Assessing the Impact of Immunosuppressive Drugs on Regulatory T Cell Therapy

Thesis submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy

Keble College

Keble College, Oxford
and
Nuffield Department of Surgical Sciences

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation induced cell death</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>AS</td>
<td>Ag-B 1 or H-le rat strain</td>
</tr>
<tr>
<td>ATG</td>
<td>Anti-thymocyte globulin</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>BAEC</td>
<td>Bovine aortic epithelial cells</td>
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<td>BMDC</td>
<td>Bone marrow derived dendritic cell</td>
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<td>BMT</td>
<td>Bone marrow transfer</td>
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<td>BOS</td>
<td>Bronchiolitis obliterans syndrome</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>B10</td>
<td>C57BL/10</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CBA</td>
<td>Mouse strain with MHC haplotype k</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidy ester</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen induced arthritis</td>
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<tr>
<td>CNI</td>
<td>Calcineurin inhibitor</td>
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<tr>
<td>CsA</td>
<td>Cyclosporine</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte-associated antigen 4</td>
</tr>
<tr>
<td>CY</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DST</td>
<td>Donor specific transfusion</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed type hypersensitivity</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental allergic encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluourescence minus one control</td>
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<tr>
<td>FoxP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>GATA-3</td>
<td>GATA-binding protein-3</td>
</tr>
<tr>
<td>GILS</td>
<td>Graft infiltrating lymphocytes</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-induced tumour necrosis factor</td>
</tr>
<tr>
<td>GMCSF</td>
<td>Granulocyte-monocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
</tr>
<tr>
<td>GVL</td>
<td>Graft versus leukaemia</td>
</tr>
<tr>
<td>GT-DC</td>
<td>GMCSF and TGF-β cultured dendritic cell</td>
</tr>
<tr>
<td>H2</td>
<td>Mouse histocompatibility system 2</td>
</tr>
<tr>
<td>HCT</td>
<td>Haematopoietic stem cell transfer</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
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<tr>
<td>HSCT</td>
<td>Haematopoietic stem cell</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible costimulatory molecule</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IPEX</td>
<td>Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome</td>
</tr>
<tr>
<td>IS</td>
<td>Immunosuppression</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>LAP</td>
<td>Latency associated peptide</td>
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<td>LAT</td>
<td>Linker for the activation of T cells</td>
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<td>LMW</td>
<td>Low molecular weight</td>
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<tr>
<td>LTS</td>
<td>Long term survival</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
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<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
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<tr>
<td>MCA</td>
<td>Methylcholanthrene</td>
</tr>
<tr>
<td>Mek1/2</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MLR</td>
<td>Mixed lymphocyte response</td>
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<td>MMF</td>
<td>Mycophenolate mofetil</td>
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<td>MP</td>
<td>Methylprednisolone</td>
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<tr>
<td>MPA</td>
<td>Mycophenolic acid</td>
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</tbody>
</table>
MST  Median survival time
mTOR  mammalian target of rapamycin
NDS  Nuffield department of surgical sciences
NFAT  nuclear factor of activated T cells
NK  Natural killer
NOD  Non-obese diabetic
nTreg  Naturally regulatory T cell
OVA  Ovalbumin
PAMP  Pathogen associated molecular pattern
PB  Pacific blue
PBMC  Peripheral blood mononuclear cell
PBL  Peripheral blood leukocyte
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PE  R-phycoerythrin
PerCP  Peridinin chlorophyll protein
PI3K  Phosphatidylinositol 3-kinase
PIP3  Phosphatidylinositol 3-phosphate
PKC  Protein kinase C
PLC-γ  Phospholipase C-γ
PMA  Phorbol myristate acetate
PTEN  Phosphatase and tensin homolog deleted on chromosome 10
PTK  Protein tyrosine kinase
RAG  Recombinase activating gene
Rapa  Rapamycin
RPMI  Roswell Park Memorial institute
SA  Steptavidin
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SLP76</td>
<td>SRC-homology 2 (SH2)-domain-containing leukocyte protein of 76 kD</td>
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<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>Sos</td>
<td>Son of sevenless</td>
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<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
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<td>Tacrolimus</td>
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<td>T-bet</td>
<td>T box expressed in T cells</td>
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<td>T cell receptor</td>
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<td>Teff</td>
<td>Effector T cell</td>
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<td>Transgenic</td>
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<td>Transforming growth factor-beta</td>
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<td>Th</td>
<td>Helper T cell</td>
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<td>TLI</td>
<td>Total lymphoid irradiation</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
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<td>Tr1</td>
<td>T regulatory cells 1</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSDR</td>
<td>Treg-specific demethylation region</td>
</tr>
<tr>
<td>VPD</td>
<td>Violet proliferation dye</td>
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Abstract

Immunosuppressive drugs have facilitated the progression of solid organ transplantation from experimental therapy to routine practice, however transplant recipients are still susceptible to chronic rejection and co-morbidities. The emergence of regulatory T cells (Treg) as a key regulator of the immune system, together with an abundance of evidence from experimental transplant models, has led to clinical trials asking whether Treg can improve transplant outcomes. However, given that Treg cellular therapy will only be acceptable if introduced into current immunosuppressive regimens, a critical question is how Treg will respond in the presence of concomitant immunosuppression. Whilst in vitro data are available, very few credible experiments have been done asking whether individual immunosuppressive drugs have a positive, a neutral or a detrimental impact on the Treg function in vivo. Thus the aims of this thesis were firstly to generate sufficient numbers of adaptive Treg for extensive experimental use and secondly to evaluate their ability to control transplant rejection in vivo in the presence of biologically valid doses of individual, clinically relevant immunosuppressive drugs. Importantly, the model chosen was the heterotopic heart transplant model in lymphoreplete mice to avoid possible artefacts that can occur in cell reconstituted lympho-depleted mice. The model also has the added advantage that by dealing with an intact immune system, it perhaps represents a small step closer to the clinical situation.

Generating sufficient numbers of stable Treg was necessary for planned in vivo experiments. Incubating CD4+ T cells with anti-CD44 antibody, prior to driving them with bone-marrow derived dendritic cells, enriched for a stable population of Treg and importantly yielded sufficient numbers of cells for in vivo experiments. It is frequently stated that alloantigen-driven Treg are more efficacious than activated autologous nTreg, however there was no difference in rejection kinetics in either a skin or heart allograft model when comparing alloantigen-driven Treg with nTreg. As generating alloantigen-driven Treg is less efficient than nTreg, pursuing the
former as a potential therapy might therefore be unnecessary. This could have a considerable impact on the logistics and the practicality of clinical Treg cellular therapeutics.

The timing of Treg administration is an important consideration to maximise efficacy. Pre-transplant administration led to the longest graft survival times, suggesting that this is the most effective time for cell delivery.

Preclinical models provide a useful tool to ask how immunosuppressive drugs will affect adoptively transferred Treg. The data presented in this thesis suggest that combining Treg with Rapamycin, Mycophenolate Mofetil (MMF) or Tacrolimus did not completely prevent Treg function. However, Methylprednisolone (MP) did prevent Treg function, suggesting it cannot be used with adoptively transferred Treg. Overall, these results provide important data for the design of immunosuppressive regimens for future clinical trials assessing the efficacy of Treg in transplant recipients.
iv. Disclaimer

All of the experimental work presented in this thesis was performed by myself.
Chapter 1: Introduction

1. Brief history of transplantation

Since the first successful solid organ transplant was performed between syngeneic twins in 1960 [1] it has become the best treatment option for patients with end stage organ failure. Advances in immunosuppressive drugs transformed the experimental procedure into a routine therapy. For example, more than 90% of kidney transplant recipients have a functioning graft one year post-transplant [2, 3] and 57% of these patients have a functioning graft 25 years post-transplant (living donation) [4]. These impressive survival rates are unfortunately blighted by episodes of rejection and co-morbidities such as diabetes [5, 6], increased risk of infections such as CMV [7, 8], nephrotoxicity [9] and increase risk of malignancies especially of the skin [10-12] due to prolonged exposure to broad acting immunosuppressive drugs.

Enormous improvements have been made preventing acute rejection of transplants, however preventing chronic graft rejection has proved to be a far greater challenge [13-16]. Chronic rejection is characterised by various clinical pathologies including transplant arteriosclerosis, interstitial fibrosis and fibrointimal arteriolar proliferation [17, 18].

A key aim for transplantation researchers is to find a way to reduce the exposure of patients to the harmful side effects caused by long term exposure to broad acting immunosuppressive drugs whilst also reducing damage to the organ associated with chronic rejection. Cellular therapies that modify the immune response in a more targeted fashion, such as Treg, are being assessed in clinical trials to ask whether these cells can reduce the long term side-effects of broad acting immunosuppressive drugs [19-21].
2. Transplant rejection

The immune system prevents pathogenic microorganism invasion and stops the proliferation of oncogenic host cells. Mechanisms have evolved to enable discrimination of “self” from “non-self or altered-self”, as well as sensing danger signals associated with pathogens or tissue damage. These mechanisms similarly inhibit autoimmunity and limit damage to self during an immune response.

Solid organ transplantation between allogeneic patients results in a robust immune response that causes the rejection and ultimately the destruction of the transplant. Peter Gorer initially described the immune system’s role in rejecting allogeneic tumour cells [22]. Peter Medawar published seminal studies of syngeneic and allogeneic skin grafts in rabbits, identifying that allogeneic grafts were rejected in a process involving graft infiltration by monocytes and lymphocytes, whereas syngeneic grafts were tolerated [23, 24]. Medawar also demonstrated that rabbits transplanted with a second allogeneic graft, after rejecting a primary graft, rejected their second grafts more quickly. However, rabbits that had previously rejected an allograft, rejected a second allograft from a third party donor with the same rejection kinetics as the primary allograft. These data suggested that the rejection of an allograft was caused by cells of the immune system and that the immune system generated donor specific memory.

a. T cell mediated transplant rejection

Animals that are lacking T cells, such as thymectomised mice or mice that have been irradiated and reconstituted with T cell depleted bone marrow, do not reject allografts. Adoptive transfer of T cells into these mice results in graft rejection [25-28]. In clinical transplantation, depleting T cells has been a focus of therapies as a method of preventing graft rejection [29].

The elucidation of the roles of CD4+ and CD8+ T cells in graft rejection has utilised a variety of methods. It has been extensively reported that skin, kidney and heart allografts can be sufficiently rejected by CD4+ T cells alone, whereas CD8+ T cells failed to reject allografts or did
so with slower kinetics in some experiments, whilst in others CD8^+ T cells were sufficient to reject allografts without CD4^+ T cell help [27, 28, 30-35].

Homeostatic proliferation is a key concern with these experimental systems. Researchers have taken advantage of mouse models which are genetically deficient in CD8^+ T cells or CD4^+ T cells to avoid caveats associated with homeostatic proliferation [36-38]. Allograft rejection in the absence of CD4^+ T cells was delayed or absent in these experimental models, whereas in CD8^+ T cell deficient mice allograft rejection showed similar kinetics to controls, although Yamada et al. demonstrated that chronic vasculopathy was evident in CD8^+ T cell deficient mice [39-46].

Cardiac allograft rejection is prevented when lymphoreplete mice are treated with αCD4-mAb, however skin allografts required depletion of CD8^+ and CD4^+ T cells to prevent rejection. In summary, CD4^+ T cells appear to be indispensable for allograft rejection, whereas CD8^+ T cells are less important but can contribute towards allograft rejection with CD4^+ T cell help, with the exception of MHC class I mismatched allografts with which CD8^+ T cells did not require help with rejection [47-55].

b. T cell allore cognition

i. The Major Histocompatibility complex (MHC)

MHC genes were discovered in research involving tissue rejection in mice and rabbits, these experiments indicated that rejection of tissues was genetically controlled [56-58]. Medawar et al. discovered that the immune system was responsible for rejection [23, 24] and this was advanced by Snell who discovered the histocompatibility genes which are responsible for immune mediated rejection [59-61].

Mouse MHC genes are located on chromosome 17, whereas in humans (MHC are often referred to Human Leukocyte Antigens – HLA) they are encoded on the short arm of chromosome 6. The MHC is thought to be the most polymorphic region of the human genome and it is this polymorphism that facilitates the theoretical ability to present virtually any molecular structure
The MHC family can be divided into two structurally similar, yet functionally different classes; MHC class I and MHC class II. MHC class I molecules consist of an α-chain and β₂-microglobulin, linked by non-covalent interactions. Only the α-chain spans the membrane, forming three of the four domains that constitute MHC class I, the fourth domain is provided by β₂-microglobulin [62]. MHC class II has two transmembrane chains, α and β. These assemble to form a four domain heterodimeric structure of 2 α and 2 β domains.

Both classes consist of a peptide binding domain formed from a β-pleated sheet supporting two parallel α-helices, which provide a peptide binding groove or cleft. MHC class I molecules are expressed to varying degrees on all nucleated cells, whereas MHC class II are largely confined to professional antigen presenting cells (APCs) such as; DCs; B-cells; Macrophages; and non-APCs such as endothelial cells [63-67].

MHC molecules are the most important alloantigen involved in the activation of immune responses and the rejection of an MHC-mismatched allograft. However, transplants between MHC matched individuals can still be rejected due to minor-histocompatibility antigens.

**ii. Direct pathway**

APCs transferred in the donated organ can traffic to the recipient’s lymph nodes and activate recipient alloreactive T cells, these T cells can then traffic to the transplant, contributing to rejection [68-70]. Seminal work by Lechler and Batchelor demonstrated that donor derived passenger leukocytes were indispensable for allo-sensitisation following re-transplantation of long surviving rat kidney grafts [70, 71]. Re-transplant of long term surviving (ASXAUG)F1 kidney grafts survived long term in secondary AS recipients, unless 1x10⁴ to 5x10⁴ donor strain DCs were adoptively transferred [70]. This process is known as the direct pathway of allorecognition and is thought to be the central cause of acute rejection due to the high pre-cursor frequency of alloreactive T cells which become activated via the direct pathway [72-77]. The precursor frequency of alloreactive T cells is between 1 and 10% [75, 78-81], which is up to 3 logs greater...
than the precursor frequency calculated for responses to viruses 1:10⁴-10⁶ [82, 83]. This results in as much as 90% of the T cell repertoire becoming activated by directly presented antigen in the context of donor MHC during acute rejection [76, 77].

Two hypotheses were proposed to explain the high precursor frequency of alloreactive T cells. These focused on the relative contribution of donor MHC molecules and donor peptides to allorecognition. MHC:peptide complexes are recognised with high specificity by TCRs, a phenomenon described as MHC restriction [84, 85]. Theoretically, heterogeneity of peptides or MHC molecules could lead to the activation of T cells. Immune responses to pathogens result in TCR clones recognising pathogen antigens presented in the context of MHC, leading to the propagation of the immune response. During allogeneic transplantation however, T cells recognise a diverse array of peptides presented by allogeneic MHC molecules. Matzinger and Bevan hypothesised that the high frequency of direct pathway activated alloreactive T cells means that each of the myriad of allogeneic MHC presented peptides has the potential to activate at least one T cell clone. This was termed the multiple binary complex model [81].

The second hypothesis is the high determinant density model which predicts that the alloresponse is primarily towards donor MHC molecules [86]. This model suggests that low affinity allogeneic TCRs can respond to MHC molecules due to the density of MHC cell surface expression [86]. Felix et al. showed that alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes. The authors stimulated 60 IE³⁻-alloreactive T cell hybridomas with Chinese hamster ovary (CHO) cells transfected with IE³ [87]. 32 of the hybridomas were unresponsive to the IE³ transfected CHO cells and therefore were selected for screening with 83 endogenous peptides isolated from IE³ molecules. 6 hybridomas responded to a single peptide, 2 responded to 2 peptides and 1 hybridoma responded to 3 peptides. To confirm that the alloreactivity of the TCR was specific for the MHC it was responding to, either the peptide or IE³ were mutated, resulting in a loss of hybridoma responsiveness [87]. The 28 hybridomas that
responded to $\text{IE}^k$ transfected CHO cells in the absence of peptide suggested that alloreactive T cells can respond to MHC irrespective of bound peptide. Unfortunately, this is complicated by the possibility that the hybridomas were responding to endogenous CHO cell peptides presented in the context of $\text{IE}^k$.

As there are a finite number of donor derived passenger leukocytes within a transplanted organ, the role of direct recognition of allogeneic peptides diminishes post-transplant and the indirect pathway of recognition becomes more predominant.

iii. Indirect pathway

In 1992, three groups showed that after the transplantation of an allograft, recipient APCs are able to process and present donor derived peptides, including MHC molecules, to recipient T cells. This process resulted in the activation of the T cells which traffic to the allograft and cause rejection [88-91]. A limited number of T cell clones with discrete TCRs for antigens were shown to be mainly responsible for the indirect allo-response. Using a mouse skin transplant model, Benichou et al. showed that indirect allo-responses were limited to only a few dominant determinants on donor MHC antigen, in the polymorphic regions of donor MHC [91]. Early indirect responses are limited to a few dominant peptides, although evidence has emerged that cryptic epitopes (epitopes unable to elicit the response of the naïve repertoire against MHC molecules) are presented at later time points [92, 93]. This has been termed, antigen spreading and it appears to be an important factor of chronic rejection [92, 94, 95].

iv. Semi direct pathway

A third method of alloantigen recognition has been identified which proposes that DCs have the ability to acquire intact MHC-peptide complexes from donor APCs and endothelial cells, and then subsequently present these to alloreactive T cells [96-98]. This phenomenon is described as the semi-direct pathway of allore cognition, or “cross dressing”. Effectively this allows a single
DC to present allogeneic peptides directly through the acquired donor MHC class I molecule and indirectly via self-MHC class II to CD4+ T cells.

Semi-direct presentation provides a “three cell model” for CD8+ T cell activation, whereby the CD8+ T cell is activated when it comes into contact with its cognate peptide, presented in the context of MHC class I by the corresponding DC and is simultaneously stimulated by the CD4+ T cell, which is stimulated by the same APC [99]. This three cell model overcomes the criticism of the four cell model in which recipient CD8+ T cells are partially activated by donor APCs and then receive help to become fully activated, in an unconnected manner, by indirectly activated recipient CD4+ T cells.

c. T cell activation

For a T-cell to become activated and undergo clonal expansion the T-cell requires 3 signals. Firstly, the TCR must recognise its cognate peptide presented in the context of MHC by an antigen presenting cell. This interaction is enhanced by the involvement of co-stimulatory molecules and finally the expression of cytokines leads to full activation of the T cell.

i. TCR

The T cell receptor complex (TCR) is most commonly formed of two polypeptide chains (α and β), linked by a disulphide bond. These chains each have a constant (C) and variable (V) region with a short stalk segment connecting the constant regions to the cell membrane [100]. The variable regions harbour three hyper-variable sections, also known as complementarity-determining regions (CDRs) that form a complementary molecular structure to recognise their cognate antigen peptide presented on the MHC. 2 CDRs are located on the V-α chain, with the third located on the V-β chain.
The short intracellular domains of the TCR necessitate the formation of a complex with CD3, which is formed of 6 polypeptide chains (εδ and εγ heterodimers and a homodimer of ζ chains) [101] enabling intracellular signalling.

T cells are divided into two functionally distinct subsets according to the expression of the co-receptors CD4 or CD8 on the cell surface. CD4+ “helper” T cells recognise peptides presented in the context of MHC class II and can provide additional stimulation (or help) to B-cells and CD8+ T cells. CD8+ T cells recognise peptides presented in the context of MHC class I. CD8+ T cells are often termed cytotoxic T-cells as they kill infected and malignant cells. CD4 and CD8 receptors bind non-polymorphic proximal domains of MHC class II and I respectively, forming a vital part of the immunological synapse by attracting signal transduction proteins into the TCR-CD3 complex.

ii. Costimulation

The hypothesis that TCR stimulation required the presence of costimulatory molecules arose from experiments in which Th1 clones were stimulated with anti-CD3 antibody, inducing unresponsiveness to antigen stimulation. This was prevented by adding accessory cells supporting the hypothesis that T cell activation required T cell stimulation (signal one) in the presence of a costimulatory molecules (signal two) [102, 103]. The type of response that the T cell initiates is intimately linked with the stimulation received by costimulatory molecules expressed on the surface of antigen presenting cells. Blocking costimulatory signals has been used to render T cells anergic and has been used as a method to prevent allograft rejection [104-106], and induce tolerance when administered in combination with a donor specific antigen transfusion (DST) [107]. These data indicate that costimulatory signals have a central role in the rejection of allogeneic transplants.
iii. TCR signal transduction

a. Proximal signalling

Research involving the utilisation of protein kinase (PTK) inhibitors revealed their important role in TCR signalling. Lck and Fyn, which form part of the Src-family of PTKs, and the Syk-family kinase ζ-associated protein of 70 kDa (ZAP-70) are activated during TCR ligation [108-110]. The activation of Lck results in the phosphorylation of ITAMS of CD3 proteins [111].

Other research has also identified the importance of Fyn for the phosphorylation of ITAMS. Samelson et al. demonstrated that using the foxglove plant extract digitonin, as a mild non-ionic detergent to solubilise a murine T cell hybridoma, that Fyn was co-precipitated with the TCR in the presence of antibodies binding to the extracellular domain of the TCR but this did not happen when antibodies were directed to the intracellular TCR region [112].

Phosphorylated ITAMS form docking sites for a number of proteins including PTKs. ZAP-70 is recruited by phosphorylated ITAMS on the ζ chains of CD3, via SH2 domains, leading to the phosphorylation and activation of ZAP-70 by Lck. Irving et al. demonstrated that ζ truncations, containing a 17 amino acid motif, were sufficient for the proximal and distal events involved with T cell activation [113]. ZAP-70 association with phosphorylated, activated TCR complexes leads to recruitment of multiple proteins and adaptors resulting in the formation of a multinucleated signalling complex.

Phosphorylated ZAP-70 leads to the phosphorylation of two crucial adaptor proteins; Linker for the activation of T cells (LAT) and the SH2 containing leukocyte phosphoprotein of 76kDa (SLP-76) [114-116]. LAT and SLP-76 form a proximal signalling complex which recruits other effector proteins [116, 117]. Phosphorylated LATs recruit SH2 domain bearing proteins such as PLCγ1, the p85 regulatory subunit of PI3K, Grb2 and Gads [118, 119]. SLP-76 binds Gads and PLCγ1 to form part of the complex with LATs. LAT and SLP-76 deficient mouse models demonstrate reduced activation of Ras, due to an impaired proximal signalling complex formation [118, 120].
Another important factor for the activation of Ras is Grb, which is constitutively bound with Son of Sevenless (Sos), a GTP exchange factor for Ras and Rho GTPases. Ras is activated when the Grb-Sos complex binds to phosphorylated tyrosines on LAT.

SLP-76 is also phosphorylated by ZAP-70 resulting in recruitment of Vav-1, a GEF, IL-2-induced tyrosine kinase (Itk, a Tec family of PTK) and other adaptor proteins such as non-catalytic tyrosine kinase (Nck) and ADAP [121]. Vav1 recruitment to SLP-76 via the SH2 domain activates the Rho family of GTPases such as Rac1, promoting actin reorganisation [122].

PLCγ1 connects the proximal signalling pathways to the distal signalling pathways. Sommers et al. demonstrated that mutating LAT (Y136F) disrupts PLCγ1 activation and Ca\(^{2+}\) influx, blocking optimal T cell activation [123]. Activated PLCγ1 hydrolyses membrane bound phosphatidylinositol 4, 5-bisphosphate (PIP\(_2\)) into two second messengers; inositol 3-phosphate (IP\(_3\)) and diacylglycerol (DAG). DAG can activate the major signalling pathways such as RasGRP1, PKC\(\theta\) and PDK1 pathways. IP\(_3\) is important for the activation of Ca\(^{2+}\)-dependent calcineurin-NFAT signalling pathway.

b. Distal signalling

i. Ras-Erk1/2-AP1 pathway

Ebinu et al. identified a Ras guanyl nucleotide releasing protein (RasGRP) as one of the key guanine nucleotide exchange factors responsible for T cell activation of Ras. Ras-GRP-1 binds to DAP, inducing membrane translocation and activation [124]. Ras-GRP null mice were identified to have normal numbers of immature thymocytes but mature CD4\(^+\) and CD8\(^+\) T cells were reduced by 76% and 90% respectively, demonstrating an important role in the maturation of thymocytes for Ras-GRP [125]. Importantly, over expression of RasGRP in Jurkat T cells resulted in enhanced Ras-Erk signalling, suggesting a key role for this molecule in the propagation of distal signalling [124].
A second key guanine nucleotide exchange factor known to activate Ras is Sos, which appears to promote Ras activation in a RasGRP dependent manner [126, 127]. Ras, a small protein coupled receptor, when bound to GTP activates the serine/threonine kinase Raf-1 [128] which phosphorylates mitogen-activated protein kinase kinase (Mek1/2). Activated Mek1/2 then serves to phosphorylate Elk-1 [129]. Activated ELK-1 initiates the expression of the transcription factor c-fos, resulting in the formation of a dimer composed of Jun/Fos, known as AP-1 which is vital for an immune response.

ii. **IP3-Ca2+-NFAT pathway**

Engagement of the TCR results in the phosphorylation of phospholipase C (PLC-γ) which hydrolyses phosphatidylinositol-3,4-trisphosphate (PIP3) to Inositol trisphosphate (IP3) and DAG. IP3 binds to its receptor (InsP3R) located on the endoplasmic reticulum (ER), resulting in the release of Ca2+. Ca2+ release activates the protein phosphatase calcineurin which dephosphorylates NFAT, enabling translocation to the nucleus. Additionally, the production of DAG activates the Ras-MAPK and PKC pathways.

d. **Effector mechanisms involved with allograft rejection**

i. **IFN-γ**

Allograft rejection is closely linked with IFN-γ expression and Th1 responses have been identified to play an important role in cytotoxic lymphocyte activation and delayed type hypersensitivity (DTH) responses, both of which are important aspects of allograft rejection [130]. For example, increased expression of IFN-γ and IL-2 were identified in rats with rejecting heart allografts, compared with non-rejecting hearts [131], whereas intra-graft IFN-γ expression increased in unmodified rejecting cardiac allografts [132]. In a tolerising protocol in which mice received anti-CD4 mAb and DST, with cyclosporine administration, expression of IFN-γ and IL-2 was reduced >90% in grafts from tolerant recipients [133]. These data all suggest that IFN-γ has a major role in acute rejection of cardiac allografts. Contrastingly, IFN-γ has also been identified
to have an immunoregulatory role. Mice with experimental encephalomyelitis (EAE) and collagen induced arthritis (CIA) that are deficient for IFN-γ or IFN-γR, developed EAE or CIA at an accelerated rate or more severely [134-136]. Additionally, in skin and heart transplant models, IFN-γ−/− mice were resistant to tolerance induction using co-stimulation blockade of CD28 and/or CD40L [137]. Additionally, IFN-γ plays an important role in Treg function in vivo [138] and can also be used to induce Treg in vitro [139, 140].

ii. TNF-α

TNF-α is a proinflammatory cytokine implicated in major inflammatory processes such as GVHD, autoimmunity and transplant rejection [141]. Raised serum levels of TNF-α were measured in acute rejection episodes of renal transplant patients [142]. TNF-α was subsequently implicated in promoting acute heart allograft rejection in rats [143] and inhibition of TNF-α using a neutralising antibody reduced the development of transplant arteriosclerosis in murine aortic allografts. The effects of TNF-α on Treg remains controversial. Treg have been shown to constitutively express TNF-α receptor and TNF-α inhibits the suppressive function of Treg [144], however others have shown that TNF-α can actually promote Treg generation [145, 146]. Similarly to IFN-γ, TNF-α deficient mice experience exacerbated diseases profiles in lupus, EAE and diabetes [147-149]. Neutralising TNF-α in patients with MS led to more severe symptoms [150]. In rheumatoid arthritis, TNF-α exacerbates disease by inducing increasing expression of PP1, which dephosphorylates Ser418, reducing FoxP3 binding to DNA, impairing suppressive function [151]. These conflicting results suggest that TNF-α is instead intimately involved in the balance between Teff and Treg, whereby Teff upregulate various cytokines, such as TNF-α and IFN-γ, during the initiation of an immune response, resulting in the activation of Treg which act to resolve the immune response.
e. Cytotoxic T cell response

CD8$^+$ T cells from rejecting allografts have been shown to kill donor cells in vitro [152, 153] and can cause the rejection of MHC class I mismatched skin grafts [154]. In an allogeneic skin transplant model, allografts were rejected by CD8$^+$ T cells independently of CD4$^+$ T cells [155]. Interestingly, using αCD154 mediated tolerance induction, CD8$^+$ T cells occasionally caused rejection, therefore requiring the addition of either a depleting anti-CD8 mAb or DST [156-160]. The unreliability of αCD154 mediated tolerance induction was due to differences in Treg numbers generated, ultimately identifying CD8$^+$ T cells as a target of Treg mediated suppression in vivo [161].

f. Innate Immune response to transplantation

The innate immune system rapidly reacts to conserved features of pathogens and also activates after physical and metabolic trauma. The surgical procedures (including heat stresses) involved in organ transplantation lead to tissue damage which activates the innate immune response, which contribute to rejection [162, 163]. One of the key responses is the activation of the complement system, which usually protects against microbial infection. The conversion of the complement protein, C3, via a variety of pathways (classical, lectin and alternative) to the active form is at the core of the complement cascade. The two peaks of activation of the complement system during organ transplantation happen during ischaemia-reperfusion injury and acute rejection [164, 165]. Ischaemia-reperfusion injury leads to conversion of C3 to C5b-9, leading to necrosis of tissue parenchyma in many transplant models. Mice and rats lacking complement suffer less severely from ischaemia reperfusion injury [166-171] with a reduction of up to 50% loss in renal function in mice lacking complement [171] and almost 50% reduction in cardiac infarct size in the heart allograft model [172]. Peng et al. demonstrated that the proinflammatory fragments C3a and C5a were important mediators of ischaemia reperfusion...
injury as mice lacking C3a or C5a, or their receptors, were protected from kidney damage, with C5a being dominant [173].

Complement protein cascade activation can also activate T cell alloreactivity via the C3a and C5a dependent activation of APCs, generating a Th1 response to alloantigen [174, 175]. C3a and C5a also act directly on T cells providing costimulatory and survival signals, and enhancing effector T cell expansion by reducing apoptosis [176, 177]. Importantly, C3aR and C5aR activation can inhibit Treg generation, contributing to skin allograft rejection [178].

A second key aspect of the innate immune system activated during the process of organ transplantation is the Toll like receptor system (TLR), which was initially discovered in insects [179] and was later described in mice displaying increased susceptibility to gram-negative bacterial infection [180]. TLRs recognise pathogens through their ability to bind to pathogen-associated molecular patterns (PAMPs). Once a TLR recognises and binds to a PAMP, signal transduction is commonly mediated via the adapter molecule myeloid differentiation faction 88 (MyD88) [181]. The damage to tissues, inevitable during solid organ transplantation, leads to the release of danger associated molecular patterns (DAMPs), which are also recognised by TLRs and contribute to transplant rejection.

Strom et al. identified that macrophages contribute to the rejection of solid organ transplants [182] and have been shown to have a role in both cell and antibody mediated rejection, and in the process of vascular disease within the graft which is known to contribute to chronic rejection [183, 184]. Mechanisms by which macrophages contribute to acute transplant rejection include the production of reactive oxygen species and proinflammatory cytokines such as TNF-α and IL-18 [185, 186]. Interestingly, macrophages may also play a regulatory role [187, 188] particularly via expression of IL-10 [189], although the exact details of this remain to be elucidated and interaction of macrophages with Treg appears to “steer” their development towards a regulatory phenotype [190].
Sub-optimal or lack of self-MHC class I recognition by NK cells leads to activation, which has become known as the “missing self” hypothesis [191-196], although NK cells were thought to be unimportant in graft rejection, as depletion of these cells did not change the rejection of skin or heart allografts in murine models [197-199]. More recently NK cells have been identified to acquire cytolytic effector functions and release IFN-γ and TNF-α to contribute towards graft rejection [200, 201]. NK cells may also contribute towards graft acceptance and immune tolerance by expressing IL-10 [202] and killing donor APCs [203, 204]. The role of NK cells in transplantation is likely to be dependent upon the subset of NK cell and the milieu.

NKT cells comprise a heterogeneous group of T cells characterised in mice by their expression of a TCR and NK1.1 (CD161c in humans) [205]. Ikehara et al. identified that the subset of NKT cells (Vα14 NKT cells) are essential for tolerance induction using an anti-CD4 mAb [206]. In a subsequent study blockade of either ICAM-1 or CD28/B7 interactions led to long term acceptance of heart allografts in wild type mice but not in Vα14 NKT cell deficient mice, importantly adoptive transfer of Vα14 NKT cells restored tolerance [207]. Both of these studies suggest that Vα14 NKT cells have an important regulatory role in organ transplantation. NKT cells have also been shown to have an early role in ischaemia reperfusion injury by expression of IFN-γ [208]. Models in which NKT cell activity was reduced resulted in significantly less ischaemia reperfusion injury in part via IFN-γ dependent activation of Kupffer cells, neutrophils and hepatocytes [209-211]. These publications highlight the heterogeneity of this subset of cells.

**g. Leukocyte recruitment**

Inflammation and ischaemia reperfusion injury are currently unavoidable consequences during the invasive surgical procedure required for organ transplantation. Inflammation upregulates the expression of chemokines and chemokine receptors by activated leukocytes. This enables the trafficking of leukocytes to the graft via a chemo-attractant gradient [212].
CXCR1 and CXCR2 are important chemokine receptors that facilitate the trafficking of granulocytic leukocytes to sites of ischaemia reperfusion damage. Bertini et al. and Cugini et al. demonstrated the importance of CXCR1 and CXCR2 using the small molecule inhibitor, Repertaxin. Both studies identified reduced granulocyte infiltration after treatment with Repertaxin, protecting allografts against ischaemia reperfusion injury and improving renal function [213, 214]. In CXCR2−/− mice or mice treated with anti-CXCR2 antisera, heart allograft survival was improved by a minimum of 3-4 days [215]. CXCR2 ligands CXCL1, 2 & 3 were upregulated in a rat lung transplant model of cold ischaemia reperfusion injury (6 hours, 4°C). The upregulation of these ligands paralleled lung neutrophil infiltration and graft damage. Blockade of CXCR2 ligands using an anti-CXCR2 antibody resulted in a significant reduction in neutrophil infiltration and damage to the lung architecture, although graft survival data was not reported [216]. This is an important observation as lung transplantation is particularly susceptible to acute rejection with early ischaemia reperfusion injury a common contributing factor.

Glycosaminoglycan (GAG) structures present chemokines on endothelial surfaces, these structures form an important mediator of early leukocyte graft infiltration. Bedke et al. generated a human dominant-negative CXCL8-based antagonist with the aim of generating a dominant negative mutant protein which would bind to GAG structures with higher affinity. Testing this in a rat model of acute renal damage treatment with the dominant negative CXCL8 antagonist reduced granulocyte, monocyte and CD8+ T cell infiltration and proximal tubular damage [217].

Blocking CCR1 and CCR5 using the agonist Met-RANTES reduced signs of acute renal rejection in a rat renal allograft model [218]. A later study identified that early application of met-RANTES was able to reduce the development of chronic allograft damage [219]. CCR1 and CCR5 share many ligands and are expressed by many of the same leukocytes. As identified by experiments
involving met-RANTES, both chemokine receptors are involved in acute allograft rejection, although it appears they are important at different stages of leukocyte recruitment as CCR1 was identified to be predominantly involved with RANTES induced arrest of leukocytes, whereas CCR5 was mostly involved with cell spreading in sheer flow [220].

CCR7 is expressed by passenger DCs and macrophages in donor organs and facilitates trafficking of these DCs to directly present donor antigen in the secondary lymphoid tissue. This was initially thought to be an important mechanism for allograft rejection via direct antigen presentation [212]. Mice which were CCR7−/− that received a heart allograft displayed only moderately increased graft survival compared with wild type controls [221]. Interestingly, CCR7 appears to have an important role in tolerance induction as Liu et al. identified that CCR7−/− mice were unable to prolong cardiac allograft survival when treated with a tolerance induction regimen. This appeared to be a result of a reduction in the number of plasmacytoid DCs and a significant reduction in the numbers of Treg within the lymph nodes, suggesting an important role for CCR7 in DC and Treg migration to lymph nodes [222]. Interestingly, an increased frequency of CCR7+ Treg in human lung transplant recipients protected from bronchiolitis obliterans syndrome (BOS) [223].

3. Transplant tolerance

One of the key advantages of the adaptive immune system is its potential to recognise an almost limitless number of antigens through the process of somatic recombination. This process is capable of generating a T cell repertoire able to recognise an estimated $10^{15}$ different antigens [224]. Due to the random process of somatic recombination, T cells are generated that recognise self-antigens, which has led to the generation of mechanisms to delete T cell clones that have a high affinity for self-MHC, thereby avoiding auto-immunity. Harnessing these
natural protective mechanisms would be highly beneficial in allogeneic transplantation to induce tolerance towards the graft. Transplant tolerance has been defined as “the specific absence of a destructive immune response to a transplanted tissue in the absence of immunosuppression”. “Specific” refers to the requirement of the transplant recipient remaining capable of responding to pathogens and malignant cells [225]. The maintenance of immunological tolerance requires deletion, anergy, regulation/suppression and ignorance, much like induction of tolerance towards an allograft [226], with the additional complication that the frequency of MHC alloreactive T cells is relatively high (1-10%) in mice [75]. Billingham, Brent and Medawar were the first to describe acquired tolerance to a foreign antigen in 1953, when they described that mouse and chickens could be inoculated as foetuses to render them tolerant to skin grafts from the original donor in adult life [227]. This breakthrough led to a vast number of methods of generating tolerance in animal models. These methods can be separated into those that rely on deletion and those that rely on active immunoregulation.

a. Central tolerance

Intrathymic T cell deletion is the key mediator of central T cell tolerance [228] and injection of donor antigen into the thymus induces central T cell tolerance, however this tolerance is temporary as the source of donor antigen is finite [229]. Haematopoietic cell transplantation (HCT) is considered the most effective method to induce T cell tolerance as a continuous supply of haematopoietic stem cell derived donor antigen is present in the thymus, providing indefinite negative selection of donor reactive T cells [230]. Ildstad and Sachs described a method of inducing specific transplant tolerance in adult recipients using full body irradiation and a T cell depleted bone marrow transplantation with host and donor components to induce donor chimerism [231]. Indeed many experimental models have been described that overcome the obstacle of intrathymic allogeneic rejection by methods that involve a combination of irradiation and cell depleting therapies to induce a state of mixed donor chimerism [232, 233]. These data resulted in a trial in 5 renal transplant patients who received an HLA single haplotype
mismatched organ from a living relative, along with cyclophosphamide anti-CD2 mAb, cyclosporine A and thymic irradiation (700cGy) and haemodialysis [234]. 4 out of 5 patients were able to discontinue all immunosuppressive drugs by 14 months post-transplant. These data demonstrate the important breakthroughs that have been possible by manipulating central tolerance mechanisms.

b. Peripheral tolerance

In experimental models, depletion of T cells has proved to be a successful method to induce tolerance, leading to research asking whether lymphocyte depletion at the time of transplantation could induce donor-specific immunological hyporesponsiveness [226]. Myburgh et al. demonstrated that operational tolerance (normal graft function >1 year post-transplant) could be achieved in half of baboon kidney transplants when the recipients received total lymphoid irradiation (TLI) and anti-CD3/4-idarubicin conjugates [235]. Clinical trials, similar to this approach, in which 28 deceased donor renal transplant recipients received TLI and ATG with low dose corticosteroids, reported that 9/28 patients showed evidence of donor-specific hyporesponsiveness, judged by MLRs. 3/28 patients were able to cease immunosuppressive therapy and did not undergo rejection, these patients were reported to be operationally tolerant [236, 237]. Another study, in which rhesus monkeys received a renal allograft after T cell depletion using anti-CD3 mAb combined with a mutated diphtheria toxin retained renal function for over 200 days [238]. There were however signs of chronic rejection in biopsies taken at 100 days post-transplant [239].

Campath (Alemtuzumab) is an anti-CD52 specific mAb that causes profound and sustained lympho-depletion. A systematic review of ten randomised controlled trials (RCTs) in kidney transplantation compared Campath with induction therapies using rabbit anti-thymocyte globulin (rATG), basiliximab and daclizumab. Although, Campath induction had a lower risk of biopsy-proven acute rejection compared with basiliximab and daclizumab, there was no
difference between Campath induction and rATG. Perhaps most significantly these authors identified two trials comparing alemtuzumab with no induction and neither trial reported a significant reduction in biopsy-proven acute rejection at 12 months [240]. Furthermore one of the studies reported that there was no difference in acute rejection episode incidences after five years between Campath induced patients and controls [241]. Overall these data suggest that peripheral T cell depletion at the time of organ transplantation is not sufficient to establish tolerance to the allograft.

**c. Immunoregulation**

The relative lack of success in inducing peripheral tolerance, in addition to the potential complications that can arise as a result of central deletion and myeloablative therapies have led to other methods of controlling and preventing transplant rejection. The *ex vivo* expansion of immunoregulatory cell populations for use as clinical therapies has become a focus for researchers to enhance and minimise current immunosuppressive regimens and several clinical trials have resulted [19-21]. There is a vast array of evidence from animal models suggesting that Treg will be key in suppressing the rejection of allografts [161, 242-249] and Treg have been identified as a vital population for the maintenance of immune homeostasis, actively preventing autoimmunity by suppressing unwarranted immune responses against self-antigens [250-253].

**4. Regulatory T cells**

A population of T cells with the ability to suppress other thymocytes were first described by Gershon et al. in 1972 [254]. This controversial discovery led to the emergence of Regulatory T cells (Treg), which have become the focus of attention in many diseases. Seminal work by Powrie and Mason in which they reconstituted T cell deficient rats with either; CD45RC*low*CD4*+* cells or CD45RC*high*CD4*+* identified that rats reconstituted with CD45RC*low*CD4*+* cells were
protected from lymphocyte mediated organ wasting disease and the development of anti-thyroglobulin autoantibodies. Importantly, rats reconstituted with unfractionated CD4\(^+\) T cells were also free of autoimmune disease, providing evidence that CD4\(5\)RC\(^{\text{low}}\)CD4\(^+\) cells might be conferring immunoregulation [255]. A few years later, Fowell and Mason identified that autoimmune diabetes could be prevented by injecting CD4\(^+\)CD4\(5\)\(^{\text{low}}\) T cells, providing further evidence of the presence of a suppressive T cell population [256].

