Role of OX40-OX40L interactions in the immune response to solid organ allografts

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor Philosophy

2012

Acknowledgements

I would firstly like to thank my supervisors Dr Nick Jones and Professor Kathryn Wood for giving me the opportunity to work in TRIG where I have learnt numerous transferable skills. Dr Nick Jones provided me with invaluable intellectual scientific advice. He also encouraged me to work harder when novel data was just around the corner, and importantly encouraged me to drown my sorrows with red wine when experiments went wrong. Professor Wood has provided additional support and encouragement since Dr Nick Jones moved to Birmingham. My supervisors at UCB, Drs Diane Marshall and Farnaz Fallah-Arani have constantly given me encouragement and opened up avenues to access other reagents and techniques.

Over the years, a number of colleagues in TRIG have been invaluable; Dr Andrew Bushell has provided me with humour, sweeties and sound scientific advice, Kate Milward for her help sweet talking the ARIA into sorting nicely and Dr Joanna Hester for her guidance and encouragement. I would like to acknowledge all the staff in the BMS and in particular those who have worked in Wing D for providing a high standard of animal care and invaluable expertise. The help given to me by Remi Okoye and Matt Page at UCB has been invaluable and I appreciate their time and assistance with the histological analysis.

Oxford would not have been the same without the Chan family, who have become incredible friends and who have introduced me to lots of great food and shared some fantastic memories. Karen, Shruti and Sam have become great friends and have

celebrated successes with me and encouraged me to keep going through the tough days. I thank you all for listening to my moans and groans and yet always highlighting the positives.

My colleagues at UCB have also been a great support and have travelled through this journey with me and I appreciate their encouragement and support. Dr Matt Catley must be thanked for encouraging me to start this adventure and believing in my abilities when few others did.

Oxford University Golf has been an amazing experience and I have loved every moment. There are too many people to individually thank but you have all played a key part in keeping me sane. The matches, the places, the people have all played a key role in my career which culminated in receiving my fourth Oxford Blue in 2012. May the pinkies and perkies never stop flowing in Vinnies' and the memories of Varsity matches never be forgotten.

I would also like to thank the MRC and UCB-Celltech for providing financial support which allowed me to conduct this research. In addition thanks must go to BSI, BTS, TTS and UKRC for awarding me travel scholarships which allowed me to attend conferences.

Finally, I must thank all my friends and especially my parents for their constant support and for always believing in me.

Abstract

Transplantation is the treatment of choice for end stage organ failure however current immunosuppressive therapies whilst effective at preventing acute allograft rejection, fail to prevent late graft loss due to chronic rejection and are associated with an increased risk of infection and malignancy. Therefore there is a clear unmet clinical need for improved strategies to prevent allograft rejection.

OX40 is a member of the TNFR superfamily that has potent costimulatory properties. Although the impact of blockade of the OX40-OX40L pathway has been well documented in models of autoimmune disease, its effect on the rejection of allografts is less well defined. Therefore the aim of this thesis was to determine the impact of OX40 blockade on conventional and regulatory T cell responses to allografts.

We found that activation of $CD4^+$ and $CD8^+$ naïve and memory T cells resulted in the induction of OX40 expression and that blockade of OX40-OX40L interactions partially inhibited the response of alloreactive T cells *in vitro* and prevented skin allograft rejection but did not result in the induction of tolerance. OX40 blockade was found to have no effect on the activation and proliferation of T cells but rather effector T cells failed to accumulate and migrate to skin allografts. This was shown to be the result of an enhanced degree of cell death amongst proliferating effector cells.

In addition, blockade of OX40-OX40L interactions at a time of exposure to alloantigen resulted in a pool of Treg with an enhanced ability to suppress T cell responses to alloantigen *in vitro* and *in vivo*. Counter-intuitively, OX40 blockade was found to increase the potency of alloreactive Treg by promoting survival following re-activation.

Finally, although OX40 blockade impacted both conventional and regulatory T cell responses, anti-OX40 administration did not promote skin or heart allograft survival in immunocompetent recipients and failed to synergise with blockade of other costimulatory molecules to prevent allograft rejection.

In conclusion, these data demonstrate that blockade of OX40-OX40L interactions can attenuate naïve and memory T cell responses to alloantigen whilst promoting the survival of alloreactive Treg. Therefore, we propose that anti-OX40 would be a worthwhile adjunct to pre-existing strategies to induce tolerance.

Abbreviations

7-AAD	7-Aminoactinomycin D
ACS	Acute coronary syndrome
AICD	Activation induced cell death
Aire	Autoimmune regulator
AP-1	Activator protein-1
APC	Antigen presenting cell
ATG	Anti-thymocyte globulin
BAL	Bronchoalveolar lavage
BM-DC	Bone marrow derived dendritic cells
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
cLN	Contralateral lymph node
CNS	Central nervous system
CTLA-4	Cytotoxic lymphocyte antigen-4
CTX	Cyclophosphamide
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
DD	Death domain
dLN	Draining lymph node
DST	Donor specific transfusion
DTH	Delayed type hypersensitivity
-D	DKK
EAE	Experimental allergic encephalitis
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FoxP3	Fork head box P3
FPLC	Fast performance liquid chromatography

GIC	Graft infiltrating cells
GvHD	Graft versus host disease
HTLV-1	Human T lymphotropic virus type 1
HVEM	Herpes virus entry mediator
ICAM	Intracellular adhesion molecule
ICOS	Inducible costimulator
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin
IPEX	Immune dysregulation polyendocrinopathy, enteropathy, X linked syndrome
Irf1	Interferon regulatory factor 1
i.v.	Intravenous
ITAM	Immunoreceptor tyrosine based activation motif
iTreg	Inducible regulatory T cell
IVC	Inferior vena cava
LAT	Linker for activation of T cells
LFA-1	Leukocyte function-associated molecule 1
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer cell
mAb	Monoclonal antibody
MAP	Mitogen activated protein
MHC	Major histocompatibility complex
miHC	Minor histocompatibility antigen
mLN	Mesenteric lymph node
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
MMP	Matrix metalloproteinase

MOG	Myelin oligodendrocyte glycoprotein
MSD	Mesoscale discovery
MST	Median survival time
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor-κ-B
NHP	Non-human primate
NK	Natural killer cells
NKT	Natural killer T cells
NOD	Non-obese diabetic
nTreg	Naturally occurring regulatory T cells
OVA	Ovalbumin
PA	Pulmonary artery
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed death 1
PEG	Poly ethylene glycol
PKB	Protein kinase B
PKC	Protein kinase C
PLC-γ	Phosholipase C-γ
PMN	Polymorphonuclear cells
PTK	Protein tyrosine kinase
PTLD	Post transplant lymphoproliferative disease
ROI	Regions of interest
rpm	Revolutions per minute
s.c	Subcutaneously
scFV	single chain variable fragment
SD	Standard deviation

