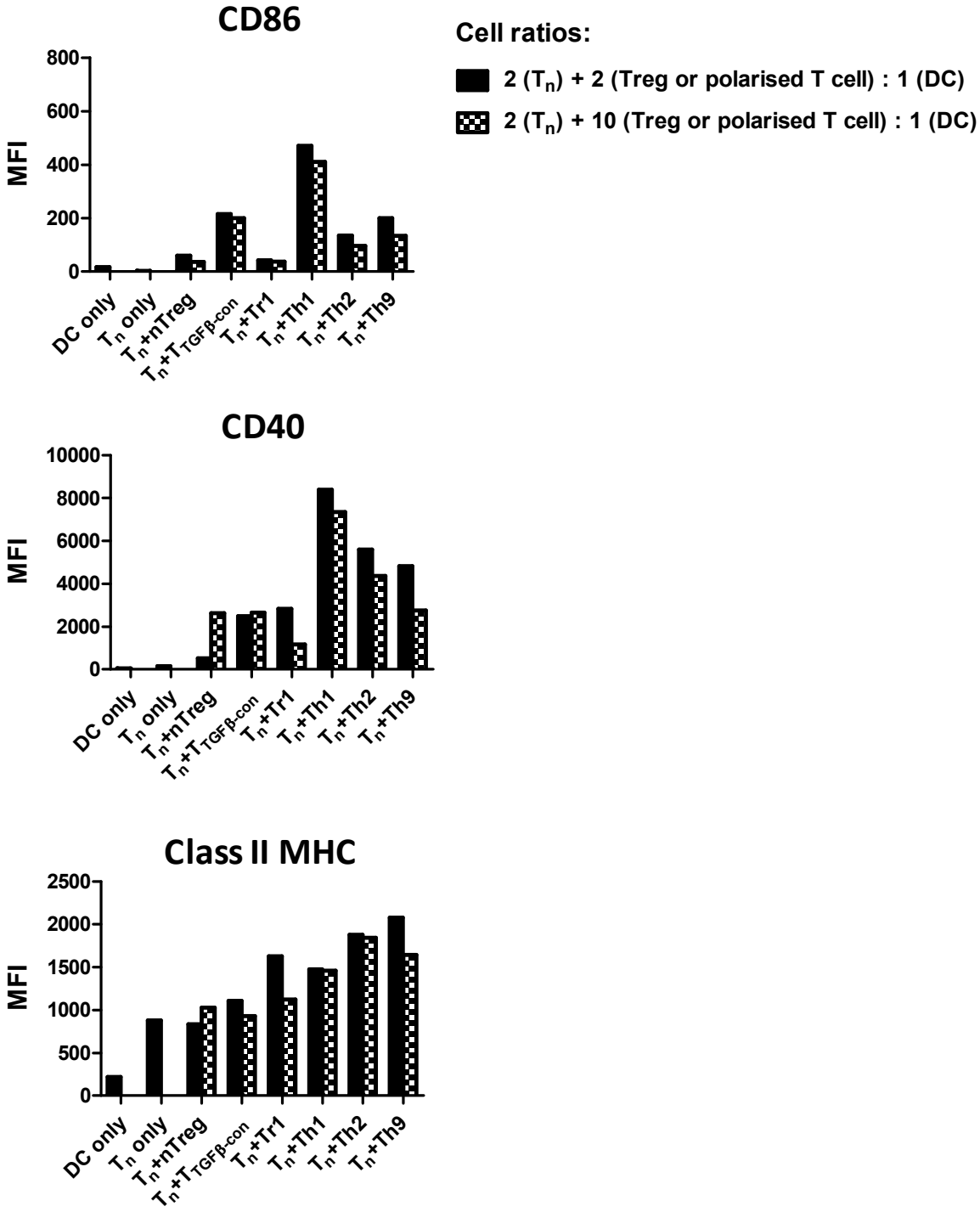
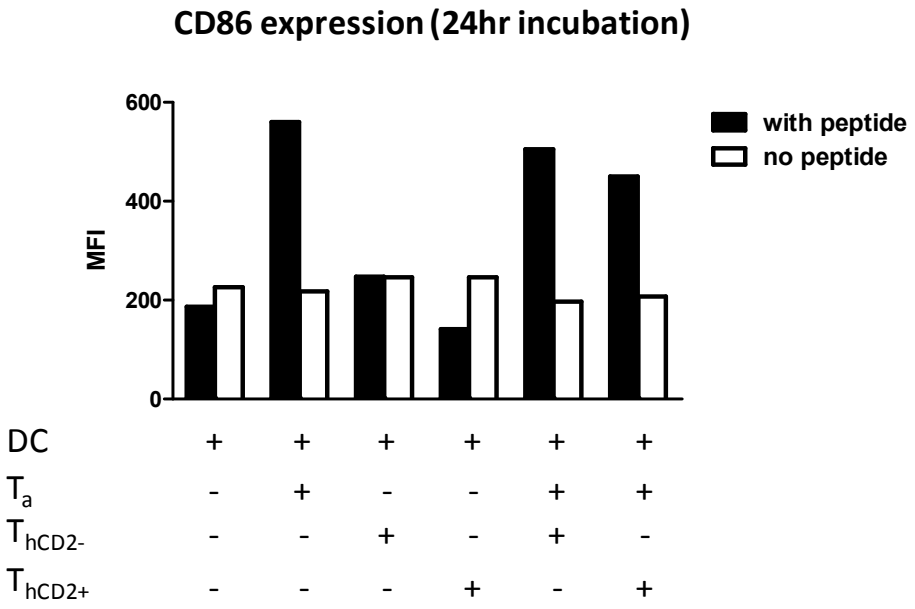