A reciprocal population of cells was also found in mice by Sakaguchi et al., who identified that a CD4\(^+\) T cell population expressing the IL-2 receptor \(\alpha\)-chain (CD25) was able to prevent a multitude of organ specific auto-immune diseases and GVHD [253]. This established that populations of cells existed in mice and rats that actively suppressed immune responses to self-antigens in otherwise healthy rodents.

a. Phenotypic markers of Treg

Despite the discovery that CD4\(^+\) T cells contain a subset with regulatory capacity, the identification of a cell surface marker specific to these cells has remained elusive. Although CD25 is predominantly expressed on mouse Treg, it is also upregulated on activated T effectors in humans [257]. Therefore, the CD4\(^+\)CD25\(^+\) cell population cannot purely be ascribed to Treg, however CD4\(^+\) cells with CD25\(^{\text{high}}\) expression do predominantly have suppressive function [257]. The lack of a specific maker for Treg has led to the exploration of many cell surface markers that are expressed differently between Treg and Teff. Unfortunately, none have proved to be specific to the Treg subset (see table 1), [258] however there are differential levels of expression of these markers, enabling some discrimination between T cells.
Table 1: Phenotypic markers used to discriminate Treg from Teff

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression in Treg</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD25</strong></td>
<td>Increased in mouse and human</td>
<td>High affinity α-chain of the IL-2 receptor [253, 259]</td>
</tr>
<tr>
<td><strong>CD27</strong></td>
<td>Increased</td>
<td>Lymphocyte specific TNFR superfamily member [260]</td>
</tr>
<tr>
<td><strong>CD45RB</strong></td>
<td>Decreased in mice only</td>
<td>Leukocyte Common Antigen (LCA) [242, 261]</td>
</tr>
<tr>
<td><strong>CD45RA</strong></td>
<td>Increased in naive human cells only</td>
<td>CD45RA^FoxP3^lowCD25^high^ denoted as naïve or resting Treg, CD45 is down regulated upon activation [262]</td>
</tr>
<tr>
<td><strong>CD45RO</strong></td>
<td>Increased in human cells only</td>
<td>CD45RO^ Treg increase with age in adults [263]</td>
</tr>
<tr>
<td><strong>CD62L</strong></td>
<td>Increased in mouse cells only</td>
<td>L-selectin (cell adhesion molecule) [264]</td>
</tr>
<tr>
<td><strong>CD103</strong></td>
<td>Increased in activated human and mouse cells</td>
<td>αβ7 integrin [265, 266]</td>
</tr>
<tr>
<td><strong>CD127</strong></td>
<td>Decreased in human and mouse</td>
<td>IL-7 α-chain receptor [267, 268]</td>
</tr>
<tr>
<td><strong>CD152</strong></td>
<td>Increased in human and mouse</td>
<td>Cytotoxic T Lymphocyte Antigen (CLTA-4), inhibits APC activation [269-272]</td>
</tr>
<tr>
<td><strong>CD195</strong></td>
<td>Increased</td>
<td>CCR5 chemotactic mediator for Treg [273, 274]</td>
</tr>
<tr>
<td>Marker</td>
<td>Expression in Treg</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CD304</td>
<td>Increased in human and mouse</td>
<td>Neuropilin 1, thought to distinguish peripherally induced and thymus derived (nTreg) [275, 276]</td>
</tr>
<tr>
<td>CD39</td>
<td>Increased in human and mouse</td>
<td>Ectonucleoside triphosphate diphosphohydrolase 1, converts ATP to AMP, generating adenosine [277, 278]</td>
</tr>
<tr>
<td>FR4</td>
<td>Increased</td>
<td>Folate receptor [279]</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Increased in mouse and human</td>
<td>Forkhead/winged helix transcription factor [280-283]</td>
</tr>
<tr>
<td>GITR</td>
<td>Increased</td>
<td>Glucocorticoid-induced TNF receptor [265, 284, 285]</td>
</tr>
<tr>
<td>Helios</td>
<td>Increased in human and mouse</td>
<td>Ikaros family transcription factor [286-288]</td>
</tr>
</tbody>
</table>

b. FoxP3

The identification that a mutation in the X-linked, forkhead/winged helix transcription factor gene, FoxP3 was responsible for the fatal autoimmune-like disease, observed in scurfy mice [281], provided a vital clue as to the possible genetic mechanism involved in immune regulation. Mutation of the X-linked FoxP3 mutation in hemizygous male mice leads to CD4+ T cell mediated lymphoproliferative disease, resulting in wasting and multi-organ lymphocytic infiltrates [280]. In humans, this mutation manifests itself as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) and causes a vast array of diseases including: neonatal autoimmune diabetes, increased infection susceptibility, inflammatory bowel disease, anaemia, atopic dermatitis and autoimmune disease in endocrine organs [289]. Mice genetically modified to be FoxP3- succumbed to fatal lymphoproliferative disease, similar to that seen in scurfy mice [282],
due to an absence of Treg. Furthermore, Fontenot et al. demonstrated that mice which were lethally irradiated and then given equal amounts of bone marrow from wild type mice and FoxP3 KO mice generated CD4⁺CD25⁺ T cells derived entirely from the bone marrow from wild type donors, identifying FoxP3 as a critical regulator of Treg development and function [282]. Additionally, it has been shown that ectopic expression of FoxP3 confers suppressive capacity in CD4⁺CD25⁻ T cells [282, 283, 290]. Hori et al. demonstrated that CD4⁺CD25⁻ T cells retrovirally transduced to express FoxP3 upregulated CD25, GITR and CTLA-4, cell surface markers associated with Treg, and these cells suppressed the proliferation of CD4⁺CD25⁻ T cells *in vitro* and prevented CD4⁺CD45RD<sup>high</sup>CD25⁻ effector T cell mediated autoimmune gastritis and inflammatory bowel disease in SCID mice [283].

Furthermore, Kim et al. and Lahl et al. engineered mice with diphtheria toxin receptor under the control of the FoxP3 gene locus enabling the conditional depletion of Treg. Depletion of Treg using diphtheria toxin led to fatal autoimmune disease similar to the scurfy-like phenotype [291, 292]. These data in combination with the previous research identified that FoxP3 was essential for the function of Treg and hence the maintenance of immune homeostasis in mice.

FoxP3 was considered to be exclusively expressed in mouse Treg and not upregulated upon activation [282, 283, 293], unlike in human cells in which FoxP3 can be transiently upregulated in activated Teff [294-298]. Zhou et al. engineered a mouse in which genetic fate mapping could be visualised to identify cells that had previously expressed FoxP3 by crossing FoxP3 bacterial artificial chromosome (BAC) transgenic mice with ROSA26-yellow fluorescent protein (ROSA26<sup>YFP</sup>) Cre-reporter mice. Surprisingly, 10-20% of YFP⁺CD4⁺ T cells were FoxP3⁻ suggesting a large proportion of T cells transiently upregulated FoxP3 during their development [299]. These results suggested that FoxP3 was not confined to Treg in mice. Miyao et al. built upon this hypothesis when they identified that a minor population of eFoxP3 Th cells can develop and accumulate in inflammatory cytokine milieu and lymphopenic environments [300].
Although FoxP3 cannot be entirely relied upon to distinguish Treg from Teff, the vast majority of FoxP3+ cells have a regulatory phenotype.

c. FoxP3 function

FOXP3 is a 431 amino acid protein coded by 11 exons and a member of the forkhead/winged-helix family of transcription factors. It contains a forkhead domain at its carboxyl-terminus [281, 295], which allows it to bind to DNA and localises it to the nucleus [301]. Early studies focused on its role as a transcriptional suppressor but it has also been identified to activate transcription [302-304].

d. FoxP3’s interaction partners

FoxP3 suppresses the expression of a variety of cytokines through interactions with a few known binding partners. Many of the genes regulated by FoxP3 are also under the influence of the Nuclear Factor of Activated T cells (NFAT). IL-2, IL-4 and IFN-γ expression is suppressed by FoxP3 and activated by NFAT, whereas CTLA-4 and CD25 are up-regulated by both transcription factors [283, 305, 306]. Observations by Schubert et al. suggested that FoxP3 controlled IL-2 expression by competitively binding to the same region of DNA as NFAT after it was discovered that the IL-2 promoter contains a binding site for FoxP3 and NFAT [293, 307-309]. Analysis of cell lysates identified that NFAT and FoxP3 were interacting, suggesting that FoxP3 suppressed NFAT dependent cytokine transcription by preventing NFAT from interacting with DNA [310]. Evidence that NFAT:FoxP3 forms a complex influencing the expression of cytokines such as IL-2 via the disruption of NFAT:AP-1 was provided by Wu et al. [311].

Another key interaction in FoxP3 dependent suppression of cytokine genes is that of FoxP3 and NF-κB. Betelli et al. identified that FoxP3 interacted with NF-κB to repress A20, a well-known NF-κB target gene [310], normally induced by TNF-α [312]. Additionally, FoxP3 binds to the transcription factor AML1 (acute myeloid leukaemia 1)/Runx1 (Runt-related transcription factor 1), resulting in the suppression of IFN-γ and IL-2 expression [313]. The interaction between
FoxP3 and AML1/Runx1 was not dependent on the FoxP3 DNA binding domain, suggesting that FoxP3, NFAT and AML1/Runx1 act as a complex to suppress IL-2 expression [313, 314].

TGF-β induced FoxP3 is also important for the preventing Th17 cell development and expression of Th17, via a DNA-independent reaction with ROR-γt (RORC2 in humans) [315]. The exact mechanism is unknown, however Zhou et al. provided evidence that the interaction between ROR-γt and FoxP3 was dependent on the N-terminal region of exon 2 and the FKH domain of FoxP3 [315], contrastingly Yang et al. found that FoxP3 dependent repression of Th17 cell development was not dependent on the FKH domain and a single amino acid mutation in the leucine zipper region of FoxP3 did not prevent its inhibitory interaction with ROR-γt [316].

FoxP3 also interacts with proteins important for epigenetic modifications that confer stability of FoxP3 expression and lineage commitment. Tat-interactive protein, 60kDa (TIP60) in combination with Histone Deacetylase 7 (HDAC7) co-operate with FoxP3 to suppress IL-2 expression during T cell activation [317]. In contrast HDAC9:FoxP3 interactions appear to be involved with prevention of FoxP3 interactions with DNA via the deacetylation of FoxP3. HDAC \(^{-/-}\) mice had approximately 50% more Treg in lymphoid tissues and a 3-4 fold increase in suppressive capacity than wild type controls, further supporting the HDAC9 dependent control of FoxP3 [318]. Administration of the HDAC inhibitor, trichostatin A (TSA) reduced the severity of colitis and doubled the time taken to reject a fully MHC disparate cardiac allograft in mice [318, 319].

e. **TSDR demethylation as a marker of Treg lineage commitment**

Evidence of epigenetic mechanisms determining the stability of the Treg lineage in mice was published by Floess et al. CD4\(^+\)CD25\(^+\) T cells, of which approximately 95% were FoxP3\(^+\), were fully demethylated in the conserved non-coding region of the FoxP3 gene known as the Treg cell-specific demethylation region (TSDR) [320]. A phenomenon confirmed in human Treg shortly after [321]. The DNA methylation status at the TSDR is similar between thymically
derived Treg and peripherally induced Treg, however \textit{in vitro} induced Treg tend to have a TSDR that is more methylated [320, 322, 323]. Floess et al demonstrated that 50% of Treg generated in the presence of TGF-\(\beta\) \textit{in vitro} lost FoxP3 expression when stimulated in the absence of TGF-\(\beta\), this corresponded with a partially methylated TSDR. Approximately 7% of nTreg when restimulated in identical conditions lost FoxP3 expression, corresponding with a fully demethylated TSDR [320]. These data demonstrated the importance of a heavily demethylated TSDR on the stability of FoxP3 expression.

5. Location of T cell priming post-transplant

Experiments in miniature swine, measuring the specific lysis of lymphocytes showed that graft infiltrating lymphocytes (GILS) from renal allografts were more likely to respond to third party MHC class I and II than recipient PBL, suggesting that GILs were contributing to rejection [324]. Graca et al. identified that grafts from mice tolerised using \(\alpha\)CD4 and \(\alpha\)CD8 mAbs, were not rejected when they were transplanted on to lymphopenic mice transfused with splenocytes, concluding that this is most likely due to a Treg in the graft [325]. It was subsequently shown that Treg that have infiltrated the allograft prevent graft destruction by inhibiting donor reactive memory CD8\(^+\) T cell generation [326]. Zhang et al. demonstrated that Treg initially migrated to an islet allograft, where they became activated, upregulating effector molecules, before trafficking to draining lymph nodes. In the graft and draining lymph nodes, Treg were shown to be responsible for prevention of effector T cell migration (Teff), accumulation and proliferation, and prevented DC migration in a TGF-\(\beta\) and IL-10 dependent manner [327].

Treg also prevent T cell activation in organ-draining lymph nodes in allotransplantation. Bousso et al. showed that T cell-DC interactions in lymph nodes were important for T cell activation [328-330] but whether Treg were inhibiting this activation in the context of allograft protection
was unknown. Tang et al. used two-photon laser-scanning microscopy to investigate whether Treg could prevent the priming of diabetogenic T cells in the draining lymph nodes of NOD mice. Using this approach the authors demonstrated that Treg prevented T cell activation via interactions with antigen bearing DCs, thereby preventing the priming of autoreactive CD4⁺CD25⁻ Th cells [331]. Tadokoro et al. utilised an EAE model to demonstrate that Treg inhibit stable contacts between CD4⁺ T cells and DCs in lymph nodes [332]. The prevention of stable contacts between DCs and T cells by Treg in lymph nodes is likely to be important for the reduction of T cell activation [326]. Walker et al. demonstrated that antigen dependent proliferation of Treg in lymph nodes was also important for suppression of Teff [333] and Treg mediated suppression was also measured in a skin allograft model [334].

6. Molecular mechanisms of suppression by Treg

Treg use a variety of mechanisms to suppress a variety of immune responses, such as the inhibition of proliferation, activation and cytokine production by CD4⁺ and CD8⁺ T cells, DC activation and B-cell antibody production and class switching [245, 335-339]. Treg utilise a variety of mechanisms including: anti-inflammatory cytokines; disrupting metabolic pathways; expression of cell surface proteins and cytolysis [340].

a. CTLA-4

Initial experiments blocking CTLA using a mAb did not prevent Treg suppression in vitro [259]. This was contradicted by two subsequent papers that concluded that a blocking αCTLA-4 mAb prevented Treg suppression in vitro [269, 341]. Administration of an αCTLA-4 mAb abrogated regulation mediated by Treg in a skin transplant model [243]. Mice in which CTLA-4 was knocked out in Treg were less able to down regulate CD80 and CD86 on APCs. They were also unable to
maintain self-tolerance and immune homeostasis, identifying CTLA-4 as key for Treg function in vivo [272].

b. TGF-β

Multiple studies have demonstrated that blocking TGF-β using an αTGF-β mAb does not abrogate Treg suppression of effector cells in vitro [259, 269, 342, 343]. Despite these data, Nakamura et al. identified that CD4⁺CD25⁺ T cells were able to secrete TGF-β when stimulated with αCD3 and αCD28 in vitro and addition of αTGF-β antibodies abolished suppression by these cells [344]. Further experiments identified that TGF-β was complexed with latency-associated peptide (LAP) and expressed on the surface of activated Treg. This complex was shown to play an important role in the suppression of proliferation of activated T cells and could induce the generation of CD4⁺FoxP3⁺ cells in vitro, therefore inferring infectious tolerance in a TGF-β dependent manner [345]. Further work by Tran et al. showed that activated FoxP3⁺ T cells also express GARP (Glycoprotein A repetitions predominant) which binds to and is required for cell surface expression of the latent TGF-β complex [346]. It was subsequently shown that activated TGF-β1 is released from the GARP:latent-TGF-β complex by integrin β8 [347]. The function of the GARP:latent-TGF-β complex is unknown, however activated nTreg from GARP⁻/⁻ mice were inefficient at inducing FoxP3 expression when compared with wt Treg. It is therefore possible that Treg expression of GARP is important in tolerance induction [348], although further work is required to confirm this.

c. IL-10

Experiments performed by Thornton and Shevach demonstrated that CD4⁺CD25⁺ cells from IL-10⁻/⁻ mice were as effective at mediating suppression as wild type cells in vitro and that the suppression by these cells was contact dependent [259]. This conflicted with later studies by Kingsley et al. who showed that alloantigen specific Treg were unable to prevent the rejection of a skin allograft in mice that received αIL-10R antibody [243]. The importance of IL-10 for Treg
function in vivo was corroborated in models of Treg-dependent suppression of airway hypersensitivity and inflammatory bowel disease [349, 350]. It was not known that Treg themselves were the source of the IL-10 until Uhlig et al. closely examined IL-10 secretion during the resolution of intestinal inflammation. The authors identified that the resolution of intestinal inflammation was dependent on the presence of IL-10 producing Treg, as Treg from IL-10−/− mice were unable to resolve disease [351]. Treg expression of IL-10 was supported further by Maynard et al. and Rubstov et al. [352, 353].

Additionally, both human and murine CD4+ T cells chronically activated in the presence of IL-10 give rise to a Treg population designated as Tr1 cells [354]. These cells, which interestingly are FoxP3−, were capable of preventing antigen driven proliferation of CD4+ T cells and preventing colitis in SCID mice.

d. IFN-γ

IFN-γ was initially identified as having pro-inflammatory properties and contributing to acute transplant rejection as increased IFN-γ expression had been identified in cardiac, renal and pancreatic islet allografts [133, 355, 356]. Indeed IFN-γ was shown to precipitate acute rejection in animals tolerant to donor antigens [131]. Interestingly however, in vivo neutralisation of IFN-γ did not prolong survival of allogeneic skin grafts in mice or rhesus monkeys [357, 358]. Saleem et al. also demonstrated that IFN-γ−/− mice rejected allogeneic heterotopic heart transplants at the same rate as wild type recipients [359] and IFN-γ−/− splenocytes demonstrated increased proliferation and CTL activity when stimulated with allogeneic cells, implicating IFN-γ in the down regulation of alloimmune responses in vitro [360]. By comparing the induction of long-term allograft acceptance between IFN-γ−/− and wild type mice, using an established tolerance inducing protocol, Konieczny et al. provided evidenced that IFN-γ is vital for long term allograft survival by blocking the CD28 and CD40L costimulation pathways for T cells [137]. Furthermore, data published by our group demonstrated that Treg prevented skin graft rejection by their
ability to secrete IFN-γ [138]. IFN-γ triggers the STAT1-AKT signalling pathway to control the regulatory function of Treg and it is thought that IFN-γ acts on Treg in an autocrine manner [361]. Additionally, a protocol to enrich for alloantigen-reactive Treg in vitro, developed by our group, relies on the addition of exogenous IFN-γ [140]. IFN-γ acts on CD4+ T cells to induce apoptosis as cells that are IFN-γ−/− were resistant to AICD and IFN-γ was shown to act directly on CD4+ T cells during mycobacterial infection to promote apoptosis [362, 363], suggesting another possible mechanism of IFN-γ by Treg.

e. **IL-35**

Collison et al. identified that mouse FoxP3+ Treg cells but not CD4+ Teff cells expressed Epstein-Barr-virus-induced gene 3 (Ebi2, which encodes IL-27β) and IL-12α, and that a novel cytokine consisting of a heterodimer of Ebi3 and IL-12α is secreted by Treg and not Teff. This heterodimeric cytokine of the IL-12 family was designated IL-35 [277]. Expression of IL-35 by Treg as well as addition of recombinant IL-35 suppressed T cell proliferation in vitro. Additionally Collison et al. identified that Ebi3 and IL-12α deficient Treg were unable to control IBD in mice. IL-35 induced a stable regulatory phenotype in human and mouse T cells, independent of FoxP3 expression. These cells were termed iT(R)35 cells [364] and remained suppressive in a variety of in vivo models. Taken together these data showed that IL-35 has potent suppressive effects and that it can induce a regulatory population of T cells. Furthermore it also appears to be important for optimal suppression by Treg.

f. **Adenosine and cAMP suppression**

Bopp et al. identified that nTreg contain high levels of cyclic adenosine monophosphate (cAMP), a second messenger previously described as a potent inhibitor of T cell proliferation and IL-2 synthesis in T cells [365]. Bopp et al. showed that cAMP mediated suppression by Treg was abolished by a cAMP antagonist or when a gap junction inhibitor was used, suggesting that
cAMP mediated suppression by Treg relies on cell to cell and is transferred via gap junctions to suppress IL-2 production by Teff [366].

Hydrolysis of ATP to ADP or AMP by the ectoenzyme CD39 and CD73 resulted in the production of soluble adenosine [367] which binds to the extracellular adenosine receptor A2a and inhibits T cell activation and expansion [368]. CD39 expression on human Treg is expressed by highly suppressive subset [369] and Treg in CD39 knock-out mice have reduced suppressive capacity in vitro and in vivo [370]. In human T cells ATP has also been shown to directly increase cAMP via the P2Y G protein coupled nucleotide receptor [371]. Zarek et al. provided evidence that A2a receptor signalling induced Treg generation and made T cells anergic [372]. These data suggest that adenosine is an important molecule used by Treg to suppress T cell responses.

g. Galectins

Galectin-1 is a member of the highly conserved family of β-galactoside binding proteins and had been implicated in tumour immune privilege, suggesting an influence over regulatory arms of the immune response [373]. It was discovered that Treg preferentially express this homodimer compared with CD4⁺CD25⁻ cells [374]. Interestingly, blockade of galectin-1 reduced Treg mediated suppression in vitro and Treg purified from galectin-1 null mice exhibited reduced regulatory activity, suggesting an important suppressive effect of this molecule. Galectin-1, potentially expressed from Treg has also been identified to endow DCs with tolerogenic properties, namely by promoting IL-10 mediated T cell tolerance and suppressing autoimmune neuroinflammation. DCs lacking galectin-1 were less capable of resolving autoimmune pathology [375].

h. Killing of target cells

Mechanisms of Treg-induced apoptosis have emerged. Firstly, cytolysis of target cells by granzymes A and B have been described. CD4⁺CD25⁺ T cells from granzyme B⁻ mice had 2-3 fold reduced capacity to suppress CD4⁺CD25⁺ T cell proliferation compared with wild type
CD4⁺CD25⁺ T cells in vitro, however no reduction in Treg suppressive capacity was seen in mice that were perforin deficient [376]. Grossman showed that activation of CD4⁺ T cells with anti-CD3/CD28 or anti-CD3/CD46 to generate Tr1 cells induced expression of granzyme B but not granzyme A and that these granzyme expressing Tr1 cells were able to kill target cells in a perforin dependent manner [377]. Interestingly models of GVHD have identified that granzyme B is not essential for Treg mediated suppression of GVHD [378], however granzyme A is [379]. Velga et al. analysed the functional role of granzyme A in a mouse model of GVHD. Adoptive transfer of CD4⁺CD25⁺ Treg from granzyme A⁻⁻ mice, which were highly suppressive in vitro, could not prevent GVHD even though they were able to migrate to inflammatory sites [379]. Taking these data into consideration it appears the context of the immune response determines whether granzyme A or B is necessary for Treg function.

Secondly, there’s evidence that Treg compete with T effector cells for IL-2 [259]. It has been shown that CD4⁺CD25⁻ cells undergo apoptosis during in vitro suppression assays including Treg [380]. Addition of IL-2 to the cultures reduced apoptosis of effector cells by approximately 70% and Treg were consuming large amounts of IL-2. Pandiyan et al. also showed that Teff deficient in Bim (a caspase activating protein that promotes cytokine deprivation-induced cell death) were spared from Treg induced apoptosis in this system. Additionally killing was not dependent on granzyme or TNF-α [380].

1. Suppression of Antigen presenting cells (APCs)

Larson et al. discovered that (ASxWF)F1 rat transplanted with (ASxAUG)F1 kidneys, which had previously been transplanted into AS recipients, did not lead to acute graft rejection, however adoptive transfer of (ASxAUG)Fx DCs at the time of transplantation led to rejection [70], uncovering a vital role for DCs in transplant rejection. Treg have been shown to down regulate important DC costimulatory molecules, such as CD80 and CD86, and the inhibition of stable contacts between CD4⁺ cells and DCs have been identified as important mechanisms of Treg
mediated suppression of DCs in vitro [338, 339]. Co-culture of CD4⁺CD25⁺ T cells with bone marrow derived DCs for 2 days in vitro showed that CD4⁺CD25⁺ T cells could reduce DC expression of CD80 and CD86 in a dose dependent manner. CD4⁺CD25⁺ Treg also down regulated CD80 mRNA levels in DCs but not CD86, suggesting that the cell surface expression of this molecule may be regulated by an alternative mechanism [338]. It has also been shown that CD4⁺CD25⁺ nTreg induced the down-modulation of CD80 and CD86 expression in vitro, in a CTLA-4 dependent manner. Oderup et al. cultured splenic DCs with anti-CD3 antibody and CD4⁺CD25⁺ T cells in the presence or absence of CD4⁺CD25⁺ Treg for 42 hours. CD80 and CD86 expression in cultures in which Treg were present revealed between 50%-150% reduction in expression of both co-stimulatory molecules, depending on the time spent in culture. Addition of 100µg/mL anti-CTLA-4 antibody reversed the decrease in expression of CD80 and CD86, identifying an important role for CTLA-4 [381]. Treg from a NOD background were shown to inhibit DC induced activation of 8.3-CD8⁺ T cells in a CTLA-4 dependent manner as measured by a reduction in the expression of IFN-γ from the T cells [382]. Treg expressing CTLA-4 have been shown to remove CD80 and CD86 from opposing cells via a process known as trans-endocytosis, these ligands are then degraded in the Treg thereby preventing costimulation via CD28 [383].

Another mechanism of Treg mediated control of DC responses is by the induction of the enzyme indolamine-2,3-dioxygenase (IDO) via a CTLA-4 dependent mechanism [384]. In the presence of IFN-γ, IDO catalyses the conversion of tryptophan to N-formylkynurenine, therefore starving T cells of the essential amino acid. Additionally kynurenine and other catabolites of tryptophan can induce T cell apoptosis [385].

Previous work suggested that Treg suppression of DC activation was cell contact dependent [259, 343, 386-388], however it wasn’t until Tang et al. utilised a combination of advances, particularly two-photon laser-scanning microscopy (TPLSM), that this was confirmed. Tang et al. identified visually that Treg were able to prevent the priming of diabetogenic CD4⁺CD25⁺ T
cells in a NOD mouse by directly interacting with islet antigen presenting dendritic cells within the lymph nodes [331]. The prevention of T cell priming appears to be, at least in part, a result of increased periods of interaction between Treg and immature DCs (iDCs), which is dependent on Neuropilin-1 expression by Treg [389]. Additionally, development of video microscopy and imaging flow cytometry techniques recently described by Juvet et al. [390] enabled the analysis of DC activation in different situations of Treg delivery. T cell and DC interactions were compared between naïve and tolerised mice. The frequency of prolonged T cell and DC interactions was increased 2.6 fold in mice that had not been tolerised to alloantigens, which could be useful as a predictor of graft outcome [390].

Human Treg express both granzyme A (thymically derived Treg) and B (induced Treg) and display perforin-dependent killing of allogeneic and autologous APCs in vitro [391]. They have also been shown to kill B cells in a contact dependent manner dependent on perforin and granzymes [336]. Misra et al. identified that human Treg inhibit the maturation and function of immature monocyte derived dendritic cells, independently of IL-10 and TGF-β suggesting that this mechanism may also be a result of Treg mediated killing [339]. Kryczek et al. in 2006 identified that Treg can trigger high levels of IL-10 production by APCs stimulating the induction of B7-H4 which in turn renders the cells immunosuppressive [392].

The mechanisms utilised by Treg to mediate suppression are varied and enable Treg to exert suppression over a wide range of cells, facilitating their role as masters of regulation.

7. Treg as a cellular therapy in transplantation

   a. Alloantigen-driven Treg and polyclonally expanded nTreg

Due to the low frequency of Treg in peripheral blood, expansion will be required prior to clinical use. Tang et al estimated that adult humans have approximately $13 \times 10^9$ Treg, which equates to
less than 10% of the CD4⁺ T cell pool. Mouse models of transplantation suggest that a ratio of 1:1 or 1:2 of Treg to effectors is required to regulate transplant rejection [242, 246], although these observations were made in lymphopenic mice and might not be representative of a lymphoreplete animal. Indeed, in animals tolerised with preconditioning using non-depleting αCD4 mAb and DST, no increase in Treg number was observed [393] suggesting that a small number of alloantigen-reactive cells might be sufficient to control allograft rejection.

The most efficacious population of Treg will not be known until randomised clinical trials have been concluded, which will take many years. There are a number of different populations of Treg requiring consideration; including polyclonally expanded nTreg, alloantigen-driven Treg, induced Treg and umbilical cord blood (UCB) derived Treg.

Perhaps the most successful method of expanding Treg *in vitro* has been using polyclonal stimulation using either soluble or plate bound αCD3 antibody with or without αCD28. Levings et al. were the first to describe the *ex vivo* expansion of magnetically isolated human CD4⁺CD25⁺ T cells from peripheral blood. They were able to achieve a forty-fold expansion when stimulating these cells with soluble αCD3 antibody and autologous PBMCs [388]. Three years later Hoffman et al demonstrated that stimulating CD4⁺CD25<sup>high</sup> T cells with artificial APCs presenting αCD3 and αCD28 in high doses of IL-2 yielded a forty-thousand fold expansion of Treg [394]. Hippen et al. reported that 4 rounds of stimulation of human nTreg, from peripheral blood, with artificial APCs, expressing high affinity Fc receptor and CD86, could achieve a 50 million fold expansion with minimal loss of functional activity in a model of GVHD [395]. Importantly, Hoffman, Hippen and Levings demonstrated that Treg were not an anergic population and could be expanded *in vitro*, therefore paving the way for clinical trials.

Unfortunately, expanding mouse Treg *ex vivo* has been more difficult. Although a 7,000 fold expansion of mouse nTreg has been reported by stimulating cells with bound αCD3 and αCD28 antibodies in the presence of IL-2 (10ng/mL) for 11 days [396], it was only possible to achieve a
10 fold expansion of mouse nTreg when replicating this method. 1x10^5 of these expanded nTreg were unable to prevent 1x10^5 CD4^+CD25^- Teff rejecting fully MHC mismatched grafts, whereas freshly isolated nTreg prolonged graft survival to >100days (personal communication with Thomas Chan).

Alloantigen-driven Treg are an attractive alternative to polyclonally expanded nTreg as they may be more potent [397, 398] and have fewer side effects due to previously published data showing that alloantigen-driven Treg have a more focused TCR repertoire [397] which theoretically should be less likely to broadly immunosuppress the immune system. It is however more difficult to expand cells using alloantigen and it is unlikely that they will be useful for cadaveric donation, at least at the time of transplantation. De novo induction of Treg (iTreg) from CD4^+CD25^- T cells is possible using TGF-β [399-401] and this has since become the gold standard method to generate iTreg in vitro. However, these iTreg readily lose FoxP3 expression upon restimulation and have the capacity to develop an effector memory phenotype and become pathogenic in vivo. Our group has developed two methods of generating Treg in vitro, using IFN-γ or a PDE3 inhibitor, cilostamide [140, 247, 402]. These protocols use bone marrow derived DCs, cultured in GMCSF and TGF-β, prior to their use as APCs to stimulate CD4^+ T cells in IFN-γ or cilostamide enriched media. IFN-γ efficiently enriches for Treg which prevent the rejection of allogeneic heart and skin grafts in lympho-depleted mice. PDE3 inhibition using cilostamide generates 2-5 fold more Treg than the IFN-γ method but it is less reliable, therefore methods to improve the PDE3 inhibition protocol were explored to facilitate direct comparisons between alloantigen-driven Treg and nTreg.

Few direct comparisons of alloantigen-driven Treg and activated nTreg have been made. Sagoo et al. stimulated human nTreg with allogeneic CD1c^+ DCs and used activation markers CD69 and CD71 on Treg to identify cells which were responding to alloantigen. These alloantigen-reactive Treg were more potent suppressors of donor specific responses by T cells than third party Treg.
and were more suppressive than polyclonally activated nTreg. *In vivo* experiments identified that human skin grafts on NOD.SCID.γc−/− mice were more protected by alloantigen-driven nTreg than polyclonally expanded nTreg [398]. Although Sagoo et al. tried to ensure that the cells were activated to the same degree, it is impossible to know whether or not the difference in potency demonstrated by alloantigen-driven nTreg is due to differences in activation status or whether their improved precursor frequency for alloantigen improved efficacy. They also described a mean of 1000 fold expansion over a 4-6 week period [398]. Compared to the 50million fold expansion described by Hippen et al., this demonstrates that it is much less efficient to expand Treg using alloantigen than using polyclonal stimulus. Putnam et al. demonstrated that CD4+CD25+CD127lo nTreg could be expanded using allogeneic B cells and that these cells were more efficacious than polyclonally expanded nTreg *in vitro* and in the same skin allograft model used by Sagoo et al. [397]. Unfortunately neither of these studies reported graft outcome data.

The first in human trial of Treg used expanded autologous Treg. Trzonkowski et al. expanded CD4+CD25+CD127lo Treg, on the basis that the vast majority of Treg can be isolated by FACS in this way [268], using αCD3 and αCD28 mAb labelled beads in high concentration of IL-2 [20] prior to administration to two HSCT patients. One of the patients, suffering from chronic GVHD had steroid responsive bronchiolitis obliterans but was intolerant of steroid withdrawal. The patient received one dose of 1x10⁵/kg expanded Treg, after which immunosuppressive drugs were stopped and lung function improved. The second patient who had acute GVHD showed a slight improvement following 3 doses of approximately 6x10⁷ nTreg. Immunosuppression was continued however, until the patient died. Trzonkowski et al. concluded that expanded nTreg were safe for patients with GVHD and they may indeed have efficacy in humans. A later trial assessing the efficacy of unexpanded nTreg from donor leukapheresis products in 28 high risk leukaemia patients demonstrated that doses up to 4x10⁶ nTreg/kilo were not only safe but only 2 out of the 28 patients experienced Grade II or higher GVHD and none developed chronic GVHD
These two trials suggest not only that Treg therapy is safe in humans but that adoptively transferred Treg may be able to control immune responses in humans.

b. Timing of administration

A key area of uncertainty for Treg as a clinical therapy is the optimal time of administration of the cells and whether multiple treatments will be required. The timing may also be determined by the type of transplantation (cadaveric versus living). Work by our group has demonstrated that expanded nTreg can be cryopreserved without any loss of function [248], facilitating flexible treatment using polyclonally expanded nTreg. However, this has not been applied to alloantigen-reactive Treg, possibly limiting this type of Treg’s application in the clinic, at least in the immediate period post-transplant. A key question for consideration was the timing of Treg in this thesis.

8. Immunosuppression

a. Symphony Study

Since the Symphony Study, most UK centres now use a regimen based on anti-CD25 induction, low dose Tacrolimus, MMF and steroids [403, 404], with Sirolimus (Rapamycin) recommended for patients who do not tolerate CNIs (http://guidance.nice.org.uk/TA85). One of the most important factors to consider with regard to the use of Treg in transplant patients is how immunosuppressive drugs will affect Treg function, as it will not be possible to trial them in humans without immunosuppression. Therefore mouse models of transplantation must be used to answer this question.

b. Rapamycin

Rapamycin (also known as Sirolimus) is a macrolide antibiotic produced by streptomyces hygroscopicus and is also a potent immunosuppressant. It was discovered on Easter Island by
Suren Sehgal and is used extensively in solid organ transplant recipients [405, 406]. Rapamycin suppresses proliferation of $\text{CD}4^+$ $\text{FoxP3}^-$ T cells by blocking mammalian target of Rapamycin (mTOR) and interestingly appears to preferentially suppress $\text{CD}4^+$ $\text{FoxP3}^-$ T cells in mice and humans. Rapamycin is commonly added to in vitro Treg expansion protocols [395, 407] and prolonged Rapamycin administration in vivo appears to increase Treg proportions in human renal transplant recipients [408]. This preferential targeting of non-Treg appears to be due to differences in intracellular signalling pathways. Treg are less dependent on the PI3K/mTOR pathway than Tconv as Treg constitutively express PTEN [409]. Additionally, Basu et al. identified that Treg express the protein kinase, PIM2 and introducing $\text{FoxP3}$ into $\text{CD}4^+\text{CD}25^-$ T cells leads to expression of PIM2 enabling cells to proliferate in the presence of mTOR inhibition, potentially preferentially allowing Treg over Tconv to avoid the suppression by mTOR inhibitors [410]. Interestingly, culture of T cells with a PI3K inhibitor resulted in similar proliferation profiles for Treg and Teff. As the proliferation benefit reported in the presence of Rapamycin was lost in the presence of a PI3K inhibitor, these data suggest that PI3K is the dominant protein in the regulation of this intracellular signalling pathway [411].

Zeiser et al. provided the first evidence that Treg could be used in combination with Rapamycin in a model of GVHD [412]. Alloantigen-driven Treg were capable of preventing the rejection of a cardiac allograft in lymphoreplete mice when used in combination with Rapamycin [413] and importantly this synergistic effect was also seen in a humanised mouse model using human Treg [414]. These data suggest that Rapamycin might be synergistic with Treg therapy in human transplant recipients.

c. Tacrolimus

Tacrolimus (FK-506) was one of the first macrolide immunosuppressants discovered in 1987 from the fermented broth of Streptomyces tsukubaensis [415]. Tacrolimus belongs to a group of drugs known as calcineurin inhibitors (CNIs), which also includes cyclosporine (CsA).
Tacrolimus acts as a prodrug, binding the immunophilin, FK506 binding protein (FKBP) [416], preventing the activation of the protein phosphatase, calcineurin [417]. This in turn prevents the dephosphorylation of the nuclear factor for activated T cells (NF-AT) and the activator protein 1 (AP-1) [418, 419]. NF-AT is prevented from entering the nucleus and the transcription of genes involved in T-cell activation, particularly IL-2 is suppressed [420]. Although Tacrolimus binds to the same intracellular protein family as Rapamycin, its mechanism is markedly different, possibly explaining the differences in the effects of each drug on Treg. CNIs have been associated with reduced numbers and decreased function of Treg in renal transplant recipients, when compared with patients who received Rapamycin [408, 421, 422]. GVHD models also suggest that Tacrolimus will not be synergistic with Treg therapy [412], however other in vivo evidence suggests that CsA, at low concentrations, can be used in addition to Treg therapy to achieve long term graft survival [423]. Therefore, CNIs might not be entirely prohibitive to Treg function in vivo. Additionally, low doses of CsA in a model of tolerance induction, facilitated Treg generation and enabled long term survival of a heart allograft [424], whereas higher doses prevented Treg induction. In a cross-sectional study of the effects of CNI and Rapamycin on Treg in children with stable grafts, patients on CNI monotherapy, with CNI levels of >3.6ng/mL, had Treg that were less effective compared with patients with CNI levels of <3.6ng/mL [425]. This lack of clarity necessitates further investigation to ascertain whether Tacrolimus can be used in combination with Treg therapy.

d. Mycophenolate Mofetil (MMF)

Nephrotoxicity caused by CNIs led to the development of MMF, a prodrug of mycophenolic acid (MPA). Nearly a century passed between the discovery of MPA by Bertolomeo Gosio and its approval by the FDA for use in transplant recipients [426]. MMF is converted to its active form MPA in the liver, which then inhibits an isoform of inosine monophosphate dehydrogenase (IMPDH), mainly expressed in T and B lymphocytes. This prevents the synthesis of guanosine nucleotides, leading to cytostasis. Other methods of inhibition include preventing glycosylation,
reducing expression and function of adhesion molecules and inducing apoptosis in human T-lymphocytes [427, 428]. Similarly to Rapamycin, Zeiser et al. showed that MMF preserved Treg function in a model of GVHD [412] and reverted CNI attenuation of Treg function in vitro [429], suggesting that MMF could be used in combination with Treg therapy.

e. Methylprednisolone (MP)

Corticosteroids have been an important part of maintenance immunosuppression protocols since the early 1960s, initially as a supplement therapy to azathioprine (AZA) [430, 431]. However, the side effects associated with long term usage necessitate more targeted therapies. Unfortunately, there is a lack of transplant-based literature of their effects on Treg, however literature from other disciplines suggest that steroids are not permissive to Treg therapy [432-437]. Chen et al. showed that glucocorticoids could amplify Treg in vivo when administering dexamethasone (5mg/kg/day) in combination with IL-2 (300,000 U/mouse/day) i.p., however, treatment of mice with dexamethasone alone led to a reduction in the number of splenic CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, suggesting that glucocorticoids in isolation are detrimental to Treg in vivo [437]. Two further studies demonstrated that glucocorticoids decreased Treg numbers in humans and mice respectively [433, 436]. Therefore, it appears that steroids will not be permissive for adoptive transfer of Treg as a therapy in solid organ transplant recipients. However, Methylprednisolone’s impact on survival and function of adoptively transferred Treg remains poorly understood and further mouse studies may help to clarify this.

9. Effects of immunosuppression on tolerance induction protocols

A multitude of tolerance induction protocols that rely on Treg induction have been described and have since been used to question whether immunosuppressive drugs affect Treg induction in vivo. Li et al. showed that treating C3H/He recipients with αCD40L and CTLA-4lg mAbs
induced tolerance to BALB/c heart grafts. Treatment of mice with CsA in addition to tolerance induction led to blockade of tolerance as all grafts rejected (MST 29d), whereas Rapamycin led to long term survival. However, a dose of Rapamycin was used that led to long term survival in the absence of tolerance induction, therefore making the validity of the Rapamycin data questionable [438]. Treatment of non-human primates with αCD154 prevented acute renal allograft rejection, however this was abrogated by tacrolimus or MP plus MMF. Contrasting, a short course of MP plus long term MMF dosing did not have a negative effect on graft survival [439]. Stock et al. showed that corticosteroids inhibited the induction of respiratory tolerance by blocking the development of Treg in vivo. BALB/c mice were exposed intranasally to 100µg/day on days 0-2 to OVA, the addition of 100µg/day dexamethasone on days -1 and +1 prevented the generation of T cells with a characteristic cytokine profile of Treg (high levels of IL-10 and low levels of IL-4 and IFN-γ), which appeared to be in part due to a lack of tolerance inducing DCs [432]. Blaha et al. tested whether short term use (1 month) of clinically relevant drugs interfered with a tolerance induction protocol involving non-myeloablative total body irradiation (3 Gy), fully allogeneic BMT and administration of αCD154 mAb and CTLA4Ig [440, 441]. CsA and Tacrolimus inhibited tolerance induction whereas Rapamycin, MP and MMF did not, suggesting that only CsA and Tacrolimus affect de novo induction of Treg in vivo in this context, although it is not clear how the authors selected the doses of drugs. This is vital to know, as excess of all drugs are likely to have a negative effect on Treg generation. De novo induction of Treg is likely to be an important aspect of successful Treg therapies, therefore drugs that do not prevent this process will be needed for preventing graft rejection. Additionally, drugs that do not prevent Treg generation in vivo are more likely to be less detrimental to adoptively transferred Treg. It would appear that Rapamycin and MMF are favourable for de novo Treg induction in vivo, whereas CNIs are not. However there appears to be a lack of clarity surrounding steroids.
10. Aims of Thesis

a. Global Aim

To study the effects of clinically relevant immunosuppressive drugs on adoptively transferred Treg in a heterotopic heart allograft model, for the purpose of guiding drug regimens in future clinical trials involving Treg as a cellular therapy in solid organ transplantation.

b. Specific Aims

i. To compare the efficacies of alloantigen-driven Treg and polyclonally activated autologous Treg in vivo.

ii. To ask when is the optimal time to adoptively transfer Treg.

iii. To titrate suboptimal doses of Rapamycin, Tacrolimus, MMF and MP to use in combination with adoptively transferred Treg to ask whether Treg can be used as a combination therapy with clinically relevant immunosuppressive drugs.
Chapter 2: Materials and methods

2.1 Mice

Sex matched mice, 6-12 weeks of age were used in all experiments. Animal care was conducted in accordance with the Animals (Scientific Procedures) Act 1986. D. Kioussis, Division of Molecular Immunology, National institute for Medical Research, Mill Hill, London, UK kindly provided CBA-Rag⁻ mice.