SH2	Src homology-2
SLE	Systemic lupus erythematosus
SLP-76	SRC-homolgy 2 domain-containing leukocyte protein of 76kD
T_{CM}	Central memory
TCR	T cell receptor
T_{EM}	Effector memory
Th	T helper cell
TLR	Toll-like receptor
Tm	Memory T cell
TMB	3,3´,5,5´-tetramethylbenzidine
TNF-α	Tumour necrosis factor-α
TNFR	Tumour necrosis factor receptor
TRADD	Tumour necrosis factor receptor type 1-associated death domain
TRAF	Tumour-necrosis factor receptor associated factors
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
VLA4	Very late antigen-4
ZAP70	Zeta-chain associated protein of 70kD

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Chapter 1: Introduction

1.1 Clinical Transplantation

1.1.1 The history of solid organ transplantation

In December 1954 Joseph Murray and colleagues performed the first successful kidney transplant between identical twins, which subsequently functioned for 8 years, paving the way for the further clinical implementation of transplantation as a therapy for end-stage organ failure (1). However, the success of this kidney transplant between monozygotic twins was in part due to the fact that no immunosuppression was required. Transplants that were subsequently attempted between genetically distinct individuals therefore failed due to uncontrolled acute rejection episodes.

Transplantation has evolved in the last 70 years from an experimental therapy to the main treatment for end-stage organ failure, as a result of a number of seminal observations which have been key to the understanding of how and why foreign allografts are rejected. One of the key advances was the demonstration that the immune system was involved in the rejection of allogeneic tumour cells (2). Subsequently Medawar published studies investigating the differences between syngeneic and allogeneic skin grafts in rabbits. In these studies, syngeneic autografts were accepted, whilst allografts were rejected in a process characterised by infiltration of lymphocytes and monocytes into the graft (3, 4). These data suggested that the rejection process has marked specificity and generates donor-specific memory, as well as infiltration of leukocytes into the graft thus confirming the immunological basis of allograft rejection.

Moreover, the work suggested that the manipulation of the immune system would be key to the induction of long term graft acceptance, a concept reinforced by our current understanding of the immunological mechanisms involved in allograft rejection.

Concurrent with the aforementioned studies, Owen *et al* demonstrated that dizygotic twin calves produced permanent haematopoietic chimeras, due to the free exchange of blood cells and precursor cells (5, 6). Furthermore, Anderson *et al* and Billingham *et al* found that twin dizygotic cattle were unable to reject skin allografts if transplanted between each other as they shared a single placenta (7, 8). Perhaps more importantly, Billingham, Brent and Medawar introduced the first experimental evidence that transplantation tolerance was achievable by injecting donor alloantigen into neonatal mice and demonstrating that as adults such mice accepted skin grafts from the same alloantigen donor (9). The summation of this research gave rise to the idea of acquired immune tolerance and gave hope that tolerance to allografts could be achieved post-natally. Indeed, the induction of 'operational' tolerance (defined as long-term acceptance of an allograft without the need for the chronic administration of immunosuppressive drugs) is now generally considered to be the ultimate goal in the field of transplantation.

Several other factors have been fundamental in advancing the field of transplantation; improved surgical methods, ABO blood group and major histocompatibility complex (MHC) matching, as well as the development and use of potent immunosuppressive drugs which target the immune system. These advances have led to a huge increase in

the number of successful transplants which are performed between non-identical individuals in a variety of different organs; kidney (10), liver (11), heart (12, 13), lung (14), pancreas (15) and most recently small bowel (16). Between April 2010 and March 2011 in the UK alone, there were 3,740 organ transplants carried out from 2,055 organ donors. However, at the end of March 2011, there were still 7,636 patients on the UK transplant waiting list (17).

1.1.2 Current immunosuppression in transplantation

In recent years, the rapid and sustained development of immunosuppressive agents and their translation to routine clinical practice have revolutionised the clinical management and mortality rates in autoimmune disease and solid organ transplantation.

The development of immunosuppressive pharmacological agents, such as azathioprine (18), have created substantial opportunities to target the immune system in response to an allograft and such agents are responsible for translating transplantation from an experimental therapy to the clinic. Azathioprine is an anti-proliferative agent which interferes with DNA synthesis and prevents cell cycle progression which, in the context of allograft rejection, impairs the clonal expansion of alloreactive T cells.

Another powerful immunosuppressive agent, cyclosporine, was pioneered by Calne and colleagues for the prevention of acute rejection. Cyclosporine was the first agent to selectively inhibit T cell activation and has had a profound impact of graft survival

rates. The first year survival rate of kidney, heart, liver and lung transplants has been improved from 50% using azathioprine and steroids to 80% with cyclosporine (19, 20). Cyclosporine binds to the cytoplasmic immunophilin and forms complexes which are able to inhibit calcineurin, a key enzyme in T cell signal transduction. This prevents translocation of nuclear factor of activated T cells (NFAT) to the nucleus therefore inhibiting cytokine production (e.g. Interleukin (IL-) -2, -4, Tumour Necrosis Factor-α (TNF-α) and Interferon-γ (IFN-γ)). Despite the impressive improvements in graft survival cyclosporine is associated with numerous side-effects including nephrotoxicity and hypertension.

The risk of acute graft rejection is greatest during the initial 3 months following transplantation therefore current immunosuppression strategies have been influenced by a recent clinical trial; SYMPHONY. This trial in *de novo* adult kidney transplant recipients compared standard cyclosporine based regimens with low dose cyclosporine, tacrolimus or rapamycin in combination with mycophenolate mofetil (MMF), daclizumab (anti-IL-2Rα) and steroids over a 3 year period (21). The trial demonstrated a regimen with an induction therapy of daclizumab, MMF and steroids with a low dose of tacrolimus was beneficial for renal function, graft and patient survival as well as acute rejection rates at 1 and 3 years post-transplant (21, 22).

More recent therapeutic strategies are based on induction therapies which concentrate on profound immune cell depletion at the time of transplant, when immune activation is most intense. Anti-thymocyte globulin (ATG) is a lymphocyte-depleting polyclonal

gamma immunoglobulin (IgG), preparation with specificity towards human thymocytes. The polyclonal nature of ATG enables it to display specificity towards a wide variety of antigens expressed on the surface of T cells, B cells, dendritic cells (DC), natural killer cells (NK) and endothelial cells. However, the precise mechanism of action underlying the immunosuppressive efficacy of ATG in solid organ transplantation recipients is unknown at present, although it has been primarily attributed to apoptosis of T cells in the circulation and lymph nodes (23).