Figure 5.10A Failure to show a reduction in DC activation when TGFβ-conditioned T cells are co-incubated with naïve T cells

Naïve A1.RAG$^{-/-}$ CD4^{+} T cells were co-incubated with either TGFβ-conditioned cells ($T_{TGFβ\text{-}con}$) or polarised T cell clones in the presence of CBA female bmDCs and HYE^{k} peptide (100nM). nTreg were CD4^{+}CD25^{+} cells isolated from A1.RAG$^{-/-}$ x CBA F1 mice. Incubation ratio (naïve T cell+Treg or polarised T cell:DC) was 2+2:1 or 2+10:1 in each well of a 96 well plate. Per well, 2x10^{4} DCs were cultured with the T cells. The data shown are representative of two independent experiments.

Figure 5.10B


CD86 expression (24hr incubation)
600
400
200
0
MFI
with peptide
no peptide
DC + + + + + +
Ta - + - - + +
ThCD2- - - + - + -
ThCD2+ - - - + - +

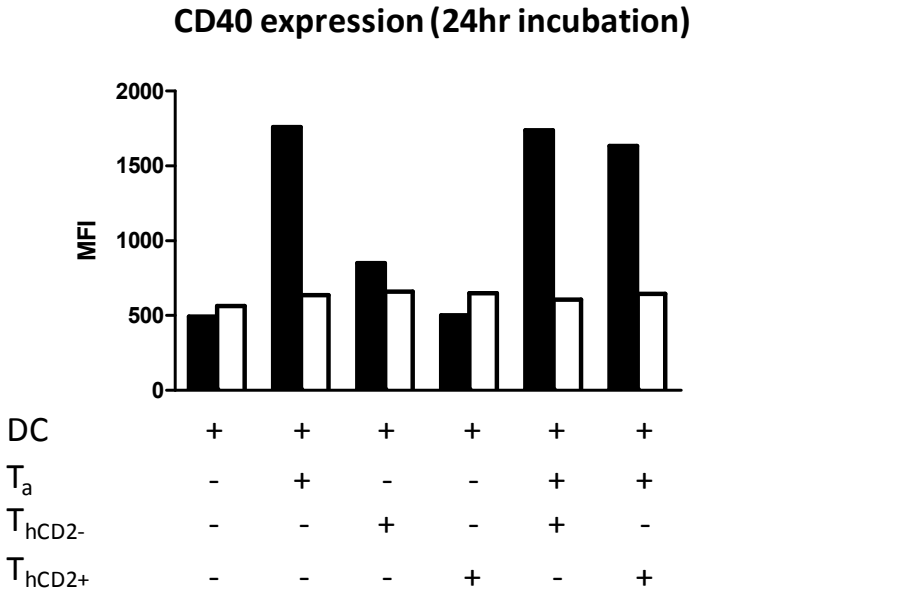

CD40 expression (24hr incubation)
2000
1500
1000
500
0
MFI
DC + + + + + +
Ta - + - - + +
ThCD2- - - + - + -
ThCD2+ - - - + - +

Figure 5.10B (cont'd)