Table 2: Mouse strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Abbreviation</th>
<th>H2 Haplotype</th>
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<th>Class II</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>K</td>
<td>D</td>
</tr>
<tr>
<td>CBA/Ca</td>
<td>CBA</td>
<td>k</td>
<td>k</td>
<td>k</td>
</tr>
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<td>b</td>
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<tr>
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<td>CBA Rag</td>
<td>k</td>
<td>k</td>
<td>k</td>
</tr>
<tr>
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<td>B6 GFP</td>
<td>b</td>
<td>b</td>
<td>-</td>
</tr>
<tr>
<td>CBA/ Ca FoxP3 GFP</td>
<td>CBA GFP</td>
<td>k</td>
<td>k</td>
<td>k</td>
</tr>
<tr>
<td>C57BL/6 Rag⁻/⁻</td>
<td>B6 Rag</td>
<td>b</td>
<td>b</td>
<td>-</td>
</tr>
<tr>
<td>BALB/c</td>
<td>-</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
</tbody>
</table>

2.2 General anaesthesia and analgesia

Domitor/Ketaset anaesthetic was prepared by diluting Ketamine hydrochloride (Ketaset, Fort Dodge Animal Health Ltd.) to a final concentration of 7.6µg/mL and medetomidine hydrochloride was diluted at a final concentration of 100µg/mL in sterile 0.9% w/v saline. Metecam was diluted in 0.9% w/v sterile saline to a working concentration of 250µg/mL.
Vetergesic analgesic working solution was prepared to a final concentration of 10mg/mL by diluting buprenorphine hydrochloride (Vetergesic, Reckitt Benckiser Healthcare Ltd.) in sterile 0.9% w/v saline.

Antisedan anaesthetic reversal working solution was prepared at a final concentration of 100µg/mL by diluting atipamezol hydrochloride (Antisedan, Pfizer Pharmaceuticals Ltd.) in sterile 0.9% w/v saline.

For removing of bandages on day 7 post skin grafting, 200uL Domitor/Ketaset working solution was injected subcutaneously. For heterotopic cardiac transplantation and skin transplantation, 300µL Domitor/Ketaset working solution and 300µL Vertergesic analgesic working solution was injected subcutaneously. Post-operation, 150µL Metecam working solution was provided as analgesia and 300µL Antisedan was used as the reversal agent.

2.3 Skin grafting

CBA-Rag mice were reconstituted with cells intravenously via the tail vein the day prior to transplanting full-thickness B6 tail skin allografts onto graft beds prepared on the flanks of reconstituted mice. The skin graft was prepared by removing tail skin from sacrificed donor mice and cutting it into grafts of approximately 10mm in length. The graft, once placed on the graft bed was secured by wrapping the mouse with plasters. The dressing was removed 7 days post-transplant and grafts were monitored for graft rejection regularly. Graft survival was compared using the Kaplan-Mayer log-rank test using PRISM software.
2.4 Heterotopic heart transplant (adapted from Corry and Russel method [442])

2.4a Donor heart harvesting
Donor mice were lethally anaesthetised by injecting 500µL Domitor/Ketaset anaesthetic subcutaneously. A midline incision in the abdomen was made and the bowels were reflected to the side of the mouse. The mouse was exsanguinated with a heparinised syringe via the inferior vena cava (IVC) and approximately 800µL heparinised saline (15U/mL heparin in sterile 0.9% w/v saline) was injected. The ribs were then incised laterally and the thoracic wall pulled up towards the head of the mouse and secured in place with autoclave tape. The IVC, the superior vena cava and azygous vein were ligated with 6-0 silk. The pulmonary artery and ascending aorta were cut with surgical scissors and carefully separated to mobilise them. All remaining vessels were ligated by making a loop around them with 6-0 silk. The heart was stored in ice cold sterile 0.9% w/v saline solution.

2.4b Recipient operation
The mouse was anaesthetised and a midline abdominal incision was made, the abdomen was held open using retractors. The contents of the abdomen were reflected onto the chest of the mouse and kept moist by covering with gauze moistened with sterile saline. Branches of the descending aorta were ligated using 6-0 silk monofilament suture. Aneurysm clips were applied to isolate a section of aorta and IVC. A venotomy was made in the aorta using a 26 gauge needle and extended to desired length using microsurgical scissors. The donor heart was then positioned to allow stay sutures to be placed at the proximal and distal apexes of recipient abdominal aorta with the donor’s aorta. The anastomosis was made using continuous sutures. This was then repeated to anastomose the donor’s pulmonary artery to the recipient’s vena cava. After the anastomosis is completed the distal clip was flashed to check leakage. Any leakage was controlled using Spongostan™ (Johnson & Johnson). The abdominal contents were returned to the abdominal cavity and the muscle and skin were closed in two layers using 4-0
continuous sutures. The anaesthetic was reversed using 300µL Antisedan working solution and given analgesia immediately post-operation and then after 24 hours.

2.5 Cell culture

All cells were cultured in R10 media which consisted of 450mL RPMI medium (Roswell Park Memorial Institute, PAA laboratories GmbH, Austria), 50mL heat inactivated FCS (Fetal Calf Serum, PAA laboratories gmbH, Austria), 2mM L-glutamine (PAA laboratories gmbh, Austria), 100units/mL of penicillin and streptomycin (Sigma-Aldrich) and 0.5mM 2-mercaptoethanol (Sigma-Aldrich).

2.5a Generation of bone marrow derived dendritic cells (GT-DCs)

Bone marrow derived dendritic cells were generated from B6 or B6 IL-6−/− (kind gift from Professor Kathryn Else, The University of Manchester) donors. Mouse femurs were removed and placed in a 60mm Petri dish with PBS (2% FCS). Both ends of the bone were cut and bone marrow was flushed with approximately 5mL PBS (2% FCS) using a 10mL syringe and 29G needle inserted into the cavity. RBCs were lysed by hypotonic lysis. B cells, T cells and MHC class II positive cells were depleted using specific antibodies (RA3.6B2, YTS 3.1, YTS 169, TIB120) followed by negative selection using anti-rat magnetic Dynabeads. 1×10⁶ enriched DC precursor cells were placed into 24 well plates in 1mL of R10 medium supplemented with 2ng/mL each of recombinant mouse GMCSF and recombinant human TGF-β (PeproTech, London, UK); 75% of the medium was changed every 48 hours. After 6 days, DCs were harvested, washed in R10 and counted prior to use or cryopreserved for later use.

2.6 Cell isolation

2.6a Enrichment of CD4+ cells

Single cell splenocyte suspensions were prepared by mashing spleens through sterile 0.7µm cell strainers (Falcon) and washing in ice cold PBS with 2% Heat inactivated FCS. Red blood cell lysis
was performed by adding 4mL, room temperature Tris-buffered ammonium chloride (TBAC) and incubating at room temperature for 4 minutes, followed by addition of 10 times volume of ice cold PBS with 2% Heat inactivated FCS. CD4+ cells were isolated with Miltenyi CD4 (L3T4) MicroBeads by positive selection according to manufacturer’s instructions.

2.6b Negative selection with DynaBeads
Single cell suspensions were resuspended at 2x10^8 /mL in MACS buffer (consisting of PBS, 2% heat inactivated FCS and 2mM EDTA). Rat anti-mouse mAbs added were: anti-CD8 (YTS 169), anti-B220 (RA3.6B2), anti-MHC class II (TIB120) and CD11b (M1/70). Rat anti-mouse mAbs used in BMDC precursor negative selection were anti-B220 (RA3.6B2), anti-CD4 (YTS 3.1), anti-CD8 (YTS 169), anti-MHC class II (TIB120). Rat anti-mouse mAbs were added to a final concentration of 100µg/mL, in a total volume of 1mL and incubated on ice for 20 minutes. The cells were washed in MACS buffer, resuspended at 10^8 cells/mL in MACS buffer and added to sheep anti-rat Ig-coated Dynabeads (Invitrogen Ltd.) at a ratio of 1 bead per cell. Cells were placed on a rotor for 20 minutes at 4°C. Unlabelled cells were removed by placing the cells on a magnet and pipetting off the supernatant.

2.6c Treg isolation
CD4+ cells selected by negative selection using Dynabeads were resuspended at 10^8 cells/mL in MACS buffer and incubated with 50µL anti-CD25PE antibody for 20 minutes on ice. Cells were washed with MACS buffer and resuspended at 10^5/mL. 50µL anti-PE Microbeads were added and cells were incubated on ice for 20 minutes. Cells were washed and applied to an MS column (Miltenyi Biotec). Cells that run through the column were collected and cells bound to the column were ejected. Only cell populations >95% purity were used for experiments.

2.6d nTreg activation
nTreg were activated by stimulating with plate bound αCD3e and αCD28 antibodies. αCD3e and αCD28 antibodies were diluted to 5µg/mL in 0.1M Borate buffer (boric acid pH adjusted to 8.5). This solution was added to a 60mm Petri dish (bacterial grade) and incubated at room
temperature for at least 2 hours. Prior to use, plates were washed with PBS 3 times and then 10% BSA blocking solution was added for an hour. Up to $5 \times 10^6$ bead isolated CD4+CD25+ nTreg, of which 95% were FoxP3+, were added to the Petri dish in 5mL R10 media supplemented with 50U/ml Proleukin (Novartis, UK). Cells were incubated at 37°C, 5% CO₂ for 4 hours.

2.7 Ex vivo conditioning protocols (IFN-γ, cilostamide, αCD44)

5x10⁵ bead isolated CD4+ cells were co-cultured with 5x10⁴ allogeneic GT-DCs in 24 well plates in 2mL of R10 medium in the presence of either; 5ng/mL IFN-γ (Peprotech), or 5µM cilostamide (Sigma-Aldrich). After 7 days, 1 mL of media was removed and the cells were restimulated with 5x10⁴ allogeneic GT-DCs in 1mL media supplemented with either IFN-γ or cilostamide. αCD44-Treg were enriched for by incubating 5x10⁵ bead isolated CD4+ T cells with αCD44 antibody for 20 minutes on ice and then culturing the cells with 5x10⁴ allogeneic BMDCs in 24 well plates in 2mL of R10 medium. On day 7, 1 mL of media was removed and the T cells were restimulated with 5x10⁴ allogeneic GT-DCs. All Treg were harvested on day 14 and analysed based on CD4 and GFP expression using FACS.

2.8 Bisulphite sequencing

Bisulphite sequencing was performed using the EZ DNA Methylation™ kit (Zymo Research, United States). Briefly, genomic DNA was isolated from cells FACS sorted to >95% purity, genomic DNA was isolating using Proteinase K according to manufacturer’s protocol. Sodium bisulphite treatment was performed to convert unmethylated cytosines to uracil by the process of deamination. PCR amplification was performed, resulting in the conversion of uracil to thymidine. PCRs were performed in a final volume of 50µL containing 1-U Taq DNA polymerase, 200µM dNTPs, 10µM of forward and reverse primers and 10ng of bisulphite treated genomic DNA. The amplification conditions were 95°C for 10 minutes followed by 40 cycles of: 95°C for 1 minute, 55°C for 45 seconds and 72°C for 1 minute, with a final extension step for 10 minutes at 72°C. PCR products were cleaned up using ExoSAP-IT (USB Corp, Staufen, Germany) and
sequenced in the Weatherall Institute of molecular medicine (WIMM, University of Oxford) using a 48 capillary ABI-3730 DNA analyser (Thermo Scientific™). The primers used were amplicon 1 of the mouse FoxP3 TSDR: forward AGGAAGAGAAGGGGTAGATA; reverse AAACCTACTTTCTCTTCTAAC. Amplicon 2 of the mouse FoxP3 TSDR: forward: ATTTGAATTGGATATTGT; reverse: AACCTTAAACCTCTAAT [320].

2.9 Heat inactivated FCS

FCS was thawed at 4°C and then placed in a water bath at 60°C for 30 minutes. It was frozen in 50mL aliquots at -20°C until needed.

2.10 Cryopreservation of cells

10⁷ Cells were pelleted and re-suspended in 1mL media containing 45% R10 media, 45% HI FCS and 10% DMSO. Cells were placed in Mr Frosty™ freezing containers (Thermo Scientific™) and placed at -80°C for 24hours prior to transfer to liquid nitrogen.

2.11 Antibodies

All mAbs used in flow cytometry were purchased from eBioscience, USA and were conjugated to fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), pacific blue (PB), PE-Cyanine 7 (PeCy7), APC-Cyanine 7 (APC-Cy7) or biotin.
Table 3: List of antibodies used for FACS

<table>
<thead>
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<th>Specificity</th>
<th>Clone</th>
<th>Species</th>
<th>Isotype</th>
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<td>Armenian hamster</td>
<td>IgG1, κ</td>
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<td>RM4-5</td>
<td>Rat</td>
<td>IgG2a, κ</td>
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<td>CD11b</td>
<td>M1/70</td>
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<td>FoxP3</td>
<td>FJK-16s</td>
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**2.11a Antibodies used for cell purification**

Hybridomas YTS 177 and YTA 3.1 were kind gifts from Professor Herman Waldmann (Sir William Dunn School of Pathology, Oxford). They were grown in roller bottles and purified using protein G columns by FPLC by Dr Andrew Bushell (NDS, Oxford).
Table 4: Cell purification antibodies

<table>
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<th>Specificity</th>
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2.12 Flow cytometry

2.12a Cell sorting
Cells were sorted using a BD FACSAria. Cells were resuspended in FACS buffer at a concentration of $1 \times 10^7$ cells/mL and sorted into collection tubes, maintained at $4^\circ C$, containing PBS with 10% HI FCS.

2.12b Cell surface marker staining
All staining was done in FACS buffer (2.6mM Na$_2$HPO$_4$, 145mM NaCl, pH 7.2, Oxoid Ltd, UK, plus 2% HI FCS, PAA laboratories, Austria). Staining antibodies were diluted in FACS buffer and added to cells, diluted to a concentration of $10 \times 10^6$ cells/mL to make a total incubation volume of 100µL. Incubations were performed at $4^\circ C$ for 20 minutes. Cells were subsequently washed in FACS buffer and re-suspended in 200µL FACS buffer for immediate analysis or fixed by transferring cells to FACS buffer containing 1% v/v formaldehyde (Sigma, USA) and incubating for 30 minutes before washing in FACS buffer and leaving in the fridge for future analysis. Cells were analysed using a BD FACSCanto II and analysed using DIVA software (Becton Dickinson, USA).
2.12c Intracellular cytokine staining
Stimulation of cells was performed in R10 media enriched with 50ng/mL Phorbol myristate acetate (PMA, Sigma, USA), 500ng/mL Ionomycin (Sigma) and 2µM Monensin (Sigma) for 4 hours. Cells were harvested and washed in FACS buffer before fixing according to instructions outlined in the FoxP3 / transcription factor staining kit (eBioscience, USA). Briefly, cells were fixed in Fixation/Permeabilisation buffer for 30 minutes on ice before transferring to Permeabilisation buffer. Antibody stains were made in Permeabilisation buffer and added to cells resuspended at a concentration of 1x10^7 cells/mL for 30 minutes. Cells were then washed in Permeabilisation buffer and then in FACS buffer before analysis using BD FACSCanto II (Becton Dickinson, USA).

2.13 VPD labelling
Violet proliferation dye (VPD) was purchased from BD. Cells were washed twice in PBS and pelleted and re-suspended at 3x10^7 cells/mL in pre-warmed (37°C) PBS. VPD was added at a final concentration of 1µM and cells incubated at 37°C for 10 minutes. Cells were washed twice with 10mL PBS and then re-suspended in complete media. Staining was checked using the BD FACSCanto II (Becton Dickinson, USA) and then used in stimulation experiments or re-suspended in PBS for adoptive transfer into mice.

2.14 IL-6 ELISA
IL-6 was analysed in supernatants using an ELISA Max deluxe set: IL-6 mouse (BioLegend, USA).

2.15 Immunosuppressive drugs
CellCept® (MMF, 1g/5mL oral suspension), Roche, was diluted in sterile saline and dosed via oral gavage. Prograf® (Tacrolimus, 5mg/mL solution), Astellas, was diluted in sterile saline and dosed i.p. Methylprednisolone, 500mg powder, Beacon Pharmaceuticals was resuspended in sterile saline and all dilutions made in sterile saline, mice were injected i.v., via the tail vein. 440μL Rapamune® (Sirolimus/Rapamycin, 1mg/mL oral solution), Pfizer, was dissolved in 1mL ethanol,
1mL cremaphore and 2mL sterile saline. This solution was further dissolved in 16mL sterile saline. Aliquots were stored at -20°C. All drugs were purchased through the John Radcliffe Pharmacy, Oxford Universities NHS foundation trust, UK.
Chapter 3: Regulatory T cells

Introduction

To obtain sufficient Treg for clinical or experimental use, ex vivo expansion will almost certainly be required. Although in vitro studies initially showed that Treg were an anergic population of cells [259, 443], transfer of these cells into lymphopenic hosts suggested otherwise [444] and adoptive transfer into lymphoreplete mice provided evidence that Treg could proliferate in response to their specific antigen. Using Treg bearing a transgenic TCR specific for OVA, Walker et al. identified that CFSE labelled Treg adoptively transferred into OVA expressing mice proliferated in response to their antigen [333].

Tang and Lee estimated that 5-8x10^10 Treg/kg in humans will be required to have any improvement on transplant outcome. Their estimate is based on extrapolations from lymphopenic mouse models and human studies. Typically a ratio of Treg to conventional T cells (Tconv) of 1:1 to 1:2 is required to prevent skin transplant rejection, therefore they suggest that 33-50% of cells would need to be Treg at the time of transplant to prevent rejection [242, 246]. A young adult human weighing 70kg is estimated to have a total lymphocyte pool of approximately 46x10^10 [445-447] and the total number of CD4^+ T cells in various tissues has been estimated at 16.5x10^10 [445, 448-450]. Additionally, percentages of Treg present in a variety of tissues and organs have been calculated and a 70kg young adult human is estimated to have 13.1x10^9 Treg [450-457]. Extrapolating from this, and assuming that no lymphodepletion is used, then at least 5x10^10 Treg will be required to reach a ratio of 1 Treg to 2 Tconv [448]. Therefore, generating sufficient numbers of Treg from CD4^+FoxP3^+ T cells, or expanding nTreg, remain important goals if sufficient numbers of Treg are to be made available on a reliable and routine basis for use in clinical trials.

It is now possible to expand human nTreg ex vivo to generate sufficient numbers of cells that remain suppressive for use in clinically relevant mouse models [248, 388, 395]. Hippen et al.
reported that 4 rounds of stimulation of human nTreg, from peripheral blood, with artificial
APCs, expressing high affinity Fc receptor and CD86, could achieve a 50 million fold expansion
with minimal loss of functional activity in a model of GVHD [395]. This could be a very important
result if it can be reproduced. Expanding nTreg from mice has been more difficult. It has been
reported that a 7,000 fold expansion of mouse nTreg is possible using plate bound anti-CD3 and
anti-CD28 antibodies in the presence of IL-2 (10ng/mL) for 11 days [396]. Unfortunately, this
approach led to only limited success in our lab, with typically only a 10 fold expansion of mouse
nTreg (personal communication with Thomas Chan). More importantly, when using these
expanded cells in an established skin graft rejection model, 1x10^5 expanded nTreg were unable
to prevent 1x10^5 CD4^+CD25^- effector T cells rejecting fully MHC mismatched grafts, whereas
freshly isolated Treg prolonged graft survival indefinitely (Thomas Chan, unpublished
observations).

*De novo* induction of Treg (iTreg) from CD4^+CD25^- T cells was first shown to be possible using
TGF-β [399-401] and this has since become the gold standard method to generate iTreg *in vitro.*
However, these iTreg readily lose FoxP3 expression upon restimulation and have the capacity
to develop an effector memory phenotype and become pathogenic *in vivo* [299, 320]. The
stability of FoxP3 expression has been shown to be dependent on the methylation status of the
TSDR (Treg-specific de-methylated region). In an analysis of FoxP3 TSDR methylation in various
populations of mouse T cells, Floess et al. demonstrated that whilst CD4^+CD25^- T cells had a
TSDR that was essentially 100% methylated, CD4^+CD25^- nTreg had a TSDR that was virtually
100% de-methylated. Importantly, when these nTreg were re-stimulated, TSDR de-methylation
was retained - implying constitutive FoxP3 expression [288]. In contrast, although TGF-β
induced Treg initially had a de-methylated TSDR, re-stimulation resulted in almost complete
methylation of the TSDR and was associated with a profound loss of FoxP3 expression. These
differences at the epigenetic level appear to be vital for the stability of FoxP3 expression in
human and mouse Treg [320, 321]. Therefore, these data suggest that using TGF-β to generate
Treg is less desirable for clinical use and the stability of the Treg population that is used must retain FoxP3 to prevent conversion to an effector phenotype, potentially contributing to graft rejection. Our group has developed two methods of generating Treg in vitro, using IFN-γ or a PDE3 inhibitor, cilostamide [140, 247, 402]. Both protocols use immature bone marrow derived dendritic cells, which have been cultured in GMCSF and TGF-β, for 6 days prior to their use as alloantigen stimulators to generate alloantigen-reactive Treg. Stimulating CD4⁺ T cells in the presence of IFN-γ efficiently enriches for Treg which are capable of preventing the rejection of both fully mismatched heart and skin grafts in lympho-depleted models, serving as important proof-of concept models [139, 140]. Crucially, if Treg therapy is to become routine, then it is imperative to ask whether Treg can prevent graft rejection in lymphoreplete hosts and what impact concurrent immunosuppression has on the survival and ultimately the function of adoptively transferred Treg. Unfortunately, the yield of Treg using IFN-γ is not sufficient for lymphoreplete models. Based on the estimation that 1 mouse will provide enough cells for 2 x 24 well plates and each 24 well plate will yield approximately 1x10⁵ Treg in the IFN-γ protocol then 5 mice would be required to achieve the 1x10⁶ Treg intended for each lymphoreplete graft recipient. Therefore, to test the function of Treg in lymphoreplete models, a protocol that achieves a greater yield of Treg is required. PDE3 inhibition typically generates 2-5 fold more Treg than the IFN-γ method but it is less reliable, therefore methods to improve the PDE3 inhibition protocol were explored with the ultimate goal of producing sufficient numbers of stable and potent Treg for subsequent study.

**Chapter aims:** The chapter aims were firstly, to find a method to improve the consistency of enrichment and potentially the yield of alloantigen-driven Treg using the PDE3 inhibitor, cilostamide, to provide sufficient cells for in vivo experiments in lymphoreplete mice. Secondly, a comparison of different types of Treg was to be conducted using the allogeneic skin graft...
model in Rag−/− recipients to identify the most efficacious population of Treg for further experiments in lymphoreplete animals.
3.0 Results

3.1 Generating Treg using a Phosphodiesterase (PDE) 3 inhibitor is unreliable

Gavin et al. demonstrated that among genes that are regulated by FoxP3, the gene encoding the enzyme phosphodiesterase 3b (Pde3b) was the most repressed [458]. Previous work from our group had shown that stimulation of CBA (H2k) CD4+ T cells in vitro with B6 (H2b) bone marrow derived dendritic cells (BMDCs) in the presence of the phosphodiesterase 3 inhibitor, cilostamide, results in a functional enrichment of FoxP3+ Treg that can prevent skin graft rejection in adoptive transfer mouse models [402]. Importantly, this is not a phenomenon restricted to mice. Culturing human CD45RO CD25 CD4+ T cells with GM-CSF/IL-4/TGF-β differentiated allogeneic monocyte derived DCs in the presence of cilostamide, resulted in the generation of human iTreg that were capable of preventing vasculopathy in a human artery transplant model, previously established in our laboratory [247]. However, Figure 3.1 illustrates the inconsistency of FoxP3 enrichment using cilostamide. B6 (H2b) BMDCs were cultured for 6 days in complete media supplemented with GMCSF and TGF-β to generate GT-DCs. These cells were then used to stimulate total CD4+ T cells from CBA FoxP3(GFP) (H2k) mice in the presence of cilostamide. CD4+ T cells were re-stimulated with GT-DCs on day 7 and analysed on day 14 for GFP(FoxP3) expression. As shown in Figure 3.1, the enrichment for FoxP3 is highly variable. In the series of replicate experiments shown the mean percentage of FoxP3+ cells ranges between 10.5% and 66.8% and the mean number of cells varies between 6.5x10^3 and 1.2x10^5. Given that this lack of reliability would frustrate any attempt to evaluate the function of Treg in lymphoreplete hosts, a series of experiments was conducted aimed at understanding what variables may be contributing to this inconsistency.
3.2 Sorted MHC class II\textsuperscript{hi}CD11c\textsuperscript{hi} DCs more efficiently enrich for Treg than unsorted GT-DCs and sorted MHC class II\textsuperscript{int/lo}CD11c\textsuperscript{hi} DCs

Both Treg enrichment protocols developed in our laboratory involve stimulating CD4\textsuperscript{+} T cells with GT-DCs previously cultured in GMCSF and TGF-\beta for 6 days [140, 247]. This generates a heterogeneous population of cells that act as allogeneic APCs. Idoyago et al. demonstrated that migratory DCs have a superior ability, compared with blood-derived lymphoid-resident DCs, to generate Treg \textit{in vivo}. Importantly, when tested in an EAE model, Treg generated using these migratory DCs were the most effective at controlling disease onset [459]. These migratory DCs were predominantly found within the MHC class II\textsuperscript{hi}CD11c\textsuperscript{hi} DC population, whereas lymphoid resident DCs reside within the MHC class II\textsuperscript{int/lo}CD11c\textsuperscript{hi} DC population. Therefore, to ask whether variability in the overall proportion of resident vs. migratory DC in the input GT-DC population might explain the inconsistency of the cilostamide protocol, the GT-DC input population was flow sorted on the basis of MHC class II and CD11c expression (Figure 3.2a) and these cells were used to stimulate H-2\textsuperscript{k} CD4\textsuperscript{+} T cells \textit{in vitro} in the presence of cilostamide. As shown in Figure 3.2, after 14 days in culture, the mean percentage of CD4\textsuperscript{+} GFP\textsuperscript{+} Treg generated using either unsorted GT-DCs, or MHC class II\textsuperscript{int/lo} cells was approximately the same (21.8\% and 18.9\% respectively, n=4, p=0.2), whereas the MHC class II\textsuperscript{hi} CD11c\textsuperscript{hi} population increased the mean percentage of CD4\textsuperscript{+} GFP(FoxP3)\textsuperscript{+} Treg to 32.2\%. Perhaps more importantly, the MHC class II\textsuperscript{hi} CD11c\textsuperscript{hi} yielded significantly more Treg (1.68x10\textsuperscript{5}) than unsorted DCs (8.0x10\textsuperscript{4}) and MHC class II\textsuperscript{int/lo} DCs (2.7x10\textsuperscript{4}).

These results suggested that the input GT-DC stimulatory population used in the cilostamide protocol is a heterogeneous mix of antigen presenting cells with different capabilities to enrich for Treg, the data also supports work published by Idoyaga et al. that migratory DCs are the most efficient at generating Treg [459]. The process of flow sorting the cells was labour intensive and required a 6-10 fold increase in mice to provide enough GT-DCs to obtain the
numbers required to use as APCs, therefore it was decided that although it might be possible to improve the enrichment of Treg in the presence of cilostamide by this method, it would not be explored further. Instead, other methods to improve the generation of Treg using cilostamide were investigated.

3.3 IL-6⁺ GT-DCs improve the enrichment but not the yield of Treg in the presence of the PDE3 inhibitor, cilostamide

Experiments by Bettelli et al. demonstrated that the addition of IL-6 halved the generation of FoxP3⁺ Treg by TGF-β [460]. It was later discovered that nTreg stimulated with plate bound anti-CD3 and anti-CD28 antibodies, in media supplemented with IL-6 differentiated into Th17 cells in the absence of exogenous TGF-β [461]. Additionally, IL-6 has previously been identified to be important in reducing the efficiency of enrichment for Treg in the IFN-γ protocol [140]. Unlike BMDCs cultured in GMCSF and TGF-β, BMDCs cultured in GMCSF alone are unable to promote the enrichment of adaptive Treg in the IFN-γ protocol [37]. Significantly, BMDCs cultured in GMCSF alone produce almost three times more IL-6 than their GMCSF plus TGF-β counterparts. Additionally, wild type (wt) GT-DCs were shown to reduce the enrichment of Treg in vitro using IFN-γ compared with IL-6 deficient GT-DCs [139]. The available data thus suggested that IL-6 production might explain the variability of Treg yield in the cilostamide protocol. Due to the similarities between the IFN-γ protocol and cilostamide protocol, experiments were planned to ask whether variable IL-6 production by the GT-DC APC population could be an explanation for the variability of Treg enrichment identified in the cilostamide protocol. Therefore, the enrichment of Treg, in the presence of cilostamide was compared using GT-DCs from either IL-6⁺ or wt mice.

BMDCs (H2b) from wt or IL-6⁺ mice were cultured in parallel, in media supplemented with GMCSF and TGF-β for 6 days. The resultant GT-DC populations were used to stimulate total
CD4+ T cells, magnetically isolated from CBA GFP(FoxP3) reporter mice (H2b) in the presence of 5µM cilostamide, as described previously. After 7 days, fresh GT-DCs of the same type used for the initial stimulation were added, in media supplemented with 5µM cilostamide. After a further 7 days, the cultures were harvested and assayed for CD4 and FoxP3(GFP) expression. As shown in Figure 3.3, the mean enrichment of Treg was significantly increased with IL-6/- DCs compared to wt GT-DCs (48.2% versus 28.3% respectively, p=0.017, n=8). The yield of Treg was somewhat reduced when IL-6/- GT-DCs were used as stimulators instead of wild type GT-DCs (17,567 Treg versus 9,867 Treg respectively, p=0.06, n=3), although this was not significant. Assuming that everything else, except for the expression of IL-6, is the same between these two GT-DC populations, these data support the idea that DC derived IL-6 reduces the efficiency of Treg enrichment using PDE3 and suggest that manipulation of IL-6 production might be a useful way of enhancing the generation of adaptive Treg in vitro. However, subsequent attempts to use anti-IL-6 antibody proved unsuccessful in that virtually all cells in anti-IL-6 cultures died by the end of the 14 day culture period, perhaps because as hinted at by the data in Figure 3.3c, a certain amount of IL-6 may actually be required for cell survival. Therefore, an alternative approach for influencing IL-6 production was examined.

3.4 CD44hi CD4+ cells express more IL-6 than CD44lo/int CD4+ cells but increasing the proportion of CD44hi CD4+ T cells does not reduce Treg enrichment using PDE3 inhibition

As shown in Figure 3.3, IL-6 expressing GT-DCs are less effective for generating Treg than their IL-6 deficient counterparts, however the input CD4+ T cell population is also a potential source of IL-6 which suggests IL-6 from this aspect must also be considered. Indeed, Miyao et al. suggested that the presence of CD4+CD44hi T cells in Treg induction protocols could explain the sparse numbers generated in many such systems because of the potential, upon stimulation, for CD4+CD44hi T cells to produce unfavourable concentrations of pro-inflammatory cytokines
such as IL-4, IL-6 and IFN-γ [300]. As IL-6 is known to be prohibitive for FoxP3 induction [139, 460, 462] and DC expression of IL-6 was unfavourable for the efficient enrichment of Treg in both the cilostamide and IFN-γ protocols (Figure 3.3 and [139]), IL-6 expression from CD44hi T cells was investigated.

The CD4⁺ T cell input population used in the cilostamide protocol typically consists of approximately 10% CD44hi cells (Figure 3.4a) and as shown in Figures 3.4b and c, these cells express greater levels of IL-6, upon stimulation with PMA and ionomycin, than their CD4⁺CD44lo/neg counterparts. To determine whether these CD44hi cells might be responsible for a reduction in the efficiency of Treg enrichment under some circumstances, CD4⁺ T cells were flow sorted on the basis of CD44 expression. CD4⁺CD44hi T cells (H2⁺) were titrated into a CD44⁺ CD4⁺ T cell population with the aim of identifying whether there was a ratio at which CD44hi cells become detrimental for Treg enrichment. The cells were stimulated with GT-DCs (H2b) for 7 days in the presence of 5µM cilostamide. On day 7, fresh GT-DCs (H2b) were added in culture medium supplemented with 5µM cilostamide. Contrary to the original hypothesis, increasing the number of CD44hi CD4⁺ T cells did not change Treg enrichment as the absolute number and percentage of FoxP3⁺ cells did not significantly alter as the percentage of CD44hi T cells increased from 0-30% (Figure 3.5). These data led to a revision of the original hypothesis and instead, speculation arose that addition of the flow-sorting anti-CD44 antibody resulted in reduced IL-6 expression from CD44hi CD4⁺ T cells and that this in turn over- rode any negative effects of increasing the percentage of CD44hi CD4⁺ T cells. In other words, it seemed possible that CD44 ligation with the sorting antibody inhibited IL-6 production.

3.5 Anti-CD44 antibody reduces IL-6 expression from CD44hi CD4⁺ T cells

A subsequent literature search revealed that Bollyky et al. had previously shown that CD44 cross-linking on human Treg using plate bound anti-CD44-antibody in combination with plate
bound anti-CD3 antibody resulted in up-regulation of TGF-β, IL-2 and IL-10, and promoted the expression of FoxP3 [463]. To ask whether anti-CD4 antibody could also reduce IL-6 expression from mouse CD44hiCD4+ T cells, CBA CD4+ T cells (H2b) were magnetically isolated and stimulated with B6 (H2b) GT-DCs for three days. Interestingly, stimulation of CD4+ T cells in the presence of anti-CD44 antibody reduced IL-6 expression in CD44hi CD4+ T cells (Figure 3.6aii) when measured using FACS. There also appeared to be a modest decrease in IL-6 expression from the CD44lo/int CD4+ T cells in the presence of anti-CD44 antibody (Figure 3.6aii, lower panel). It had been previously shown that stimulation of human nTreg with plate bound anti-CD3, anti-CD28 and anti-CD44 antibodies was important for optimal activation. However, addition of soluble anti-CD44 antibody in combination with plate bound stimulation with anti-CD3 and anti-CD28 antibodies did not result in an increase of FoxP3+ Treg persistence, suggesting the importance of cross-linking of CD44 for the mechanism [463]. To further evaluate whether CD44 cross-linking was important for the reduction in IL-6 expression, as suggested in Figure 3.6a, CD4+ T cells were stimulated with plate bound anti-CD3, anti-CD28 and anti-CD44 antibodies for 7 days, and IL-6 was measured using ELISA. Stimulating CD4+ T cells with plate bound anti-CD3, anti-CD28 and anti-CD44 antibodies reduced IL-6 expression to a mean concentration of 277pg/mL from 738pg/mL compared with when cells were stimulated with plate bound anti-CD3 and anti-CD28 antibodies alone. Interestingly, when cells were pre-incubated with anti-CD44 antibody, prior to stimulation with plate bound anti-CD3 and anti-CD28 antibodies, there was also a reduction in IL-6 expression compared with plate bound anti-CD3 and anti-CD28 antibodies (mean 587pg/mL). This result demonstrates that cross-linking of CD44 with antibody results in a significant reduction in IL-6 expression from the input CD4+ T cell population and that effective cross-linking of CD44 using plate bound anti-CD44 is important for the optimal reduction of IL-6 expression identified in Figure 3.6b. It seems possible that the reduction of IL-6 expression from CD4+ T cells stimulated with plate bound anti-CD3, anti-CD28 and anti-CD44 antibodies is, at least in part, due to anti-CD44 antibody mediated cross-linking of CD44, however cross-linking
may not be entirely responsible. Bollyky et al. reported that addition of soluble anti-CD44, in combination with anti-CD3 stimulation did not lead to the activation of FoxP3+ Treg [463]. As shown in Figure 3.6b, stimulation of total CD4+ T cells with soluble anti-CD44 antibody prior to stimulation with plate bound anti-CD3 and anti-CD28 did reduce IL-6 expression, however this was less effective compared with plate bound anti-CD44 antibody (Figure 3.6b). It is possible that once anti-CD44 is bound to cells, or any anti-CD44 left unbound in the media, can bind to the culture plate and effectively cross-link CD44 on the cell surface.

CD44 cross-linking has been shown to increase the expression of TGF-β by nTreg [463] and Walia et al. showed that TGF-β down regulates IL-6 expression by suppressing the activation of STAT-3, ultimately preventing ICAM-1 expression [464]. This provides a potential mechanism for the CD44-mediated reduction in IL-6 expression. Additionally, culturing BMDCs in TGF-β in combination with GMCSF leads to a 3 fold reduction in IL-6 expression, compared with BMDCs that were cultured in GMCSF alone [139]. Therefore, it is likely that any increase in TGF-β expression as a result of anti-CD44 cross-linking, as shown by Bollyky et al. [463], may act to reduce IL-6 expression. Overall, this result suggests that cross-linking is important for the optimal IL-6 reduction shown in Figure 3.6 and it is possible that activation of nTreg in the CD4+ T cell input population leads to the expression of cytokines such as TGF-β which ultimately leads to the suppression of IL-6 from CD44hi CD4+ T cells.

The clear effect of anti-CD44 antibody on IL-6 production prompted us to test whether CD44 blockade, without PDE3 inhibition, could enrich for Treg when CD4+ T cells are stimulated with GT-DCs.
3.6.1 Anti-CD44 antibody can be used to enrich for Treg

To test the idea that anti-CD44 antibody might enhance Treg enrichment in the absence of cilostamide, total CD4+ T cells (H2b) were pre-incubated with increasing concentrations of anti-CD44 antibody and then stimulated with GT-DCs (H2b) for 7 days. On day 7, fresh GT-DCs were added and cells were harvested on day 14. As shown in Figure 3.7, there was a dose dependent effect of anti-CD44 antibody on the enrichment and yield of Treg compared with the isotype control, IgG2a. Pre-incubation with anti-CD44 antibody at and above 1 µg/ml provided the greatest enrichment and yield of CD4+GFP(FoxP3)+ Treg. Although 4 µg/mL anti-CD44 antibody provided a larger mean yield of CD4+GFP(FoxP3)+ Treg compared with 1 µg/mL, 1 µg/mL anti-CD44 antibody was chosen as the concentration to use for future experiments to keep the experiments economically viable. Importantly, the yield achieved appeared to be improved compared with the cilostamide protocol, which was extremely promising as planned in vivo experiments required relatively large numbers of Treg. To identify which Treg enrichment protocol yielded the most Treg, the protocols were compared directly.

3.6.2 Anti-CD44 antibody blockade yields more Treg than PDE3 inhibition using cilostamide

In order to determine whether CD44 blockade would be a more reliable and robust method for the generation of alloreactive Treg than the cilostamide protocol [247] the two methods were compared directly. To provide an external comparator, the same input and APC populations were used in a TGF-β/IL-2 protocol which can be regarded as a prototypic method for the generation adaptive Treg in vitro [400, 465, 466]. Treg enrichment was relatively modest when T cells were incubated with anti-CD44 antibody in comparison with cilostamide and TGF-β/IL-2 protocols (mean Treg percentage; anti-CD44 27.3%, cilostamide 49%, TGF-β 47.6%, n=3 independent experiments, Figure 3.8), however the yield of Treg was substantially higher using the anti-CD44 antibody protocol (mean Treg number; anti-CD44 protocol 3.1x10⁶, cilostamide protocol 9.7x10⁵, TGF-β protocol 1.3x10⁶). Although the difference in yields between the anti-CD44 and TGF-β/IL-2 protocols did not reach statistical significance, it has been demonstrated
that enriching for Treg using TGF-β/IL-2 leads to an unstable phenotype [299, 320]. If enriching for Treg using anti-CD44 antibody provided more stable Treg than the TGF-β/IL-2 protocol, then this method could be extremely promising.

CD44 is a cell surface receptor whose expression facilitates T cell attachment to the extracellular matrix component hyaluronic acid (HA), ultimately providing anchorage for T cells to access inflammatory sites [467]. Modulation of CD44 using high molecular weight (HMW) HA has previously been shown to increase the potency and promote the persistence of human FoxP3+ nTreg [463, 468]. In these published experiments, incubation of FoxP3+ Treg with 20µg/mL HMW-HA for 7 days resulted in the increased expression of FoxP3, whereas incubating cells in LMW-HA had a less profound effect on FoxP3 expression. Additionally, nTreg incubated with 20µg/mL HMW-HA overnight were approximately 3 times more suppressive than nTreg that had been incubated overnight in media alone, at a ratio of 1 Treg to 1 Tconv, in a [3H]thymidine incorporation assay [468]. Although ligation of CD44 on human nTreg with either anti-CD44 antibody or HMW-HA in combination with plate bound anti-CD3 antibody had been previously shown to promote the in vitro persistence and function of polyclonal FoxP3 regulatory T cells [463], it was not known whether ligation of this receptor using anti-CD44 antibody could be used to promote the enrichment of functional, alloantigen-reactive Treg. The data in Figure 3.8 show that in the presence of alloantigen stimulation, anti-CD44 antibody can enrich for and yield a large number of Treg. The ability to generate large numbers of Treg was essential to address the core aim of this thesis, which was to determine whether current clinically relevant immunosuppressive drugs, recommended by the Symphony Study [403, 404], have a detrimental impact on Treg function in vivo. Despite the limitations of murine transplant models, such an evaluation can only be made in an experimental setting. Although lymphopenic mouse models have provided invaluable data on the function of Treg, they are most frequently used to assess the ability of Treg to control a few key components of the immune system that can contribute to rejection, in isolation. It was decided that lymphopenic mouse models were
not a suitable surrogate for the investigation of the effects of immunosuppression on Treg therapy. Additionally, lymphopenic mouse models are hampered by a number of factors, such as homeostatic proliferation of transferred cell populations and the difficulty in reconstituting a complete, “in-proportion” immune system, particularly in humanised mouse models. It was felt that a lymphoreplete mouse transplant model could provide the most relevant insights into the impact of clinically relevant immunosuppressive drugs on Treg function.