Monoclonal antibodies (mAb) used in transplantation include alemtuzumab (anti-CD52) and daclizumab. Alemtuzumab is a humanised rat gamma immunoglobulin (rat IgG2b) directed against the CD52 antigen, which is expressed on 95% of peripheral blood T and B cells, monocytes and macrophages (24). The profound and long-lasting lymphopenia produced after the administration of 2 doses of alemtuzumab is explained by the abundant expression of CD52 by leukocytes**.** It should be noted that examination of peripheral blood lymphocytes from human recipients post-alemtuzumab induction has identified a subset of T cells, predominantly $CD4^+$ central memory cells, which survive despite alemtuzumab induction and appear largely resistant to depletion (25).

Basiliximab and daclizumab are now commonly used in solid organ, namely renal, transplantation in low-risk recipients (as defined as first allograft, living-related donor or no delayed graft function) (26). As these drugs specifically target activated T cells, they do not cause significant lymphocyte depletion and are not associated with major adverse effects when compared to lymphocyte-depleting agents. However, it is

important to note that other subsets of T cells, including foxp3^{+} regulatory T cells (Treg) also express CD25, and therefore the use of these agents may impact some of the natural mechanisms of immunoregulation.

These induction therapies are then followed by maintenance immunosuppression which is a combination of therapies aimed at reducing the side effects of individual agents, whilst maintaining overall immunosuppression by manipulating multiple steps in T cell activation. Combinations include a calcineurin inhibitor (such as tacrolimus (27)), an anti-proliferative agent (MMF, a reversible inosine monophosphate dehydrogenase inhibitor (28, 29)), and low-dose corticosteroids (such as prednisone). Corticosteroids have both immunosuppressive and anti-inflammatory properties. They act principally by binding to cytoplasmic glucocorticoid receptors, which can translocate to the nucleus where they are able to alter the expression of multiple cytokines through DNA-binding and by targeting transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB). Corticosteroids reduce the expression of many molecules important in the immune response including IL-1, -2, -3 and -6, TNF-α, IFN-γ and chemokines. By inhibiting cyclooxygenase, corticosteroids are also able to reduce the production of inflammatory mediators such as leukotrienes and prostaglandins.

Provided there are no episodes of acute rejection, the doses of these agents are gradually reduced and then maintenance immunosuppression is continued indefinitely. This dose reduction is based on the concept that as graft inflammation subsides and donor-derived antigen presenting cells (APC) are cleared, the transplanted organ progressively adapts

to the host, decreasing in immunogenicity and risk of rejection. Improvements in clinical strategies such as tissue matching, surgical advances and immunosuppression have greatly reduced the number of acute rejection episodes and increased the one year transplant survival rates, but there has been little improvement in long-term survival rates and instances of chronic rejection (30). Despite the success of clinical transplantation, two key challenges still remain; 1) shortage of suitable organ donors and 2) the adverse effects and associated risks of long-term administration of current immunosuppressive drugs. These factors are entwined as long-term graft failure results in patients returning to the waiting list, adding to the burden on the healthcare system.

Certainly, current immunosuppressive agents are far from ideal therapies to combat the rejection of transplanted cells/tissue, mainly attributable to associated side-effects that are responsible for significant mortality and morbidity amongst the transplant population. Firstly, current immunosuppression lacks specificity, inhibiting immune responses necessary to clear pathogens and tumours in addition to suppressing immune responses to the allograft (31, 32). Secondly, a number of commonly used immunosuppressive agents have toxic side-effects and can cause irreversible damage to the allograft, for example the long-term use of cyclosporine has clear nephrotoxic side-effects and is believed to be a major contributor to chronic allograft dysfunction, the leading cause of graft failure in renal transplantation (33). Finally, these therapies must be taken for the lifetime of the allograft, demanding a high compliance rate by the recipient.

It is therefore clear that there is an unmet clinical need for the development of improved therapies to prevent rejection that could be administered in the short-term but provide long-term allograft-specific immunosuppression without debilitating side-effects. However, to achieve this goal, a better understanding of the immunology of rejection must be acquired in order to develop improved therapeutic modalities.

1.2 Immunobiology of allograft rejection

The immune system has developed mechanisms to clear invading micro-organisms and malignant, apoptotic or infected cells, which are recognised as foreign as they express antigens that are not normally expressed by the adult host. Transplantation between allogeneic individuals results in the immune system of the recipient recognising the antigens (alloantigens) on the transplant as foreign which initiates the complex process of rejection.

The immune response directed against the transplanted organ is a highly orchestrated process involving both innate and adaptive immunity which can result in hyperacute, acute or chronic rejection. It has been clear for many years that the adaptive immune system, and in particular T cells, play a key role in the response against alloantigens. Animals which lack T cells (due to thymectomy or lethal irradiation and reconstitution with syngeneic bone marrow for example) are unable to reject full-MHC mismatched allografts (34-36). But this alloresponse can be fully restored if such animals receive T cells (syngeneic to the recipient) (34-36). Therefore understanding the biology of T cells and how they become activated and recognise alloantigen will allow improved

therapeutic strategies to be developed to prolong graft survival through the modulation of the alloresponse.

1.2.1 Pathways of Allorecognition

Allorecognition in the presence of costimulation (signal 2) results in the activation and expansion of alloreactive T cells i.e. T cells that recognise polymorphic donor antigens. Allorecognition is executed by the T cell receptor (TCR), which is associated with the CD3 surface molecule (TCR-CD3 complex). Recipient T cells recognise donor antigens which are products of either the MHC or minor histocompatibility (miHC) antigen loci, which stimulate the process of rejection and are the main targets responsible for triggering a cascade of immunological events that result in rejection (37). The MHC complex in the mouse was described by Gorer (38, 39) and was later characterised by Snell (40). The function of the MHC in adaptive immunity is to process and present peptides. T cells expressing CD8 co-receptor preferentially interact with MHC class I molecules presenting antigenic peptides, and T cells expressing CD4 interact with MHC class II-peptide complexes.

MiHC antigens alone cannot cause rapid rejection, but when there are multiple miHC mismatches rejection can occur as rapidly as when there is a MHC mismatch (41). MHC class I and class II molecules present peptide antigens to T cells and are polymorphic members of the immunoglobulin family. MHC class I molecules consist of an α-chain which is stabilised by non-covalent association with the related molecule β2-microglobulin. The α-chain α1 and α2 domains of MHC class I form a cleft able to

bind peptides of 8-11 amino acids in length. MHC class II molecules on the other hand are heterodimers of an α and β chain that form a peptide binding cleft which is open at both ends and can therefore accommodate longer peptides (13-18 amino acids).

It is widely accepted that there are 3 non-mutually exclusive and concurrent mechanisms by which MHC alloantigens can be recognised; direct, indirect and semi-direct pathways (Figure 1). They differ in the origin of the APC, kinetics of the initiation of recognition as well as longevity of and contribution to the alloresponse over time.