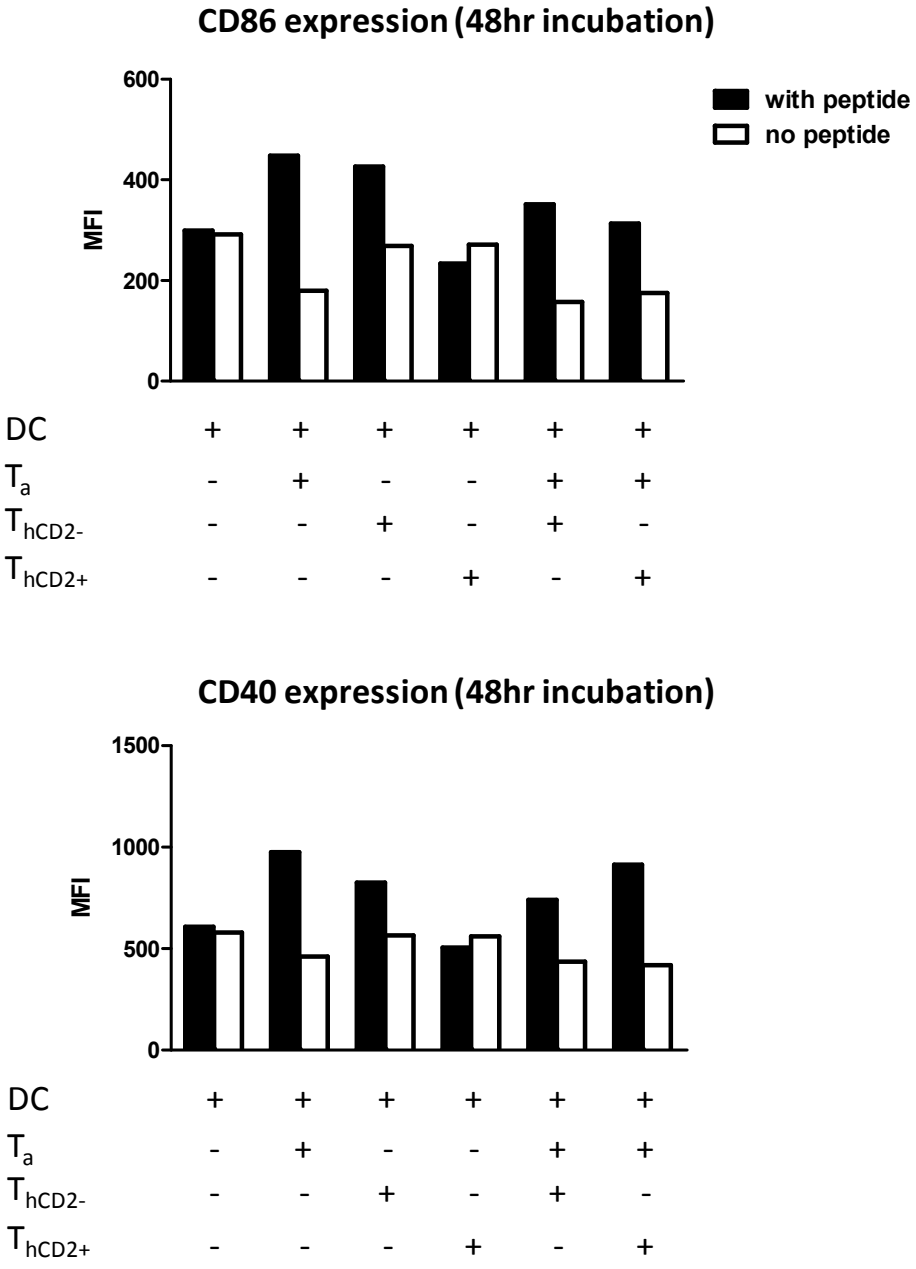


Figure 5.10B TGFβ-conditioned T cells do not affect activation of dendritic cells by activated T cells

TGFβ-conditioned T cells were generated from Marilyn.hCD.2.KI mice and sorted into $hCD2^+$ ($Foxp3^+$) and $hCD2^-$ ($Foxp3^-$) populations. Activated $CD4^+$ cells were peptide activated for 7 days but were not conditioned in TGFβ. Peptide-activated $CD4^+$ cells, $hCD2^+$ or $hCD2^-$ cells were incubated with B6 female DCs alone at 2:1 (T cell: DC) ratio, or co-incubated together at a ratio of 2(activated T cell)+2($hCD2^+$ or $hCD2^-$ cell):1 (DC). Incubation was for 24 or 48hrs. $4x10^4$ DCs were used per incubation.

Discussion

In vitro, nTreg appear to function in a contact-dependent manner (Shevach 2009). One potential contact-dependent mechanism of nTreg-mediated suppression is their physical 'displacing' of responder T cells around DCs, resulting in reduced DC activation and subsequent activation of effector T cells (Onishi et al. 2008). Here, it was shown that both cTreg and iTreg, like nTreg, can also physically out-compete naive T cells. However, their ability to 'out-aggregate' naïve cells did not appear to be unique, and was shared by other antigen experienced cell types such as polarised $CD4^+$ cells. Onishi et al., having demonstrated that nTreg out-compete naïve T cells, speculated that competitive aggregation may be a Treg-intrinsic property (Onishi et al. 2008). However, it appears from these experiments that antigen experience, rather than Foxp3 expression, may be the pre-requisite for the property of competitive aggregation. Consistent with this, $Foxp3^+$ TGFβ-conditioned cells were no better at out-competing naïve T cells than $Foxp3^-$ cells. cTreg were also no better at outcompeting naïve T cells than empty-vector-transduced $CD4^+$ cells. Therefore, Onishi et al.'s findings may reflect the antigen experienced state of nTreg due to their thymic origin, and their constant stimulation by self-antigen in the periphery (Apostolou et al. 2002; Fisson et al. 2003).

Whilst physically out-competing naïve T cells, TGFβ-conditioned T cells did not appear to activate re-plated bmDCs to the same extent as activated or polarised inflammatory T cells. Inflammatory polarised cells such as Th1 and Th9 cells can both out-compete naïve T cells in aggregation and also induce bmDCs to upregulate high levels of CD40 and CD86 expression. TGFβ-conditioned T cells upregulated CD40 and CD86 expression on DCs to a higher level than naïve T cells, but levels were lower than those of proinflammatory polarised cell clones. Onishi and colleagues found that nTreg could inhibit *splenic* DC activation in a DO11.10

system (where T cells are specific for the OVA peptide) (Onishi et al. 2008). In the current system (A1.RAG$^{-/-}$), incubation of naïve T cells with bmDCs for as long as 48hrs was not found to lead to a marked upregulation of DC activation markers, thus rendering the study of iTreg or cTreg inhibition of DC activation difficult. It was clear, however, that TGFβ-conditioned T cells co-incubated with naïve CD4^{+} cells only led to a modest increase in DC activation markers, whereas polarised inflammatory cells co-incubated with naïve CD4^{+} cells resulted in a markedly upregulated expression.