3.7 Treg enriched using anti-CD44 antibody can prolong allograft survival

To determine whether FoxP3+ T cells, enriched for using anti-CD44 antibody, could regulate allograft rejection, 1x10^5 (H2k) anti-CD44 antibody generated Treg together with 2x10^5 CD4+CD25- (H2k) T cells, as an effector population, were transferred into CBA Rag-/- (H2k). The next day the mice received a full thickness skin graft from a B6 (H2b) mouse (Figure 3.9a). Mice reconstituted with 2x10^5 CD4+CD25- cells only, rejected their grafts acutely (MST = 12.5 days, n = 5) but co-transfer of 1x10^5 CD44-Treg prevented rejection and 5 out of 8 grafts survived beyond 100 days (MST >100 days, n = 8) (Figure 3.9b). Mice reconstituted with activated nTreg and CD4+CD25- cells had an MST of 57 days (n=7), although this was not significantly different from mice that received CD44-Treg (p=0.9, Log-rank test, Figure 3.9b), suggesting that these two populations of Treg have similar efficacies in vivo. Therefore, Treg enriched for in the presence of anti-CD44 antibody are able to control the rejection of fully mismatched skin grafts.

3.8 Treg enriched using anti-CD44 antibody are alloreactive

To avoid the plethora of side-effects involved with globally immunosuppressing transplant patients using modern immunosuppressive drugs, such as an increased frequency of malignancy and infections [469, 470], it is imperative that adoptively transferred Treg do not also broadly
immunosuppress transplant patients. Therefore, it would be desirable to deliver Treg that recognise antigens presented in the graft, on the basis that specific stimulation in vivo might lead to fewer “off-target” effects than the delivery of polyclonally stimulated nTreg.

To test the specificity of anti-CD44 antibody generated Treg, total CD4+ T cells from CBA FoxP3(GFP) mice were magnetically isolated, pre-incubated with 1µg/mL anti-CD44 antibody for 10 minutes and then stimulated with B6 (H2b) GT-DCs for 7 days. On day 7, fresh GT-DCs were added and on day 14, Treg were harvested. CD44-Treg (H2h) were flow sorted to >95% purity and 1x10⁵ were adoptively transferred into a CBA (H2k) Rag−/− mouse together with 1x10⁵ CD4+CD25− (H2b) as an effector population (Figure 3.10).

The following day, mice received full thickness skin grafts from B6 (H2b) or 3rd party BALB/c (H2d) donors. Mice that received CD4+CD25− (H2b) effectors only, rejected both B6 and 3rd party skin grafts acutely, both with an MST of 10 days (n=6 for both groups). All mice that received effectors plus CD44-Treg accepted H2b donor skin long term (MST >100 days, n=5), whereas 5 out of 7 mice that received the third party (H2d) skin grafts were rejected acutely (MST 12 days). The extended graft survival in two of the mice that received 3rd party skin grafts is possibly due to the adoptive transfer of an activated population of Treg. Thornton and Shevach first described the phenomenon that activated Treg could inhibit T cell responses non-specifically in vitro experiments in which pre-activated Treg were able to inhibit the antigen-specific responses of transgenic cells for a distinct antigen [471]. Karim et al. showed that antigen specific Treg could regulate the rejection of a third party allograft if the Treg were reactivated by their cognate antigen [472]. These two studies suggest that activated Treg can suppress immune responses towards antigens that they do not directly recognise in a process referred to as ‘bystander regulation’ [471]. It is perhaps surprising that only 2 of 7 mice responded in this way but it could be that these mice received slightly more Treg, or slightly fewer Teff, than the rest of the mice in the group and this in conjunction with the by-stander regulation may be
responsible for the extension of graft survival in mice that received third party grafts in combination with Treg generated in the presence of anti-CD44 antibody. Ultimately, it remains unclear what the exact reason is for this, although this phenomenon has been reported before by our group [140].

With the caveat that only a single third-party strain was used to explore specificity, the data do indicate that regulation mediated by CD44-Treg is specific to the strain of APC used to drive Treg enrichment.

3.9 Treg enriched using anti-CD44 maintain FoxP3 expression when re-stimulated in vitro

The stability of Treg is an important concern for their use as a cellular therapy because Treg that lose FoxP3 can develop an effector memory phenotype and exacerbate GVHD in mice [299, 320]. This observation was made using induced Treg (iTreg) generated in the presence of TGF-β and IL-2 but the theory that nTreg are a committed lineage has raised debate. nTreg were initially thought to exhibit stable expression of FoxP3, although when Duarte et al. adoptively transferred 2-5x10^5 CD4^+ GFP^+ Treg, purified from FoxP3(GFP) reporter mice, into Rag^/- mice, 65-70% of previously FoxP3^+ lost FoxP3 expression after 4 weeks in both the lymph nodes and spleen. This challenged the notion that Treg were a committed lineage [473].

Komatsu et al. adoptively transferred CD4^+GFP^+ nTreg from FoxP3(GFP) reporter mice into Rag2^/- mice either alone, or mixed at 1:1 or 1:10 ratios with CD4^+GFP^+ cells. Using congenic markers Ly5.2 and Ly5.1 the authors were able determine that 50% of cells in mice that received only CD4^+GFP^+ Treg lost GFP expression. However, CD4^+GFP^+ Treg in Rag2^/- mice that also received CD4^+GFP^- cells at a 1:10 ratio (termed lymphoreplete conditions by the authors) were shown to be subject to a loss of 8% GFP expression at 4 and 8 weeks post transfer. These data suggest that only a small proportion of FoxP3^+ Treg lose FoxP3 expression in a normal physiological
process linked to the delicate homeostasis established by the immune system [474]. More recently, elegant experiments from Miyao et al. sought to clarify whether nTreg are a stable committed lineage or whether they lose FoxP3 expression in lymphopenic and proinflammatory conditions. By taking advantage of genetic fate mapping with FoxP3$^{\text{GFPCre}}$ knock-in ROSA26$^{\text{RFP}}$ mice and adoptive transfer experiments, Miyao et al. showed that exFoxP3 T cells do not reflect reprogramming of committed Treg but rather a minor population of uncommitted FoxP3$^+$ T cells that promiscuously express FoxP3 at some stage during their development [300]. Therefore, it appears that unstable FoxP3 expression is mainly a concern with Treg induced in vitro, specifically iTreg generated in the presence of TGF-β and IL-2 (TGF-β iTreg). This was elegantly demonstrated by Floess et al. in 2007 who showed that stimulating TGF-β iTreg, that were initially >97% FoxP3 positive, with plate bound anti-CD3 and anti-CD28 antibodies for 6 days resulted in over 90% of the TGF-β iTreg losing FoxP3 expression, compared with approximately 7% loss of FoxP3 in nTreg [320]. Interestingly, this was very similar to the loss of FoxP3 expression by Treg adoptively transferred into “lymporeplete” mice by Komatsu et al. [474].

The approach used by Floess et al. [320] was therefore utilised to assess the stability of FoxP3 expression in the anti-CD44 antibody generated Treg (CD44-Treg). CD44-Treg, TGF-β iTreg and nTreg were flow sorted to ≥99% purity and stimulated with plate bound anti-CD3 and anti-CD28 antibodies for 6 days. The vast majority of nTreg remained FoxP3 positive as determined by flow cytometry (≈90%), compared with a 60% loss of FoxP3(GFP) expression in TGF-β iTreg (Figure 3.11). Approximately 10% of CD44-Treg lost FoxP3(GFP) expression which was not significantly different to the loss observed with nTreg (p=0.2, Student’s t test), demonstrating that the stability of FoxP3 expression is similar between these two Treg populations. Importantly, the stable expression of FoxP3 in CD44-Treg suggests that they would be unlikely to revert to an effector memory phenotype in vivo and therefore should not contribute to graft rejection.
3.10 CD44-Treg exhibit stable expression of FoxP3 because of a demethylated TSDR

Floess et al. demonstrated that upon re-stimulation in vitro with plate bound anti-CD3 and anti-CD28 antibodies, the vast majority of nTreg retain FoxP3 expression (92%), whereas the majority of TGF-β iTreg lose FoxP3 expression (~90% loss) [320]. In a subsequent epigenetic analysis, it was shown that stable FoxP3 expression in nTreg correlated with a fully de-methylated TSDR, implying constitutive ‘hard-wired’ FoxP3 expression in this population of cells, known to be essential for normal immune homeostasis. In stark contrast, in TGF-β iTreg the TSDR was partially demethylated [320]. These observations are contextualised by the fact that CD4⁺CD25⁺ T effector cells, which were sorted to a purity of >99% FoxP3⁺, were shown to have a fully methylated TSDR. These methylation differences were identified in CpG sequences within amplicons 1 and 2 of the FoxP3 locus. Amplicons 3 and 4 showed no significant differences between CD25⁺ and CD25⁻CD4⁺ T cells [320] (see Figure 3.12i). This has since become the gold standard method to identify the stability of FoxP3 expression and the phenomenon also extends to human nTreg [321], therefore the TSDR demethylation status of CpG sequences in amplicons 1 and 2 of CD44-Treg was analysed and compared with nTreg, CD4⁺CD25⁺GFP(FoxP3⁻) T effectors and TGF-β iTreg.

Total CD4⁺ T cells were magnetically isolated from CBA(FoxP3GFP) mice. CD44-Treg were generated by pre-incubating CD4⁺ T cells with 1µg/mL anti-CD44 antibody for 10 minutes prior to stimulation with GT-DCs from B6 (H2b) mice. TGF-β Treg were generated by stimulating CD4⁺ T cells with GT-DCs in media supplemented with TGF-β (5µg/mL) and IL-2 (100U/mL). After 7 days, all cultures were re-stimulated with fresh GTDCs. CD44-Treg and TGF-β Treg were harvested after 14 days in culture with H2b GT-DCs and all cells were then sorted to a minimum purity of 99% (Figure 3.12iiia). The genomic DNA was isolated and methylation analysis was performed at amplicons 1 and 2 of the TSDR region. As expected, nTreg exhibited a TSDR which was almost fully de-methylated, whereas CD4⁺ CD25⁻ FoxP3⁻ cells (Teff) exhibited a TSDR which was fully methylated (Figure 3.12iib). Importantly, CD44-Treg had a TSDR that was de-
methylated to a similar extent of the TSDR in nTreg. The similar TSDR methylation profile identified in nTreg and CD44-Treg suggests that stability of FoxP3 expression in Treg enriched for using anti-CD44 antibody, upon re-stimulation (shown in Figure 3.11), is as a result of a heavily de-methylated TSDR. This is an important result as the safety of Treg in humans is paramount to their success as a clinical therapy and the stability of FoxP3 expression is critical for the retention of inhibitory functions [299].

These data demonstrate that enriching for Treg using anti-CD44 antibody generates a population of CD44-Treg that maintain FoxP3 expression upon re-stimulation in vitro. The retention of FoxP3 expression in over 90% of the cells correlates with a de-methylated TSDR which is similar to that found in the stable, committed nTreg lineage. Therefore, enriching for Treg using anti-CD44 antibody provides an extremely promising method to provide a high yield of stable Treg for the utilisation of these cells in experiments exploring the effects of immunosuppressive drugs on adoptively transferred Treg.

**3.11 Enrichment of Treg using anti-CD44 antibody is predominantly due to nTreg proliferation and not de novo FoxP3 induction in non Treg**

The stable expression, upon re-stimulation of FoxP3 by CD44-Treg demonstrated in Figure 3.11, and the similarity of TSDR de-methylation profiles between nTreg and CD44-Treg (Figure 3.12), raised the possibility that driving Treg enrichment in the presence of anti-CD44 antibody is a result of nTreg proliferation and not de novo FoxP3 expression in CD4^+^ FoxP3^−^ T cells. To test this, CD4^+^ FoxP3^−^ T cells from CBA FoxP3(GFP) mice were flow sorted to >99% purity, incubated with anti-CD44 antibody and stimulated with H2^b^ GT-DCs for 7 days. After 7 days fresh GT-DCs were added and cells were analysed after a total of 14 days. The de novo expression of FoxP3(GFP) was compared with Treg from the TGF-β protocol, as this has been shown to induce de novo FoxP3 expression in FoxP3^−^ CD4^+^ T cells [400]. Removal of nTreg from the CD4^+^ T cell
input population resulted in relatively poor enrichment for Treg (mean GFP(FoxP3)+ cells, 2.3%) in the presence of anti-CD44 antibody. In comparison, cultures that were not depleted of Treg had a mean of 34.4% GFP(FoxP3)+ cells when driven by allogeneic DCs in the presence of anti-CD44 antibody (Figure 3.13). Removal of Treg from the TGF-β protocol did reduce the mean percentage of Treg present at the end of the culture period, in comparison with cultures in which Treg were not removed, however this was not statistically significant (p=0.11, Student’s t test). These data suggest that enriching for Treg using anti-CD44 antibody relies on the presence of nTreg in the initial culture and there is little de novo induction of FoxP3.

3.12 anti-CD44 antibody is predominantly facilitating the proliferation of FoxP3+ T cells

As shown in Figure 3.13, the enrichment of Treg using anti-CD44 antibody is predominantly due to nTreg present in the initial CD4+ T cell pool and not de novo FoxP3 expression. To ask whether anti-CD44 antibody was favouring the proliferation of nTreg over FoxP3− cells, total CD4+ cells were magnetically sorted and labelled with violet proliferation dye (VPD). The cells were incubated with anti-CD44 antibody and stimulated with allogeneic (H2b) GT-DCs for 7 days. After 7 days, fresh GT-DCs were added and cells were analysed on day 14. ~48% of FoxP3+ cells incubated with anti-CD44 antibody proliferated, compared to only ~15% in the absence of antibody (p=0.001, n=3). Although FoxP3− anti-CD44 incubated cells proliferated more than untreated FoxP3− cells (~8% versus ~4.8% respectively), this was not significant (p=0.17, n=3). These results support data from Figures 3.13 that anti-CD44 antibody leads predominantly to the proliferation of nTreg and this is responsible for the enrichment of Treg in this protocol.
3.13 Activated nTreg are as effective as alloantigen-driven Treg at preventing graft rejection in a skin allograft model

Assessing the relative efficacies of nTreg and alloantigen-reactive Treg is increasingly important as they enter human trials. Sagoo et al. identified alloantigen-reactive Treg by their up-regulated expression of the activation markers CD69 and CD71 when stimulated with allogeneic dendritic cells in vitro. Using a humanised mouse model, Sagoo et al. concluded that these alloantigen responding Treg were more effective than polyclonally expanded nTreg at reducing the clinically relevant indicators of dermal tissue injury in a allogeneic skin transplant model [398]. Putnam et al. also demonstrated that alloantigen-reactive Treg, expanded using CD40L stimulated allogeneic B cells, were more efficacious than polyclonally expanded nTreg. BALB/c.Rag2\(^{+/−}\)γc\(^{−/−}\) mice transplanted with human skin prior to adoptive transfer of CD25\(^{+}\) depleted allogeneic PBMCs showed intense CD45\(^{+}\) mononuclear cell infiltration, signs of active inflammation and loss of dermo-epidermal integrity mediated by human leukocytes [397]. Addition of polyclonally expanded nTreg reduced signs of inflammation by approximately half, however addition of alloantigen-driven Treg nearly completely protected grafts from histological features of injury. There were also significantly more FoxP3\(^{+}\) infiltrates in grafts of mice that received alloantigen-driven Treg compared with polyclonally expanded, suggesting that alloantigen-driven Treg are more efficacious [397]. Interestingly, neither of these studies assessed graft survival as an outcome.

One of the aims of this thesis was to compare the efficacy of alloantigen-driven Treg with nTreg. In order to make this a comprehensive comparison activated and freshly isolated nTreg were compared with different methods of generating alloantigen-driven Treg. CD4\(^{+}\)CD25\(^{−}\) effector T cells were adoptively transferred at ratios of 1:1, 2:1 and 3:1 with either: freshly isolated nTreg, activated nTreg, TGF-β Treg, cilostamide Treg or CD44-Treg. A full thickness skin allograft was transplanted the following day. Previous work by our laboratory has shown that titrating effectors is a very sensitive way of comparing Treg efficacy, whereas titrating Treg does not
provide the required sensitivity [475]. Mice reconstituted with effectors only rejected their grafts acutely. At a 1:1 ratio of effectors to Treg, all Treg populations protected graft survival to a median survival time (MST) of >100 days, although there were some rejection events in mice that received TGF-β, freshly isolated and activated nTreg (Figure 3.15b).

At a ratio of 2 effectors to 1 Treg (Figure 3.15c), mice that received freshly isolated nTreg had an MST of 58.5 days, with only one mouse maintaining its skin graft beyond 100 days (n=6), whereas mice that received activated nTreg had an MST of >100 days (n=7), with 4 mice retaining their grafts past 100 days. These differences were not statistically significant however (p=0.19, Log-rank test). At the highest ratio of effectors to Treg (3:1) tested, the MST of mice that received freshly isolated nTreg decreased to 26 days (n=6) and mice that received activated nTreg had an MST of 53 days (n=6) (Figure 3.15d). The difference in survival were statistically significantly different at this ratio of cells (p=0.048, Log-rank test). This observation agrees with the conclusions of many others, that Treg are most effective when recently activated [259, 476, 477].

TGF-β Treg proved to be the least effective at controlling graft rejection, for example 3/5 mice rejected their grafts at a ratio of 1:1 (Teff:Treg) and grafts rejected with an MST of 30 days, at a ratio of 2:1 (Teff:Treg) (Figure 3.15c). All grafts rejected by day 85 at the highest number of Teff used. The observation that TGF-β Treg were not less effective than other populations at a ratio of 1:1 but became so as the number of Teff was increased validates the experimental approach as a sensitive method to distinguish differences in Treg efficacies. Activated nTreg had an MST of >100 days at this ratio, compared to an MST of 85 and 78 days for CD44-Treg and cilostamide Treg respectively (Figure 3.15c). The differences between activated, cilostamide Treg and CD44-Treg were statistically non-significant. At a 3:1 ratio of effectors to Treg, mice that received TGF-β Treg rejected their skin grafts at control rates (MST = 11 days) but in striking contrast, mice that received CD44-Treg and activated nTreg had graft MST of 52 and 53 days respectively, and
mice that received cilostamide Treg had a slightly improved MST of 75 days (Figure 3.15d). The differences in MST between cilostamide Treg, CD44-Treg and activated nTreg were not statistically significant, suggesting that there is no intrinsic difference in efficacy between these three types of Treg. Despite the theoretical advantage of alloantigen-driven Treg there appears to be no real advantage as demonstrated in this sensitive comparative transplant model. This is an important observation because if supported by other studies it could mean that generating alloantigen-driven Treg is unnecessary.

As expected, activated nTreg were more suppressive than freshly isolated nTreg. A possible explanation for the reduced efficacy of TGF-β Treg is that a large percentage of TGF-β Treg, as demonstrated in Figure 3.9 and in the literature, lose FoxP3 expression when re-stimulated or exposed to inflammatory conditions due to a demethylated TSDR and can contribute to graft rejection [299, 320]. Cilostamide Treg, CD44-Treg and activated nTreg all controlled graft rejection with similar kinetics regardless of the ratio of Treg to effectors. All three of these Treg populations exhibit a TSDR which is more demethylated than found within TGF-β Treg and >90% of CD44-Treg, cilostamide Treg and nTreg retained FoxP3 expression upon restimulation in vitro (Figure 3.12, [247, 320]). The fidelity of FoxP3 expression is a plausible reason for their increased efficacy in comparison with TGF-β Treg as stable Treg are unlikely to revert to an effector phenotype and should not contribute to graft rejection. Interestingly, there was no difference between alloantigen-driven Treg and activated nTreg in controlling rejection in this skin graft model despite it being previously reported in other models that alloantigen-driven Treg are more efficacious [397, 398]. The activated nTreg were not rested after activation, which differs to previous work by Sagoo et al., who rested both the polyclonally activated nTreg and the alloantigen-driven Treg for 2 days prior to adoptive transfer into BALB/c.Rag2<sup>−/−</sup>γc<sup>−/−</sup> mice [398]. It is possible that this rest period affects the two populations of Treg differently and that the polyclonally stimulated nTreg used by Sagoo et al. [398] are less activated than their alloantigen-driven counterparts when adoptively transferred, or alloantigen-driven Treg are able to
reactivate more rapidly than polyclonally stimulated nTreg in vivo. This would correlate with the requirement for Treg to be activated for optimal suppression, as demonstrated by the increased efficacy of activated nTreg compared with freshly isolated nTreg in this skin graft model.

The importance of activation in determining the efficacy of the nTreg population raises an important question regarding the relative efficacies of activated nTreg and alloantigen stimulated Treg. Due to the important nature of activation, it is possible that although alloantigen-driven Treg have a higher precursor frequency for alloantigen, this advantage is abrogated by the increased activation status of activated nTreg. To address the activation status issue it would be desirable to look at a panel of activation markers (e.g. CD69 [478, 479], and CD71 [398, 480]) and phosphorylation of signalling molecules associated with Treg function (e.g. STAT-3 [481] and STAT-5 [482]) in vitro before Treg are adoptively transferred into mice. This could be used to rule out a difference in activation status for the similar efficacies of nTreg and alloantigen-driven Treg.
3.14 Discussion

This chapter demonstrates that the generation of Treg using the PDE3 inhibitor cilostamide is sensitive to IL-6, and that both CD4\(^+\) T cells and GT-DCs produce IL-6 at concentrations that are not permissive for optimal alloantigen-driven Treg generation in the presence of cilostamide. However, data in this chapter have shown that it is possible to ameliorate the negative effects of IL-6 expression to generate large numbers of Treg using anti-CD44 antibody in the absence of PDE3 inhibition. These CD44-Treg are capable of protecting a fully MHC mismatched skin graft and do so largely in a donor specific manner. This may be explained by the fact that these cells resemble nTreg, with regards to their fidelity of FoxP3 expression in the presence of a robust polyclonal stimulus, and this appears to be due to a highly demethylated TSDR, similar to that found in nTreg.

The discovery that Treg can regulate allograft rejection in multiple models has facilitated their path towards clinical trials [19, 21, 248, 483]. Although modern immunosuppressive drugs have produced outstanding short term survival results, long term survival outcomes of organ transplants have not seen the same improvements, leading to clinical trials of Treg as a potential solution to this problem [484, 485].

Although clinical trials are investigating alloantigen-reactive Treg, most trials are focusing on polyclonally expanding Treg *ex vivo* [21, 486]. Due to the potential of polyclonally expanded nTreg to recognise a wide array of antigens within the patient, these cells could potentially lead to the same side effects associated with modern immunosuppressive drugs, which broadly dampen the immune system, leaving the host susceptible to an increased risk of co-morbidities [12, 487]. Vajdic et al. compared the incidence of cancer in patients receiving immunosuppression after kidney transplantation with the incidence of cancer in the same group of patients whilst they were on dialysis during end-stage kidney disease. After transplantation, the patients were reported to be 3 times more likely to develop cancer than when on dialysis.
Another study, conducted by Zwald et al., showed that non-melanoma skin cancer in solid
organ transplant recipients receiving immunosuppression had an incidence 200 times greater
than the general population [489]. Therefore, it would be desirable to treat the patient with a
population of Treg that do not broadly immunosuppress the patient.

It is possible to enrich for and expand alloreactive Treg by stimulating recipient CD4\(^+\) T cells with
donor APCs under favourable conditions for Treg and alloantigen-driven Treg have been shown
to be more potent than their polyclonally expanded counterparts [397, 398]. The comparison
of different Treg populations using the Rag\(^{-}\) skin allograft model was done with the expectation
that activated nTreg would be more suppressive than freshly isolated nTreg, and in turn,
alloantigen-driven Treg would be more suppressive than activated nTreg. It was also important
to compare the suppressive function of CD44-Treg with other Treg populations to identify the
most suppressive population for future experiments in lymphoreplete mice. Activated nTreg
did indeed protect skin allografts more effectively than their freshly isolated counterparts,
although this difference was only significant at a ratio of 3 effectors to 1 Treg. Due to the
limitations of other data assessing the relative efficacies of different Treg populations this was
an important set of experiments. Titrating un-manipulated and therefore consistent Teff offers
a sensitivity advantage over many other studies that have attempted to compare Treg
populations. Titrating in Teff but keeping the numbers of Treg constant showed that
alloantigen-driven Treg were not more suppressive than activated nTreg in this skin graft model.
This is potentially an important result as generating alloantigen-reactive Treg is typically more
challenging than generating polyclonally expanded nTreg and alloantigen-driven Treg will not
be applicable in all types of organ donation. For example, alloantigen-driven Treg will not be
available at the time of transplant with deceased donors. These issues therefore, make
determining whether alloantigen Treg are more efficacious than nTreg an important question
to address and so far there is little evidence to suggest this is the case although it is often stated
[397, 398]. A key theoretical advantage of alloantigen-driven Treg is the possibility that they
will be more specific for antigens present in the donor allograft and are less likely than nTreg to be broadly immunosuppressive. This not just a theoretical issue because polyclonally expanded nTreg have been shown to recognise a broader repertoire of antigens than their antigen-driven counterparts [397]. However, pre-clinical models of graft versus host disease (GVHD) have identified that adoptive transfer of nTreg can prevent GVHD whilst retaining the desirable graft-versus-leukaemia (GVL) response [469, 470], suggesting that they are not broadly suppressing the immune response. Co-transfer of $1 \times 10^4$ A20-\textit{luc/yfp} cells with T cell depleted (TCD) Bone marrow (BM) results in leukaemia in B6 mice and death before day 36. Animals that received A20-\textit{luc/yfp}, TCD BM and Tconv died sooner, due to GVHD. However, mice that also received polyclonally expanded nTreg survived past the observation period of 60 days [469]. Although Edinger et al. did not look for evidence of increased infections in mice that received Treg, it is reasonable to assume that because they did not report any adverse events then the Treg did not suppress the normal immune response to infections. Additionally, in the Treg titration experiments (Figure 3.15) in Rag^{-/} mice, no increase in complications associated with infections were observed with any of the Treg populations, again suggesting that nTreg do not prevent protective immune responses towards infections. These data are extremely promising as they suggest that polyclonally expanded nTreg may not broadly immune-suppress the recipient, and may not leave patients susceptible to common problems associated with global immunosuppression [469, 470].

Budd et al. demonstrated that female mice, immunised with male minor transplant antigen, H-Y, contained CD8$^+$ T cells that were responsive to the H-Y antigen and these cells were CD44$^{hi}$ (Pgp-1$^+$) [490]. Butterfield et al. later demonstrated that this phenomenon extended to CD4$^+$ T cells when they showed that mice immunized with sperm whale myoglobin or keyhole limpet hemocyanin had antigen-specific CD4$^+$ memory T cells that were exclusively Pgp-1$^+$ (CD44$^{hi}$) [491]. CD44 is a type I transmembrane glycoprotein which is expressed by many types of cell. It is subjected to alternative splicing and post-translational modifications, generating multiple
variants of CD44. These have the potential to bind a variety of ligands including: collagen [492], fibronectin [493], osteopontin [494], galectin-9 [495] and the best characterised hyaluronic acid (HA) [496]. HA is an extracellular matrix component (ECM), which is known to exist in two forms; high molecular weight (HMW) and low molecular weight (LMW). HMW-HA is known to have anti-inflammatory properties in a variety of settings by signalling through CD44 [467, 497]. Incubation of bovine aortic endothelial cells (BAEC) with HMW-HA did not induce proliferation, whereas LMW-HA did. Importantly, incubation of BAEC with HMW HA inhibited the induction of early response gene expression by LMW-HA, in a dose dependent fashion, and this was shown to be dependent on the expression of CD44 by BAEC cells [498]. Stimulation of nTreg with plate bound anti-CD3 antibody in combination with 20µg/mL HMW-HA has been shown to promote the suppressive effects of CD4+CD25+ regulatory T-cells [468]. LMW-HA is a fragment of the ECM which is generated during infection and injury, and in contrast to HMW-HA, it has inflammatory properties and can stimulate angiogenesis [468, 499]. Bollyky et al. demonstrated that CD44 cross-linking promoted FoxP3 expression in human nTreg, in part via IL-2 production [463]. CD44 mediated activation also stimulated the expression of TGF-β and IL-10 [463]. CD44 blockade has also been shown to reduce IL-17 expression from Th1 cells, although IFN-γ expression was not affected [500]. The effects of manipulating CD44 on Th1 cells and nTreg, previously reported in the literature, in combination with a reduction in IL-6 expression from CD44hi cells identified in Figure 3.6 suggests that anti-CD44 antibody generates a favourable environment for the expansion of nTreg in a combination of ways.

Interestingly, it has been demonstrated that cross-linking CD44 on nTreg with HA renders them resistant to the commonly used CNI, Cyclosporine (CsA) [463, 501]. Stimulation of Treg in vitro with anti-CD3, anti-CD28 and anti-CD44 antibodies for three days in media containing 50ng/mL CsA resulted in 87% of Treg remaining FoxP3+ and CD25+, whereas stimulation of nTreg in identical conditions with the omission of anti-CD44 resulted in only 32% of cells retaining FoxP3 and CD25 [501]. Cross-linking CD44 was found to promote FoxP3 expression in an IL-2
independent manner, however STAT-5, which has been identified to be essential for maintaining FoxP3 expression and Treg function in vivo [502, 503], was indispensable [501]. Stimulation of nTreg in this way could provide a method of generating Treg that are more resistant to CNIs in vivo, which is a particularly interesting discovery as CNIs may be detrimental to Treg function and survival in vitro and in vivo [412, 429]. Zeiser et al. showed that 10mg/kg of CsA, dosed from day 0 until the end of the observation period, in addition to 8x10^5 CD4^+CD25^{high} Treg reduced overall survival from GVHD to approximately 37% compared with approximately 80% of mice that received CD4^+CD25^{high} Treg [412]. This could be a serious problem for Treg implementation in the clinic, as CNIs from an important part of the immunosuppressive regimens given to transplant recipients [403, 404]. The generation of CNI resistant Treg would thus be highly advantageous.

Wu et al. recently provided evidence that another key CD44 ligand, Galectin-9, interacts with CD44 and serves to increase the stability and function of adaptive Treg [504]. Purified GFP^+iTreg from wt and Galectin-9 knockout mice (Lgals^-/-) were transferred into Rag2^-/- Lgals^-/- mice and the presence of FoxP3^+ (GFP) cells were analysed on day 2 and 5. Only 11.4% of cells were still FoxP3(GFP) after 5 days in mice that received iTreg that were Lgals^-/- compared with 27.8% of cells retaining FoxP3(GFP) from wt mice. These data suggest that signalling through Galectin-9 is important for the stability of de novo of FoxP3 expression in Treg [504]. This is promising as even though there is a small de novo induction of FoxP3 expression when enriching for Treg in the presence of anti-CD44 antibody, (Figure 3.13) they appear to be stable.

The observation in Figure 3.5 that increasing proportions of CD44^{hi} CD4^+ T cells did not affect Treg enrichment driven by PDE3 inhibition, led to the hypothesis that anti-CD44 antibody could be used to enrich for alloantigen-reactive Treg. An interesting point that arises from these data is that anti-CD44 antibody, in combination with cilostamide, led to a lower yield of Treg than either cilostamide or anti-CD44 antibody alone. This reduction in yield when combining two
methods of generating Treg is not unique to this combination. Combining either IFN-γ or TGF-β with cilostamide also reduces Treg yield when cells are stimulated with alloantigen (personal communication with Thomas Chan, unpublished data). The reasons for this are unclear but the combination of different reagents could be affecting the T cells detrimentally. Generating and enriching for Treg using IFN-γ was shown to be, in part, dependent on dominant toxicity and apoptosis of FoxP3+ cells and preferential survival of FoxP3+ cells [139]. Feng et al. showed that driving CBA CD4+ T cells with GMCSF/TGF-β GT-DC in the presence of IFN-γ enhanced cell death in the FoxP3+ population by approximately 3 fold, compared with cells that were not cultured in IFN-γ [139]. Although there is no direct evidence that cilostamide is toxic towards T cells, it is reasonable to assume that there is some level of toxicity caused by cilostamide due to the observation that fewer cells are harvested after the culture period, than are added to the culture at the beginning and in vitro concentrations of 20µM cilostamide led to nearly a 4 fold reduction in yield of Treg compared with 5-10µM cilostamide [247]. With this in mind, it is possible that the combined toxicity of cilostamide, with IFN-γ or anti-CD44 antibody, prevents the generation of useful yields of Treg. Carefully titrating combinations of these reagents could lead to an efficient method of generating Treg in vitro but this has not yet been explored.

As MHC class II+ GT-DCs were the most efficient APC used to enrich for Treg in the cilostamide protocol, attempts were made to increase the proportion of these cells within our GT-DC input population, as sorting these cells was extremely mouse intensive. The bone marrow derived cells used in these protocols are cultured in the presence of GMCSF and TGF-β for 6 days prior to using them as APCs. TGF-β is added to cultures as it restricts the maturation of the BMDCs by reducing the expression of CD80, CD86, CD40 and IL-6 [505] [139]. IL-10 has also been identified as a modulator of dendritic cell activation, with variable reports on its effects on expression of CD80, CD86 and MHC class II dependant on the timing of exposure to IL-10 and the variety of dendritic cell studied [506-510]. Ozawa et al. showed that culturing Langerhan cells in media supplemented with 20ng/mL IL-10 for 72 hours, inhibited the expression of CD80.
Only a partial inhibitory effect on the up-regulation of CD86, CD54 and CD40 expression was shown [506]. The majority of others have reported that IL-10 differentially regulates CD80 and CD86 expression, down-regulating the expression of CD80 but not CD86 [507-510]. Therefore, to ask whether more “migratory” like dendritic cells, that have been shown to be more effective at generating Treg in vitro (Figure 3.2) and in vivo [459], could be generated in the presence of IL-10, TGF-β was substituted for IL-10 when conditioning BMDCs in the presence of GMCSF. The proportions of MHC class IIhi cells was not changed significantly in the presence of IL-10 (data not shown) after 6 days in culture and therefore this was not explored further. It might have been interesting to use these cells as APCs and compare the efficiency of Treg generation in the presence of cilostamide with our GMCSF and TGF-β conditioned DCs but other avenues of generating Treg were explored instead.

IL-6 expression from CD4+ T cells is often ignored but there are few reports concerning CD4+ expression of IL-6 in the literature. Sofi et al. identified that when T cells were stimulated with plate bound anti-CD3 antibody, IL-6 originating from CD4+ T cells was responsible for inducing IL-4 expression and polarising the cells towards the Th2 lineage [511]. IL-6 expression from CD4+ T cells clearly has an important function in cultures that are dependent on specific conditions for T cell lineage commitment. The ~3 fold reduction in IL-6 expression from CD4+ T cells when plate bound anti-CD44 antibody is used to stimulate the cells appears to contribute to a favourable environment for Treg enrichment. However, it must be considered that anti-CD44 antibody binding to CD4+ T cells is also having an effect on BMDC expression of IL-6. CD44 cross-linking also activates nTreg, as demonstrated by Bollyky et al. and stimulates them to generate IL-2, TGF-β and IL-10 [463]. It is therefore unclear whether the reduction in IL-6 expression from CD4+ T cells is the only effect of CD44-crosslinking, or whether a subsequent increase in the expression of IL-2, TGF-β and IL-10 from activated nTreg, present in the input culture, also has an impact on the expression of cytokines such as IL-6 from the allogeneic GT-DCs, facilitating the enrichment of Treg further. A combination of some or all of these factors is also possible.
However, it is unlikely that unbound anti-CD44 antibody is having a profound effect on the APCs, as there was no difference in Treg enrichment when anti-CD44 antibody was left in the cultures or washed away after the incubation period with CD4+ T cells.

In conclusion, the data presented in this chapter suggest that activated autologous nTreg are as effective as two different populations of alloantigen-driven Treg and are more effective than TGF-β Treg. This is an important observation due to previously published data suggesting that alloantigen-driven Treg are more efficacious than activated nTreg [397, 398]. Further experiments validating this result, as well as asking whether nTreg more broadly suppress protective immune responses, will be important to address whether pursuing the more difficult to expand alloantigen-driven population is worthwhile.
Chapter 3: Figures
Figure 3.1 Stimulation of purified CD4\(^+\) T cells with allogeneic BMDCs combined with PDE3 inhibition leads to variable enrichment of Treg.

Total CD4\(^+\) T cells, from CBA(GFPFoxP3) (H2\(^k\)) mice, were magnetically isolated and co-cultured with B6 (H2\(^b\)) GMCSF and TGF-β “matured” DCs (GT-DCs) in the presence of 5µM cilostamide, a PDE3 inhibitor. 5x10\(^5\) CD4\(^+\) T cells and 5x10\(^4\) GT-DCs were added per well of a 24 well plate. On Day 7, 5x10\(^4\) fresh GTDCs were added in media supplemented with 5µM cilostamide. Cells were analysed after 14 days of culture. Treg were gated on CD4, TCR-β and GFP(FoxP3) and analysed using FACs. 

a) Representative plots showing variation of enrichment.  
b) Quantification of Treg at the end of the 14 day culture period. Each experiment shows mean number and percentage of Treg from 4 wells of a 24 well culture plate. Data from six separate experiments (p value for one-way ANOVA analysis).
Figure 3.2 MHC class II\textsuperscript{hi} CD11c\textsuperscript{hi} more efficiently enrich for Treg than unsorted GT-DCs and MHC class II\textsuperscript{int/lo}CD11c\textsuperscript{hi}

a) GT-DC sorting strategy; after 6 days in culture supplemented with GMCSF and TGF-\(\beta\), cells were gated and sorted into MHC class II\textsuperscript{hi}CD11c\textsuperscript{hi} (MHC\textsuperscript{hi}) and MHC class II\textsuperscript{int/lo}CD11c\textsuperscript{hi} (MHC\textsuperscript{int/lo}) sub-populations.  

b) CD4\textsuperscript{+} T cells, from CBA(GFPFoxP3) (H2\textsuperscript{k}) mice were co-cultured in media supplemented with 5\(\mu\)M cilostamide, with one of three populations of B6 (H2\textsuperscript{b}) GT-DCs: 1) Unsorted GT-DCs (Total DCs), 2) MHC class II\textsuperscript{hi}CD11c\textsuperscript{hi} (MHC\textsuperscript{hi}), or 3) MHC class II\textsuperscript{int/lo}CD11c\textsuperscript{hi} (MHC\textsuperscript{int/lo}).  

5x10\textsuperscript{5} CD4\textsuperscript{+} T cells and 5x10\textsuperscript{4} BMDCs were added per well of a 24 well plate.  

On Day 7, 5x10\textsuperscript{4} freshly sorted GT-DCs were added in media supplemented with 5\(\mu\)M cilostamide.  