The direct pathway involves the recognition of intact donor MHC and peptide complexes, on the surface of donor APC or the allograft itself (**Figure 1A**). This pathway of recognition is unique to allograft responses, and the proportion of the T cell repertoire that is reactive to alloantigen is in the region of 10% (42). Pietra *et al* provided the first evidence to support the idea that T cells with direct allo-specificity can mediate transplant rejection (43). The reconstitution of immunodeficient $Rag^{-/-}$ or SCID (severe combined immunodeficiency) mice with syngeneic $CD4^{+}$ T cells led to the rejection of MHC class $II^{+/+}$ grafts, but not MHC class $II^{-/-}$ grafts. In addition $Rag^{-/-}$ MHC Class $II^{-/-}$ mice were also able to reject cardiac allografts when reconstituted with $CD4^{+}$ T cells. These mice lack $CD8^{+}$ T cells and lack the ability to reject allografts via the indirect pathway. Thus demonstrating $CD4^{+}$ T cells utilising the direct pathway of allorecognition are sufficient and required to mediate allograft rejection. The number of donor APC available to prime recipient T cells is limited, as they will be destroyed by primed T cells and other effector mechanisms. Therefore it is likely that the direct

pathway only mediates rejection for a finite time after transplantation. Evidence from clinical studies supports this theory as T cells with direct alloreactivity were not detectable years after transplantation in the peripheral blood of renal allograft patients despite chronic allograft dysfunction (44).

Normally T cell recognition of a foreign antigen is self-MHC restricted i.e. foreign peptides are presented in the context of self-MHC molecules. This route of allorecognition is known as the indirect pathway in the context of transplantation (**Figure 1B**). In this pathway, donor MHC (or other polymorphic proteins) are processed by APC and presented in the context of recipient MHC molecules to recipient T cells (45, 46). The presence of the indirect pathway of allorecognition was first demonstrated by Auchincloss *et al* who showed that MHC class $I^{-/-}$ recipients were able to reject MHC class $II^{-/-}$ skin allografts. In this model, the only available pathway of MHC allorecognition was the indirect pathway where recipient $CD4^+$ T cells recognise donor MHC class I presented in the context of recipient MHC class II molecules (47). It has been proposed that the indirect pathway remains active throughout the life of the graft due to continuous acquisition of donor peptides derived from the allograft (44, 48). For this reason, it is likely that the indirect pathway is responsible for immune responses associated with chronic rejection (49, 50).

More recently a semi-direct pathway for recognition of alloantigen has been described. This involves the recognition of intact donor MHC+peptide complexes presented by recipient APC following acquisition from exosomes secreted by donor APC or through cell to cell contact (**Figure 1C**) (51-53). This pathway may provide a link between the

direct and indirect pathways as a single APC may present both intact and processed alloantigen, however there is no direct *in vivo* evidence to support this hypothesis to date and the significance of this pathway in transplantation has yet to be elucidated.

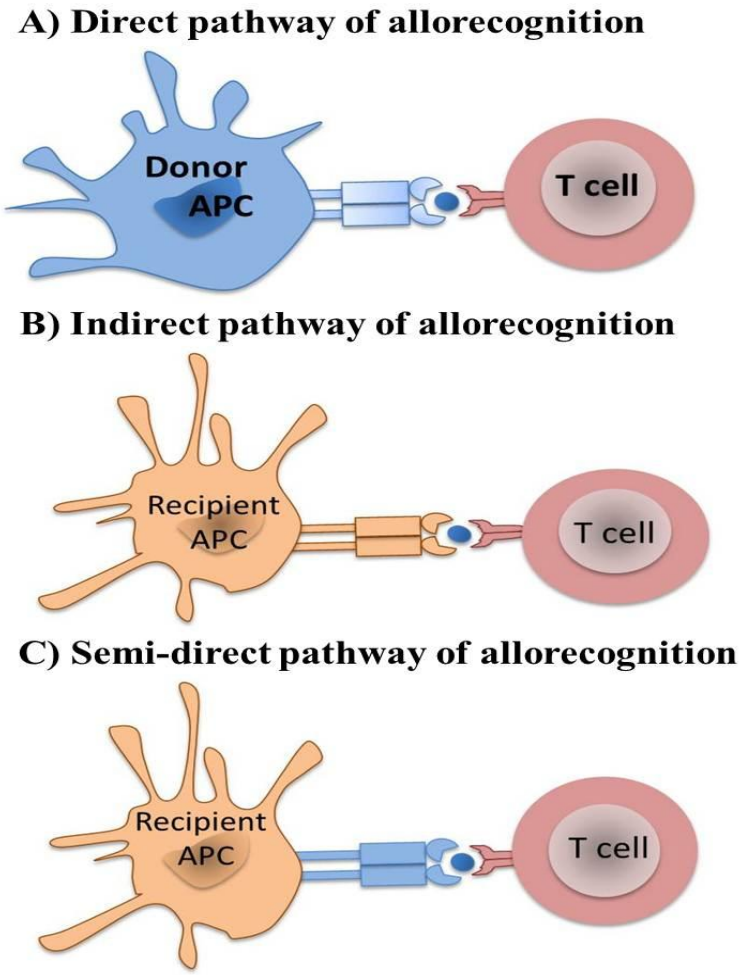


Figure 1: Pathways of Allorecognition

A) Direct pathway; interaction of recipient T cells (via TCR) with intact MHC-peptide complexes, which are presented by donor APC. B) Indirect pathway; Donor MHC are processed and presented by recipient APC and recognised by recipient T cells. C) Semi-direct pathway; recipient APC acquire intact donor MHC-peptide complexes from exosomes which are secreted by donor APC or through cell to cell contact. These are then presented directly to recipient T cells.

1.2.2 T cell activation

Naïve $CD4^+$ and $CD8^+$ T cells must become activated in order to differentiate into effector cells that elicit damage to allografts. The TCR is central to this activation process. The TCR is a heterodimer, consisting of predominantly αβ chains but in some cases γδ chains. The peptide chains are bound together by a disulphide bond and form a binding site with variable regions, which enables TCR specificity (54). Activation of naïve T cells is a tightly regulated process which requires 3 distinct signals. Signal 1 involves the interaction between a given TCR on a naïve T cell and a MHC-peptide complex on an APC which generates a signal that is transmitted through the adjacent CD3 complex (55, 56). This recognition mechanism is responsible for the specificity of the immune response.

T cell activation is not solely dependent on the antigen-dependent signals but requires a second signal generated through other cell surface molecular interactions, known as costimulation or signal 2 (57). Costimulatory molecules can be sub-divided into those providing a positive signal (i.e. results in proliferation, differentiation and survival of the T cell) or a negative signal (i.e. results in termination of the immune response). Costimulation will be discussed in further detail in Section 1.4.