In summary, whilst iTreg generated from TGFβ-conditioning and inflammatory polarised T cells share the ability to preferentially aggregate around DCs, they differ in their functional impact on DCs: iTreg appear to induce only modest activation of the DCs, but inflammatory polarised cell clones can lead to strong activation of DCs. This is consistent with the 'civil service model' of regulation proposed previously to explain therapeutically-induced tolerance – that is, the physical displacement of responder T cells from DCs along with subdued DC activation could be a mechanism by which regulatory T cells dampen the immune response (Waldmann et al. 1998). The aggregation assay, however, appeared to have limited sensitivity in the systems that were studied. For example, there was little or no upregulation of CD40 and CD86 on DCs cultured with naïve T cells compared to DCs that were cultured alone. This made it difficult to study the suppressive effect of iTreg on the activation of DCs by naïve T cells. In the future, the peptide concentration and duration of incubation will be titrated to enhance the sensitivity of the system. Moreover, the effect of iTreg on other types of DCs (eg. splenic DCs or LPS-treated DCs) could be examined. These types of DCs may have a higher sensitivity to activation and therefore provide a better system for gauging the suppressive effects of regulatory T cells. Finally, the effect of cTreg on

the activation of DCs *in vitro* will also be investigated to examine whether Foxp3-transduction can also lead to subdued DC activation.

CHAPTER 6 GENERAL DISCUSSION

The potential power of therapeutically-induced regulation to mediate long-term antigen-specific tolerance was first described by Qin et al. (Qin et al. 1993). However, investigation into induced regulatory T cells has been hampered by their numerical paucity and a lack of reliable discriminating markers to separate them from natural Tregs. As a result, knowledge of the relative contributions of nTreg and iTreg to self- and non-self tolerance in the conventional mouse is sparse.

The importance of iTreg for tolerance induction has been questioned by reports suggesting that they were phenotypically plastic and prone to conversion under the influence of their immediate cytokine environment unlike nTreg (Bettelli et al. 2006; Veldhoen et al. 2006). Moreover, nTreg were shown to be potent suppressor cells in *in vitro* assays whereas iTreg sometimes failed to demonstrate the suppressive capacity *in vitro* (Hill et al. 2007). However, the *in vitro* assays commonly used to examine Treg function often simplify the complexities of physiological microenvironments that can impact on the function and stability of iTreg (Waldmann et al. 2009). Moreover, culture conditions for iTreg vary between laboratories. These factors have contributed to the challenge of studying the behaviour of iTreg.

I set out to investigate how iTreg function *in vivo* using transplantation as a read-out system. Specifically, I sought to understand the factors important for their suppressive activity and their phenotypic stability *in vivo*. I also examined if iTreg can mediate their suppression by competitive clustering around DCs and modulating their function. In order to have a ready source of $Foxp3^+$ iTreg, I used iTreg generated from the established protocol of *in vitro* TGFβ-conditioning of $CD4^+$ cells (Chen et al. 2003; Fantini et al. 2004; Daley et al. 2007). Certain

investigators are dismissive of *in vitro*-generated iTreg in favour of '*in vivo*-generated' iTreg produced in systems of parenteral or DC-targeted antigen delivery, arguing that the latter represents a more realistic representation of physiological processes in the conventional animal (Apostolou et al. 2008). However, this bias misses the fact that '*in vivo*' systems of iTreg-generation merely represent a different but no less experimental approach to the study of iTreg. Hence, studying the behaviour of *in vitro* generated iTreg in a transplantation setting would provide equally invaluable insights into their mechanism of action *in vivo*, and would be valuable for patients with autoimmune conditions or graft rejection.