Quantification of FoxP3\textsuperscript{+} T cells was by CD4\textsuperscript{+} and GFP(FoxP3)\textsuperscript{+} expression, on day 14 after two rounds of stimulation (n= 4, p value for Student’s t test).
Figure 3.3 IL-6−/− GT-DCs improve the enrichment of Treg in the presence of the PDE3 inhibitor cilostamide

BMDCs isolated from wild type (wt) or IL-6−/− mice were cultured in the presence of GMCSF and TGF-β for 6 days to generate GT-DCs. Total CD4⁺ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and co-cultured with wild type (wt) or IL-6−/− GT-DCs in the presence of 5µM cilostamide for 7 days. 5x10⁵ CD4⁺ T cells and 5x10⁴ GT-DCs were added per well of a 24 well plate. On Day 7, fresh GT-DCs were added in media supplemented with 5µM cilostamide. Cells were harvested on day 14 and Treg were enumerated using FACs on the basis of GFP(FoxP3)⁺ and CD4⁺ expression. a) Representative dot plots of cilostamide Treg enrichment in the presence of IL-6−/− or wt GT-DCs. b) Cilostamide Treg enrichment in the presence of IL-6−/− or wt GT-DCs (n=8 separate experiments). c) Cilostamide Treg yield in the presence of IL-6−/− or wt GT-DCs (n=3 separate experiments, p value for Student’s t test).
Figure 3.4 CD44<sup>hi</sup> CD4<sup>+</sup> T cells express more IL-6 than CD44<sup>int/lo</sup> CD4<sup>+</sup> T cells

Total CD4<sup>+</sup> T cells, from CBA(GFPFoxP3) (H2<sup>k</sup>) mice, were magnetically isolated and stimulated with PMA, Ionomycin and Monensin for 5 hours. Cells were stained with CD4, TCR-β and CD44 surface markers prior to intracellular cytokine analysis of IL-6 expression. Cells were analysed using FACs. FMO refers to Fluorescence minus one control.

a. FACS plot identifying CD44<sup>hi</sup> population.

b. Representative histograms of IL-6 expression in CD44<sup>hi</sup> versus CD44<sup>int/lo</sup> CD4<sup>+</sup> T cells

Bar chart comparing median fluorescence intensity (MFI) of IL-6 for CD44<sup>hi</sup> and CD44<sup>int/lo</sup> CD4<sup>+</sup> T cells (n=3, p=0.0001, Student’s t test).
Figure 3.5 Increasing the proportion of CD44hi CD4+ T cells does not affect cilostamide dependent Treg enrichment

Total CD4+ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and flow sorted on the basis of CD44, CD4 and TCR-β expression. Purified CD4+ CD44hi cells were titrated into the CD4+ CD44int/lo population at increasing proportions (0-30%). A total of 5x10^5 CD4+ T cells were added to each well. 5x10^4 B6 (H2b) GMCSF and TGF-β “matured” DCs (GT-DCs) were used as APCs in media supplemented with 5µM cilostamide. After 7 days fresh GT-DCs were added in media containing 5µM cilostamide. Treg were analysed on the basis of CD4+ and FoxP3(GFP)+ expression after 14 days in culture.

a. Representative dot plots of Treg enrichment after 14 days of stimulation.
b. GFP(FoxP3)+ Treg enrichment with increasing proportions of CD44hi cells (n=3).
c. GFP(FoxP3)+ Treg yield with increasing proportions of CD44hi cells (n=3).
Figure 3.6 anti-CD44 antibody reduces IL-6 expression from CD4+ T cells

a. Total CD4+ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and stimulated with GT-DCs for 3 days. Cells were stained with CD4, TCR-β and CD44 surface markers prior to intracellular cytokine analysis of IL-6 expression using FACs.

i) Gating strategy of CD44hi and CD44int/lo. ii) Histograms comparing IL-6 intracellular cytokine signal intensity in cells that were stimulated with GT-DCs +/- anti-CD44 antibody.

b. Total CD4+ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated to a purity of >95% and stimulated with either: plate bound anti-CD3 and anti-CD28 antibodies (CD3/28), pre-incubated with 1µg/mL anti-CD44 antibody and then stimulated with plate bound anti-CD3 and anti-CD28 antibodies (CD3/28 + 44), or stimulated with plate bound anti-CD3, anti-CD28 and anti-CD44 antibodies (CD3/28/44) for 7 days. Supernatants were collected and analysed using an ELISA for IL-6 and results were normalised to 1x10^5 input cells per well (n=3 separate experiments, Student’s t test).
Figure 3.7 Anti-CD44 antibody titration

Total CD4⁺ T cells, from CBA(GFPFoxP3) (H2⁺) mice were magnetically isolated and incubated for 10 minutes with increasing concentrations of anti-CD44 antibody or isotype control (IgG2a). The cells were then co-cultured with B6 (H2ᵇ) GT-DCs, for 7 days. 5x10⁵ CD4⁺ T cells and 5x10⁴ GT-DCs were added per well of a 24 well plate. On Day 7, 5x10⁴ fresh GT-DCs were added in media. Cells were gated on CD4 and TCR-β for analysis using FACS. Each experiment shows mean number and percentage of Treg from 2 wells of a 24 well culture plate. Data from six separate experiments.

a. Illustrative dot plots of anti-CD44 antibody titration showing enrichment for CD4⁺GFP(FoxP3)⁺ Treg.
b. Percentage of GFP(FoxP3)⁺ Treg with increasing concentrations of anti-CD44 antibody.
c. GFP(FoxP3)⁺ Treg number with increasing concentrations of anti-CD44 antibody.
Figure 3.8 The anti-CD44 protocol yields more Treg than using TGF-β or cilostamide

Total CD4+ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and incubated for 10 minutes with 1µg/mL anti-CD44 antibody (CD44-Treg), or were added to media supplemented with TGF-β (5µg/mL) and IL-2 (100U/mL) to generate TGF-β Treg, or media supplemented with 5µM cilostamide. All cells were co-cultured with GMCSF and TGF-β differentiated B6 (H2b) GT-DCs, for 7 days. On Day 7 fresh GT-DCs were added. Cells were harvested on day 14 and gated on CD4, TCR-β and GFP(FoxP3) for analysis using FACs.

a. Comparison of percentage of GFP(FoxP3)+ Treg from cultures containing TGF-β and IL-2 (TGF-β), cilostamide or anti-CD44 antibody (CD44) after 14 days in culture (n=3).

b. Yield of GFP(FoxP3)+ Treg from cultures containing TGF-β and IL-2 (TGF-β), cilostamide or anti-CD44 antibody (CD44) after 14 days in culture (n=3, Student’s t test).
**Figure 3.9** Alloantigen-driven Treg enriched for in the presence of anti-CD44 antibody prevent skin allograft rejection

Total CD4+ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and incubated for 10 minutes with anti-CD44 antibody (1µg/mL). The cells were co-cultured with GMCSF and TGF-β differentiated B6 (H2b) GT-DCs, for 7 days. On Day 7 fresh GT-DCs were added. Cells were harvested on day 14 and sorted to >95% purity based on CD4 and GFP expression. nTreg were activated with 5µg/mL plate bound anti-CD3 and anti-CD28 for 4 hours in the presence of 250U/mL IL-2. 2x10^5 CD4+CD25- effectors with, or without, 1x10^5 activated nTreg or CD44-Treg were adoptively transferred into CBA Rag-/- (H2k) mice on day -1. A full thickness B6 (H2b) skin allograft was transplanted on day 0.

a. Experimental outline

(Figure continued on the next page)
b. Skin allograft survival. Mice that received $2 \times 10^5$ effectors only, rejected their skin grafts acutely (MST 12.5 days, n=5), co-transfer of activated nTreg or CD44-Treg increased MST to 57 days and >100 days respectively (n=7/8).

c. Representative images of a skin grafts from mice that received, i) CD44-Treg (100 days post-transplant) and an ii) acutely rejected skin graft that received effectors only (day 11 post-transplant). (**p value = 0.0001, Log-rank test).
**Figure 3.10 CD44-Treg regulate skin allograft rejection in a donor specific manner**

Total CD4+ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and incubated for 10 minutes with anti-CD44 antibody (1µg/mL). The cells were co-cultured with GMCSF and TGF-β differentiated B6 (H2b) GT-DCs, for 7 days. On day 7 fresh GT-DCs were added and cells were harvested on day 14. Cells were sorted to >95% purity based on CD4 and GFP expression. 1x10^5 CD4+CD25- effectors with, or without 1x10^5 anti-CD44 Treg were adoptively transferred into CBA Rag-/- (H2k) mice on day -1. A full thickness B6 (H2b) or 3rd party BALB/c (H2d) skin allograft was transplanted on day 0.

a. Experimental outline.

b. Skin allograft survival. Mice that received 1x10^5 effectors only, rejected B6 (MST 10 days, n=6) and BALB/c skin grafts (MST 10 days, n=6) acutely. Co-transfer of CD44-Treg increased MST of B6 skin grafts to >100 days (n=5), whereas co-transfer or CD44-Treg did not increase survival of BALB/c skin grafts (MST 12 days, n=7).
Figure 3.11 Treg enriched in the presence of anti-CD44 antibody maintain FoxP3 expression when restimulated in vitro

Total CD4⁺ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and incubated for 10 minutes with anti-CD44 antibody (1µg/mL), or media was supplemented with TGF-β and IL-2 (5µg/mL and 100U/mL, respectively) for TGF-β iTreg. The cells were co-cultured with GMCSF and TGF-β differentiated B6 (H2b) GT-DCs, for 7 days. On Day 7 fresh GT-DCs were added and cells were harvested on day 14. nTreg were bead enriched prior to flow sorting. All cells were flow sorted to ≥99% purity based on CD4 and GFP(FoxP3) expression. All populations were stimulated with plate bound anti-CD3 and anti-CD28 (5µg/mL of each) for 6 days. GFP(FoxP3) expression was measured using FACS.

a. Representative histograms for each Treg population before (left plots) and after stimulation (right plots) with plate bound anti-CD3 and anti-CD28 antibodies for 6 days.

b. Comparison of FoxP3 retention by nTreg, TGF-β Treg and CD44 Treg after stimulation with plate bound anti-CD3 and anti-CD28. nTreg FoxP3 mean retention 90.8% (n=3), TGF-β FoxP3 mean retention 43.25% (n=2) and CD44-Treg FoxP3 mean retention 93.5% (n=3).
**Figure 3.12i** Schematic view on the FoxP3 locus identifying exon-intron structure and the locations of amplicons (Amp 1–4). The position of each individual CpG motif within amplicons is shown.

Figure taken from Plos Biol. 2007 Feb;5(2):e38 “Epigenetic control of the FoxP3 locus in regulatory T cells.” Floess et al., with permission from Professor Jochen Huehn.
Figure 3.12ii Treg enriched in the presence of anti-CD44 have a heavily demethylated TSDR

Total CD4+ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and incubated for 10 minutes with anti-CD44 antibody (1µg/mL) or media was supplemented with TGF-β & IL-2 (5µg/mL and 100U/mL respectively). The cells were co-cultured with GMCSF and TGF-β differentiated B6 (H2b) GT-DCs, for 7 days. On Day 7 fresh GT-DCs were added and cells were harvested on day 14. nTreg were enriched for using magnetic beads. All Treg were flow sorted to ≥99% purity, based on CD4 and GFP(FoxP3) expression. Methylation status was measured using bisulphite sequencing (see materials and methods for details).

a. Representative sort purities for nTreg, CD4+GFP(FoxP3)-cells (Teff), TGF-β Treg and CD44-Treg.

b. Methylation patterns of amplicons 1 and 2 of the FoxP3 locus. Methylation status of individual CpG motifs are separated by horizontal lines, with each box representing each individual motif. Replicate populations from at least two independent experiments were analysed with a minimum of three replicates per population for each experiment.
Figure 3.13 Enrichment of Treg using anti-CD44 antibody is largely dependent on the presence of nTreg at the beginning of the culture.

Total CD4$^+$ T cells, from CBA(GFPFoxP3) (H2$^b$) mice were magnetically isolated. Half of the cells were then flow sorted on the basis of GFP(FoxP3) expression to remove FoxP3$^+$ (GFP$^+$) Treg. Purified CD4$^+$GFP$^-$ T cells were then incubated with anti-CD44 antibody (1µg/mL) for 10 minutes, or the cells were added to media supplemented with TGF-β and IL-2 (5µg/mL and 100U/mL, respectively). In parallel, cultures were set up with the CD4$^+$ T cells that had not been depleted of Treg (Total CD4$^+$). All cells were co-cultured with GMCSF and TGF-β differentiated B6 (H2$^b$) GT-DCs, for 7 days. On Day 7 fresh GT-DCs were added. Treg were measured by GFP(FoxP3) using FACS after 14 days in culture.

a. Representative plots identifying de novo FoxP3 induction using anti-CD44 antibody or TGF-β and IL-2

(Figure continues over the page)
b. Mean Treg percentages:

- anti-CD44 antibody cultures, CD4\(^+\)GFP\(^-\): 2.3\% GFP\(^+\). Total CD4\(^+\): 34.4\% GFP\(^+\) (n=3, Student’s t test)

- TGF-\(\beta\)/IL-2 cultures, CD4\(^+\)GFP\(^+\)TGF-\(\beta\): 66.9\% GFP\(^+\). Total CD4\(^+\) TGF-\(\beta\): 81.7\% GFP\(^+\), (n=3, Student’s t test).
Figure 3.14 anti-CD44 antibody facilitates the proliferation of FoxP3+ Treg

Total CD4+ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated, VPD labelled and incubated for 10 minutes with anti-CD44 antibody (1µg/mL). The cells were co-cultured with GMCSF and TGF-β differentiated B6 (H2b) GT-DCs for 7 days. On day 7, fresh GT-DCs were added and cells were harvested on day 14. Treg were gated on the basis of CD4 and GFP(FoxP3) expression.

a. Illustrative histograms of Treg proliferation.

b. 48.9% (+/- 6%) of nTreg (GFP+) incubated with anti-CD44 antibody (+44) proliferated at least once, compared with 12.9% (+/- 1.5) in the absence of anti-CD44 antibody.

c. Illustrative histograms GFP(FoxP3) proliferation.

d. 7.9% (+/- 1.5%) of GFP(FoxP3) T cells incubated with anti-CD44 antibody (+44) proliferated at least once, compared with 4.8% (+/- 0.9%) in the absence of anti-CD44 antibody (p value Student’s t test, n = 3 separate experiments).
Figure 3.15 Comparison of different Treg populations in a skin allograft model

Total CD4+ T cells, from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and incubated for 10 minutes with 1µg/mL anti-CD44 antibody (CD44-Treg), or isolated CD4+ T cells were added to media supplemented with TGF-β (5µg/mL) and IL-2 (100U/mL) to generate TGF-β Treg, or media supplemented with 5µM cilostamide (cilos Treg). All cells were co-cultured with GMCSF and TGF-β differentiated B6 (H2b) GT-DCs, for 7 days. On Day 7 fresh GT-DCs were added. Cells were harvested on day 14. nTreg were magnetically isolated from CBA(GFPFoxP3) (H2k) mice. Freshly isolated nTreg were kept on ice in media supplemented with 100U/mL IL-2 while activated nTreg were stimulated with plate bound anti-CD3 and anti-CD28 antibodies for 4 hours in the presence of 250U/mL IL-2. All Treg populations were sorted to >90% purity based on CD4 and GFP expression. 1-3x10^5 CD4^+CD25^- effectors, with or without: 1x10^5 activated nTreg, freshly isolated nTreg, CD44 Treg, cilos Treg or TGF-β Treg were adoptively transferred into CBA Rag/- (H2k) mice on day -1. A full thickness B6 (H2b) skin graft was transplanted on day 0. Graft survival was monitored on a regular basis.

a. Experimental outline.  
b) Skin allograft survival 1x10^5 effectors.

(Figure continues on the next page)
c) Skin allograft survival, 2x10^5 effectors.

d) Skin allograft survival, 3x10^5 effectors.
Chapter 4: *In vivo* efficacy of Regulatory T cells

**Introduction**

Clinical trials have started investigating the potential for Treg to control GVHD in HSCTx [19, 20] and rejection in solid organ transplantation [21]. Preclinical models have provided a vast amount of data facilitating the start of clinical trials assessing Treg efficacy in humans. These models provide a useful and ethical way of comparing different administration times of Treg to identify when they will be most efficacious. It is also important to address this issue as certain types of transplantation will require the administration of Treg post-transplant, as there is often not enough time to expand Treg to sufficient numbers prior to the transplant procedure, primarily in cadaveric donors. A variety of pre-clinical models of solid organ transplantation have administered cells pre-transplant, however there is a lack of data in which Treg were administered in the period post-transplant, without any other treatment. This suggests a lack of efficacy when administering Treg in the post-transplant period [512].

Feng et al. showed that CBA Rag²⁻/⁻ mice co-transferred with $2 \times 10^5$ IFN-γ conditioned Treg and $2 \times 10^5$ CD4⁺CD25⁻ Teff, the day before the transplant of a B10 skin graft went onto long term survival, whereas mice that received $2 \times 10^5$ CD4⁺CD25⁻ cells only, rejected their grafts acutely [140]. Using a different model, Warnecke et al. co-transferred CD25⁺CD4⁺ Treg cells, from CBA mice that had been pre-treated with a DST from B6 mice, with CD25⁻CD4⁺ (H2k) Teff the day before mice received a B6 aorta graft. Mice that received Treg in combination with Teff had significantly reduced luminal occlusion of the aorta graft compared with mice that received Teff only [513]. Models of GVHD have also identified that Treg administration pre-transplant is effective. Lethal GVHD in BALB/c recipients, induced by B6 CD4⁺CD25⁻ T cells, was shown to be prevented by the co-injection of B6 CD4⁺CD25⁺ cells after irradiation and reconstitution of mice with B6 T cell depleted bone marrow [514]. However, there are a lack of data directly comparing the efficacy of different administration times of Treg therapy, especially post-transplant. There
is also a lack of data indicating whether adoptively transferred Treg are sufficient to prolong allograft survival, in lymphoreplete animals, without any other treatment. Zheng et al. demonstrated that $10^7$ TGF-β induced alloantigen-driven Treg administered on both D-1 and D+5, relative to the graft, led to the long term survival of cardiac allografts in mice [515]. This is the only example of Treg preventing allograft rejection in otherwise lymphoreplete animals in the literature and is another example of Treg being used effectively when the initial dose is adoptively transferred pre-transplant.

The timing of Treg administration, and the type of Treg used (nTreg versus alloantigen-reactive), is likely to be largely determined by the type of transplant (cadaveric versus living donor). Administration of nTreg pre-transplant may be a feasible option for all types of transplantation as preclinical data has demonstrated that nTreg can be expanded and cryopreserved without loss of function [248, 516, 517]. However, there are major caveats associated with alloantigen-reactive Treg. Firstly, it is unlikely that they will be available until after the transplant, particularly in cadaveric donors as the donor is not known until immediately before the transplant. Secondly, generating large numbers of stable alloantigen-driven Treg is still more challenging than polyclonally expanding nTreg, however protocols achieving thousand fold levels of expansion have been described [518]. Sagoo et al. sorted alloantigen-activated human Treg, based on up-regulation of CD69 and CD71 after stimulation with allogeneic DCs, and expanded them in the presence of IL-2 (250U/mL) without further restimulation. They described a mean of 1000 fold expansion (range of 340-5130 fold) over a 4-6 week period [398]. However, Hippen et al. have published data suggesting that flow sorted human nTreg, stimulated with cell based artificial APCs (expressing anti-CD3, CD86 and CD64) in the presence of IL-2 (300U/mL) led to a 50 million fold expansion of nTreg [395]. These two examples reporting Treg expansion in vitro demonstrate the disparity in the levels of expansion achievable between the two types of Treg.
Studies comparing alloantigen-reactive Treg with nTreg suggest that alloantigen-reactive or induced Treg are more efficacious than nTreg [397, 398, 519, 520]. Dai et al. showed that antigen-induced, but not naïve Treg can suppress allograft rejection mediated by CD8+ memory T cells in splenectomised H2b mice that received BALB/c (H2d) skin grafts [521]. It must be considered that the activation status of the alloantigen-reactive Treg might have been an important confounding factor in these data, as the authors compared freshly isolated naïve T reg with antigen-induced Treg and the data in Figure 3.15 suggests that the activation of nTreg is important for their efficacy in vivo. Using humanised pre-clinical models, Sagoo et al. stimulated Treg with allogeneic DCs and used the up-regulation of CD69 and CD71 to determine which Treg were alloantigen-reactive. In contrast, Putnam et al. demonstrated that it was possible to expand and enrich for alloantigen-reactive Treg using CD40L stimulated allogeneic B cells [397, 398]. Both of these methods showed that alloantigen-reactive Treg were more effective at preventing the early signs of graft rejection in a skin allograft model in BALB/c.Rag-1-/-γc-/- mice. Interestingly, neither of these two studies assessed graft survival, instead focusing on early histological signs of rejection [397, 398]. Although their data does support the hypothesis that alloantigen-driven Treg are more efficacious, the impact on graft survival is the most important factor and early signs of rejection may not reveal a complete picture, although it is likely to be indicative.

Whilst the elegant experiments described by Sagoo et al. and Putnam et al. suggest that alloantigen-reactive Treg are more suppressive in vivo, the results are also complicated by the humanised mouse model system that they have used. In this model, the vast majority of cells that engraft are CD3+CD4+ and therefore it is similar to the experimental system in Figure 3.15, which demonstrated that activated nTreg were not significantly different in their ability to control skin allograft rejection when compared with alloantigen-driven Treg. Additionally, the adoptive transfer of naïve T cells into lymphopenic mouse models results in homeostatic proliferation and the acquisition of a memory-like phenotype [36-38], therefore these models
are unlikely to be representative of graft rejection in the clinical setting. Importantly, they are not asking whether alloantigen-driven, or polyclonally activated Treg can control a multifaceted immune response that leads to the rejection of an allograft, instead they are asking whether alloantigen-driven or polyclonally activated Treg can control homeostatic proliferation and prevent rejection mediated by a memory-like population of T cells. It is possible that alloantigen-driven Treg have an advantage over polyclonally activated nTreg in their control over the memory-like T cells that arise from homeostatic proliferation in a lymphopenic setting [38, 522] but this may not be an accurate reflection of their true efficacy in vivo. Furthermore, these humanised mice have impaired lymph node development, poorly developed germinal centres and impaired humoral immune responses, questioning the usefulness of these models when considering the ability of Treg to suppress a multifaceted immune response to an allograft [523, 524]. Therefore, one of the aims of this chapter was to ask whether Treg could prolong the survival of an allogeneic heterotopic heart graft in lymphoreplete mice and secondly to try to delineate any differences in efficacies between activated nTreg and alloantigen-driven Treg in the lymphoreplete mouse.

One of the key reasons for pursuing Treg as a novel therapy to control rejection in solid organ transplant recipients is that in theory Treg will have fewer side effects. Although vast improvements have been made regarding the sophistication of immunosuppressive drug regimens, used to control transplant rejection, there is still a huge problem with the increased incidence of co-morbidities in these patients as a result of broad acting immunosuppressive drugs. These problems include an increased risk of infection, vascular disease and cancer [525-527]. For example, from a database of 798 liver transplant recipients, the second most common cause of death in the first year was malignancy (22%), with cardiovascular disease and infections causing 11% and 9% of the deaths respectively. Rejection accounted for 28% of deaths [526]. These findings are similar to those in a study involving long term follow up (>25years) of renal transplant recipients in Leiden, in which the most frequent cause of death within the first year
of transplant was infection (36%), however death after the first year was predominantly caused by cardiovascular disease (44%) with malignancy being the second largest cause of death (22%) [527]. These studies highlight that malignancies account for a large proportion of deaths in transplant recipients. Increasingly, endogenous nTreg have been identified to have a detrimental effect on the immune clearance of tumours [528-536].

Due to the increased incidences of cancer in transplant recipients, as a result of the requirement to use broad acting immunosuppressive drugs to prevent transplant rejection, it is important to ask whether adoptively transferred Treg could impair protective immune responses. Edinger et al. and Trenado et al. both used models of GVHD to show that adoptively transferred polyclonally activated nTreg do not prevent the graft versus leukaemia (GVL) response [469, 470], however there is a lack of data regarding whether polyclonally activated nTreg or alloantigen-driven Treg can impair a protective immune response against a tumour in the setting of solid organ transplantation and whether either population is less likely to impair protective immune responses.

B16F10 is a B6-derived melanoma cell line that has been used extensively to develop new metastatic agents. Several variants were isolated during the development of this model, with B16F10 being the most commonly used [537]. B16F10 has been described as a low-immunogenic tumour due to the difficulty of inducing protection against B16 challenge [538]. However, Ngiow et al. identified that DEREG mice, inoculated s.c with 5x10^4 B16F10 melanoma cells and treated with diphtheria toxin to deplete Treg, had a mean tumour size of approximately 10mm^2 compared with a mean tumour size of greater than 150mm^2 in mice that did not receive diphtheria toxin. Treg depletion was shown to significantly increase immune cell infiltration, including the infiltration of CD4^+ and CD8^+ T cells into tumours [536]. Therefore, the B16F10 melanoma model was used to investigate whether adoptively transferred Treg could impair the protective immune response against the growth of a tumour.
Chapter Aims: The first aim of this chapter was to identify whether adoptively transferring a specified number of either activated nTreg or alloantigen-driven CD44-Treg could prolong the survival of allogeneic heterotopic heart transplants. Secondly, the timing of Treg administration relative to the time of transplant was compared in order to explore the impact of timing on transplant outcome. The final aim of this chapter was to ask whether alloantigen-driven Treg or polyclonally expanded nTreg could impair protective immune responses against tumours.
4.0 Results

4.1 Comparison of activated nTreg and alloantigen-driven Treg using the heterotopic heart allograft model

The heterotopic heart model has been an invaluable tool for studying allograft rejection since its development by Corry and colleagues in 1973 [442], providing a model to analyse the immune response during chronic and acute rejection. The inherent contraction of the heart allows for a convenient way to monitor graft rejection, via palpation. Zheng et al. generated CD4+ and CD8+ Treg from total T cells by stimulating T cells from DBA/2 (H2d) mice with irradiated B6 (H2b) APCs in media enriched with TGF-β and IL-2. 10^7 Treg were adoptively transferred into B6 (H2b) mice on D-1 and D+5 relative to an allogeneic (H2d) heterotopic heart transplant. All mice that received a heart transplant rejected their hearts by day 12, whereas mice that received 2 doses of 10^7 Treg had an MST of approximately 50 days and 2 out of 6 mice went on to long term survival (>100days) [462]. Previous work from this laboratory had shown that when transferred at day -3, relative to heart transplant, 1x10^6 IFN-γ conditioned alloantigen-driven Treg could lead to a modest but significant prolongation of transplant survival (MST ~15 days), relative to untreated controls (MST ~ 8days) (unpublished data, personal communication with Thomas Chan). This model therefore provided a useful platform with which to compare the efficacy of activated nTreg versus alloantigen-driven Treg and to explore the impact of delivery timing on graft outcome. 1x10^6 activated nTreg or alloantigen-driven Treg (CD44-Treg) were adoptively transferred into CBA mice, 3 days pre or post-transplant (Figure 4.1a). Mice were transplanted with fully vascularised B6 hearts. Neither activated nTreg, nor alloantigen-driven CD44-Treg significantly increased graft survival when given 3 days post-transplant (Figure 4.1b, MST 9 and 8 days respectively. n=5 for both), when compared with untreated mice (Figure 4.1b, MST 7 days. n=5). However, when either of the Treg populations were given 3 days pre-transplant, both significantly increased graft survival (activated nTreg MST, 18 days. n=5. Alloantigen-driven CD44-Treg MST, 20 days. n=7). These data suggest that both types of Treg
are most efficacious when given pre-transplant. They also support previous work in the laboratory that suggested that as few as $1 \times 10^6$ IFN-γ Treg, adoptively transferred pre-transplant could prolong the survival of an allograft. This is an important result as requiring a combined total of $20 \times 10^6$ Treg per transplant, as used by Zheng et al. in two doses on D-1 and D+5 [462], would have made future experiments in lymphoreplete mice unfeasible. Furthermore, this result also supports observations made in Figure 3.15, using a different model, that neither type of Treg is more efficacious than the other.

This result demonstrates for the first time, that despite the theoretical advantages of alloantigen-driven Treg such as specificity and increased precursor frequency, when given at the same time and in the same number in otherwise lymphoreplete mice, activated nTreg are equally effective when judged using a functional, primary allograft model. This is of critical importance because if replicated in other studies and in other models, the clear implications are that the cost and the logistical limitations which will hamper the use of alloantigen-driven Treg in the clinical setting can simply be ignored. Robust protocols for the isolation, expansion, characterization and cryopreservation of activated nTreg already exist in forms that have been approved by regulatory authorities in the UK, some European countries and in the US, and since such cells have already been used in small-scale clinical trials, the data provided in Figure 4.1 support the use of activated nTreg and suggest that larger trials of these cells should follow.

4.2 Administration of Treg pre-transplant results in increased numbers of GFP⁺ Treg in the spleen

The proportion of Treg to effector T cells is an important consideration for the success of Treg therapy, as previous studies suggest that a ratio of at least 1 Treg to 2 effector T cell is required to prevent transplant rejection [242, 246, 518]. Hara et al. showed that $1 \times 10^5$ adoptively transferred CD45RB<sup>low</sup> but not CD45RB<sup>high</sup> CD4⁺ T cells from previously tolerised mice prevented
allogeneic skin graft rejection by $1 \times 10^5$ naive CD45RB$^{high}$ CD4$^+$ T cells. As not all mice went on to long term survival, it suggests that a ratio of greater than 1:1 Treg to effector T cell is required to control rejection although a titration was not performed [242]. Figure 4.1b suggests that this may not be the case. It has been estimated previously that a healthy adult human weighing 70kg has $16.5 \times 10^{10}$ CD4$^+$ T cells [448]. Extrapolating from this, an average adult mouse weighs 25g and therefore if the proportions of immune cells are similar, this would mean that a mouse has a total pool of approximately $5 \times 10^6$ CD4$^+$ T cells. Of these cells it can be estimated from previously published work that the frequency of T cells that will respond to alloantigen might be as high as 1:10 [78, 80, 81]. Therefore, if the higher estimates of precursor frequency of T cells for alloantigen are accurate, the adoptively transferred Treg in Figure 4.1 would be out-numbered by potentially alloantigen-reactive CD4$^+$ T cells by as many as 5 times. Typically, there are approximately two thirds the number of CD8$^+$ T cells in a mouse relative to CD4$^+$ T cells, which based on earlier assumptions would mean that there are approximately $33 \times 10^6$ CD8$^+$ T cells in a 25g mouse. This would mean that the adoptively transferred Treg used in Figure 4.1 are out-numbered by potentially alloreactive T cells by $>8:1$. Figure 4.1 suggests that much smaller doses of Treg may be required to have an effect on graft survival. Additionally, if Treg are administered in combination with immunotherapy that does not prevent Treg function, they could potentially be used at lower doses.

The absolute number of adoptively transferred GFP$^+$ Treg were enumerated in the spleens of mice that received $1 \times 10^6$ CD44-Treg 3 days pre-transplant or post-transplant to identify if one of the reasons for the improvement in graft survival in mice that received Treg pre-transplant (Figure 4.1) was due to an increase in the number of Treg. As shown in Figure 4.2b and c, when Treg were given 3 days pre-transplant there was an approximate 13 fold increase in the number of Treg detected at POD 5 compared with Treg administration 3 days post-transplant. It is logical to assume that the increased number of Treg in the spleens of mice that received cells 3 days pre-transplant is due to the extra three days that the cells are exposed to alloantigen in vivo. It
is possible that these extra 3 days allows the cells to proliferate to a greater extent than when the Treg are transferred post-transplant, resulting in greater numbers in the spleens of these mice.

4.3 Treg are most effective at reducing dendritic cell activation when adoptively transferred pre-transplant

The data in Figure 4.1 suggest that both activated nTreg and alloantigen stimulated Treg can prolong graft survival in a lymphoreplete mouse but only when adoptively transferred pre-transplant. It appears that this is in part due to greater numbers of Treg in the spleens of mice 5 days post-transplant, as shown in Figure 4.2c. The suppression of DC activation by Treg has been shown to be an important part of their mechanism when controlling graft rejection [539, 540], therefore the activation status of DCs was compared between different treatment groups.

DCs harness a variety of ways to initiate graft rejection and are well understood to be key mediators of this process [541]. This was elegantly discovered by Lechler and Bachelor when (ASxWF)F1 rats were transplanted with (ASxAUG)F1 kidneys, which had previously been transplanted into AS recipients. Acute graft rejection did not occur unless (ASxAUG)Fx DCs were injected at the time of transplantation [70], identifying a crucial role for DCs in transplant rejection. The down regulation of DC costimulatory molecules, such as CD80, and the inhibition of stable contacts between CD4+ cells and DCs have been identified as important mechanisms of Treg mediated suppression of DCs in vitro [338, 339]. Cederbom et al. co-cultured CD4+CD25+ T cells with bone marrow DCs for 2 days in vitro and showed that CD4+CD25+ T cells could decrease the expression of CD80 and CD86 on DCs in a dose dependent manner. CD4+CD25+ Treg also down regulated CD80 mRNA levels in DCs, however, CD86 mRNA levels were not decreased, suggesting that the cell surface expression of this molecule may be regulated by an alternative mechanism [338]. Oderup et al. demonstrated that CD4+CD25+ nTreg induced the
down-modulation of CD80 and CD86 expression *in vitro*, in a CTLA-4 dependent manner. The authors cultured splenic DCs with anti-CD3 and CD4+CD25- T cells in the presence or absence of CD4+CD25- Treg over a 42 hour period. Analysis of CD80 and CD86 expression in cultures in which Treg were present revealed a range of approximately 50%-150% reduction in expression of both costimulatory molecules, depending on the time spent in culture. The addition of anti-CTLA-4 antibody (100µg/mL) reversed the previously described decrease in expression of CD80 and CD86, identifying an important role for CTLA-4 [381]. *In vivo* experiments by Mahnke et al. identified that dendritic cells were less able to prime CD8+ T cells and failed to induce the proliferation of CD4+ T cells in mice that were injected with Treg [540]. Although previous work had suggested that Treg suppression of DC activation was cell contact dependent [259, 343, 386-388], it wasn’t until Tang et al. utilised a combination of advances, namely two-photon laser-scanning microscopy (TPLSM), that this phenomenon was confirmed. Tang et al. utilised Treg populations expanded from BDC2.5 TCR transgenic mice, which were able to prevent and also occasionally reverse autoimmune diabetes in NOD mice [331]. To determine whether BDC2.5 Treg interacted with islet antigen bearing DCs, *in vivo*, Treg were labelled with the orange dye chloromethyl benzoyl amino tetramethylrhodamine (CMTMR) and transferred into mice carrying an enhanced GFP transgene under the control of the mouse insulin I promoter (MIP.GFP). Imaging pancreatic lymph nodes 24 hours later revealed that BDC2.5 Treg swarmed in the MIP.GFP mice and the observation that some of these swarms were localised with GFP+DCs suggested that Treg were interacting directly with DCs *in vivo* [331].

To determine whether the prolongation of graft survival, shown when Treg are given pre-transplant but not post-transplant (Figure 4.1), was due to a reduction in dendritic cell activation, 1x10^6 activated nTreg were adoptively transferred into mice 3 days pre or post-transplant and mice were harvested on post-operative day 5 (Figure 4.3a). DCs were identified according to CD11c and MHC class II expression, and their activation status was determined by examining relative levels of the costimulatory marker CD80. As shown in Figure 4.3c, there was
approximately a 15% reduction in the percentage of CD80hi expressing DCs when Treg were given 3 days pre-transplant (mean CD80hi 67.5%, n=3), compared with mice that received Treg 3 days post-transplant (mean CD80hi 78.8%, n=3, p=0.0001). Interestingly, there was also a reduction in CD80 expression when Treg were given 3 days post-transplant compared with heart only controls (mean CD80hi 96.5%, n=2, p=0.002), however this was evidently not sufficient to prolong graft survival. These data suggest that Treg administered 3 days pre-transplant reduces the activation of DCs compared with post-transplant delivery and considering the importance of DCs in transplant rejection, it is likely that this contributes to the prolongation of graft survival identified in Figure 4.1.

The smaller but still significant reduction in CD80 expression when Treg were administered post-transplant is encouraging as this will be the only available method for certain types of transplant and this effect on DC activation in combination with immunotherapy could lead to improved transplant outcomes, regardless of the timing of Treg therapy. This is of course dependent on the immunosuppressive drugs not preventing Treg function. What is encouraging, is that there does seem to be a correlation between DC activation as measured by CD80 expression and Treg effectiveness in vivo. If this is confirmed by further experiments, then CD80 expression could be a valuable tool for assessing different populations of Treg and perhaps most importantly, whether delivery of Treg at multiple time-points post-transplant could overcome the deficit of a single delivery (Figure 4.1). Furthermore, development of the video microscopy and/or imaging flow cytometry techniques, recently described by Juvet et al. [390] could allow a functional analysis to be made of DC activation in different situations of Treg delivery. In combination, these phenotypic and functional analyses could provide a sensitive way of optimising and understanding the use of Treg in mouse models before moving to further clinical trials.
The overall aims of the first half of this chapter were to: 1) determine whether Treg could improve graft survival in a lymphoreplete mouse; 2) ask whether adoptively transferring Treg before or after a transplant was optimal for graft survival and to; 3) determine whether there was a difference in efficacy between activated nTreg and alloantigen-driven Treg in a lymphoreplete mouse.

There was a significant increase in graft survival when Treg were administered pre-transplant, regardless of the type of Treg used but there was no significant difference in efficacy between the two Treg populations. The experimental design of administering Treg 3 days pre or post-transplant was chosen as administering $1 \times 10^6$ IFN-γ Treg on day -3 had been shown to significantly prolong the MST of an allogeneic heterotopic heart allograft by approximately 5 days, compared with untreated controls (personal communication with Thomas Chan, unpublished data). Administering Treg 3 days post-transplant was selected as the time point for post-transplant administration of Treg to mirror the pre-transplant timing. However, this approach was considered to be less than optimal for investigating the effects of different administration times of Treg because of the extra three days that the Treg are exposed to alloantigen when Treg are administered pre-transplant.

In the clinical transplant setting, there are significant logistical and practical problems with Treg delivery pre-transplant. Even in living donor transplantation, where surgery is planned ahead of time, transplants can be and are often postponed for a number of reasons including lack of theatre availability or unexpected illness in the donor or recipient. With deceased-donor transplantation, even if the impossibility of generating donor-reactive Treg pre-transplant is ignored, delivery of pre-expanded nTreg three days before transplant would be impossible because the availability of a suitable transplant cannot be predicted in advance. Some of these problems might be overcome if it were possible to deliver Treg shortly before or shortly after
transplantation. Therefore, the next experiments were designed to look at Treg delivery one day pre or one day post-transplant.

4.4 Adoptive transfer of CD44-Treg 1 day pre-transplant significantly prolongs rejection in otherwise lymphoreplete mice

To address the concerns regarding the length of time post-transplant that Treg were adoptively transferred in Figure 4.1, the experimental design was altered to give Treg 24 hours before or after transplantation. CD44-Treg were generated as described previously and flow-sorted to >95% purity. 1x10^6 alloantigen-driven CD44-Treg were adoptively transferred into CBA (H2^k) mice, either 24 hours before or after the heterotopic transplant of a heart from a B6 (H2^b) donor (Figure 4.4a). As shown in Figure 4.4b, mice that received heart allografts but no Treg rejected their hearts acutely with an MST of 7.5 days (n=4). Adoptively transferring 1x10^6 CD44-Treg either 1 day pre or post-transplant led to a significant increase in graft survival, compared with mice that received no Treg (MST 20 days, n=5, p=0.007, and MST 11 days, n=5, p=0.007 respectively, Log-rank test). Treg adoptively transferred 1 day pre-transplant were significantly more effective than Treg adoptively transferred 1 day post-transplant (p=0.048, Log-rank test).

On the basis of the flow data shown in Figures 4.2 and 4.3, a parallel analysis was performed on mice that had received Treg on day -1 and day +1 relative to transplantation on day 0.

4.5 Treg are found in greater numbers when adoptively transferred at day -1 compared with day +1

As shown in Figure 4.5, adoptive transfer of Treg 1 day pre-transplant, compared with 1 day post-transplant, resulted in an increase in the number of GFP^+ Treg found in the spleen at POD 5 (Figure 4.5c, mean number of Treg, 14751 versus 8169, respectively, p=0.045, n=4 for both
treatment groups) but the difference in Treg number between administration times was far less than when Treg were adoptively transferred on either d-3 and d+3 (Figure 4.2c). These data support the idea that Treg delivery pre-transplant is more effective because of the increased number of Treg in the critical period shortly post-transplant when donor DC are known to migrate to the spleen to directly present antigen [542].

Interestingly, when compared in terms of absolute numbers in the spleen at POD 5, there were more Treg in mice that received the adoptive transfer on D-1 compared with D-3 (Figure 4.5c, mean Treg numbers: 14751 (n=4) versus Figure 4.2c, 6849 (n=3) respectively, p=0.043 Student’s t test). This suggests that there is a contraction of Treg number in the period between adoptive transfer of cells and the transplant of the graft. This would correspond with a previously published study suggesting that Treg numbers contract over time [543]. Singh et al. stimulated CD4+CD25+CD127lo nTreg for three rounds with plate bound anti-CD3 and anti-CD28 antibody coated magnetic beads for a total of 36 days, Rapamycin was added to the cultures for the final two days, after which cells were labelled with CFSE. Treg were then infused intravenously into macaques and Treg were quantified in the peripheral blood. Treg numbers dropped from 12.2% of the Treg pool 30 minutes after adoptive transfer, to 4.0%, 2 days post adoptive transfer. 16 days post adoptive transfer, only 0.04% of CD3+CD4+CD25+FoxP3+ cells remained CFSE positive [543]. While there are questions surrounding the usefulness of this study, namely due to the lack of alloantigen stimulation in vivo, it does support the difference observed between Figures 4.2c and Figure 4.5c suggesting that Treg numbers decline in vivo in the absence of alloantigen stimulation. This suggests that in a clinical setting, for optimal efficacy, it would be necessary to administer Treg as close to the time of transplantation of the organ as possible, if the immunosuppressive drugs that the patient receives in this period are permissive to Treg therapy.
4.6 Administration of Treg 1 day pre-transplant, compared with 1 day post-transplant, leads to an increase in proliferation of GFP+ Treg in the spleen at post-operative day 5.

After two rounds of stimulating total CD4+ T cells with GT-DCs in vitro, 1x10^6 CD44-Treg from CBA (H2^k) FoxP3(GFP) mice were flow sorted to >95% purity based on GFP expression and subsequently labelled with violet proliferation dye (VPD) prior to adoptive transfer. GFP+ VPD labelled Treg, adoptively transferred 1 day post-transplant, proliferated significantly less than Treg that had been adoptively transferred 1 day pre-transplant (Figure 4.6b, mean percentage of cells proliferated more than once: 18% versus 43% respectively, p=0.0062, n=6 for both treatment groups). Less than 2.2% of Treg adoptively transferred into mice that did not get a transplant proliferated (n=5).

These data appear to confirm that proliferation of Treg plays a critical role in the ability of transferred Treg to control acute rejection.

4.7 Delivery of Treg at D-1 results in impaired DC activation compared with delivery at D+1

Adoptive transfer of 1x10^6 CD44-Treg into CBA (H2^k) mice, 1 day prior to the heterotopic transplant of a B6 (H2^b) heart resulted in a significant reduction of CD80 expression by CD11c^+ MHC class II^+ DCs, compared with mice that received 1x10^6 CD44-Treg 1 day post-transplant (Figure 4.7). CD11c^+ MHC class II^+ DCs in mice that received Treg on day -1 had a mean of 42.9% CD80^hi expression (n=6), compared with 50.8% CD80^hi in mice that received Treg on day +1 (n=4, p=0.03 Student’s t test). Mice that received a heart transplant without Treg had a mean CD80^hi expression of 73.4% (n=3). These results support the data in Figure 4.3, which suggested that pre-transplant administration of Treg is important for optimal efficacy.
CD80, and its homologue CD86 are ligands for both CD28 and CTLA-4 (CD152) expressed on T cells. A large number of experiments and experimental models have demonstrated that when T cells encounter their cognate peptide-MHC complex on the surface of APC, ligation of CD28 tends to lead to co-stimulation thereby supporting T cell activation whilst ligation of CTLA-4 tends to deliver negative T cell signals [107, 544-550]. Several in vivo models have shown unequivocally that blockade of CTLA-4 can abolish the protective effect of adoptively transferred Treg [243, 270, 341, 551, 552] and thus, understanding which of the two CTLA-4 ligands is most relevant to the transplant situation has been of significant interest. Judge et al. compared the roles of CD80 and CD86 in heterotopic heart allografts with targeted deletion of the CD80 gene as well as with anti-B7 mAbs [553]. Previously published data from the same laboratory indicated that donors deficient of CD80 did not show an improvement in cardiac allograft graft survival but long term survival could be induced in CD80−/− mice by treatment with CTLA-4Ig [554]. Building on this, Judge et al. asked whether CD80-deficient grafts transplanted onto wt recipients could identify whether CD80 or CD86 preferentially interacts with CTLA-4 and inhibits the immune response [553]. Using CD80 sufficient mice, a single dose of CTLA-4Ig, given 2 days post heterotopic heart transplant was sufficient to induce long term survival in a BALB/c donor, 129 recipient combination. In contrast, anti-CD86 mAb alone was unable to prevent graft rejection when given instead of CTLA-4Ig. Neither anti-CD86 mAb nor CTLA-4Ig was able to induce long term survival of heart allografts in wt mice from CD80−/− donors. Administration of CD80 mAb on day 0 led to long term survival of grafts in 50% of the mice but concurrent administration of anti-CD152 (CTLA-4) mAb abrogated this effect, suggesting that CTLA-4 signalling is important for induction of long term survival, regardless of blockade of CD86.