The third signal required for T cell activation refers to the signal delivered from an APC to the T cell by means of soluble molecules, known as cytokines. The local cytokine milieu can determine the differentiation from naïve into various types of effector T cells. A number of cytokines can act as signal 3, for example IL-12 as it is able to promote Th1 differentiation (58).

The accumulation of these 3 signals results in the transcription of genes encoding soluble molecules (such as IL-2) and anti-apoptotic molecules (such as Bcl-$_{XL}$) that enable clonal expansion of antigen-specific T cells. Without one or more of these signals the cells become anergic and refractory to further stimulation.

1.2.3 T cell Receptor Signalling

The interface between a T cell and an APC is termed the immunological synapse which acts as the initial event leading to T cell activation (59). TCR triggering causes protein tyrosine kinases (PTKs) of the Src family (e.g. Lck) to become activated and phosphyorylate immunoreceptor tyrosine based motifs (ITAMs). These events lead to the recruitment and activation of PTK zeta chain-associated protein 70 (ZAP-70), which in turn phosphorylates linker for activation of T cells (LAT) and SRC-homology 2 (SH2)-domain-containing leukocyte protein 76 kD (SLP-76) resulting in the activation of phospholipase C-γ (PLC-γ) and Ras (60). Further downstream events including induction of the Ca^{2+}-calcineurin, protein kinase C (PKC)-extracellular signal-regulated kinase (ERK), and mitogen activated protein (MAP) kinase cascades. Finally the transcription factors NFAT, NF-κB and AP-1 are activated. Their activation results in the translocation of these molecules into the nucleus and promotes specific expression of genes involved in IL-2 production, T cell proliferation, survival and differentiation (61, 62).

1.2.4 T cell subsets and rejection

T cells can be divided into 2 subsets based on the expression of either CD4 or CD8 co-receptors that are expressed on mature T cells in a mutually exclusive manner (63). Animals that lack T cells, for example athymic mice (i.e. nude) (34, 35), or mice that have received depleting anti-CD4 and anti-CD8 monoclonal antibodies (64), do not reject fully MHC-mismatched allografts.

Each subset undergoes a distinct programme of differentiation which generates a helper, regulatory or cytotoxic T cell phenotype. $CD4^+$ T cells mainly elicit effector functions through the production of cytokines thus generally defined as helper T cells (Th). $CD8^+$ T cells express molecules which enable direct cytotoxic killing through granzyme production under certain conditions. However, $CD4^+$ T cells can exhibit Fas-mediated cytotoxicity (65) and $CD8^+$ T cells can help promote Th1 responses by modulating the function of APC (66). Evidence suggests that all of the different subsets of T cells utilise their distinct effector mechanisms to contribute to allograft rejection.

One mechanism of allograft rejection is the induction of apoptosis by activation of $CD8^+$ T cells, NK cells and $CD4^+$ T cells, which can act as cytotoxic T cells. Target cells are killed by the production of molecules such as perforin and granzyme B. Perforin polymerises and acts by creating pores in the target cells, which then allows granzyme B and other components to enter the target cell and results in activation of the caspase pathway, DNA fragmentation and apoptosis (67, 68). $CD8^+$ T cells can also secrete soluble molecules such as TNF-α and IFN-γ that can result in cytolysis. Fas/FasL interactions are more important for $CD4^+$ T cell mediated direct cytotoxicity

where ligation of Fas on the target cell establishes a death inducing signalling complex which subsequently activates the caspase pathway, again leading to cell death by apoptosis (69).

$CD4^+$ T cells are often necessary for the induction of naïve $CD8^+$ T cells to become activated and differentiate. This process, known as licensing, occurs when $CD4^+$ T cells activate APC via CD40-CD154 interactions, which leads to downstream signals to produce IL-12 (70), which is important for T cell differentiation. Signalling through CD40 also induces APC to upregulate MHC expression along with costimulatory molecules e.g. CD80/CD86 on their surface (71). These surface changes on the APC contribute to increased T cell recognition of antigen thus increasing T cell priming. Therefore APC can act as a temporal bridge receiving activation signals from $CD4^+$ T cells and can activate naïve antigen specific $CD8^+$ T cells (72-74).

$CD4^+$ T cells alone are sufficient to mediate allograft rejection. In 1986, seminal work by Mosmann *et al* showed that $CD4^+$ T cells could be further sub-divided into two distinct populations, termed Th1 or Th2 cells, based on the pattern of cytokine secretion *in vitro* (**Figure 2**) (75). After antigenic stimulation, under the transcription factor T-bet, Th1 cells secrete high levels of IFN-γ and IL-2, and low levels of IL-4 and IL-5 (**Figure 2**) (76). Th1 T cells act as the main mediators in delayed type hypersensitivity (DTH) responses, through the release of pro-inflammatory cytokines such as IFN-γ and TNF-α inducing macrophage activation, neutrophil infiltration and tissue necrosis. This can initiate a positive feedback loop which leads to a further increase in cytokine

secretion and secretion of other destructive factors such as nitric oxide (77). Th1 T cells also provide help to B cells to produce IgG_{2a} isotype antibodies in the mouse, which are able to fix complement and assist with phagocytosis and cell mediated immunity, leading to the clearance of intracellular pathogens (78).

Th2 cells secrete high levels of IL-4, IL-5 and IL-13 but low levels of IFN-γ (**Figure 2**) following activation as a result of the expression of the transcription factor GATA-3 (79). Activated eosinophils can be recruited to the graft by IL-4, -5 and -13 and release reactive oxygen species causing graft damage (80). Th2 responses are associated with humoral immunity, characterised by IgG_1 and IgE production and eosinophilic inflammation. These responses are important for the clearance of extracellular pathogens and dysregulation of this response results in atopy and allergic inflammation (81).

The original paradigm that $CD4^+$ T cells could differentiate into either Th1 or Th2 cells has been recently challenged by several key observations. Experimental allergic encephalitis (EAE), an animal model of multiple sclerosis, was believed to be a Th1 mediated disease, however the neutralisation or deletion of IFN-γ was found to exacerbate rather than attenuate disease (82, 83). This result was explained by the discovery of a new cytokine, IL-23, and a new subset of $CD4^+$ cells, which secrete IL-17 (termed Th17). Neutralisation of IFN-γ and IL-4 allows IL-23 to induce differentiation of Th17 cells from naïve T cells, under the transcription factor RORγt (84). Thus Th17 cells are a separate T cell lineage and do not differentiate from Th1 or Th2 cells (85, 86) (**Figure 2**). Th17 cells have been implicated in graft destruction via

the recruitment of polymorphonuclear (PMN) cells and eosinophils into the graft (87). IL-17 is thought to contribute to immune disease by functioning as a pro-inflammatory mediator, enhancing T cell priming as well as up-regulating the secretion of proinflammatory mediators such as IL-1 and TNF- α (88).