A key area of uncertainty regarding the function of iTreg has been the relative contribution of TGFβ and Foxp3 for their suppressive function. Whilst TGFβ has been shown to be important for the generation of iTreg (Chen et al. 2003; Fantini et al. 2004; Kretschmer et al. 2005; Coombes et al. 2007), it has been difficult to dissect its role for the function of iTreg from that of Foxp3 expression. iTreg were shown to express genes related to TGFβ-exposure rather than Foxp3 expression (Hill et al. 2007). On its own, TGFβ can upregulate CD73, important for the production of adenosine which can have an immunosuppressive effect on both T cells and DCs (Huang et al. 1997; Panther et al. 2003). TGFβ can also act directly on T cells via the TGFβ receptor complex to repress T cell activation and polarisation towards a Th1 pro-inflammatory phenotype (Marie et al. 2006). Unimpaired TGFβ-signalling to the T cells was shown to be critical for therapeutically-induced transplant tolerance (Daley et al. 2007). However, I found that TGFβ-exposure alone without Foxp3 expression was insufficient to render $CD4^+$ cells suppressive *in vivo*. In contrast, $Foxp3^+$ TGFβ-conditioned T cells were suppressive *in vivo* and could prevent rejection by naïve T cells at a ratio of 5:1 (suppressor cell: naïve cell) in 50% of the recipients. The modest suppressive potency of sorted $Foxp3^+$ iTreg could possibly be due to lack of extrinsic cofactors such as IL-2 upon co-

transfer with naïve T cells that could put these cells at a disadvantage *in vivo*. Indeed the Foxp3$^-$ subpopulation of TGFβ-conditioned T cells could be a source of IL-2 because when unsorted TGFβ-conditioned T cells were transferred with naïve T cells, the ratio of Foxp3$^+$ suppressor to naïve T cell could be titrated down to as low as 2.5 to 1 with >80% graft survival among recipients (A. Kendall, personal communication). Moreover, as was shown later, TGFβ-conditioned T cells are still epigenetically predisposed to plasticity at the time of transfer, and may need further experience of antigen *in vivo* to affirm their regulatory function.

Whilst TGFβ-conditioning alone was insufficient to confer a suppressive phenotype to T cells, Foxp3 expression, on the other hand, was found to be both sufficient and indispensable. Ectopic Foxp3 expression in CD4$^+$CD25$^-$ cells using a conditional retroviral expression system led to the adoption of a suppressive phenotype both *in vitro* and *in vivo*. This echoes findings by Chai et al. who used a non-conditional system of ectopic Foxp3 expression (Chai et al. 2005). Unlike Foxp3$^+$ iTreg, 'cTreg' were found to be highly potent and 500,000 could suppress rejection in a TCR-transgenic recipient that has 1-2x10^6 antigen-specific CD4$^+$ cells in its spleen alone. Moreover, stopping the conversion to or maintenance of functional cFoxp3 on day 27 post-transplant when the graft looked well-healed led to the rapid rejection of the graft within 2 weeks. This suggests that Foxp3$^+$ cells are not only important in the inductive stage of graft rejection, but also for the maintenance of long-term graft tolerance. This is consistent with reports that Foxp3$^+$ cells are found in long-term tolerant grafts, and their elimination leads to the eventual rejection of the previously well-healed graft (A. Kendall, Y. Chen et al., in submission). This continuous dynamic balance seen in the tolerant graft between effector and regulatory T cells is also seen in the maintenance of self-

tolerance, where the acute elimination of Foxp3$^+$ cells can lead to increased T cell activation and the development of systemic autoimmunity in the adult mouse (Kim et al. 2007).

Whilst Foxp3 appears to be important for iTreg function, it has been reported that its expression by iTreg is highly unstable. When *in vitro* iTreg are re-stimulated in vitro in the absence of TGFβ, Foxp3 expression is rapidly lost after 6 days (Floess et al. 2007). Furthermore, iTreg transferred into a normal mouse were also found to have significantly reduced percentage of Foxp3 expression after 14 days *in vivo* (Kretschmer et al. 2005). Unlike iTreg, the phenotype of nTreg appears to be more 'stable' – and this was attributed to the strongly demethylated state of their DNA at the *Foxp3* locus (Floess et al. 2007; Polansky et al. 2008; Lal et al. 2009). Therefore I wished to examine what if any factors *in vivo* could help towards 'stabilising' their phenotype. Given that iTreg are often generated in the context of inflammation, I hypothesised that cognate antigen recognition along with the antigen-expressing tissue microenvironment might impact on the stability of their Foxp3 expression. Previously, Kretschmer and colleagues showed that the percentage of Foxp3 expression by unsorted TGFβ-conditioned T cells injected in to a naive mouse could be maintained *in vivo* using DC-targeted delivery of their cognate antigen (Kretschmer et al. 2005). Using sorted Foxp3$^+$ iTreg and transferring into lympho-deficient recipients, I showed that cognate antigen delivered in the form of a transplanted graft led to the maintenance of a high number of Foxp3$^+$ cells in the DLN of these recipients. Furthermore, this was associated with a greater proportion of permissive histone 3 modifications at the *Foxp3* locus. This suggested that the encounter of iTreg with cognate antigen led to epigenetic changes at the *Foxp3* locus conducive to Foxp3 expression, hence 'stabilising' their suppressive phenotype. Future experiments would involve examining DNA methylation at