These data suggest that CD80 has a dominant role on donor cells in down-regulating the immune response and the authors suggest that this is probably due to the ligation of CTLA-4 [442]. Additionally, Yamada et al. transplanted CD28-deficient mice with allogeneic heterotopic heart grafts and showed that blockade of CTLA-4 or B7-1 (CD80) led to a significant increase in
the rate of graft rejection. B7-2 (CD86) blockade, in contrast, led to prolonged allograft survival, again suggesting that CD80 is the dominant ligand for CTLA-4 mediated down regulation of alloimmune responses in vivo [555]. This perhaps contradicts what has been observed in Figure 4.7 and that a reduction in DC expression of CD80 is not permissive for improved graft outcome. However, Treg mediated down-regulation of CD80 as a mechanism to suppress DCs is a well-known phenomenon, as discussed earlier in this chapter (section 4.3) and it is reasonable to conclude that experiments in which CD80 or CTLA-4 was blocked or knocked out, inhibited an important mechanism of Treg mediated suppression. Further evidence that points towards a CTLA-4 dependent mechanism and can explain the reduction in CD80 expression is the observation by Qureshi et al. that CTLA-4 can remove CD80 and CD86 from opposing cells by a process known as trans-endocytosis [383]. This would help to explain the reduction in CD80 expression on DCs, mediated by Treg observed in Figure 4.7.

4.8 T cell activation is reduced when Treg are administered D-1 versus D+1

Another key method of suppression by Treg involves controlling and preventing the activation of T cells, and an important aspect of T cell activation is the production of pro-inflammatory cytokines. The data in Figures 4.3 and 4.7 suggest that adoptively transferred CD44-Treg are able to reduce DC activation, in the form of a reduction in the percentage of CD80hi expression, when Treg are adoptively transferred before, and to a lesser extent after the transplant, compared with heart only controls. As DCs are key mediators of T cell activation, the cytokine profile of CD4+ and CD8+ T cells was assessed as a marker of their activation.

To compare cytokine expression between different treatment groups, CBA (H2k) mice were given 1x10^6 CD44-Treg, i.v., either 1 day pre, or 1 day post-transplant of a heterotopic heart allograft from B6 (H2b) donors. The mice were harvested 5 days post-transplant. As shown in Figures 4.8 and 4.9, there were no differences in either CD4+ or CD8+ T cells regarding the
expression of TNF-α or IL-17. There was however a significant reduction in CD4⁺ and CD8⁺ T cells that were positive for expression of IFN-γ when mice were given Treg 1 day pre-transplant compared with 1 day post-transplant (Figure 4.10).

It has been previously shown that Treg can reduce the expression of IFN-γ from CD8⁺ T cells in vitro [556, 557]. Piccirillo and Shevach showed that CD4⁺CD25⁺ Treg suppress CD8⁺ T cell proliferation and effector function. They demonstrated that stimulation of CD8⁺ T cells with anti-CD3, in vitro, in the presence of CD4⁺CD25⁺ Treg reduced IFN-γ expression from CD8⁺ T cells over 3 fold, at a 1:1 ratio of Treg to CD8 [556]. The authors also showed that CD4⁺CD25⁺ Treg acted via a T-T cell interaction without the direct requirement for APC involvement [556]. Qiao et al. stimulated CD8⁺ T cells and CFSE labelled iTreg with anti-CD3 antibody for 24 hours before separating the CD8⁺ T cells and measuring IFN-γ mRNA using quantitative PCR. Cultures in which iTreg were added had approximately a 3 fold reduction of IFN-γ mRNA in CD8⁺ T cells, compared with cultures in which CD8⁺ T cells were cultured in isolation or with CD4⁺CD25⁻ T cells [557].

Using a model of autoimmune diabetes Sarween et al. showed that Treg prevented CD4⁺ T cells from up-regulating IFN-γ expression. The authors transferred DO11/rag cells into RIP-mOVA/rag mice that had been injected with CD4⁺CD25⁺ or CD4⁺CD25⁻ cells 24 hours previously. Mice were immunised with 200µg of OVA/IFA, 24 hours after transfer of DO11/rag cells. Peripheral and pancreatic lymph node cells were isolated 3 or 6 days post immunisation and re-stimulated in vitro with OVA₃₂₃₋₃₃₉ for 4 hours. Intracellular cytokine analysis revealed that approximately 30% of DO11 cells in mice that were adoptively transferred with CD4⁺CD25⁻ cells were IFN-γ⁺, however, in mice that received CD4⁺CD25⁺ Treg, less than 3% were IFN-γ⁺ [558].

DiPaolo et al. identified that CD4⁺CD25⁺ prevent the development of autoimmune disease, in part by inhibiting IFN-γ expression from autoreactive T effector cells. The authors co-transferred
CD4⁺CD8-CD25⁻ thymocytes from TxA23.Thy1.1 TCR-Tg mice with splenocytes from BALB/c mice, which had either been depleted of, or enriched for CD4⁺CD25⁺ Treg. Gastric lymph nodes were removed after 7 or 14 days and stimulated in vitro with H/K-ATPase α-chain630-641 peptide. IFN-γ production in cultures from animals that received Treg was reduced to approximately 35pg/mL compared with approximately 200pg/mL in cultures of cells from mice that did not receive Treg [559].

Additionally, Sojka and Fowell showed that Treg inhibit IFN-γ synthesis in CD4⁺ T cells without blocking Th1 differentiation. Interestingly, they also showed that TNF-α production from CD4⁺ T cells was not changed in the presence of Treg [560]. Thy1.1CD4⁺Th1 effector cells were stimulated with APC and anti-CD3 mAb with, or without, Treg for 18-24 hours. Wells in which Treg were included had approximately 5% of Th1 cells producing IFN-γ, compared with approximately 30% of Th1 cells producing IFN-γ when Treg were omitted [560]. Importantly, Sojka and Fowell also identified that control of effector function is independent of differentiation in vivo. CFSE labelled naïve DO11.10⁺Thy1.2⁺CD4⁺ target T cells were transferred i.v. with, or without, DO11.10⁺FoxP3GFP⁺ Tregs into congenic Thy1.1 BALB/c mice that had been previously immunised with OVAp/IFA. After 5 days, IFN-γ expression in the adoptively transferred target T cells was assessed. This revealed about half as many IFN-γ⁺ cells in mice that received Treg compared with those that did not [560]. Sojka and Fowell showed that Treg do not prevent Th1 differentiation but instead limited the pool of cells that could produce IFN-γ.

Thus, there is a large quantity of data showing that a reduction in T cell expression of IFN-γ is an important part of the way in which Treg control T cell responses. This is entirely consistent with the data shown in Figure 4.10 which show a clear link between suppressed IFN-γ expression and the most effective time-point for Treg delivery. Again, if this link is supported by further experiments, another potential functional assay will be available to optimise yet further the
timing and mode of Treg administration in mouse transplant models. In the light of the data shown in Figure 4.10, one critical question is why Treg delivered at day -1 cannot prevent rejection completely (MST 20 days, Figure 4.4). The most likely explanation is that at the numbers given, the Treg become overwhelmed by effector populations which are able to continue to proliferate. As demonstrated in Figure 3.15 the ratio of Treg to Teff is important for Treg to have efficacy in vivo.

4.9 B16F10 sub-cutaneous melanoma model

One of the often-stated advantages of alloantigen-driven Treg is that they are less likely than polyclonally expanded nTreg to broadly suppress the immune system. As discussed previously, a key issue associated with modern immunosuppressive drugs is that they globally suppress the immune system, leaving the patient susceptible to a variety of complications, particularly an increased risk of malignancy [12, 487]. It is therefore extremely important for the success of Treg therapy that they are more targeted than previous therapies in the context of controlling allograft rejection and do not leave the patient immuno-compromised.

The immune system is vital for constant surveillance and targeted removal of cells with the potential to cause cancer. A growing number of studies have indicated that Treg can prevent this process, enabling malignant cells to propagate. Robert North demonstrated that suppressor T cells (Treg) are involved in anti-tumour immune responses using a methylcholanthrene-induced fibrosarcoma cell line (known as Meth A). North et al. identified that tumour-induced Treg activity inhibited the immune response to the tumour [529]. Other experiments indicated that suppressor T cells could be preferentially depleted by cyclophosphamide, resulting in tumour rejection [528]. North showed that infusion of tumour-sensitised spleen cells alone had no impact on the growth of Meth A, however a combination of
100mg/kg cyclophosphamide and tumour-sensitised spleen cells caused tumours to completely regress, regardless of size [528].

A variety of groups identified that depletion of CD25+ cells, of which the majority are Treg in mice, resulted in the rejection of tumour cells lines in vivo, in a T cell dependent manner [530-532]. Onizuka et al. administered a single 0.125mg dose of anti-CD25 interleukin 2 receptor α (IL-2Rα) monoclonal antibody (PC61) and showed that 6 out of 8 different tumour varieties (two spontaneously occurring leukaemias, two radiation induced leukaemias, a dimethylbenzanthracene-induced leukaemia, a mineral oil-induced leukaemia and two methycholanthrene-induced sarcomas) could effectively be completely removed from mice. Analysis of lymph nodes of mice revealed that a single 0.25mg dose of PC61 reduced CD4+CD25+ percentages from approximately 11% pre-administration, to approximately 3%, 3 days post administration. This recovered by day 7 to normal levels [530]. Jones et al. confirmed these data using the B16F10 melanoma model [532]. Furthermore, Turk et al. identified that RAG1−/− mice, adoptively transferred with CD8+ and CD4+ T cells depleted of CD4+CD25+ T cells, were able to mount a robust immune response to the poorly immunogenic B16 melanoma and importantly, re-addition of CD4+CD25+ T cells resulted in tumour progression [533]. Additionally, Ngiow et al. demonstrated that specifically depleting Treg using diphtheria toxin in DEREG mice inoculated with B16F10, led to a heterogeneous anti-tumour response [536]. Using a chemical carcinogen, methylcholanthrene (MCA), it has also been demonstrated that tumours develop less quickly and less frequently in mice depleted of CD25+ cells [534, 535]. Tawara et al. inoculated BALB/c mice with 25µg MCA and then left mice untreated or treated them with 0.25mg PC61 after 4 or 6 weeks. Mice treated with PC61 had significantly reduced tumour growth which resulted in prolonged survival [534]. Betts et al. also demonstrated that FoxP3+ CD4+ T cells are found with high abundance within tumours in mice inoculated with MCA [535].
These data provide convincing evidence for the role of Treg in the progression of tumours and therefore it seems logical to theorise that using Treg as a cellular therapy could contribute to tumour growth. Therefore, it is imperative that experiments are performed to ascertain whether Treg therapy can lead to the suppression of normal tumour immune surveillance in transplant recipients. As previously discussed, adoptive transfer of either alloantigen-driven Treg or freshly isolated nTreg, in a model of GVHD, did not prevent the T cell dependent graft versus tumour effect [469, 470]. Surprisingly, the literature on the effects of adoptively transferring Treg on protective immunity against tumours in solid organ transplantation is sparse and few direct comparisons have been made between alloantigen-driven Treg and polyclonally expanded nTreg. As organ transplant recipients are prominently predisposed to cancers, experiments were designed to ask whether adoptively transferred Treg could contribute to the progression of a tumour using the B16F10 melanoma model. Additionally, tumour progression was compared when adoptively transferring alloantigen-driven Treg or activated nTreg.

4.10 Subcutaneous injection of B16F10

Subcutaneous injection (s.c) of the B16F10 melanoma cell line has been widely used as a tumour model [538]. Upon s.c injection of $1 \times 10^5$ B16F10 cells, many studies have reported consistent growth of a palpable tumour and therefore s.c injection was used to titrate the B16F10 cells in order to identify the number required for the growth of a tumour with dimensions of $1 \text{cm}^3$ after approximately 14 days, at which point the mouse would be sacrificed to prevent unnecessary suffering as a result of tumour necrosis. B16F10 cells were harvested at approximately 50% confluency and re-suspended in sterile saline. B6 mice were sedated and B16F10 cells were carefully injected subcutaneously into the left hind limb, ensuring a defined “bleb” was visible with each injection. Any mice that did not receive a defined bleb were sacrificed. Unfortunately,
there was no significant different in the growth of the tumours between mice that received 0.5, 1.0 or 2.0x10^5 B16F10 cells (Figure 4.11). It was also not possible to achieve consistent tumour growth, although uniform growth is commonly reported in the literature, Ngiow et al. have also reported heterogeneity in tumour outgrowth [536]. Without a model that provided uniform growth, it was a concern that any subtle differences in the impact on immune surveillance of a tumour between alloantigen-driven and polyclonally expanded Treg would not be distinguishable. Therefore, an alternative administration method to give more uniform growth of B16F10 was sought.

4.11 Intravenous injection of B16F10

Due to the variability of tumour growth when B16F10 cells were subcutaneously injected, it was decided to use intravenous injection (i.v.) of the cells, which it was believed would be less technically demanding and importantly more precise. When intravenously injected, essentially all B16F10 cells migrate to the lungs and form pulmonary melanomas [538]. 1.0 or 2.0x10^5 B16F10 cells were injected per B6 mouse and mice were harvested 10 days post injection. Each black dot on the surface of the lung was recorded as an individual melanoma (Figure 4.12). Mice that received 1x10^5 B16F10 cells had a mean number of 21 melanomas (n=8), which was significantly different to mice that received 2x10^5 B16 cells (mean number of 181 melanomas, n=7, p=0.0025). Importantly, when 1.0x10^5 B16F10 cells were injected i.v. there was tight grouping around the mean, with a standard error of +/-5.4 melanomas. It was anticipated that this would be sufficient to identify any subtle effects of alloantigen-driven Treg or polyclonally activated nTreg on the protective immune response.
4.12 Comparison of B16F10 melanoma cell growth in wild type B6 and B6 Rag\(^{-/-}\) after i.v. injection

To ask whether the presence of Treg might affect the growth of B16F10 melanoma, B6 Rag\(^{-/-}\) and B6 wild type mice were injected intravenously with \(1 \times 10^5\) and \(2 \times 10^5\) B16F10 cells. Mice were harvested 10 days post injection and the lungs were analysed for pulmonary melanomas (Figure 4.13). B6 Rag\(^{-/-}\) mice that received \(1 \times 10^5\) and \(2 \times 10^5\) B16F10 cells had means of 0.8 and 1.4 melanomas, respectively (n=6/5). Wild type mice that received \(1 \times 10^5\) B16F10 cells had a mean of 3.2 melanomas (n=4), whereas mice that received \(2 \times 10^5\) B16F10 cells had a mean of 37.5 melanomas (n=4). Although these data do not comprehensively identify that Treg are involved with B16F10 growth \textit{in vivo}, they do suggest that the presence of Treg impedes tumour clearance in wild type mice. It should also be noted that the melanoma cells are being injected into mice with vastly different lymphoid architecture and composition, and therefore the growth of the tumours could also be impacted by this. However, these data are supportive of previously published data that Treg prevent NK cell mediated removal of B16F10 cells in wild type mice. Grundy et al. showed using confocal microscopy that NK cells remove B16F10 tumour cells. Mice that were depleted of NK cells had increased numbers of B16F10 cells compared with mice that did not have NK cell depletion [561], suggesting that NK cells are key for the removal of B16F10 melanomas.

4.13 Activated nTreg and alloantigen-driven CD44-Treg equally increase the number of pulmonary melanomas

Although the majority of studies have focused on the role of CD8\(^+\) T cells in tumour clearance, it has also been shown that CD4\(^+\) T cells can clear tumours, independently of CD8\(^+\) T cells [562-566]. Fernandez-Cruz et al. established that W3/25\(^+\) (CD4\(^+\)) cells were required to eliminate an established Moloney sarcoma (MST-1) with an established blood supply \textit{in vivo}. Rats were
irradiated with 400 rad whole body irradiation and inoculated with 1x10^5 MST-1 and infusion with: 5x10^4 W3/25, 1.5x10^7 unsorted immune cells or 5x10^7 T cell depleted immune cells led to a lethal outgrowth of MST-1 tumours. Rats that received an infusion of 5x10^6 W3/25+ cells completely cleared tumours by day 15 [562]. Greenberg et al. showed the same phenomenon in mice, using a different tumour model. Adult, thymectomised, irradiated, bone marrow reconstituted (ATXBM) B6 mice were inoculated with 5x10^6 cells of Friend virus-induced erythroleukaemic (FBL-3). Mice that were adoptively transferred with 10^7 immune spleen cells from congenic B6/Thy1.1 donor mice that had been sensitised to FBL-3 in vivo, treated with 180mg/kg cyclophosphamide (CY) or treated with CY in combination with Lyt-1,2+ cells died within 45 days. In contrast, over 90% of mice that received CY in combination with either unfractionated immune cells or Lyt-1,2 immune cells survived long term [563]. These two seminal papers suggest that CD4+ T cells are sufficient, without the presence of CD8+ T cells, to completely eliminate tumours. Further work by Perez-Diez et al. provided evidence that CD4+ Tg T cells were more effective at rejecting tumours than CD8+ Tg T cells in 6 different in vivo tumour models [567]. Perez-Diez et al. directly compared the abilities of CD4+ and CD8+ T cells to clear tumours by using transgenic cells which were specific for the same tumour. In all 6 tumour models tested, CD4+ T cells in isolation were more effective than CD8+ T cells, including the B16 melanoma model. Partnering of CD4+ T cells with NK cells was essential for maximal anti-tumour effect and interestingly, CD4+ T cells were more effective than CD8+ T cells in tumours solely expressing MHC class I molecules [567]. CD4+ T cells are also known to have an important role as helper T cells [568, 569]. Although Zhang et al. reported that depleting Treg using anti-CD4 antibodies did not prevent B16F10 tumour growth [570], a more recent study showed that depletion of Treg in DREG mice using diphtheria toxin led to rejection of B16F10 tumours [536], suggesting that Treg have a key role in the progression of the growth of this tumour model in vivo.
To identify whether T cells, depleted of Treg could prevent or reduce the number of pulmonary melanomas, B6 Rag\(^{-/-}\) mice were adoptively transferred with 1x10^4 CD4^+ GFP(FoxP3)^- T cells. The following day the mice received a full thickness allograft (H2\(^k\)) from CBA donors and 1x10^5 B16F10 cells i.v. Allografts were included with the intention of activating the T cells \textit{in vivo}. Compared with mice that received B16F10 cells alone, there was a reduction in pulmonary melanomas in mice that received Treg depleted T cells. To identify whether activated nTreg or alloantigen-driven CD44-Treg effect pulmonary melanoma growth, B6 Rag\(^{-/-}\) were adoptively transferred with Treg depleted T cells and either 1x10^4 activated nTreg or CD44 Treg. The following day the mice received a full thickness H2\(^k\) skin graft and 1x10^5 B16F10 cells. Mice were harvested 11 days post inoculation with B16F10 cells. The hypothesis being tested is that there will be fewer pulmonary melanomas in mice that receive alloantigen-driven Treg compared with activated nTreg. As shown in Figure 4.14c, addition of either type of Treg significantly increased the number of pulmonary melanomas compared with mice that received Treg depleted T cells (mice that received B16F10 cells only, had a mean of 152 melanomas (n=4), mice that received B16F10 cells + CD44-Treg had a mean of 192 melanomas (n=5), mice that received B16F10 cells + activated nTreg had a mean of 190 pulmonary melanomas (n=5). Mice that received B16F10 + CD25 T cells (Teff) had a mean of 109 pulmonary melanomas (n=3)). Critically, there was no significant difference in numbers of pulmonary melanomas between mice that received either type of Treg (p=0.9, Student’s t test).

The observation that both populations of Treg contributed to tumour growth compared with mice that received Teff only at first appears concerning regarding the safety of Treg therapy. However, it is important to consider that this model was designed to ensure that tumours were detectable and therefore B16F10 cells were titrated to achieve this. Secondly, the ratio of Treg to effectors is much greater than even the most ambitious clinical trials to date. Marek-Trzonkowska et al. administered 20x10^6 expanded nTreg in children with type 1 diabetes [571],
which equates to about 0.12% of the endogenous Treg in a healthy 70kg person, using Tang’s estimations of endogenous Treg number [448].

Interestingly, although alloantigen-driven Treg are thought to be less likely than polyclonally activated nTreg to recognise antigens outside of the graft, in part due to the more focused TCR repertoire [397], these data suggest that they are equally as broadly suppressive as nTreg. This is an important result when considering these results in combination with the data in Figures 3.15 and 4.2 as there appears to be no advantage, with regards to potency or in terms of their effect on tumour growth, of using alloantigen-driven Treg instead of nTreg. Coupled with the fact that alloantigen-driven Treg expand less efficiently makes a strong case for pursuing nTreg as the population of choice for therapeutic applications in man.
4.14 Discussion

4.14i Treg in lymphoreplete mice

There are relatively few publications that have assessed the efficacy of Treg in lymphoreplete mice. As previously described, adoptive transfer of $10^7$ TGF-β iTreg on day -1 and day +5 relative to a heart allograft prolonged graft survival beyond 100 days [462]. Work previously undertaken in this laboratory, in which CBA (H2k) mice were given $1x10^6$ alloantigen-driven Treg, 3 days before the transplant of an allogeneic (H2b) heterotopic heart graft could prolong graft survival by approximately 5 days (Thomas Chan, unpublished data). Figures 4.1 and 4.4 suggest that $1x10^6$ CD44-Treg, adoptively transferred on either D-3 or D-1, relative to an allogeneic heterotopic heart transplant can significantly prolong graft survival by a similar number of days. The experiments performed by Zheng at al., which used 2 doses of $10^7$, ex vivo generated TGF-β Treg, 1 day pre and 5 days post-transplant would have made future experiments, with the aim of assessing the effects of immunosuppression on adoptively transferred Treg, unfeasible as generating enough Treg ex vivo, even with the CD44-protocol, would have been too mouse intensive [515]. An interesting point to note is that Zheng et al. used TGF-β and IL-2 conditioned Treg which have subsequently been identified by Floess et al. [320] and by data presented in Figure 3.11, as exhibiting an unstable phenotype that can lead to cells converting to an effector memory phenotype in inflammatory conditions [299]. It is unlikely that 20 fold more CD44-Treg would be required to achieve the same graft survival results reported by Zheng et al., as CD44-Treg were shown to be as stable as nTreg and therefore unlikely to lose FoxP3 expression in the inflammatory environment of graft rejection [572]. In addition, CD44-Treg were also more efficacious than TGF-β Treg in the Rag²-skin graft model, shown in Figure 3.15.

Key to the design of experiments in chapter 5, involving the investigation of the effects of immunosuppression on adoptively transferred Treg, was finding a dose of Treg that could suboptimally increase graft survival, meaning that addition of Treg would not lead to long term
survival but instead lead to a moderate but significant delay of graft rejection. The reason for this was so that it would be possible to decipher whether immunosuppressive drugs were affecting Treg function by asking whether individual drugs in combination with Treg could improve graft survival, compared with mice that received either drug, or Treg, in isolation.

### 4.14ii Alloantigen-driven Treg and nTreg are equally broadly immunosuppressive

As discussed previously, the potential for Treg to globally immune-suppress the patient is a legitimate concern. Reducing the susceptibility of transplant recipients to the increased incidence of malignancies is one of the key aims of new immune-modulating therapies as the number of patients dying of cancer but with a functioning graft continues to increase [573, 574]. Treg have been implicated in a wide array of tumours and therefore it is feasible that adoptively transferred Treg could inhibit protective immune mediated clearance of oncogenic cells. Currently, Treg are being used alongside conventional immunosuppression, which could lead to over-suppressing the patient if the immunosuppressive drugs do not kill the Treg. The risk of over-immunosuppressing patients could effectively exacerbate the long term consequences associated with immune-suppressive drugs. Pre-clinical animal models are a vital tool to explore the potential side-effects that adoptively transferred Treg may have on the immune-response to a tumour in vivo. Edinger et al. and Trenado et al. asked whether Treg, in a model of GVHD, impaired the GVL effect [469, 470]. Their concern was based on data previously published by Annacker et al. [575], which suggested that there is a non-specific effect of adoptively transferred Treg on immune reconstitution. Annacker et al. showed that B6 Rag-2−/− recipients injected with $3 \times 10^6$ IL-10−/− CD25−CD4+ T cells in combination with $3 \times 10^5$ IL-10−/− CD25−CD45RBlo cells had a greater than 3 fold reduction of splenic CD4+ cells after 12 weeks, compared with mice that received IL-10−/− CD25− CD45RB cells in isolation. The reduction in CD4+ T cell numbers was less profound in lymph nodes but more profound in the blood and intestine which had a
reduction of CD4+ T cell numbers of approximately 21 fold and 49 fold respectively [575]. These data suggest that adoptively transferred Treg have a profound effect on immune reconstitution of CD4+ T cells in a variety of organs and therefore this could prevent the reconstitution of the patient’s immune system post-transplant to “normal” pre-transplant levels, exacerbating the compromised immune system that results from the use of broad acting immunosuppressive drugs.

Whilst Edinger et al. and Trenado et al. were concerned with the effects of polyclonally expanded nTreg on the protective immune response [469, 470], there has also previously been work undertaken on alloantigen-reactive Treg effects on protective immunity. Bushell et al. were concerned that induction protocols that lead to the generation of alloantigen-reactive CD25+CD4+ Treg from CD25- precursors might cause a reduced capacity to respond to environmental pathogens by tolerised individuals. To test this, CBA (H2k) mice were pre-treated with a DST and a non-depleting anti-CD4 antibody (177), and transplanted with B.10 cardiac allografts. Mice were infected intranasally 7 days post-transplant with 5 haemagglutinin units of influenza A/PR/8/34. Naïve, un-transplanted mice, were infected using the same method. Viral load was measured by ELISA for haemagglutinin. Mice tolerised to B.10 antigen, with DST/177, cleared the virus, as shown by haemagglutinin levels that were similar to those of uninfected mice. These data suggest that alloantigen-reactive Treg which have been generated in situ, by a well-established tolerising regimen, do not interfere with the immune response towards a viral pathogen [576]. This is a promising result in the context of alloantigen-driven Treg, as it suggests that this type of Treg will not interfere with protective immune responses.

Although solid organ transplant recipients are generally not as severely lympho-depleted as patients who receive bone marrow transplants, there is often still induction therapy involved [577] which leads to a significant depletion of leukocytes. In addition, the requirement for lifelong immunosuppression leads to a long-term reduction in leukocytes. This, in combination
with the potential for Treg to prevent reconstitution of immune cells could leave patients more severely immunocompromised than without Treg therapy. Therefore, to avoid potentially harmful side effects of the combination of Treg therapy and conventional immune-suppressive drugs it might be necessary to reduce the levels of conventional immunosuppressive drugs, if Treg are proven to have efficacy in human recipients. As shown in Figure 4.14c, activated nTreg and alloantigen-driven CD44-Treg are equally as broadly immunosuppressive. This is an important observation as although alloantigen-driven Treg have a smaller repertoire of TCRs [397] and are theoretically less likely to globally suppress the immune system, there appears to be no advantage of using these cells instead of activated nTreg, in this experimental setting.

4.14iii Implications of administering Treg pre-transplant

Administration of Treg prior to the transplant was more effective than giving the Treg in the period post-transplant (Figures 4.1 and 4.4). Cryopreservation of expanded nTreg, in anticipation of a donor being available, increases the flexibility of administration of cells and is likely to be the most effective way of using Treg. Fortunately, there is evidence that this is possible and experiments with human Treg have shown that they do not lose suppressive capacity after cryopreservation, although the number of viable cells is often approximately halved [248, 517]. An alternative approach to allow pre-transplant administration of Treg that has been suggested is the use of third party Treg, which would effectively create an “off the shelf” therapy [578]. This option is probably the least likely to be realised clinically due to the complications associated with the rejection of these cells by the recipient’s immune system. Finally, the most feasible option would be to treat the transplant recipient with conventional immunosuppressive drugs until the Treg have been generated ex vivo and then transfuse the cells into the patient at this time point. This option highlights the necessity to understand how concurrent immunosuppression will affect Treg function in vivo and if certain
Immunosuppressive drugs prevent Treg from functioning then it will be necessary to taper these before administration of the Treg.
Chapter 4: Figures
Figure 4.1 Activated nTreg and alloantigen-driven Treg can prolong heart allograft survival in a lymphoreplete mouse

Total CD4+ T cells from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and incubated for 10 minutes with anti-CD44 antibody (1µg/mL). 5x10^5 CD4+ T cells were co-cultured, per well of a 24 well plate, with 5x10^4 GMCSF and TGF-β differentiated B6 (H2b) GT-DCs, for 7 days. On Day 7, 5x10^4 fresh GT-DCs were added to each well. On day 14, Treg were flow sorted to >95% purity based on CD4 and GFP expression. nTreg were activated with plate bound anti-CD3 and anti-CD28 antibodies, in media supplemented with IL-2 (250U/mL) for 4 hours. 1x10^6 Treg were administered i.v., either 3 days before, or 3 days after the transplant of a heterotopic heart allograft from B6 (H2b) donor mice.

a. Experimental outline.

b. Heterotopic B6 (H2b) heart allograft survival in CBA (H2k) mice.
### 4.2 Enumeration of Treg administered 3 days pre or post-transplant.

CD44-Treg were generated by the same method detailed in figure legend 4.1. 1x10^6 CD44-Treg were adoptively transferred into CBA (H2^k) mice on either D-3 or D+3, relative to the transplant of a heterotopic heart allograft, from B6 (H2^b) donors. Spleens were harvested on POD 5 and were analysed for CD4^+FoxP3(GFP)^+ Treg.

- a. Experimental outline.
- b. Representative plots.
- c. Comparison of the mean number of GFP^+ Treg in spleens of mice that received 1x10^6 CD44-Treg, D-3: 6849 (n=3) and D+3: 518 (n=3) (p=0.0012, Student’s t test).
4.3 Adoptively transferring Treg pre-transplant leads to the greatest reduction in CD80 expression from DCs.

CD44-Treg were generated by the same method detailed in figure legend 4.1 and 1x10^6 were adoptively transferred into CBA (H2^k) mice on either D-3 or D+3 relative to the mice receiving a heterotopic heart allograft from B6 (H2^b) donors. Spleens were harvested on POD 5.

a. Experimental outline.

b. Representative plots.

c. Comparison of mean CD80^hi expression on CD11c^+ MHC class II^+ DCs. Mice that did not receive a heart transplant mean CD80^hi expression of 40.5% (n=2). Mice that received a heart transplant but not Treg, mean CD80^hi expression of 96.5%. (n=2) Mice that received Treg on D+3 relative to a heart transplant, mean CD80^hi expression of 78.8% (n=3). Mice that received Treg on D-3 relative to a heart transplant, mean CD80^hi expression of 67.5% (n=3).
Figure 4.4 Activated nTreg and alloantigen-driven Treg can prolong heart allograft survival in a lymphoreplete mouse

CD44-Treg were generated by the same method detailed in figure legend 4.1 and 1x10^6 Treg were administered i.v., either 1 day before, or 1 day after the transplant of a heterotopic heart allograft from B6 (H2^b) donors. Graft function was monitored by palpation, rejection was determined when hearts stopped contracting.
a. Experimental outline.
b. Heterotopic B6 (H2^b) heart allograft survival in CBA (H2^k) mice. (p value, Student’s t test).
4.5 Administration of Treg before transplant increases the number of cells in the spleen at POD 5

CD44-Treg were generated by the same method detailed in figure legend 4.1. 1x10^6 CD44-Treg were administered i.v., either 1 day before, or 1 day after the transplant of a heterotopic heart allograft from B6 (H2^b) donor mice. Mice were harvested on post-operative day 5.

a. Experimental outline.
b. Illustrative dot plots of adoptively transferred Treg.
c. Comparison of the absolute number of GFP^+ Treg in spleens of mice that received 1x10^6 CD44-Treg. Mean number of GFP^+ Treg in mice that received cells D-1, 14751 (n=4) and D+1, 8169 (n=4) (p=0.045, Student’s t test).
4.6 Administration of Treg pre-transplant increases the proliferation of adoptively transferred Treg in the spleen at POD 5

CD44-Treg were generated by the same method detailed in figure legend 4.1. 1x10^6 VPD labelled Treg were administered intravenously (i.v.), either 1 day before, or 1 day after the transplant of a heterotopic heart allograft from B6 (H2^b) donor mice. Mice were harvested on post-operative day 5.

a. Illustrative histograms of adoptively transferred GFP+ Treg VPD proliferation.

b. Comparison of GFP+ Treg VPD proliferation in spleens of mice that received 1x10^6 CD44-Treg. Mean number of GFP+ Treg that had proliferated more than once in mice that received cells D-1: 43% (n=6) and D+1: 18% (n=6) (p=0.0062, Student’s t test).
4.7 Treg most effectively reduce DC CD80 expression when administered pre-transplant.

CD44-Treg were generated by the same method detailed in Figure legend 4.1 and \(1\times10^6\) were adoptively transferred into CBA(H2\(^k\)) mice on either D-1 or D+1 relative to the mice receiving a heterotopic heart allograft from B6 (H2\(^b\)) donors. Mice were harvested on POD 5.

a. Representative plots.

b. Comparison of mean expression of CD80\(^{hi}\) on CD11c\(^+\) MHC class II\(^+\) DCs. Mice that did not receive a heart transplant mean CD80\(^{hi}\) expression of 32.5% (n=6). Mice that received a heart transplant but not Treg, mean CD80\(^{hi}\) expression of 73.4% (n=3). Mice that received Treg on D+1 relative to a heart transplant mean CD80\(^{hi}\) expression of 50.8% (n=4). Mice that received Treg on D-3 relative to a heart transplant mean CD80\(^{hi}\) expression of 42.9% (n=6, Student’s t test).
4.8 Treg do not alter TNF-α expression

CD44-Treg were generated by the same method detailed in figure legend 4.1. 1x10^6 were adoptively transferred into CBA(H2^k) mice on either D-1 (n=5) or D+1 (n=5) relative to the mice receiving a heterotopic heart allograft from B6 (H2^b) donors. Mice were harvested on post-operative day 5. Splenocytes were stimulated in the presence of PMA, Ionomycin and Monensin for 4 hours. Cytokine expression was measured using FACs. F-1 represents fluorescence minus 1 control.

- **a.** Representative plots of TNF-α expression in CD4^+ cells.
- **b.** Comparison of Median fluorescence intensity (MFI) between different treatment groups. p value between D+1 and D-1 not significant (ns) (Student’s t test).
- **c.** TNF-α expression in CD8^+ cells.
- **d.** Comparison of MFI between different treatment groups.
CD44-Treg were generated by the same method detailed in figure legend 4.1. 1x10^6 were adoptively transferred into CBA(H2^k) mice on either D-1 (n=5) or D+1 (n=5) relative to the mice receiving a heterotopic heart allograft from B6 (H2^b) donors. Mice were harvested on post-operative day 5. Splenocytes were stimulated in the presence of PMA, Ionomycin and Monensin for 4 hours. Cytokine expression was measured using FACs. F-1 represents fluorescence minus 1 control.

a. Representative plots of IL-17 expression in CD4^+ cells.

b. Comparison of Median fluorescence intensity (MFI) between different treatment groups. p value between D+1 and D-1 not significant (ns) (Student’s t test).

c. Representative plots of IL-17 expression in CD8^+ cells.

d. Comparison of different treatment groups.
4.10 Treg administered 1 day pre-transplant reduces CD4⁺ and CD8⁺ T cell IFN-γ expression.

CD44-Treg were generated by the same method detailed in figure legend 4.1. 1x10⁶ were adoptively transferred into CBA(H2k) mice on either D-1 (n=5) or D+1 (n=5) relative to the mice receiving a heterotopic heart allograft from B6 (H2b) donors. Mice were harvested on post-operative day 5. Splenocytes were stimulated in the presence of PMA, Ionomycin and Monensin for 4 hours. Cytokine expression was measured using FACs. F-1 represents fluorescence minus 1 control.

a. Representative plots of IFN-γ expression in CD4⁺ cells.
b. Comparison of Median fluorescence intensity (MFI) between different treatment groups (Student’s t test).
c. Representative plots of IFN-γ expression in CD8⁺ cells.
d. Comparison of MFI between different treatment groups.
B16F10 melanoma cells were harvested once cells had grown to approximately 50% confluency and resuspended in sterile saline. The left hind leg of B6 (H2b) mice was prepared by removing fur using an electric razor and then cleaned with ethanol. B16F10 cells were then injected subcutaneously, in a total volume of 100µL. Tumour size was measured using a calliper 20 days post injection (Student’s t test).

Figure 4.11 Titration of B16F10 melanoma cells after sub-cutaneous injection
B16F10 melanoma cells were harvested once cells had grown to approximately 50% confluency and re-suspended at $1 \times 10^6$ cells/mL, in sterile saline. Cells were injected intravenously via the tail vein into B6 (H2$^b$) mice. Mice were harvested 10 days post injection and lungs were examined for the presence of melanomas. Each individual black dot on the surface of the lung was recorded as a single melanoma. a) Representative pictures of mice harvested 10 days post injection of cells. i) Healthy lungs from a mouse that did not receive B16F10 cells. ii) Lungs from a mouse that received $1 \times 10^5$ B16F10 cells. b) Mice that received $1 \times 10^5$ B16F10 cells had a mean of 21 melanomas (n=8) compared with mice that received $2 \times 10^5$ B16F10 cells, which had a mean of 181 melanomas (n=7). p value for Student's t test.

**Figure 4.12 Titration of B16F10 melanoma cells after intravenous injection**
Figure 4.13 Comparison of B16F10 melanoma cell growth in wt B6 (WT) and B6 Rag\(^{-/-}\) after intravenous injection

B16F10 melanoma cells were harvested once cells had grown to approximately 50% confluency and re-suspended at 1x10^6 cells/mL in sterile saline. Cells were injected intravenously via the tail vein into B6 (H2^b) mice or B6 (H2^b) Rag\(^{-/-}\) mice. Mice were harvested 10 days post injection and lungs were examined for the presence of melanomas. Each individual black dot on the surface of the lung was recorded as a single melanoma.

Rag\(^{-/-}\) mice that received 1x10^5 and 2x10^5 B16F10 cells had means of 0.8 and 1.4 melanomas, respectively (n=6/5). Wild type mice that received 1x10^5 B16F10 cells had a mean of 3.2 melanomas (n=4), whereas mice that received 2x10^5 B16F10 cells had a mean of 37.5 melanomas (n=4). p value for Student’s t test.
Figure 4.14 Activated nTreg and CD44 Treg equally increase the numbers of pulmonary melanomas

Total CD4⁺ T cells from B6 (GFPFoxP3) (H2ᵇ) were magnetically isolated and incubated for 10 minutes with anti-CD44 antibody (1µg/mL). The cells were co-cultured with GMCSF and TGF-β differentiated CBA (H2ᵏ) GT-DCs, for 7 days. After 7 days fresh GT-DCs were added. Cells were harvested after 14 days in culture and sorted using FACs on the expression of CD4 and GFP(FoxP3) to >95% purity. nTreg were magnetically isolated and activated using plate bound anti-CD3 and anti-CD28 antibodies for 4 hours. 1x10⁴ CD25⁻ T cells, +/- 1x10⁴ Treg were injected per B6 Rag⁻/⁻ (H2ᵇ) mouse.

The following day, B16F10 melanoma cells were harvested and resuspended at 10⁶ cells/mL in sterile saline. Cells were injected via the tail vein into B6 Rag(H2ᵇ) mice. On the same day, all mice received a full thickness, CBA (H2ᵏ) skin graft. Mice were harvested 11 days post B16F10 injection and the lungs were examined for the presence of melanomas.

a. Experimental outline.

(Figure continues on the next page)
b. Representative images: i) B16F10 cells only, ii) B16F10 cells + CD44-Treg, iii) B16F10 cells + nTreg and iv) B16F10 + CD25 T cells (Teff).

c. Mice that received B16F10 cells only, had a mean of 109 melanomas (n=4), mice that received B16F10 cells + CD44-Treg had a mean of 192 melanomas (n=5), mice that received B16F10 cells + activated nTreg (nTreg) had a mean of 190 pulmonary melanomas (n=5). Mice that received B16F10 + CD25 T cells (Teff) had a mean of 152 pulmonary melanomas (n=3). p values for Student’s t test.
Chapter 5: Effects of Immunosuppression on Treg function in vivo

Introduction
The first clinical trials of Treg in kidney transplant recipients have begun [21], however Treg will be used in conjunction with conventional immunosuppression as it is not ethical to trial Treg in isolation due to the shortage of organ donors and acute rejection is uncommon in these patients. As immunosuppressive drugs have been designed to target key mediators of graft rejection, which includes T cells, it is expected that adoptively transferred Treg will also be affected by immunosuppressive drugs. It is important that drugs that have a negative impact on Treg function in vivo are identified and this information is used to guide the design of drug regimens in future clinical trials.