More recently other subsets of T cells have been described which can develop under certain conditions e.g. Th9 cells producing IL-9 (89). However, there remains significant controversy as to whether Th9 cells are a distinct lineage of T cells or whether they may be other T cell subsets that have acquired the ability to secrete IL-9 (90).

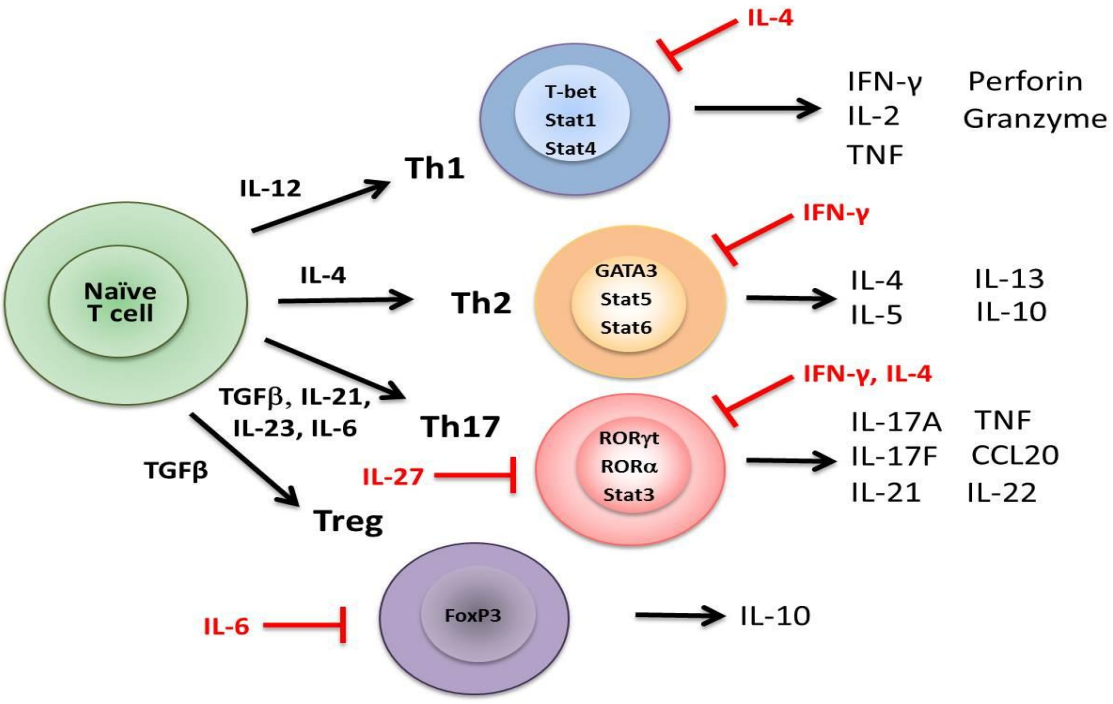


Figure 2: The cytokine milieu which determines the differentiation of $CD4^+$ T cells.

1.2.5 Memory T cells

Following the clearance of antigen by a primary immune response most effector T cells die leaving a small pool of long-lived, antigen-specific memory T cells. This process is one of the hallmarks of the adaptive immune response and results in the rapid clearance of pathogens upon re-encounter with antigen. **Table 1** summarises the properties of memory T cells (Tm) compared to naïve and effector T cells.

The formation of a memory population after the primary immune response results in a 1000-fold increase in antigen-specific precursor frequency compared to naïve animals (91). This results in an increase in the magnitude of the response upon a second exposure/encounter to antigen (92). The lag time from antigen encounter to the first cell division in memory $CD8^+$ T cells has been shown to be only twelve hours, as opposed to a twenty-seven hours for naïve cells (93, 94). Furthermore, the threshold for activation is reached faster in memory cells than naïve cells due to their lowered dependency on costimulatory molecules (95).

Two subsets of Tm (central (T_{CM}) and effector (T_{EM}) cells) have been described based on their anatomical localisation and expression of the lymph node homing molecules CCR7 and CD62L (L-selectin) (96). T_{CM} are $CCR7^+CD62L^+$ and can transmigrate through high endothelial venules to the lymph nodes and thus share some migratory routes with naïve T cells. However, they are also able to home to sites of inflammation, whereas naïve T cells are excluded (97). T_{EM} lack expression of CCR7 and express low

levels of CD62L, similar to effector T cells therefore T_{EM} rapidly transit to sites of inflammation but are excluded from the lymph nodes.

Alloreactive Tm can develop as a result of prior blood transfusion, pregnancy or previous transplants. However, they also appear to form part of the normal Tm pool even in non-sensitised individuals (98). Heeger *et al* showed that the presence of alloreactive Tm in non-sensitised individuals prior to transplantation (>30 cells per 300,000; identified by their ability to secrete IFN-γ within twenty-four hours of allostimulation) correlated with an increased incidence of acute rejection episodes (98). These data suggest that not only are alloreactive Tm present in non-sensitised patients but also that they can elicit rejection despite the administration of current immunosuppressive agents. It has been proposed that such cells are generated due to homeostatic proliferation after a period of lymphopenia (99) and perhaps more frequently following exposure to numerous environmental pathogens, where a fraction of pathogen-reactive T cells display cross-reactivity with alloantigen (100) Adams *et al* provided strong evidence to support the generation of Tm by cross-reactivity triggered by infection (101) which has been termed heterologous immunity (102).

Although Tm are beneficial in protection against infectious pathogens, they can cause significant problems to the outcome of an allograft in clinical transplantation e.g. enhanced kinetics of rejection. Therefore the development of immunosuppressive agents which specifically target Tm would be highly advantageous in the clinical setting. However, Tm have been shown to not only impact allograft survival induced

by non-specific immunosuppression but also to impede the induction of tolerance. Tolerance induction is readily achievable in rodents which are housed in pathogen free conditions (103), but in rodents pre-sensitised with donor alloantigen these protocols fail to induce tolerance (101, 104). In addition, the presence of Tm prior to kidney transplantation in a non-human primate (NHP) model, also prevented the induction of tolerance (105). Therefore developing immunosuppressive agents which can impact Tm will be important to facilitate the induction of tolerance to allografts in humans.

	Naïve T cells	Effector T cells	Memory T cells	Ref.
Markers of Identification	$CD44^{-}CCR7^{+}CD62L^{+}$		$CD44^{+}CD45RB^{+}$ T_{CM} = $CCR7^{+}CD62L^{+}$ T_{EM} = $CCR3^{+}CCR5^{+}CXCR6^{+}$	96, 106
Survival	Modest (months to years)	Very short (days)	Very long (years to life)	
Response kinetics	Slow	Intermediate	Fast	96
Costimulation requirements	High threshold	High threshold	Low threshold	95
Frequency	Low	Very high	High	91
Homing properties	Restricted to secondary lymphoid organs	Home to lymphoid organs only	Circulate through lymphoid (T_{CM}) and non-lymphoid (T_{EM}) organs	106

Table 1: A comparison between naïve, effector and memory T cells in relation to how they respond to antigen.