the *Foxp3* locus and also the role of IL-2 in promoting the stabilisation of iTreg Foxp3 expression.

Finally, it has been reported that nTreg can physically out-compete naïve T cells in clustering around DCs (Onishi et al. 2008). Along with their inability to activate their interacting DCs, this could operate as a mechanism of suppression for nTreg. I asked whether a similar mechanism would operate for iTreg. However, whilst iTreg could also out-compete naïve T cells, it was found that antigen-experienced cells such as activated T cells or polarised inflammatory T cells also shared this property. Furthermore, these activated cells were not out-competed by iTreg. Therefore, competitive aggregation may be a consequence of antigen-experience. The competitive advantage of nTreg over naïve T cells could be due to their antigen-experience during their thymic development (Apostolou et al. 2002) and also to constant activation from their encounters with self-antigen (Fisson et al. 2003).

In conclusion, I have demonstrated that TGFβ-conditioning without Foxp3 expression appears to be insufficient in conferring suppressive properties to T cells whilst Foxp3 expression is sufficient to generate suppressive T cells that can prevent graft rejection. I have shown that a non-rejected graft can still undergo rejection when functional Foxp3 expression is ceased, suggesting that graft tolerance is a dynamic state requiring the continuous input of regulatory T cells. I have demonstrated that graft antigen can lead to the enhanced survival and/or expansion of $Foxp3^+$ iTreg in the DLN of graft recipients, and this is associated with an increase in the proportion of permissive histone 3 modifications at the Foxp3 locus. This suggests that graft antigen can promote more stable Foxp3 expression in $Foxp3^+$ iTreg. Finally, I showed that iTreg can competitively 'out-aggregate' naïve T cells in clustering with DCs. Such preferential aggregation appears to be shared by other antigen-

experienced cells such as inflammatory polarised T cells. However, this may be a potential mechanism for iTreg function because, unlike inflammatory T cells, iTreg showed a limited capacity to activate the DCs.

Future experiments would further dissect the mechanism of suppression by iTreg *in vivo*. Grafts which were not rejected as a result of iTreg suppression *in vivo* have been set aside to examine potential mechanisms of suppression within the graft microenvironment. A recent study suggests that Tregs can induce the production of enzymes which consume essential amino acids within the transplanted graft. This may then induce *de novo* Foxp3 expression in T cells via reduced mTOR signalling (Cobbold et al. 2009). Skin grafts harvested from recipients that were injected with either $Foxp3^-$ or $Foxp3^+$ TGFβ-conditioned T cells will be examined for the presence of these enzymes to ask whether this potential mechanism of 'infectious tolerance' is TGFβ-conditioning dependent. Furthermore, a future experiment would involve transducing $Foxp3^-$ TGFβ-conditioned T cells with *cFoxp3* in a bid to confer suppressive function to these cells and comparing their function *in vivo* with that of cTreg. Areas of comparison may include their homing behaviour, their suppressive potency, and the generation of 'infectious tolerance'. With regard to the *in vitro* aggregation assay, its sensitivity will be enhanced in future experiments by performing a peptide titration, but also by trying other types of DCs such as splenic or LPS-treated DCs to achieve better upregulation of CD80 and CD86. If that were possible, then I would be better able to establish the extent to which iTreg could inhibit this.

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