Following the Symphony Study [404], most UK transplant centres now use a regimen based on anti-CD25 induction, low dose Tacrolimus, Mycophenolate Mofetil (MMF) and Methylprednisolone (MP), with Sirolimus (Rapamycin) recommended for patients who do not tolerate CNIs (https://www.nice.org.uk/Guidance/ta85). Tacrolimus, MMF and MP form the immunosuppressive regimen for the multi-centre clinical trial assessing Treg therapy in kidney transplantation (ONE Study) and therefore the key aim of this chapter is to assess the effects of these drugs on Treg efficacy in vivo. Rapamycin will also be included as it and MMF are generally considered to be favourable for Treg function and generation in vitro and in vivo. In contrast, calcineurin inhibitors (CNIs) such as Tacrolimus are generally considered to be unfavourable for Treg function [395, 407, 412, 425]. Battaglia et al. demonstrated that Rapamycin can be used to selectively expand CD4⁺CD25⁺FoxP3⁺ Treg by driving total CD4⁺ T cells from DO11.10 tg mice with BALB/c APCs in the presence of OVA₃₂₃₋₃₃₉ peptide and 100nM Rapamycin. Cultures enriched with Rapamycin had 62% CD25⁺CD4⁺ T cells whereas cultures with Rapamycin omitted contained 38% CD25⁺CD4⁺ T cells. There was also double the number of CD25⁺⁺ CD4⁺ T cells in cultures with Rapamycin [407]. Zeiser et al. investigated both the in vitro and in
vivo effects of CsA, Rapamycin and MPA (the active form of the pro-drug MMF) on Treg function. In vitro experiments, in which freshly isolated B6 Treg (CD4^+CD25^{high}, H2^b, Thy-1.1) were incubated with BALB/c APCs (CD11c^+) in media supplemented with either: CsA (100ng/mL), Rapamycin (10ng/mL) or MPA (50ng/mL). Treg were re-isolated by sorting and then incubated for 4 days with irradiated APCs (H2^d) and CFSE Tconv cells (CD4^+CD25^-H2^bThy-1.2^+). Treg that had been cultured in Rapamycin and MPA were more suppressive than Treg exposed to CsA (58%, 55% and 24% undivided Tconv respectively). Addition of 50U/mL IL-2 almost completely restored Treg function after exposure to CsA. In vivo experiments also suggested that Rapamycin and MPA were less harmful than CsA for Treg function. Doses of each drug that moderately increased survival of mice were identified in a model of GVHD which involved transferring 1.6x10^6 CD4^+ and CD8^+ T cells, on day 2 after lethal irradiation with 800cGy, from luciferase expressing FVB/N animals (H2^q) into MHC class I and II mismatched BALB/c recipients (H2^d), in combination with a T cell depleted bone marrow (TCD BM) transplant (5x10^6 cells) on day 0. In this model, transfer of 8x10^5 Treg led to the survival of greater than 75% of mice for 70 days, whereas in the absence of Treg mice succumbed to acute-GVHD (aGVHD) by day 30. Mice that were irradiated and received TCD BM plus T cells in combination with 8x10^5 Treg and Rapamycin (0.5mg/kg) or MMF (90mg/kg) were far more likely to survive past 80 days than mice that received CsA (10mg/kg). Over 50% of mice that received MMF and over 80% that received Rapamycin survived past 80 days, whereas all mice that received CsA in combination with Treg succumbed to aGVHD by day 40 [412]. These data suggest that Rapamycin is the least harmful to Treg function in vivo, when used at suboptimal doses. MMF appears to reduce Treg function modestly compared with mice that received Treg without immunosuppression and therefore does appear to be having an effect on Treg function. Treating mice with the suboptimal dose of CsA in addition to Treg significantly reduced Treg function compared with mice that received Treg in combination with MMF or Rapamycin [412].
Most of the research with CNIs has involved CsA but there is some evidence that Tacrolimus is less harmful to Treg [429]. Using an MLR to assess the effects of CsA or Tacrolimus on Treg function, Miroux et al. showed that low doses of CsA (20 and 40ng/mL) inhibited Treg suppression of PBMC proliferation, however even at the higher doses of Tacrolimus tested (50ng/mL) Treg did not lose suppressive capacity [429]. Additionally, Siepert et al. showed that indefinite treatment of renal allograft recipients with CNI treatment could be replaced by Treg. DA (RTlavl) kidneys were transplanted into LEW (RT1l) rats and treated with ten doses of low dose CsA (3mg/kg), starting at the time of transplant, in combination with T cell depletion. T\text{mem} were transferred 7 days pre-transplant. Splenocytes from tolerised rats, containing approximately 2x10^6 Treg, were able to inhibit T\text{mem} expansion and 5 out of 6 rats survived past 150 days, whereas 5 out of 6 rats that received CNI treatment without Treg rejected their kidneys by day 100 [423]. These data suggest that low doses of the CNI, CsA can be used in combination with Treg therapy to control T\text{mem} mediated rejection, however due to the fact that mice were depleted of T cells means that the authors did not address whether Treg are capable of preventing the rejection of an allograft in an otherwise lymphoreplete mouse.

Although corticosteroids have been a mainstay of immunosuppression regimens, there is a lack of transplant-based literature of their effects on Treg. There is a wealth of information from asthma and allergy research and these data are largely contradictory as to whether steroids are detrimental to Treg or not [432-437]. Karagiannidis et al. compared the levels of FoxP3 expression in CD4^+ T cells present in the peripheral blood of asthmatics who were, or were not, using steroids. Patients with moderate asthma who were using glucocorticoid-inhalers had approximately a 2 fold increase in FoxP3 expression in CD4^+ T cells, compared with moderate asthma suffers not using steroids [434]. Unfortunately, the authors did not use any other markers of Treg phenotype and it is possible that the up regulation of FoxP3 is not indicative of a Treg phenotype but instead a phenomenon later described in human CD4^+ T cells in which
upon activation FoxP3 is transiently upregulated [294]. Chen et al. identified that
glucocorticoids could amplify IL-2 dependent expansion of functional Treg in vivo when they
showed that short term administration of the glucocorticoid dexamethasone (5mg/kg/day) in
combination with IL-2 (300,000 U/mouse/day) i.p., increased the percentages of
FoxP3+CD4+CD25+ T cells in the spleen, inguinal and mesenteric lymph nodes by 180, 75 and 95%
respectively after 5 days of treatment, compared with mice that received no treatment.
However, treatment of mice with dexamethasone alone led to a reduction in the absolute
number of splenic CD4+CD25+ and CD4+CD25+ T cells and although the reduction in CD4+CD25+ T
cells was more pronounced, this result suggests that glucocorticoids in isolation are bad for
Treg in vivo [437]. In addition to this, Stock et al. showed that corticosteroids inhibited the
induction of respiratory tolerance by blocking the development of Treg in vivo. BALB/c mice
that were exposed intranasally to OVA (100µg/day) on days 0-2, in addition to 100µg/day
dexamethasone on days -1 and +1 did not generate T cells with a characteristic cytokine profile
of Treg (high levels of IL-10 and low levels of IL-4 and IFN-γ), which appeared to be in part due
to a lack of tolerance inducing DCs [432]. Additionally, Sbiera et al. and Olsen et al. both
identified that glucocorticoids decreased the numbers of Treg in humans and mice respectively
[433, 436]. Therefore, due to the conflicting nature of currently published data it remains
unclear whether steroids will or will not be permissive for adoptive transfer of Treg as a therapy
in solid organ transplant recipients.

As the multi-centre Phase I/IIa clinical trial designed to test the safety and feasibility of different
regulatory cell populations, including Treg, known as the ONE Study [21] is not powered to
examine efficacy of Treg therapy, it will not be capable of assessing the impact of
immunosuppression on Treg function. Therefore, the data from this chapter will form an
important guide for the drug regimen of future clinical trials involving Treg. For example, if CNIs
kill a large proportion of adoptively transferred Treg, or prevented their function, then it would
be necessary in future trials to use lower doses of CNIs such as Tacrolimus, or to design drug regimens to exclude CNIs altogether.

As shown in previous chapters, there was no significant difference in efficacy between activated nTreg and alloantigen-driven Treg in either the skin, or heart allograft models in Figures 3.15 and 4.1 respectively. As it is less mouse intensive to generate the number of Treg required for the experiments to assess the impact of immunosuppression on Treg survival and function, alloantigen rather than activated nTreg were used to investigate the effects of immunosuppression on adoptively transferred Treg.

**Chapter aims:** The aim of this chapter was to titrate suboptimal doses of: Rapamycin, Tacrolimus, MMF and MP to ask whether Treg could be used in combination with any of these drugs in isolation, with the ultimate goal to determine which drugs could be recommended for future clinical trials involving Treg therapy.
5.0 Results

5.1 Thought experiment exploring how to investigate the effect of immunosuppression on Treg function in vivo

Assessing the effect of immunosuppressive drugs on the function of Treg in vivo is complicated when the read out is graft survival and excess dosing of immunosuppressive drugs will prevent graft rejection. For example, if a given dose of immunosuppression in addition to Treg leads to long-term graft survival (>100 days), there could be a total loss of function of co-transferred Treg caused by the immunosuppressive drug but this would not be seen because of the dominant effect of the drug on the rejection process. Observing an effect of an immunosuppressive drug on Treg function relies on having an experimental model whereby one can observe a Treg mediated increase in graft survival, in addition to that conferred by the immunosuppressive drug. One way of circumventing this problem is to use a drug concentration deliberately chosen to be suboptimal and then add Treg, in order to look for an additional effect. As shown in Figure 4.1 and 4.4, Treg administered pre-transplant were able to modestly but significantly extend graft survival of an allogeneic heterotopic heart graft from a B6 (H2b) donor into a CBA (H2k) recipient. Thus, in order to look for an effect of immunosuppression on Treg, each drug was titrated to establish a suboptimal regimen that significantly increased the survival of a heterotopic heart allograft from control rate rejection of 7-8 days but importantly not too close to the limit allowed by the group’s Home Office project licence of 100 days (Figure 5.1.a – thought experiment). Then Treg would be adoptively transferred pre-transplant, in addition to the suboptimal dosing regimen of an immunosuppressive drug. Any significant increase in graft survival in mice that receive Treg in combination with an immunosuppressive drug, compared with immunosuppression alone will indicate that some Treg function is retained, suggesting that at low doses, that drug can be used in combination with Treg as a cellular therapy (Figure 5.1.b – thought experiment). If, however there was no improvement in graft survival when Treg are administered in combination with suboptimal doses of a drug compared with mice that only
receive suboptimal doses of a drug, then this would indicate that the drug was affecting Treg function (Figure 5.1.c – thought experiment).

5.2 Treg can prolong graft survival in the presence of a previously determined suboptimal dose of Rapamycin

To ask whether the timing of Treg administration affected the efficacy of Treg in the presence of immunosuppression, a previously titrated suboptimal dose of Rapamycin was used in combination with adoptively transferred Treg at D-3, D-1, D+1 or D+3 relative to the transplant of a cardiac allograft. Due to the large number of published studies suggesting that Rapamycin is favourable for Treg expansion and function [407, 412, 579], as discussed in both the introduction to the thesis and this chapter, it was decided that Rapamycin was the most suitable drug to use to ask whether Treg were still more efficacious when given pre-transplant, as suggested by Figures 4.1 and 4.4, when they are used in combination with an immunosuppressive drug.

Doses of Rapamycin that led to an MST of approximately 35 days, when CBA (H2^a) recipient mice received a B6 (H2^b) heterotopic heart allograft, were established previously by Dr Andrew Bushell (unpublished data). Using this previously determined dose of Rapamycin (500µg/kg/dose on days 0, 1, 3 and 5, i.p.), led to an MST of 29 days (n= 12, figure 5.2). This dose was therefore termed “suboptimal” and was used to test the efficacy of Treg at different time points. CBA mice that received a B6 heart allograft alone had an MST of 8 days (n=7), therefore suboptimally dosed mice had a statistically significant increase in MST compared with untreated controls (p=0.0001, Log-rank test). Mice that received 1x10^6 CD44-Treg, 1 or 3 days post-transplant in combination with the suboptimal dose of Rapamycin had an MST of 62.5 and 46 days respectively, there was no statistical difference between the two groups however (p=0.7, Log-rank test). Mice that received 1x10^6 CD44-Treg on 1 or 3 days pre-transplant both
had an MST of >100 days (n=3 or n=6 respectively), there was no statistical difference between the two (p=0.44, Log-rank test). Interestingly, statistically the survival of grafts in mice that received Treg on D-3 or D-1 compared with D+1 was not different when used in combination with suboptimal doses of Rapamycin (p=0.15 and p=0.12 respectively, Log-rank test), however there was only one rejection event in mice that received Treg on D-3 and none in mice that received Treg on D-1 compared with 3/5 mice rejecting their grafts before day 50 in mice that received Treg on D+1. Unfortunately, one mouse died with a functioning graft on day 21, of unknown causes, in the group that received Rapamycin and Treg on D-3. Both D-3 and D-1 administration led to statistically different survival times compared with D+3 administration (p=0.048 and p=0.38, respectively, Log-rank test).

Taken together, the data in Figure 5.2 indicate that a suboptimal regimen of Rapamycin is not damaging for Treg function when cells are delivered at the optimal time relative to transplant. Additionally, these data support previously published literature which suggests that Rapamycin is favourable for Treg function in vivo [407, 412, 579]. In the light of the data presented in Figures 4.1, 4.4 and 5.2, pre-transplant administration of Treg was decided upon for subsequent experiments asking whether Treg can function in the presence of suboptimal doses of immunosuppression.
5.3 Titrations and final dosing regimens of Tacrolimus, Mycophenolate Mofetil (MMF) and Methylprednisolone (MP)

Zeiser et al. compared the effects of Rapamycin, the CNI, Cyclosporin (CsA) and MMF on Treg function in a model of GVHD [412]. This provided a reference point for the concentrations and frequencies of drug administration for these drugs.

5.3.1 $1 \times 10^6$ Treg in addition to a suboptimal dosing regimen of Tacrolimus significantly increases graft survival

The titrations to find a suboptimal dose of Tacrolimus are shown in Figure 5.3. Suboptimal doses of each drug were sought that led to an MST of 20-50 days, doses that led to survival too close to 100 days were dismissed as mice that survive past 100 days are likely to be operationally tolerant and the experimental design required the observation of a clear improvement of graft survival when Treg were used in combination with immunosuppression. Mice were given Tacrolimus for 10 consecutive days, i.p., commencing immediately post-transplant (Figure 5.3a). Tacrolimus dosed at 4mg/kg/day and 2mg/kg/day led to an MST that was too close to, or passed 100 days (MST of >100 days and 93.5 days respectively, Figures 5.3b&c). In contrast, a dosing regimen of 1mg/kg/day, given immediately post-transplant, until day 9 post-transplant, led to an MST of 51.5 days (Figure 5.2d). This was therefore defined as the “suboptimal dose” and was then used to test the effect of Tacrolimus on Treg function in vivo (Figure 5.3d). When $1 \times 10^6$ CD44-Treg were adoptively transferred 3 days pre-transplant, in combination with the suboptimal dosing regimen of Tacrolimus (Figure 5.4a, 1mg/kg/day, Days 0-9, i.p.), the MST increased significantly to 75 days (Figure 5.4b, n=6), compared with that seen with a contemporaneous suboptimal regimen of Tacrolimus alone (MST 49days, n=6, p=0.0427 log rank test). Notably, half of the mice that received Treg in combination with suboptimal doses of Tacrolimus accepted their grafts long term (>100 days, Figure 5.4b). This result suggests that suboptimal doses of Tacrolimus, which clearly is sufficient to have a demonstrable biological effect (Figure 5.3d), does not abolish Treg function in vivo.
As discussed in the introduction to this chapter, CNIs are generally considered to be detrimental to Treg function, proliferation, and survival \textit{in vitro} and \textit{in vivo} \cite{408, 412, 429}. However, Siepert et al. showed that Treg in combination with low doses of CsA, could induce long term graft survival in a rat kidney allograft model \cite{423}. Additionally, there is evidence that Tacrolimus is able to reduce acute and chronic rejection more effectively than CsA in paediatric transplantation \cite{580, 581} and although Jain et al. and McDiarmid et al. didn’t attribute this to anything specific, it is possible that Tacrolimus is less detrimental to Treg function than CsA. This is supported by the data from Miroux et al. that suggested that \textit{in vitro}, low concentrations of Tacrolimus preserves Treg suppressive function, whereas CsA did not \cite{429}.

\subsection{5.3.2 1x10^6 CD44-Treg in addition to a suboptimal dosing regimen of Mycophenolate Mofetil (MMF) significantly increases graft survival}

The titrations to find a suboptimal dose of MMF are shown in Figure 5.5. Mice were given the first MMF dose 30 minutes before transplant (Figure 5.5a). All doses of MMF were at a concentration of 1mg/kg/day and were given orally via gavage needle. MMF dosed on days 0-6 inclusive, days 0, 1, 3 and 5 or days 0, 1 and 5, all led to an MST that was deemed too close to 100 days to confidently identify a difference in survival with the addition of Treg (MST of 87.5 days, 80.5 days and 73.5 days respectively, Figures 5.4b,c&d). A dosing regimen of 1mg/kg/day, on days 0-3 inclusive led to an MST of 24.5 days (Figure 5.5e). This regimen was therefore defined as the “suboptimal regimen” and was subsequently used to ask whether MMF could be used in combination with Treg \textit{in vivo} (Figure 5.5e).

When 1x10^6 CD44-Treg were adoptively transferred 3 days pre-transplant, in combination with the suboptimal MMF dosing regimen, the MST increased significantly to 45 days (n=5), compared to an MST of 26 days for mice that received suboptimal doses of MMF only (Figure 5.6b, n=6, p=0.0091 log rank sum). These data suggest that MMF at suboptimal doses does not prevent Treg function. As discussed previously, MMF has been shown to preserve the function
of Treg in a model of GVHD [412]. These data, in addition to the data in Figure 5.5, suggest that MMF does preserve the function of adoptively transferred Treg in vivo in the setting of organ transplantation.

5.3.3 1x10^6 Treg in addition to a suboptimal dosing regimen of Methylprednisolone (MP) has no effect on graft outcome

The titrations to find a suboptimal dose of MP are shown in Figure 5.7. All doses of MP, except those shown in Figure 5.7g and h, were given daily until grafts rejected, i.v. starting 15 minutes before the transplant (Figure 5.7a). MP doses of 0.1, 0.4, 0.6, 1.2, 2.4 and 6.3 mg/kg/day had very little, if any effect on transplant outcome (Figure 5.7b-g). 8.4mg/kg/day MP dosed i.v. on days 0-4 inclusive extended graft survival to an MST of 18.5 days (Figure 5.7h). Dosing mice at higher concentrations led to cessation of respiration and death of the mice, and therefore this regimen was defined as the “suboptimal regimen” and was used to test the effect of MP on Treg function in vivo.

When 1x10^6 CD44-Treg were adoptively transferred 3 days pre-transplant, in combination with the suboptimal MP dosing regimen, the MST was reduced slightly (16.5 days), compared with MP only treated mice (18 days) but this was not significantly different to mice that did not receive Treg (Figure 5.8b, p=0.96, Log-rank test). Given that mice that received 1x10^6 CD44-Treg only, on D-3, had an MST of 20 days (Figure 4.1), an MST of 16.5 days for Treg plus MP shows that at this suboptimal dose, which in itself has only a marginal impact on graft outcome, MP clearly has a negative effect on Treg function in vivo.

This result agrees with the majority of previously published literature that steroids are not compatible with Treg therapy [432, 433, 436]. This is an important observation as steroids are an important part of the immunosuppressive regimen for transplant recipients, especially immediately post-transplant. These data, if replicated by others, could have a significant impact on the shape of protocols which seek to add Treg therapy into existing drug regimens. It is also
the first time that this has been shown in solid organ transplant recipients, in otherwise lymphoreplete mice.

Although palpation of hearts is a useful read-out, simple graft outcome lacks the sensitivity to detect more subtle effects that each immunosuppressive drug might have on adoptively transferred Treg. In an attempt to increase the sensitivity, so that more subtle effects could be detected, experiments were performed to look at cell number as a measure of cell survival and proliferation to measure the response to alloantigens of adoptively transferred Treg in the presence of suboptimal doses of immunosuppressive drugs. In addition, DC activation was assessed as a measure of direct Treg function and cytokine expression was examined in T cells to look at the ability of Treg to reduce T cell activation.

5.4.1 Effect of MMF, Rapamycin, Tacrolimus or MP on Treg proliferation and number

Previous studies assessing the impact of immunosuppression on Treg function have investigated the effect of individual drugs on the ability of the cells to proliferate and have also enumerated the cells after stimulation [407, 408, 412, 425, 429, 433, 436, 437, 581-585]. Zeiser et al. CFSE labelled B6 Treg (H2b, Thy-1.1) and transferred them into BALB/c recipients (H2d) on day 6 after BMT. In animals treated with CsA, Treg expansion was markedly reduced as 48% of cells did not divide at all, compared to 23% not proliferating in mice that received Rapamycin and 31% of Treg not proliferating in mice that received MMF. In control experiments in which Treg were stimulated in the absence of any immunosuppression, 24% of Treg did not proliferate. This trend was mirrored by in vitro data [412]. These data suggest that Rapamycin has minimal, or no effect on Treg proliferation. Additionally, exposure of Treg to Rapamycin in culture has been identified as a robust way to selectively expand Treg. Battaglia et al. showed that sorted CD4+CD25+ Treg from BALB/c mice, stimulated with anti-CD3 and anti-CD28 in media enriched
with 1000U/mL IL-2 plus Rapamycin, expanded over 60% more than when cells were stimulated in the absence of Rapamycin [407].

Miroux et al. compared the effects of CsA and Tacrolimus on Treg proliferation and function *in vitro*. Stimulation of Treg with anti-CD3 and anti-CD28 in the presence of increasing doses of CsA and Tacrolimus identified a dose dependent effect of these drugs on the proliferation of Treg [429]. Although this suggests that CNIs such as Tacrolimus may not be compatible with Treg as a therapy for organ transplantation, Siepert et al. identified that prevention of rejection of an allogeneic kidney allograft using indefinite treatment with a CNI could be replaced by a short term, suboptimal dose of the CNI, CsA, starting on the day of transplantation, in combination with a single dose of splenocytes from tolerised mice on day 3 post-transplant, containing approximately 2x10^6 Treg [423]. These data suggest that *in vivo*, Treg still retain function in the presence of the CNI, CsA, although they didn’t assess the effects of CsA on Treg proliferation.

Segundo et al. compared the percentages of Treg in Rapamycin or CNI treated renal transplant recipients that had demonstrated stable renal function for a year, with healthy subjects. The percentages of Treg in Rapamycin treated patients were similar to those of healthy subjects, whereas in CNI treated patients, the percentages of Treg were significantly reduced [408]. These data suggest that CNIs are detrimental to Treg survival *in vivo* and it is possible that CNIs prevent the proliferation of Treg. However, the data in figure 5.4 suggests that a short term, suboptimal dose of Tacrolimus does not prevent the ability of Treg to extend the survival of a cardiac allograft and therefore, although proliferation may be affected compared to other immunosuppressive drugs, Treg are still able to function in the presence of a suboptimal dose of CNIs, as supported by work published by Siepert et al. [423].

Although corticosteroids have been a mainstay of immunosuppression regimens, their effects on Treg proliferation *in vivo*, especially in transplant recipients are poorly understood. Chen et
al. identified that glucocorticoids could amplify IL-2 dependent expansion of functional Treg \textit{in vivo}. As mentioned in the introduction section to this thesis and chapter, short term administration of the glucocorticoid, dexamethasone (5mg/kg/day) in combination with IL-2 (300,000 U/mouse/day) i.p., increased the percentages of FoxP3$^+$CD4$^+$CD25$^+$ T cells in the spleen, inguinal and mesenteric lymph nodes by 180%, 75% and 95% respectively after 5 days of treatment, compared with mice that received no treatment [437]. This would suggest that Treg may be able to proliferate in the presence of steroids, although the numbers of Treg reduced when exposed to Dexamethasone alone [437]. However, another study showed that corticosteroids inhibited the induction of tolerance to inhaled antigens by blocking the development of Treg. Exposure of mice to 100µg/day of OVA, intranasally on days 0-2, in addition to 100µg/day dexamethasone on days -1 and +1 prevented the generation of T cells with a characteristic cytokine profile of Treg (high levels of IL-10 and low levels of IL-4 and IFN-$\gamma$), suggesting that the induction and potentially the proliferation and survival of Treg was being inhibited [432]. Furthermore, two different groups identified that glucocorticoids decreased the numbers of Treg in humans and mice respectively [433, 436]. Again these data are suggestive that steroids are detrimental for Treg survival and proliferation.

It is feasible that the adoptively transferred Treg that recognise alloantigens present in the graft will be required to proliferate to generate significant numbers in order to effectively suppress the recipient’s immune response. Therefore, Treg were enumerated in the spleens of mice after exposure to suboptimal doses of immunosuppression and the ability of the adoptively transferred Treg to proliferate in the presence of each drug was also assessed. All mice were harvested 10 days post-transplant to allow dosing completion. It was also desirable to analyse mice before they had rejected their grafts and therefore it was decided that 10 days post-transplant would be a suitable time point. As mice were analysed 10 days post-transplant, this precluded controls in which mice would receive Treg without immunosuppression as by this
time point, although Treg only controls have an MST of 20 days (Figure 4.4) some mice are likely to have rejected their grafts before day 10 and without alloantigen stimulation, as evidenced by Chapter 4.6 and work published by Singh et al. [543], adoptively transferred Treg numbers diminish in vivo. Additionally, cells involved with rejection responses will have begun to, or have already down-regulated costimulatory molecules and cytokine expression. It was also not possible to include transplant only controls for the same reasons, as all of these mice would have rejected 2-4 days previously.

5.4.2 Effect of immunosuppression on Treg number

To ask whether different immunosuppressive drugs were affecting Treg number, CD44-Treg were generated from CBA(FoxP3GFP) (H2k) mice and sorted to >95% purity, based on GFP expression. 1x10⁶ were injected (i.v.) into CBA (H2k) mice. 3 days later the mice were given heart grafts from B6 mice (H2b) and immunosuppressive drugs were administered according to the regimens outlined in Figures 5.2, 5.4, 5.6 and 5.8. Treg were enumerated in the spleens of mice 10 days post-transplant (Figure 5.9). Mice that received 1x10⁶ Treg 3 days pre-transplant and were treated with suboptimal doses of MMF had the most GFP+ Treg per spleen (mean GFP+ cells 9474, n=6), mice treated with suboptimal doses of Rapamycin had a mean of 7500 GFP+ Treg cells (n=6), Tacrolimus treated mice had a mean of 5223 GFP+ Treg cells (n=6) and mice treated with MP had the fewest recoverable Treg with a mean of 2998 GFP+ cells (n=3).

5.4.3 Effect of Immunosuppression on Treg proliferation

To ask whether the differences in numbers of Treg, observed in the presence of each of the immunosuppressive drugs, correlated with differences in Treg proliferation, CD44-Treg were generated from CBA-FoxP3GFP mice and sorted to >95% purity, based on GFP expression and VPD labelled before i.v. injection. Mice were sacrificed 10 days post-transplant and proliferation of splenic GFP+ Treg was analysed by VPD dilution (Figure 5.10).
The proportion of infused Treg that proliferated at least once in the presence of suboptimal immunosuppression were as follows: MMF 64.6%; MP 50.9%; Rapamycin 47.1%; Tacrolimus 35.6%. Although in percentage terms, it might be tempting to conclude that MP had little impact on the ability of Treg to proliferate, the VPD histogram shows quite clearly that very few events were recovered from this group making a clear conclusion impossible. However, the VPD data and the absolute number data (Figure 5.9) suggest very strongly that MP, even at these modest doses, is highly toxic for regulatory T cells. To confirm whether MP is actively killing Treg as opposed to affecting their trafficking and therefore reducing their presence in the spleen, analysis of markers of apoptosis such as Annexin V or Bim (Bcl-like-2 protein 11) could be assessed using FACS.

5.5 Effect of immunosuppression on Dendritic Cell (DC) activation status

As discussed in the previous chapter (Chapter 4.3), DCs are well understood to be key mediators of graft rejection [70, 259, 331, 338, 339, 343, 381, 386-388, 540, 541]. The down regulation of DC costimulatory molecules, such as CD80 and CD86, and the inhibition of stable contacts between CD4+ cells and DCs have been identified as an important mechanism of Treg mediated suppression of DCs in vitro [338, 339] and in vivo [540]. Administration of Treg prior to the transplant most effectively reduced the expression of CD80 as demonstrated in Figures 4.3 and 4.7, and therefore experiments were designed to investigate whether this Treg mediated suppression of DC costimulatory molecule up-regulation was enhanced in the presence of each immunosuppressive drug.

1x10^6 CD44-Treg were adoptively transferred into CBA (H2k) mice, 3 days prior to receiving a heart transplant from B6 (H2b) mice. Mice were treated with suboptimal dosing regimens of immunosuppressive drugs according to Figures 5.2a, 5.4a, 5.6a and 5.8a. The mice were harvested 10 days post-transplant as this would ensure that DCs were analysed after the last
dose of immunosuppression was given across all of the suboptimal dosing regimens and before any mice had rejected their grafts. This unfortunately would negate the inclusion of a transplant only control as this time point is after rejection would have occurred, thereby making this control unhelpful. CD11c+ MHC class II+ dendritic cell activation was measured using the costimulatory molecules CD80 and CD86. The hypothesis was that if a drug could be used in combination with Treg therapy then DC activation would be reduced compared to mice that received immunosuppression in isolation. If there was no reduction in DC activation when Treg were used in combination with immunosuppression, then this would be indicative that the immunosuppressive drug was affecting this aspect of Treg function. Due to the fact that each drug is likely to affect DC activation differently (highlighted by the different survival times exhibited in Figures 5.2b, 5.4b, 5.6b and 5.8b), the effects of Treg mediated suppression of DC expression of CD80 and CD86 was compared with each drug separately.

5.5.1 Effect of Rapamycin on Treg suppression of DC activation

The expression of CD86 on splenic DCs in mice treated with Rapamycin and Treg, as opposed to Rapamycin only, was significantly reduced (Rapa + Treg; CD86 mean expression = 16.2%. Rapa only; CD86 mean = 20.0%. n=5/6 in each group, p=0.046 Student’s t test), CD80 expression was also reduced in splenic DCs from Rapamycin and Treg treated mice compared with mice that received Rapamycin only, however this was not statistically significant (Figure 5.11, p=0.061 Student’s t test).

5.5.2 Effect of MMF on Treg suppression of DC activation

The combination of Treg and MMF reduced CD86 expression on splenic DCs compared to when MMF was used in isolation (MMF only; CD86 expression = 28.4%, MMF +Treg; CD86 expression = 23.8%. n=6 in each group, p=0.0002), CD80 expression was not significantly different between the two groups (Figure 5.12).
5.5.3 Effect of Tacrolimus on Treg suppression of DC activation

Treg combined with Tacrolimus did not significantly reduce CD86 expression (Figure 5.13c&d, 
p=0.77 Student’s t test). CD80 expression was reduced but again this was not significant (Figure 5.13a&b, 
p=0.13 Student’s t test). Interestingly, the data shows that four out of six mice had lower CD80 expression than the mice that received Tacrolimus only. The absence of a significant reduction in DC activation could be explained by the graft survival data in Figure 5.4, in which some mice that received Treg and Tacrolimus reject their grafts sooner than mice that received Tacrolimus only. Although the mice are inbred and are theoretically identical, the variability in graft survival and “two tiered” DC expression of CD80 might be a result of a variety of inconsistencies such as; differences in cold ischaemia time of graft during harvesting, inconsistent dosing of Tacrolimus or inaccurate injection of Treg.

5.5.4 Effect of MP on Treg suppression of DC activation

One of the key mechanisms of Treg mediated suppression of immune responses is the suppression of DC activation [332, 338, 339, 382, 586-588] and therefore it is important this is not perturbed by immunosuppressive drugs, or can enhance drug reduction of DC activation. As discussed in Chapter 4 and the introduction to this thesis, several mechanisms of Treg suppression of DC activation have been described, including inhibition of stable contacts between DCs and CD4+ T cells [332], CTLA-4 dependent down regulation of CD80/CD86 binding and induction of IDO [272, 588, 589].

Treg did not significantly reduce CD80 or CD86 expression in the presence of MP (Figure 5.14, 
p=0.58 and p=0.59 respectively, Student’s t test). This reflects what was seen previously in the survival data and the quantification of Treg 10 days post-transplant, that MP is detrimental to Treg function in vivo. This is almost certainly due to the fact that nearly all of the Treg were killed by MP.
Overall these data suggest that Rapamycin and MMF can be used in combination with Treg and the increased graft survival shown in Figures 5.2 and 5.6 is probably, in part, due to a reduction in the activation of dendritic cells in the graft draining lymph organ. Treg in combination with Tacrolimus did not significantly reduce DC activation, however 4 out of 6 mice did have reduced CD80 expression and graft survival was significantly improved. MP appears to be detrimental to Treg function in vivo which is entirely consistent with the transplant outcome data.

These results suggest that at least some mechanisms of Treg mediated suppression of DC activation are not perturbed completely in the presence of suboptimal doses of MMF, Rapamycin and the majority of mice dosed with Tacrolimus.

5.6 Effect of IS on T cell cytokine expression

CD4\(^+\) and CD8\(^+\) T cells are important mediators of transplant rejection. During acute rejection of an allograft, dendritic cells residing in the graft will leave and traffic to the lymphoid tissue, where they subsequently activate T cells [68, 590, 591]. These activated T cells can traffic to the graft, become activated further and cause damage via a variety of mechanisms. As discussed in Chapter 4.8, an important mechanism of T cell activation involves the expression of cytokines and certain “proinflammatory” cytokines have been well documented to contribute to allograft rejection [556-560]. Treg can control cytokine related T cell activation using a variety of mechanisms. One key suppression mechanism of T cell activation is mediated by the release of cytokines, such as IL-10, IL-35 and TGF-β, by Treg [258]. Indirectly, Treg prevent T cell activation by suppressing DC activation, in part by a reduction of the expression of costimulatory molecules. B7 costimulatory molecules are known to play critical roles activating T cells, leading to an increased expression of cytokines involved in acute rejection such as IFN-γ and TNF-α. The observation that in the presence of Rapamycin (Figure 5.11d), or MMF (Figure 5.12d) Treg were able to significantly reduce the expression of CD86 and to reduce, although not significantly,
CD80 expression when Treg were administered together with suboptimal dosing of Tacrolimus (Figure 5.13d), in addition to the knowledge that Treg have been shown to directly suppress T cell activation, raised the question as to whether T cell activation was being affected directly. Alternatively, a lack of reduction in T cell activation would suggest that the immunosuppressive drug was impeding Treg mediated suppression of T cell activation.

5.6.1 The addition of 1x10⁶ Treg to a suboptimal dosing regimen of Rapamycin significantly reduces IFN-γ and TNF-α expression from CD4⁺ T cells

1x10⁶ CD44-Treg were injected (i.v.) into CBA (H₂kn) mice, 3 days prior to the transplant of a heart allograft from B6 (H₂kb) mice. Mice were treated with suboptimal doses of Rapamycin (as outlined in Figure 5.2), sacrificed 10 days post-transplant and their spleens harvested. Spleen cells were stimulated with PMA and Ionomycin, along with Monensin for 5 hours, after which intracellular cytokine expression was analysed using flow cytometry. IFN-γ expression was significantly reduced from CD4⁺ (Figure 5.15.a, Rapa only mean IFN-γ positive, 3.4%. Rapa + Treg mean IFN-γ % positive 2.2%, n=6 for both groups, p=0.0008 Student’s t test) but not CD8⁺ (Figure 5.15.b, Rapa only mean IFN-γ positive, 23.4%. Rapa + Treg mean IFN-γ % positive 21.2%, n=6 for both groups, p=0.37 Student’s t test) T cells when Treg were added to the suboptimal dosing regimen of Rapamycin compared to Rapamycin only controls. CD4⁺ T cell expression of TNF-α was also significantly reduced in mice that received Treg and Rapamycin (Figure 5.15.c, Rapa only mean TNF-α positive, 37.7%. Rapa + Treg mean TNF-α % positive 30.5%, n=6 for both groups, p=0.001 Student’s t test) but not in CD8⁺ T cells (Figure 5.15.d, Rapa only mean IFN-γ positive, 11.5%. Rapa + Treg mean IFN-γ % positive 10.8%, n=6 for both groups, p=0.49 Student’s t test).
5.6.2 The addition of $1\times10^6$ Treg to a suboptimal dosing regimen of MMF significantly reduces IFN-γ expression from CD8⁺ T cells

$1\times10^6$ CD44-Treg were injected (i.v.) into CBA (H₂ᵃ) mice 3 days prior to the transplant of a heart allograft from B6 (H₂ᵇ) mice. Mice were treated with suboptimal doses of MMF (as shown in Figure 5.6), sacrificed 10 days post-transplant and their spleens harvested. Spleen cells were stimulated with PMA and Ionomycin, along with Monensin for 5 hours, after which intracellular cytokine expression was analysed using flow cytometry. IFN-γ expression was not significantly reduced in CD4⁺ T cells (Figure 5.16.a, MMF only mean IFN-γ positive, 4.81%. MMF + Treg mean IFN-γ % positive, 4.78%, n=6 for both groups, p=0.91 Student’s t test), however it was significantly reduced in CD8⁺ T cells (Figure 5.16.b, MMF only mean IFN-γ positive, 33.2%. MMF + Treg mean IFN-γ % positive, 27.6%, n=6 for both groups, p=0.026 Student’s t test), in mice that received both Treg and MMF, compared with MMF alone. There was no significant difference with the expression of TNF-α from either CD4⁺ (Figure 5.16.c, MMF only mean TNF-α positive, 16.2%. MMF + Treg mean TNF-α % positive, 14.6%, n=6 for both groups, p=0.079) or CD8⁺ T cells (Figure 5.16.d, MMF only mean TNF-α positive, 2.65%. MMF + Treg mean TNF-α % positive, 2.11%, n=6 for both groups, p=0.31 Student’s t test) between the two different groups.

5.6.3 The addition of $1\times10^6$ Treg to a suboptimal dosing regimen of Tacrolimus significantly reduces IFN-γ expression from CD4⁺ and CD8⁺ T cells, and CD4⁺ T cell TNF-α expression

$1\times10^6$ CD44-Treg were injected (i.v.) into CBA (H₂ᵃ) mice 3 days prior to the transplant of a heart allograft from B6 (H₂ᵇ) mice. Mice were treated with suboptimal doses of Tacrolimus (as shown in Figure 5.4), sacrificed 10 days post-transplant and their spleens harvested. Spleen cells were stimulated with PMA and Ionomycin, along with Monensin for 5 hours, after which intracellular cytokine expression was analysed using flow cytometry. IFN-γ expression was significantly reduced in CD4⁺ T cells (Figure 5.17.a, Tac only mean IFN-γ positive, 3.43%. Tac + Treg mean IFN-γ % positive, 1.62%, n=6 both groups, p=0.0019 Student’s t test) and CD8⁺ T cells (Figure 5.17.b, Tac only mean IFN-γ positive, 7.31%. Tac + Treg mean IFN-γ % positive, 4.62%, n=6 both groups,
p=0.0005 Student’s t test), in mice that received both Treg and Tac, compared with Tac alone. There was a significant difference with the expression of TNF-α from CD4+ (Figure 5.17.c, Tac only mean TNF-α positive, 14.2%. Tac + Treg mean TNF-α % positive, 11.2%, n=6 both groups, p=0.047 Student’s t test) but not CD8+ T (Figure 5.17.d, Tac only mean TNF-α positive, 5.02%. Tac + Treg mean TNF-α % positive, 4.73%, n=6 both groups, p=0.69 Student’s t test) cells between the two different groups.

5.6.4 $1 \times 10^6$ Treg plus suboptimal dosing of MP does not affect IFN-γ or TNF-α expression from CD4+ or CD8+ T cells

1x10^6 CD44-Treg were injected (i.v.) into CBA (H2b) mice 3 days prior to the transplant of a heart allograft from B6 (H2k) mice. Mice were treated with suboptimal doses of MP (as shown in Figure 5.8), sacrificed 10 days post-transplant and their spleens harvested. Spleen cells were stimulated with PMA and Ionomycin, along with Monensin for 5 hours, after which intracellular cytokine expression was analysed using flow cytometry. Neither IFN-γ expression or TNF-α was significantly different in either CD4+ or CD8+ T cells between different treatment groups (Figure 5.18a, MP only treatment, mean IFN-γ positive CD4+ T cell, 20.9%. MP + Treg mean IFN-γ positive CD4+ T cell, 24.0% n=4/3 respectively, p=0.42 Student’s t test. Figure 5.18.b, MP only treatment, mean IFN-γ positive CD8+ T cell, 24.6%. MP + Treg, mean IFN-γ % positive CD8+ T cell, 26.7%, n=4/3 respectively, p=0.45 Student’s t test. Figure 5.18c, MP only treatment, mean TNF-α positive CD4+ T cell, 58.0%. MP + Treg mean TNF-α CD4+ T cell, 58.1%, n=4/3 respectively, p=0.91 Student’s t test. Figure 5.18d, MP only treatment, mean TNF-α positive CD8+ T cell, 39.6%. MP + Treg mean TNF-α % positive CD8+ T cell 46.3%, n=4/3 respectively, p=0.5 Student’s t test). The observation that there is no difference between mice that received Treg plus MP versus MP alone is most probably due to the MP mediated killing of Treg.
Taken together these data indicate that Treg can be used in combination with suboptimal doses of MMF, Rapamycin and Tacrolimus, whereas suboptimal doses of MP were not permissive to Treg function.
5.7 Discussion

The data presented in this chapter suggest that Rapamycin, MMF and Tacrolimus are not detrimental to Treg function in vivo as shown in Figures 5.2, 5.4 and 5.6. This demonstrates that Treg can increase the survival of heart allografts when combined with suboptimal doses of any of these three drugs compared to mice that received MMF, Rapamycin or Tacrolimus alone. However, MP is clearly detrimental to Treg function in vivo (Figure 5.8). Furthermore, data presented in this chapter indicate that MMF is the most favourable for Treg proliferation as 64.6% of the cells proliferated in the presence of MMF, compared with 47.1% Treg proliferation in the presence of Rapamycin (Figure 5.10). Tacrolimus did not completely prevent Treg proliferation (mean Treg proliferation of 35.6%) but did significantly reduce it compared with MMF treated mice. Although Treg adoptively transferred into mice that also received MP proliferated more times than Rapamycin, the small number of cells visible on the plot (Figure 5.10) and present in spleens of mice on day 10 (Figure 5.9), clearly indicates that MP is detrimental to Treg in vivo. It is interesting that compared to mice that were treated with suboptimal doses of Rapamycin or MMF, numbers and proliferation of Treg were reduced compared with mice that also received suboptimal doses of Tacrolimus but these Treg were still able to significantly prolong graft survival as shown in Figure 5.4. This result supports earlier research published by Zeiser et al. suggesting CNIs are detrimental to Treg expansion in vivo [412] but also supports findings by Siepert et al. that CNIs do not prevent Treg function in vivo [423], although Zeiser et al. showed that suboptimal doses of the CNI, CsA, did prevent the ability of Treg to control GVHD [412].

In terms of identifying an immunosuppressive drug that is most permissive for Treg in vivo, these data suggest that MMF is the most favourable, although the difference between MMF and Rapamycin were modest. The different in proliferation of adoptively transferred Treg between mice treated with MMF or Rapamycin was significant (p=0.0001, Student’s t test) but the difference in numbers of GFP+ Treg recovered 10 days post-transplant was not (p=0.12,
Student’s t test). Interpreting this result is complicated by the difference in rejection kinetics between these two drugs, especially as none of the hearts in the mice that received MMF and Treg went on to long term survival, whereas 80% of the hearts in mice that received Rapamycin and Treg went on to >100 days. This suggests that there is a difference in the inflammatory environment to which the Treg are exposed which could have an effect on the Treg proliferation and survival data. However, these results do support the observations in Figures 5.2 and 5.6, as well as being consistent with the literature, that MMF and Rapamycin can be used in combination with Treg [412].

MP is the most detrimental drug to Treg function and survival in vivo. Analysis of splenic, adoptively transferred GFP⁺ Treg after MP dosing revealed the least number of Treg per spleen (mean of 2998 Treg), which was significantly lower than the Treg per spleen in the all three other groups of suboptimally immunosuppressed mice (Figure 5.9). This correlated with the failure of Treg to increase graft survival.