1.2.6 Innate immunity and rejection

Although T cell responses to allografts are central to the process of rejection there is increasing evidence that innate responses also contribute to the adaptive immune responses following transplantation. The activation of innate cells in the early phase

post-transplant is a non-specific response to tissue damage and is independent of the MHC disparities between donor and recipient (107). The innate immune system comprises monocytes, macrophages, neutrophils, eosinophils, mast cells and NK cells and acts as an early first line of defence against pathogens whilst the adaptive immune response is activated. Tissue injury is an unavoidable consequence of the surgical procedures required for organ retrieval and re-implantation during transplantation.

Cells of the innate immune system express invariant pattern recognition receptors that recognise molecular patterns associated with tissue injury and the presence of pathogens (108). The best characterised are the toll-like receptors (TLRs) that bind to molecular features unique to microorganisms, as well as endogenous molecules that are produced as a consequence of tissue injury (109, 110). Ischaemia/reperfusion injury occurs during the surgical removal of the donor organ and during reperfusion of blood within the recipient which generates cell stress and the release of heat shock proteins, complement breakdown products and reactive oxygen species (111-114). These danger signals activate cells of the innate immune system via TLR ligation, resulting in the production of inflammatory mediators and chemokines within the donor organ. This identifies the transplanted tissue as a site of inflammation, triggering the recruitment of inflammatory leukocytes into the graft via upregulation of pro-inflammatory chemokines and selectins and integrins, as well as stimulating the maturation and migration of donor-derived passenger APC to recipient lymphoid tissue.

1.3 Transplantation Tolerance

The ultimate goal in the field of transplant immunology is to achieve a state of operational tolerance i.e. defined as a state of unresponsiveness to the donor organ whilst maintaining a protective immune system against pathogens and tumours, without the use of chronic immunosuppression. Current immunosuppressive agents prevent acute rejection responses, but are not as effective at controlling late graft loss. The non-specific nature of these agents results in a global depression of the host immune system which leaves the recipient open to pathogenic infections and malignancy. Therefore, the quest has begun to find therapeutic modalities that induce allograft specific tolerance in order to promote long-term survival of the graft and the host, as well as to eliminate adverse effects associated with chronic non-specific immunosuppression.

1.3.1 Central tolerance

The primary mechanism by which self-tissues are protected from aggressive immune responses is through the deletion of self-reactive T cells in the thymus. T cells that enter the periphery must have sufficient self-reactivity to be able to survive but should also not be capable of mounting an immune response against self-antigen. The thymus consists of a cortex and medulla, with distinct thymic epithelial cells located in each region (115). Double positive $CD4^+CD8^+$ thymocytes are positively selected on cortical thymic epithelial cells initiating downregulation of co-receptor (116). Single positive $CD4^+$ and $CD8^+$ thymocytes are then directed towards the medulla, where the deletion of self-reactive T cells is completed in the presence of medullary thymic epithelial cells and DC (117).

A small population of these medullary thymic epithelial cells express the autoimmune regulator gene (Aire), which is critical for the induction of T cell tolerance towards tissue-specific antigens (118). Expression of Aire allows medullary thymic epithelial cells to express and present tissue specific peptides to single positive thymocytes, and thus enable the deletion of any of self-reactive thymocytes. Aire is activated in medullary thymic epithelial cells via the ligation of the TNF family receptor RANK by its ligand derived from lymphoid tissue inducer cells found in the thymus (119). T cells that bind strongly to self-MHC/peptide have the potential to elicit aggressive responses against self in the periphery and are depleted by TCR-induced apoptosis in a process termed negative selection (120, 121).

In 1953 Billingham *et al* demonstrated that the infusion of allogeneic cells into neonatal mice resulted in the subsequent acceptance of skin grafts from the donor strain (9). This was the first evidence (although only shown in neonates) that tolerance to allografts could be achieved through central tolerance and the induction of mixed chimerism. The caveat to intrathymic delivery of alloantigen is that donor cells/peptides only remain in the thymus for a short defined period of time. Therefore this kind of treatment only provides a window of opportunity to perform a solid organ transplant, instead of generating persistent deletion of thymocytes.

Another way of achieving central tolerance is by generating haematopoietic chimeras through a bone marrow transplant. Engraftment of donor haematopoietic stem cells

results in the re-population of the recipient's thymus with donor derived DC, which can delete donor-reactive T cells (122). In experimental models, full donor chimerism induced by myeloablative therapy (a combination of total body irradiation and cytotoxic medication) followed by donor bone marrow transplantation induces donor-specific tolerance, without impairing immune responses to third party allografts (123). However, the establishment of full donor chimerism is not acceptable for most individuals who are on the transplant waiting list as the risk profile is not acceptable when compared to conventional immunosuppression.

An alternative approach is the induction of mixed haematopoietic chimerism (survival of both donor and recipient haematopoietic progenitor cells), which can be achieved in experimental models with less toxic induction therapy (124). Examples of induction protocols include the use of depleting anti-CD4 and anti-CD8 mAb in combination with non-myeloablative total body irradiation or costimulatory blockade. These protocols are used in combination with a bone marrow transplant to produce mixed chimerism in mice, which subsequently demonstrates tolerance to donor-strain allografts (125, 126). Similar results have been obtained in miniature swine (127, 128) and primates (129, 130), and recently, promising results have been published using similar protocols in human renal allograft recipients (131-133).

1.3.2 Peripheral tolerance

The deletion of self-reactive T cells during development in the thymus is incomplete therefore additional mechanisms are required to regulate potentially autoreactive T cells

that have escaped deletion in the thymus and emerge into the periphery. A number of mechanisms of peripheral tolerance to self-antigens have been identified including the induction of anergy, deletion or suppression of self-reactive T cells.

Anergy is the term used to describe the functional inactivation of lymphocytes. Anergic T cells fail to undergo activation after TCR stimulation due to an inability to mediate downstream signalling of the TCR (134). *In vitro*, TCR ligation by cognate peptide-MHC in the absence of costimulation has been shown to induce anergy (135). Interestingly, the costimulatory molecule programmed death 1 (PD-1) is highly expressed on anergic T cells (136) and recent evidence has highlighted the role of B7-PD-1 interactions in the induction of T cell anergy (137). Furthermore, PD-1 signalling plays a key role in the prevention of autoimmunity in mice (138, 139).