It is possible that although Tacrolimus reduces Treg number compared with MMF and Rapa, there is a minimum number of Treg required to control graft rejection and MP has reduced Treg numbers to below this threshold. Indeed, data presented in chapter 3 (Figure 3.15) identified that the proportion of Treg to Teff is an important relationship for Treg mediated control of allograft rejection. To test this theory, titrating down the number of Treg administered in conjunction with a drug that is favourable with Treg function in vivo, such as Rapamycin, might identify a point at which Treg numbers become too low to have a physiological effect. Alternatively it would be interesting to give mice that receive MP a bigger initial dose of Treg, or multiple doses of Treg to identify whether this is a simple approach to overcome the negative effect that MP has on Treg in vivo.
Immunosuppressive drugs are rarely used as a monotherapy, raising an important point regarding the synergistic effects of multiple immunosuppressive drugs on Treg function in vivo. It will be important to answer whether drugs that can be combined with Treg facilitate Treg control of graft rejection in vivo when more than one drug is used or whether the use of multiple drugs has a combined toxic effect on Treg. It will also be important to ask whether drugs that are not detrimental to Treg are able to reduce, or prevent the negative effects of MP. Alternatively, it may be that the combined toxicity of multiple drugs will prove to be detrimental to Treg function, regardless of how they act as a monotherapy. Conducting these experiments in pre-clinical models is extremely difficult for a number of reasons. Identifying a dosing regimen for combinations of drugs that has any relevance to the clinical scenario is extremely challenging if the read out is graft survival. However, surrogate markers of Treg function, outlined in this chapter, could be used. It would be interesting to adoptively transfer $1 \times 10^6$ Treg into mice, transplant a heart allograft together with various combinations of the suboptimal doses of immunosuppressive drugs and compare functions of Treg at day 10 with mice that receive immunosuppression only. If a combination of drugs was found that enabled the Treg to survive, proliferate, reduce dendritic cell or T cell activation, or reduce immune mediated damage to the graft then this would provide more important information to guide the design of drug regimens in future clinical trials.

Importantly, this is one of the first times the effect of suboptimal doses of MP on adoptively transferred Treg has been examined in an allograft model. This result adds support to previously published data suggesting that MP is detrimental to Treg function in vivo [339, 425, 435, 437, 586, 587].

Treg in mice treated with suboptimal doses of Rapamycin and MMF significantly reduced the activation of DCs, evidenced by the reduction of the costimulatory molecule CD86 (Figures 5.11d and 5.12d, respectively). Suboptimal doses of Tacrolimus in addition to Treg did not significantly
reduce DC activation (Figure 5.13), although there was a reduction in CD80 expression in 4 out of 6 mice (Figure 5.13b). It has been shown that the relative expression of CD80 and CD86 have important consequences for T cell activation. As discussed in Chapter 4, blockade of CD86 has been shown to increase the suppressive function of Treg, whereas CD80 blockade enhanced T cell responses [592]. CTLA-4 expression by Treg can inhibit dendritic cell maturation by reducing CD80 and CD86 expression [272]. The observation that Rapamycin and MMF facilitate Treg mediated reduction of CD86 expression suggests that the CTLA-4 dependent control of this process may not be abrogated in the presence of these two drugs. In contrast, Treg that were exposed to Tacrolimus did not reduce CD86 expression. This result could be explained by the lower numbers of Treg in mice treated with Tacrolimus, or it might indicate that Tacrolimus could be reducing CTLA-4 expression on Treg, or otherwise be having some other effect on CTLA-4 such as preventing down-stream signalling from CTLA-4. Further experiments could be done to assess the relative expression of CTLA-4 on Treg after exposure to each immunosuppressive drug to identify if this is the cause of these observed differences.

The reduction in T cell activation observed, specifically cytokine expression, indicates that Rapamycin, MMF and Tacrolimus did not prevent Treg suppression of important pro-inflammatory cytokines involved in acute allograft rejection. It is possible that the Treg mediated suppression of dendritic cell activation observed in Rapamycin and MMF treated mice contributed to this reduction, although it is likely that there is also direct suppression of T cells by Treg, due to the observation that Tacrolimus did not significantly reduce dendritic cell activation but there was still a reduction in T cell activation in these mice. This is perhaps not surprising considering the variety and plasticity of Treg suppressive mechanisms that have been previously described and discussed in the introduction to this thesis [258, 593].

IFN-γ promotes allograft rejection via multiple pathways including the induction of cytotoxic T lymphocyte (CTL) and delayed type hypersensitivity (DTH) responses [130, 594, 595], and nearly
double the number of IFN-γ transcripts were identified by Northern blot analysis in LEW.1A recipients with rejecting heart allografts from LEW.1W donors, compared with rats that had been tolerantised with 2mL donor blood 14 and 7 days pre-transplant [131]. As discussed in Chapter 4.8, a key mechanism previously ascribed to Treg is the reduction of IFN-γ expression from CD4+ and CD8+ T cells [556-560]. Therefore, it is an important observation that Treg in combination with Rapamycin or Tacrolimus reduced the expression of IFN-γ from both CD4+ and CD8+ T cells, and that in the presence of MMF, CD8+ T cell expression of IFN-γ was significantly reduced compared with mice that received the drugs without Treg.

TNF-α is a pleiotropic cytokine that has important anti-inflammatory roles including the activation of Treg through the constitutively expressed TNF-α receptor, GITR [265]. McHugh et al. used DNA microarray analysis of CD4+CD25− and CD4+CD25+ T cells, and CD4+CD25+ T cells showed increased expression of GITR. Importantly, addition of goat anti-GITR antibody prevented CD4+CD25+ T cell suppression of CD4+CD25− T cell proliferation, suggesting an important role for this receptor for Treg function [265]. Expression of TNFR2, one of two cell surface receptors for TNF-α has been shown to identify a subset of Treg which are highly suppressive [596, 597]. Chen et al. demonstrated that the majority of tumour infiltrating Treg are TNFR2+ and suppression assays identified them to be more suppressive than both CD4+CD25+ Treg and the highly suppressive CD4+CD103+ Treg subset [596]. However TNF-α has also been identified in kidney transplant patients with acute rejection [142] and in heart allograft rejection in rats [143]. Maury et al. collected serum samples from 10 patients following cadaveric renal transplantation and showed that serum levels of immunoreactive TNF-α were <10pg/mL in healthy subjects compared to a median peak level of 140pg/mL during acute renal allograft rejections [142].

Imagawa et al. showed that in a rat cardiac transplant model using buffalo donors and lewis recipients, rejection of grafts in untreated recipients had an MST of 10.8 days, whereas
treatment of rats with a monoclonal anti-TNF-α antibody increased MST of grafts to 17.4 days. Addition of recombinant TNF-α accelerated graft rejection to 7.4 days [143]. Yokota et al. showed that TNF-α can promote T cell activation [598]. Addition of recombinant TNF-α to in vitro cultures enhanced anti-CD3 antibody driven CD8⁺ and CD4⁺ T cell proliferation [598]. TNFR2 was also identified to be costimulatory to TCR mediated T cell activation in human T cells as stimulation of non-adherent PBMCs with beads coated with anti-TCR mAb proliferated significantly more in the presence of TNF-α than when TNF-α was omitted from the cultures. Additionally, cultures in which TNF-α was included had vastly more IFN-γ after 5 days [599]. TNFR2 KO mice confirmed this phenomenon and TNFR2 deficiency resulted in reduced proliferation of T cells and also a reduction in the expression of IFN-γ [600, 601]. This suggests that during acute inflammatory responses, the concentration of TNF-α is contributing to graft rejection, in part, via the stimulation of T cells and therefore the reduction of TNF-α expression mediated by Treg in the presence of Rapamycin or Tacrolimus potentially contributes to the increase in graft survival seen in Figures 5.2 and 5.4. There was also a reduction in CD4⁺ T cell expression of TNF-α in the presence of MMF but this wasn’t significant. Unfortunately, there hasn’t been the scope to investigate the effects of immunosuppressive drugs on Treg immunosuppressive mechanisms in vivo but such experiments will be important for future development of drug regimens in human trials involving Treg.

One of the aims was to sort Treg and then test their function in vitro, after they had been exposed to immunosuppression in vivo because it is unlikely that exposing Treg to immunosuppression in vitro accurately represents the milieu experienced by the Treg in vivo, as evidenced by the contrasting literature on the effects of CNIs on Treg function, for example [412, 423, 429]. However, it was not possible to obtain enough cells for these experiments because a group of 6 mice, adoptively transferred with 1x10⁶ Treg and dosed with MMF, would only yield, when pooled, approximately 5x10³ cells after flow sorting to high purity (>95%). These experiments would have allowed direct comparison of Treg suppression after exposure
to immunosuppressive drugs in vivo. As the yield of recovered alloantigen-driven Treg is always likely to be low, the effects of immunosuppression on the native nTreg could instead be assessed as a surrogate. Another issue arising with in vitro studies is the use of blood trough levels of immunosuppressive drugs, from transplant recipients, to inform what concentrations to use to assess the effects on Treg function. However, this often stated “clinically relevant” dose is likely to be significantly higher than the concentration of drug that the cells are exposed to in tissues and the organ itself, and could be contributing to the conflicting literature.

Zeiser et al. demonstrated that the CNI, CsA prevented Treg dependent control of rejection in a model of GVHD [412]. The irradiation of the hosts prior to adoptive transfer of Teff and Treg effectively means that this model is assessing the ability of Treg to control homeostatic proliferation, as well as preventing GVHD. A potential complication of using this model is that different drugs might affect homeostatic proliferation differently, which is not something the authors addressed. It is also likely that the T cells that were adoptively transferred by Zeiser et al. are effectively exposed to a higher concentration of CsA than in a lymphoreplete mouse, due to an increased drug half-life because there are fewer cells to metabolise it [412]. It is therefore reasonable to suggest that using a lymphoreplete mouse is the best pre-clinical mouse transplant system in which to test the effects of immunosuppression on adoptively transferred Treg to avoid these complications.

Previously published data assessing the effects of CNIs on Treg function have used CsA, which in vitro and in vivo has been shown to reduce or prevent Treg function and proliferation [407, 589]. This was in contrast to data published by Siepert et al. who showed that low doses of CsA could be used in conjunction with adoptively transferred Treg [339]. The conclusion from the data presented in chapter 5 is that the CNI, Tacrolimus can be used in combination with Treg, at suboptimal doses. There is evidence that Tacrolimus reduces rejection episodes in humans compared with CsA and the mean annual death rate for transplant patients who received
Tacrolimus was significantly reduced compared to patients who received CsA-based immunosuppression (0.14% versus 0.8% respectively, p=0.001) [580], which might be attributable to lower toxicity of Tacrolimus compared with CsA with regards to Treg [429]. A direct comparison of whether Tacrolimus is less toxic than CsA for Treg function in vivo could be achieved using the suboptimal dosing strategy outlined in this chapter.

Due to the complexity of the experiments and the time taken for extensive titrations, it was not possible to determine whether optimal doses of any of the Treg permissive drugs were detrimental to Treg function in vivo. In this instance, graft outcome would not be useful, however the surrogate markers, such as DC activation and T cell activation, which have been validated in this chapter as a measure of Treg function in vivo could be used.
Chapter 5: Figures
Figure 5.1 Thought experiment outlining the possible effects of suboptimal doses of immunosuppression on Treg prolongation of graft survival as a method to ask whether Treg can be used in conjunction with clinically relevant immunosuppressive drugs

a. CBA (H2k) mice that received a heterotopic heart allograft from B6 (H2b) donors reject their grafts with an MST of 8 days (black dotted line, n=5). Mice that received 1x10^6 CD44-Treg i.v., 3 days pre-transplant have an MST of 18 days (Red line, n=5). Mice that received a previously titrated suboptimal dose of immunosuppression significantly extend graft survival compared with untreated controls (blue line).

b. Example of experimental outcome if mice that received Treg in addition to suboptimal dosing regimen of immunosuppression retain Treg function (green line).

c. Example of experimental outcome if mice that received Treg in addition to suboptimal dosing regimen of immunosuppression lose Treg function (black solid line).
5.2 Treg can prolong graft survival in the presence of a suboptimal dose of Rapamycin.

Total CD4+ T cells from CBA(GFPFoxP3) (H2k) mice were magnetically isolated and incubated for 10 minutes with anti-CD44 antibody (1µg/mL). In each well of a 24 well plate, 1x10⁵ CD4+ T cells were co-cultured with 1x10⁴ GMCSF and TGF-β differentiated B6 (H2b) GT-DCs, for 7 days. On Day 7, 1x10⁴ fresh GT-DCs were added and cells were harvested on day 14. Treg were flow sorted to >95% purity based on CD4 and GFP expression. 1x10⁶ CD44-Treg were adoptively transferred into CBA (H2k) mice on either: D-3, D-1, D+1 or D+3 relative to the mice receiving a heterotopic heart allograft from B6 (H2k) donors. Graft function was monitored by palpation of the heart, rejection was determined when hearts stopped contracting.

- **Experimental outline.**

- **Untreated mice rejected their heart allograft acutely (MST 8 days, n=7).** Mice treated with suboptimal dosing regimen of Rapamycin (500µg/kg/day, D0,1,3,5. i.p.) had an MST of 29days (n=12). Mice treated with suboptimal dosing regimen of Rapamycin + 1x10⁶ CD44-Treg on D-1 or D-3 (n=3/n=6) both had an MST >100days. Mice treated with suboptimal dosing regimen of Rapamycin + Treg on D+1 (n=5) or D+3 (n=6) had an MST of 62.5 days and 46 days respectively.
5.3 Tacrolimus dose titrations in the heterotopic heart allograft model.

CBA (H2^k) mice received a heterotopic heart allograft from B6 (H2^b) donors. Graft function was monitored by palpation of the heart, rejection is determined when hearts stop contracting. All tacrolimus doses were given i.p., starting immediately post-transplant, once per day, days 0-9 inclusive.

a. Schematic of dosing regimen.
b. Tacrolimus dosing 4mg/kg/day, MST >100days, n=4.
c. Tacrolimus dosing 2mg/kg/day, MST 93.5days, n=4.
d. Tacrolimus dosing 1mg/kg/day, MST 51.5days, n=4.
5.4 Treg can prolong graft survival in the presence of a suboptimal dose of Tacrolimus

Treg were prepared exactly as detailed in Figure legend 5.2.

a. Experimental outline.

b. Untreated mice rejected their heart allografts acutely (MST 8 days). Mice treated with suboptimal dosing regimen of Tacrolimus, starting immediately post-transplant, (1mg/kg/day, D0-9, i.p.) + 1x10^6 CD44-Treg had an MST of 75 days (n=6) which was significantly increased compared with Tacrolimus only treated mice (MST 49 days. n=6, p=0.0427, Log-rank test).
5.5 Mycophenolate Mofetil (MMF) suboptimal dose titrations in the heterotopic heart allograft model.

CBA (H2k) mice received a heterotopic heart allograft from B6 (H2b) donors. Graft function was monitored by palpation of the heart, rejection is determined when hearts stop contracting. MMF was dosed orally by gavage. All doses were 1mg/kg/day commencing 30 minutes pre-transplant.

a. Schematic of dosing regimen.

b. MMF dosing. Days 0-6 inclusive. MST 87.5 days, n=4.

c. MMF dosing. Days 0,1,3,5. MST 80.5 days, n=4.

d. MMF dosing. Days 0,1,5. MST 73.5 days, n=4.

e. MMF dosing. Days 0-3 inclusive. MST 24.5 days, n=4.
5.6 Treg can prolong graft survival in the presence of a suboptimal dose of MMF.

Treg were prepared exactly as detailed in Figure legend 5.2.

a. Experimental outline.

b. Untreated mice rejected their heart allograft acutely (MST 8.5 days). Mice treated with suboptimal dosing regimen of MMF (1mg/kg/day, D0-3, by oral gavage starting 30 minutes pre-transplant) + 1x10^6 CD44-Treg had an MST of 45 days which was significantly increased compared with MMF only treated mice (MST 26 days. p=0.0091, Log-rank test).
5.7 Methylprednisolone (MP) suboptimal dose titrations in the heterotopic heart allograft model.

CBA (H2k) mice received a heterotopic heart allograft from B6 (H2b) donors. Graft function was monitored by palpation of the heart and rejection was determined when hearts stopped contracting. All MP doses were given i.v. in a total volume of 200µL. Mice were dosed daily until rejection, except for mice that received 6.3mg/dose or 8.4mg/dose. These mice received doses on days 0-4 inclusive.

a) Schematic of dosing regimen.

b) MP dosing 0.1mg/kg/day, MST 8 days, n=4.

c) MP dosing 0.4mg/kg/day, MST 8.5 days, n=4.
d) MP dosing 0.6mg/kg/day, MST 10 days, n=4. (Figure continues on next page)

e) MP dosing 1.2mg/kg/day, daily dosing, MST 12.5 days, n=4.

f) MP dosing 2.4mg/kg/day, daily dosing, MST 12 days, n=4.

g) MP dosing 6.3mg/kg/day, D0-4, MST 11 days, n=4.

h) MP dosing 8.4mg/kg/day, D0-4, MST 18.5 days, n=4.

e) MP dosing 1.2mg/kg/day, MST 12.5 days, n=4.

f) MP dosing 2.4mg/kg/day, MST 12 days, n=4.

g) MP dosing 6.3mg/kg/day, MST 11 days, n=4.

h) MP dosing 8.4mg/kg/day, MST 18.5 days, n=4.
5.8 Treg cannot prolong graft survival in the presence of a suboptimal dose of Methylprednisolone (MP).

Treg were prepared exactly as detailed in Figure legend 5.2.

a. Schematic of dosing regimen.

b. Untreated mice rejected their heart allograft acutely (MST 8.5 days, n=4). Mice treated with suboptimal dosing regimen of MP (8.4mg/kg/day, D0-4. i.v.) +1x10^6 CD44^-Treg had an MST of 16.5 days (n=4) which was not significantly different compared with MP only treated mice (MST 18 days. p=0.96, Log-rank test).
5.9 Effect of suboptimal doses of Immunosuppressive drugs on number of adoptively transferred Treg in the spleen

Treg were prepared exactly as detailed in Figure legend 5.2. 1x10^6 Purified CD44-Treg cells were adoptively transferred (i.v.) into CBA (H2^k) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H2^b) donors. Mice were treated with previously identified suboptimal doses of Rapamycin, Tacrolimus, MMF or MP. Spleens were harvested 10 days post-transplant and adoptively transferred GFP^+ Treg were analysed with flow cytometry and enumerated using counting beads.

a. Representative plots.

b. Enumeration of adoptively transferred CD4^+GFP^+ Treg 10 days post heart allograft transplant. Rapamycin treated mice, mean number of Treg: 7500 (+/-865, n=6). Tacrolimus treated mice, mean number of Treg: 5223 (+/-663, n=6). MMF treated mice, mean number of Treg: 9474 (+/-804, n=6). MP treated mice mean number of Treg, 2998 (+/-464, n=3).
5.10 Effect of suboptimal doses of Immunosuppressive drugs on Treg proliferation

Treg were prepared exactly as detailed in Figure legend 5.2. On day 14, Treg were flow sorted to >95% purity based on CD4 and GFP expression. Purified CD4+Treg were labelled with VPD and 1x10^6 cells were adoptively transferred (i.v.) into CBA (H2^k) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H2^b) donors. Mice were treated with previously identified suboptimal doses of Rapamycin, Tacrolimus, MP or MMF and harvested 10 days post-transplant. Adoptively transferred GFP+ Treg proliferation was measured using VPD dilution in the spleen by FACS.

a. Histograms representative of VPD dilution. Cells were gated on CD4+GFP+ Treg.
b. Proliferation of adoptively transferred CD4+GFP+ VPD labelled Treg 10 days post heart allograft transplant. Mean percentage of adoptively transferred Treg proliferated: Rapamycin 47.1% (+/-1.3%, n=6); Tacrolimus 35.6% (+/-1.9%, n=6); MMF 64.6% (+/-1.1%, n=6); MP 50.9% (+/-3.7%, n=3).
5.11 Treg reduce dendritic cell activation in the presence of a suboptimal dosing regimen of Rapamycin

Treg were prepared exactly as detailed in Figure legend 5.2. 1x10^6 purified CD44-Treg cells were adoptively transferred (i.v.) into CBA (H2<k>) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H2<b>) donors. Mice were treated with suboptimal doses of Rapamycin. Mice were harvested 10 days post-transplant and splenic dendritic cell activation was measured using flow cytometry. Dendritic cells were gated on CD11c^+MHC class II^+ expression.

a. Representative plots showing CD80 expression on dendritic cells. No Tx refers to mice that had no treatment (n=3).

b. CD80 expression on dendritic cells (gated on CD11c^+MHC class II^+ expression). Treg plus suboptimal doses of Rapamycin reduced mean CD80 expression to 30.9%, compared to 38.1% mean CD80 expression in mice treated with Rapamycin only (n=5/6 both groups, p=0.06).

c. Representative plots showing CD86 expression on dendritic cells.

d. CD86 expression on dendritic cells. Mean CD86 expression was 16.2% in mice treated with Treg and Rapamycin, compared to 20% mean CD86 expression in Rapamycin only treated mice (n=5/6 both groups, p=0.046, Student's t test).
5.12 Treg reduce dendritic cell activation in the presence of a suboptimal dosing regimen of MMF

Treg were prepared exactly as detailed in Figure legend 5.2. 1x10⁶ Purified CD44-Treg cells were adoptively transferred (i.v.) into CBA (H₂k) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H₂b) donors. Mice were treated with suboptimal doses of MMF. Mice were harvested 10 days post-transplant and splenic dendritic cell activation was measured using flow cytometry. Dendritic cells were gated on CD11c+MHC class II+ expression.

a. Representative plots showing CD80 expression on dendritic cells. No Tx refers to mice that had no treatment (n=3).

b. CD80 expression on dendritic cells (gated on CD11c+MHC class II+ expression). Treg plus suboptimal doses of MMF reduced mean CD80 expression, 41.6%, compared with 45.2% mean CD80 expression in mice treated with MMF only (n=6 both groups, p=0.12).

c. Representative plots showing CD86 expression on dendritic cells.

d. CD86 expression on dendritic cells. Mean CD86 expression was 23.8% in mice treated with Treg and MMF, compared with 28.4% mean CD86 expression in MMF only treated mice (n=6 both groups, p=0.0002).
Treg do not significantly reduce dendritic cell activation in the presence of a suboptimal dosing regimen of Tacrolimus

Treg were prepared exactly as detailed in Figure legend 5.2. 1x10^6 Purified CD44-Treg cells were adoptively transferred (i.v.) into CBA (H2^k) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H2^b) donors. Mice were treated with suboptimal doses of Tacrolimus. Mice were harvested 10 days post-transplant and splenic dendritic cell activation was measured using flow cytometry. Dendritic cells were gated on CD11c^+MHC class II^+ expression.

a. Representative plots showing CD80 expression on dendritic cells. No Tx refers to mice that had no treatment (n=3).

b. CD80 expression on dendritic cells (gated on CD11c^+MHC class II^+ expression). Treg plus suboptimal doses of Tacrolimus reduced mean CD80 expression to 41.6%, compared with 45.2% mean CD80 expression in mice treated with Tacrolimus only (n=5/6 both groups, p=0.13).

c. Representative plots showing CD86 expression on dendritic cells.

d. CD86 expression on dendritic cells. Mean CD86 expression was 64.9% in mice treated with Treg and Tacrolimus, compared with 65.5% mean CD86 expression in Tacrolimus only treated mice (n=5/6 both groups, p=0.77).
5.14 Treg do not change dendritic cell activation in the presence of a suboptimal dosing regimen of MP

Treg were prepared exactly as detailed in Figure legend 5.2. 1x10^6 Purified CD44-Treg cells were adoptively transferred (i.v.) into CBA (H2^k) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H2^b) donors. Mice were treated with suboptimal doses of MP. Mice were harvested 10 days post-transplant and splenic dendritic cell activation was measured using flow cytometry. Dendritic cells were gated on CD11c^+MHC class II^+ expression.

a. Representative plots showing CD80 expression on dendritic cells. No Tx refers to mice that had no treatment (n=3).

b. CD80 expression on dendritic cells (gated on CD11c^+MHC class II^+ expression). Treg plus suboptimal doses of MP mean CD80 expression, 67.1%, compared with 65.4% mean CD80 expression in mice treated with MP only (n=4/3 both groups, p=0.58).

c. Representative plots showing CD86 expression on dendritic cells.

d. CD86 expression on dendritic cells. Mean CD86 expression was 87.5% in mice treated with Treg and MP, compared with 87.3% mean CD86 expression in MP only treated mice (n=4/3 both groups, p=0.59).
5.15 Treg reduce cytokine expression from CD4⁺ T cells in the presence of suboptimal doses of Rapamycin

Treg were prepared exactly as detailed in figure legend 5.2. 1x10⁶ Purified CD44⁻ Treg cells were adoptively transferred (i.v.) into CBA (H2k) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H2b) donors. Mice were treated with previously identified suboptimal doses of Rapamycin (Figure 5.2). Mice were harvested 10 days post-transplant and spleen cells were stimulated with PMA and Ionomycin in the presence of Monensin for 5 hours. Cells were harvested, intracellular cytokine staining was performed and cells were analysed using flow cytometry. F-1 identifies fluorescence minus 1 control.

a. Representative plots illustrating CD4⁺ T cell IFN-γ expression. Mean IFN-γ expression in mice treated Rapa only, 3.4%, Rapa + Treg, 2.2% (n=6 both groups).

b. Representative plots illustrating CD8⁺ T cell IFN-γ expression. Mean CD8⁺ T cell IFN-γ expression in mice treated with Rapa only, 23.4%, Rapa + Treg 21.2% (n=6 both groups).
c. Plots illustrating CD4⁺ T cell TNF-α expression. Mean CD4⁺ T cell TNF-α expression in mice treated with Rapa only, 37.7%, Rapa + Treg, 30.5% (n=6 both groups).

d. Plots illustrating CD8⁺ T cell TNF-α expression. Mean CD8⁺ T cell TNF-α expression in mice treated Rapa, 11.5%, Rapa + Treg, 10.8% (n=6 both groups).
Treg were prepared exactly as detailed in figure legend 5.2. 1x10^6 Purified CD44-Treg cells were adoptively transferred (i.v.) into CBA (H2^d) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H2^b) donors. Mice were treated with previously identified suboptimal doses of MMF (Figure 5.6). Mice were harvested 10 days post-transplant and spleen cells were stimulated with PMA and Ionomycin in the presence of Monensin for 5 hours. Cells were harvested, intracellular cytokine staining was performed and cells were analysed using flow cytometry. F-1 identifies fluorescence minus 1 control.

a. Representative plots illustrating CD4^+ T cell IFN-γ expression. Mean IFN-γ expression in mice treated MMF only, 4.81%, MMF + Treg, 4.78% (n=6 both groups).

b. Representative plots illustrating CD8^+ T cell IFN-γ expression. Mean CD8^+ T cell IFN-γ expression in mice treated with MMF only, 33.2%, MMF + Treg, 27.6% (n=6 both groups).
c. Plots illustrating CD4\(^+\) T cell TNF-\(\alpha\) expression. Mean CD4\(^+\) T cell TNF-\(\alpha\) expression in mice treated with MMF only, 16.2\%, MMF + Treg, 14.6\% (n=6 both groups).

d. Plots illustrating CD8\(^+\) T cell TNF-\(\alpha\) expression. Mean CD8\(^+\) T cell TNF-\(\alpha\) expression in mice treated MMF, 2.65\%, MMF + Treg, 2.11\% (n=6 both groups).
5.17 Treg reduce cytokine expression from CD4$^+$ and CD8$^+$ T cells in the presence of suboptimal doses of Tacrolimus

Treg were prepared exactly as detailed in figure legend 5.2. 1x10$^6$ Purified CD44-Treg cells were adoptively transferred (i.v.) into CBA (H2$^k$) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H2$^b$) donors. Mice were treated with previously identified suboptimal doses of Tac (Figure 5.4). Mice were harvested 10 days post-transplant and spleen cells were stimulated with PMA and ionomycin in the presence of Monensin for 5 hours. Cells were harvested, intracellular cytokine staining was performed and cells were analysed using flow cytometry. F-1 identifies fluorescence minus 1 control.

a. Representative plots illustrating CD4$^+$ T cell IFN-γ expression. Mean IFN-γ expression in mice treated Tac only, 3.43%, Tac + Treg, 1.62% (n=6 both groups).

b. Representative plots illustrating CD8$^+$ T cell IFN-γ expression. Mean CD8$^+$ T cell IFN-γ expression in mice treated with Tac only, 7.31%, Tac + Treg 4.62% (n=6 both groups).
c. Plots illustrating CD4⁺ T cell TNF-α expression. Mean CD4⁺ T cell TNF-α expression in mice treated with Tac only, 14.2%, Tac + Treg, 11.2% (n=6 both groups).

d. Plots illustrating CD8⁺ T cell TNF-α expression. Mean CD8⁺ T cell TNF-α expression in mice treated Tac, 5.02%, Tac + Treg, 4.73% (n=6 both groups).
5.18 Treg have no effect on the expression of IFN-γ or TNF-α in the presence of suboptimal doses of MP

Treg were prepared exactly as detailed in figure legend 5.2. 1x10⁶ Purified CD44-Treg cells were adoptively transferred (i.v.) into CBA (H2k) mice. 3 days later the mice received a heterotopic heart allograft from B6 (H2b) donors. Mice were treated with previously identified suboptimal doses of MP (Figure 5.8). Mice were harvested 10 days post-transplant and spleen cells were stimulated with PMA and Ionomycin in the presence of Monensin for 5 hours. Cells were harvested, intracellular cytokine staining was performed and cells were analysed using flow cytometry. F-1 identifies fluorescence minus 1 control.

a. Representative plots illustrating CD4⁺ T cell IFN-γ expression. Mean IFN-γ expression in mice treated MP only, 20.9% (n=4), MP + Treg, 24.0% (n=3).

b. Representative plots illustrating CD8⁺ T cell IFN-γ expression. Mean CD8⁺ T cell IFN-γ expression in mice treated with MP only, 24.6% (n=4), MP + Treg 26.7% (n=3).
c. Plots illustrating CD4$^+$ T cell TNF-α expression. Mean CD4$^+$ T cell TNF-α expression in mice treated with MP only, 58.0% (n=4), MP + Treg, 58.1% (n=3).

d. Plots illustrating CD8$^+$ T cell TNF-α expression. Mean CD8$^+$ T cell TNF-α expression in mice treated with MP, 39.6% (n=4), MP + Treg, 46.3% (n=3).
Chapter 6: Discussion

The experiments described in this thesis have revealed a novel method for enriching alloantigen-reactive Treg using a combination of anti-CD44 antibody and allogeneic DCs. Furthermore, the data demonstrate that adoptive transfer of Treg pre-transplant can be combined safely and effectively with Rapamycin, Tacrolimus and MMF in order to improve survival, whereas Methylprednisolone is detrimental to Treg function.

6.1 Mechanism of Treg enrichment using anti-CD44 antibody

The aim of chapter 3 was to improve the enrichment of alloantigen-driven Treg in a previously established protocol using the PDE3 inhibitor cilostamide [247]. Interrogation of this protocol revealed that anti-CD44 antibody can enrich for alloantigen-driven Treg with the ability to generate a larger number of cells.

The data presented in chapter 3 suggest that incubation of CD4⁺ T cells in the presence of anti-CD44 antibody prior to stimulating the cells with allogeneic GT-DCs, reduces IL-6 expression from CD44⁺ CD4⁺ T cells in the input population of T cells. GT-DC expression of IL-6 had previously been identified to reduce Treg enrichment using IFN-γ [402] and similarly is detrimental to Treg enrichment in the presence of cilostamide (Figure 3.3, [247]). Whereas the expression of IL-6 from APCs is well understood, the ability of the T cell population to produce pro-inflammatory cytokines in Treg-inducing protocols is often overlooked, as discussed by Miyao et al. [300]. Therefore, the reduction of IL-6 expression from CD44⁺ CD4⁺ T cells using anti-CD44 antibody may create an environment that favours Treg enrichment. Additionally, previously published data have indicated that stimulation of human nTreg with plate-bound anti-CD44 antibody activates nTreg and stimulates the production of IL-2, IL-10 and TGF-β [463]. This in combination with a reduction in IL-6 expression may contribute towards a favourable environment for Treg enrichment.
Although it is not known whether alloantigen-reactive Treg can be generated using anti-CD44 antibody, data published by Bollyky et al. showed that activating human nTreg with plate bound anti-CD3, anti-CD28 and anti-CD44 antibodies led to increased expression of anti-inflammatory cytokines and increased expression of FoxP3 in nTreg [463]. It seems reasonable to conclude that alloantigen-driven human Treg could be enriched for in a similar manner, providing a new method for the generation of stable alloantigen-reactive human Treg.

*In vivo* data are also supportive of this activity of CD44 ligation. In a study by Li and colleagues, a short course of anti-CD44 mAb treatment at the time of Treg adoptive transfer *in vivo* improved the reversal of diabetes in mice by over 90% in comparison with Treg transfer alone [602]. Furthermore, administration of anti-CD44 antibody alone delayed diabetes onset and increased CD4\(^+\) T cell frequency producing IL-2 and TGF-β but did not affect Treg numbers [602]. These data complement previous work by Bollyky et al. and show that anti-CD44 antibody can also activate nTreg *in vivo*, although there does not appear to be an effect on Treg number *in vitro* [463] or *in vivo* [602]. This contradicts what was shown in Chapter 3, however Bollyky et al. used plate bound antibodies instead of APCs which could be crucial for Treg enrichment using this method.

Others have shown that signalling via the CD44 ligand, galectin-9, using anti-CD44 antibody increases TGF-β-dependent induction of Treg *in vitro* [504]. This is in contrast to the data presented in chapter 3 which identified little *de novo* generation of Treg in the presence of anti-CD44 antibody. It is possible that this is due to low concentrations of TGF-β in the cultures and that adding exogenous TGF-β, as demonstrated by Wu et al. [504], could increase the enrichment for Treg using CD44-antibody. Low levels of TGF-β could also be preventing *de novo* Treg generation *in vivo* in the presence of anti-CD44 antibody [602].

Pursuing protocols to generate alloantigen-reactive Treg has remained an attractive line of research due to previously published data suggesting they are more efficacious [397, 398].
However, comparison of alloantigen-driven Treg and activated nTreg in lymphopenic and lymphoreplete animals in non-vascularised and vascularised allograft models (Figures 3.15 and 4.1) suggest that this is not the case. This is the first time that alloantigen-driven Treg and activated nTreg have been directly compared using lymphoreplete mice. Additionally, alloantigen-driven Treg are theoretically less likely to cross react onto antigens presented outside of the transplant [397], compared with polyclonally expanded autologous nTreg and therefore less likely to result in “off target” suppression. Using a well-established melanoma model in which Treg have been shown to facilitate the progression of the tumour [536], both alloantigen-driven Treg and nTreg equally suppress the immune response to pulmonary melanomas (Figure 4.14). These data in combination with the observation that neither population of Treg is more efficacious, indicate that the pursuit of protocols that enrich for alloantigen-driven Treg may be unnecessary. This is important to consider, given that the expansion of alloantigen-driven Treg is currently less efficient [395, 398] and it is logistically challenging to produce such cells in a timely manner in the case of cadaveric donor transplants.

6.2 Alloantigen-reactive Treg – new methods

Although the data in this thesis suggest that activated autologous nTreg are as effective as alloantigen-driven Treg in both the skin and heart allograft models, there are exciting advances that are currently being applied to T cells to make them “specific” for a particular antigen. One technique involves T cell transduction using a lentivirus carrying gene sequences for a particular TCR [603-605]. Alternatively, lentiviruses can be used to genetically engineer Treg to express extracellular single-chain antibody (scFv) antigen-binding domains fused to intracellular signalling domains, commonly referred to as chimeric antigen receptors (CARs) [606-609]. It has recently been demonstrated that this technology can be used to redirect Treg toward a transplant-relevant antigen [610]. MacDonald et al. created an HLA-A2-specific CAR (A2-CAR)
and expressed it on human Treg. Using a humanised mouse model the authors demonstrated that A2-CAR expressing Treg could prevent xenogenic GVHD caused by HLA-A2+ T cells and were more effective than Treg expressing an irrelevant CAR [610]. Applying this technology to transplantation will not be simple and many questions remain, such as: what is the optimal affinity of the CAR for the target antigen; will the co-expression of the TCR and the CAR affect the function or stability of Treg; will the intracellular signalling complex associated with the CAR affect Treg function; can CAR engineered Treg lead to linked epitope suppression or infectious tolerance and which target antigens will be optimal.

Additionally, transduction of Treg that could potentially lose FoxP3 in inflammatory conditions with a TCR directed to antigens present in the graft is a concern as these cells could become pathogenic and contribute to rejection.

6.3 Interactions of Treg with immunosuppressive drugs

Administration of Treg as the sole immunosuppressive therapy would be unethical given the likelihood of transplant rejection [75, 78-80]. Therefore, drug regimens will initially be required that can be combined with Treg therapy. It is therefore important to understand the effects of immunosuppression on adoptively transferred Treg. Pre-clinical models can provide insight regarding whether certain drugs may have a negative impact.

The data in this thesis are the first assessing the effects of clinically-relevant immunosuppressive drugs on adoptively transferred Treg in otherwise lymphoreplete animals. Treg in combination with Rapamycin and MMF, both of which have been identified not to be harmful to Treg function in a variety of models [412-414, 425, 543], were able to improve graft outcome compared with drugs alone. CNI inhibitors are generally considered to be detrimental to Treg function and survival [408, 412, 425, 438], however low doses of CsA in addition to Treg could replace
indefinite CsA treatment [423], suggesting that low doses of CNIs may be used in combination with Treg therapy. Indeed, data presented in this thesis using the heterotopic heart allograft model demonstrate that Treg therapy in addition to low doses of Tacrolimus improves allograft survival compared with Tacrolimus alone (Figure 5.4).

Steroids form an important part of immunosuppressive regimens and although some data suggest that glucocorticoids might not be detrimental to Treg function in vivo [611], the vast majority of data suggest the opposite [432-437]. Data from chapter 5 confirm this finding, as Treg were unable to improve graft outcome when used in combination with MP (Figure 5.8).

6.4 Timing of Treg administration

Experiments comparing pre and post-transplantation administration of Treg identified that Treg are most effective when given pre-transplantation (Figures 4.1 and 5.2). However, in the presence of the suboptimal Rapamycin regimen, post-transplantation administration of Treg still improved graft survival compared with Rapamycin alone (Figure 5.2). These data are important for two reasons: firstly, Treg will not always be available at the time of transplantation; and secondly, steroids which are frequently administered in the peri-transplant period may still be used and Treg administered once steroid levels permit.

With data from the Phase I/IIa ONE Study confirming the safety and feasibility of nTreg therapy in renal transplantation, the next phase of investigation will be a Phase IIb study of efficacy (the TWO Study, Dr Fadi Issa personal communication). In this study, patients will be converted to Rapamycin from CNI inhibition at 3 months and then receive $5-10 \times 10^6$ per kilogram body weight polyclonally-expanded nTreg 6 months post-transplantation. The control group will remain on MMF and Rapamycin, whilst patients receiving Treg will be converted to Rapamycin
monotherapy. According to the data presented in Figure 5.2, such a regimen would be a permissive environment in which to administer Treg therapy.

6.5 Multiple Treg dosing regimens

Singh et al. identified that two doses of $10 \times 10^6$ Treg on D-1 and D-5 relative to the transplant could lead to indefinite survival of cardiac allografts in otherwise lymphoreplete mice [515]. Multiple doses of Treg were not explored in this thesis, however it is unknown whether this is the optimal method to administer Treg and although this is not planned in the TWO Study, it is possible that in future clinical trials this is considered. However, the economics of multiple doses of Treg may be limiting. Current estimates are that Treg therapy during the ONE Study cost 15,000-45,000 Euros (currently £12,700-38,000) [486], whereas kidney transplants in the UK typically receive basiliximab induction followed by tacrolimus, MMF and MP which costs approximately £6000 per year [518]. However, the health and economic benefits to reducing the immunosuppressive burden, including a reduction in associated complications, morbidity, and re-transplantation rates are likely to make Treg therapy viable. In addition, Treg therapy is scalable like any evolving technology and production costs are likely to drop in the future. Considerable investment is required to pursue Treg therapy but the cost savings of patients that are operationally tolerant or need very low doses of maintenance immunosuppression and potentially are less susceptible to co-morbidities could significantly reduce indirect costs, leading to Treg therapy being economically viable.

6.6 Withdrawing immunosuppression

An interesting issue that arises with Treg therapy is when it is safe to withdraw immunosuppression. Previously developed methods, such as the mixed leukocyte reaction
(MLR), which measures donor antigen driven recipient T cell proliferation is poorly predictive [236, 612] and other techniques such as cytokine enzyme-linked immunospot and limiting dilution assays may not accurately identify donor reactivity or are impractical [390, 613-618]. Juvet et al. recently described a method to measure donor reactivity by measuring interactions between recipient CD4+ T cells and donor DCs using in vitro time-lapse microscopy. The authors identified that allograft rejection and tolerance were associated with changes in the proportion of interaction events greater than 500 seconds [390]. Unfortunately, it was not assessed if this technique was sensitive enough to assess T cell interactions with autologous DCs via the indirect pathway, which is more relevant to chronic rejection [619, 620] and the weaning of patients off immunosuppression if Treg therapy is successful. However, they did utilise FACS to detect synapse formation between DCs and T cells which could provide a high throughput method to measure rare indirect-antigen presentation events [390]. Ultimately, to remove immunosuppression it would be desirable to have a robust assay that distinguishes operationally tolerant patients from patients who need to remain on immunosuppression.

6.7 Testing Treg efficacy

Early clinical trials of Treg in kidney transplant recipients have provided invaluable information regarding the feasibility of adoptive Treg therapy, however due to the low rates of rejection in these patients, determining whether Treg are efficacious may take up to a decade. Therefore, it would be desirable to begin trials of Treg in solid organ transplants that have poorer early prognosis, such as lung transplants, to identify whether Treg can improve graft survival in humans more rapidly.
6.8 Conclusion

Although alloantigen-driven Treg have a more focused TCR repertoire [397] and it has previously been shown [397, 398] that they are more efficacious than polyclonally expanded autologous nTreg, no evidence for this could be shown. Therefore, although there are still questions regarding the efficacy and safety of nTreg, the relative ease with which nTreg can be expanded means that using these cells for Treg therapy is recommended. Importantly, apart from steroids, the majority of immunosuppressants used in clinical transplantation appear to not be detrimental to Treg activity in vivo. Treg given post-transplantation in combination with rapamycin were able to improve graft survival suggesting that if corticosteroids are required in the immediate post-transplant period, Treg could be administered once steroids have been tapered. Further work assessing the impact of combinations of drugs on Treg function in vivo is required to enhance the understanding of drug interactions on Treg therapy in humans.
7.0 References


The effect is manifested only at the differentiation, CXCR562.