One of the main barriers to tolerance induction is the high precursor frequency of alloreactive T cells. Therefore a key mechanism for the induction of tolerance is the deletion of alloreactive T cells. Passive death is triggered by growth factor deprivation, cytotoxic drugs or irradiation and is regulated by the Bcl-2 family. Overexpression of Bcl-$_{XL}$ in mice results in resistance to passive cell death (140). Active deletion of T cells is mediated by activation induced cell death (AICD), which involves death of activated T cells that have been restimulated by TCR engagement after the engagement of specific receptors such as Fas (CD95) or upon exposure to reactive oxygen species (141). AICD can involve signalling through CD95 (142), TNFR1 (143), and TRAILR in the case of $CD8^+$ T cells activated in the absence of $CD4^+$ T cell help (144).

Deficiencies in these pathways can lead to autoimmunity in mice (145, 146), and autoimmune lymphoproliferative syndrome in humans (147). The induction of AICD involves a complex series of signalling events which ultimately lead to caspase activation, DNA fragmentation, cytoskeletal degradation, and cell death.

1.3.3 Regulatory T cells and the induction and maintenance of tolerance

The immune system has developed mechanisms which are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammation. Gershon and colleagues were the first to suggest that there was a subset of T cells which may exert suppressive activity, and termed these cells "suppressor cells" (148). They showed using an adoptive transfer model, T cells isolated from animals tolerised to a specific foreign antigen were able to suppress the antibody response against that specific antigen in secondary recipient animals (149, 150). However, these cells and the proposed soluble mediators which they secreted were hard to characterise and therefore the concept of suppressor cells was highly criticised and questioned (151).

The field was reignited by Sakaguchi *et al* who showed that the depletion of a small population (5-10%) of $CD4^+$ T cells which co-expressed CD25 (IL-2Rα), in naïve mice, induced multi-organ autoimmune disease when adoptively transferred into immunodeficient recipients (152). Furthermore, autoimmune disease caused by pathogenic effector T cells was prevented by adoptive transfer of purified $CD4^+CD25^+$

T cells from naïve adult mice (152, 153). Thus a subset of T cells had been identified in mice that were $CD4^+CD25^+$ which were capable of actively suppressing immune responses against self-antigens. These cells were termed "regulatory" T cells (Treg).

Further phenotypic analysis of Treg has identified a number of cell surface markers that are differentially expressed between Treg and conventional T cells. Although these markers vary greatly between mouse and human, only mouse Treg will be discussed here. The forkhead/winged helix family transcription factor (Foxp3) is selectively expressed by $CD4^+CD25^+$ regulatory cells, and therefore is thought to act as a master regulator or lineage specification factor in the development of Treg (154). Several studies have shown FoxP3 is required to convert naïve $CD4^+$ T cells towards cells with a regulatory phenotype, and mice with a mutation in the *foxp3* gene leads to defective thymic generation of Treg (154, 155). Furthermore, Foxp3 is not significantly upregulated in $CD4^+CD25^-$ T cells following activation in mice, and is not expressed in differentiated Th1 or Th2 cells (154, 155), thus it is unique to Treg. Other markers that are currently used to help distinguish between Treg and conventional T cells (however, are not exclusive to Treg); GITR (156, 157), CTLA-4 (cytotoxic lymphocyte antigen-4; (158, 159)), CD103 (157, 160) and Helios (161, 162).

Lack of Treg, either due to congenital deficiency of *foxp3* (163, 164) or selective depletion of FoxP3 expressing cells (165) results in fatal autoimmune disease; in humans Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX), and in scurfy mice. IPEX is characterised by the neonatal onset of autoimmune

diabetes, increased susceptibility to infections, thrombocytopenia, atopic dermatitis and autoimmune disease in multiple endocrine organs (166). This highlights the importance of Treg in modulating the balance between immunity and tolerance, and therefore protecting the host against autoimmune diseases.

Two broad categories have been defined for $CD4^+$ Treg; thymic derived naturally occurring (nTreg) and those differentiated from $CD4^+CD25^-$ conventional T cells (inducible Treg; iTreg). nTreg are indispensable for the maintenance of self-tolerance and develop within the thymus through selection and stimulation with autoantigens via the TCR, which provides a self-reactive repertoire of TCRs and cross reactivity to multiple alloantigens (167). nTreg mature in the thymus before migrating into the periphery. Once they have migrated out of the thymus, nTreg are anergic and fail to produce IL-2 upon stimulation (168).

iTreg is the term used collectively to refer to naïve T cells which gain regulatory activity in the periphery. This subset consists of $CD4^+CD25^+FoxP3^+$ T cells that are generated in the periphery from naïve conventional T cells after being exposed to antigens (e.g. in the gut) and/or under certain conditions (e.g. IL-2 and TGF-β) (169-171). This subset of Treg has a number of interchangeable names with no common nomenclature, often leading to confusion; inducible, adaptive or antigen-experienced Treg.

In addition, it should be noted that immunoregulation consists of more than just Treg and that there are many other T cell subsets which have been shown to possess regulatory activities such as $CD8^+$ (172), $CD8^+CD28^-$ (173), $TCR^+CD4^-CD8^-$ (174), Tr1 (175) and natural killer T (NKT) cells (176).

1.4 T-cell costimulation - Potential targets for the induction of tolerance

1.4.1 Importance of costimulation

The importance of costimulation was first demonstrated by Bretscher and Cohn, who proposed that the provision of signal one alone (TCR-MHC-peptide interaction) was not sufficient to optimally activate T cells and that without accessory signals, T cells were rendered anergic or unresponsive (177). Anergic T cells cannot produce IL-2 upon re-exposure to antigen or costimulation, however this state can be reversed with the addition of exogenous IL-2 (178). Costimulation blockade can render alloantigen-reactive T cells unresponsive, which may promote tolerance induction. However the identification of numerous costimulatory molecules, suggests a degree of redundancy exists within the immune system.

1.4.2 Diversity of costimulatory molecules

Costimulatory molecules can have opposing roles in the immune response. Some costimulatory molecules provide a positive signal, that results in the proliferation and expansion of T cells e.g. CD28 and OX40. However, other costimulatory molecules provide a negative signal which results in termination of the immune response e.g.

CTLA-4. The CD28-CD80/CD86 axis was the first costimulatory pathway to be defined and is therefore the best characterised to date (179-181).

In addition to the CD28-CD80/CD86 pathway, numerous other molecules have been described with costimulatory potential that can be divided into 3 sub-groups namely, integrins (e.g. lymphocyte function antigen-1; LFA-1), Ig superfamily members (including CD28, ICOS) and the Tumour Necrosis Factor Receptor (TNFR) superfamily members (including CD154 (CD40L), OX40 (CD134), 4-1BB (CD137) and herpes virus entry mediator (HVEM)). Although different costimulatory molecules have been shown to have over-lapping functions, which molecules act to costimulate T cell responses depends on the stage of differentiation of the responding T cell as well as the availability of corresponding ligands.

1.4.2.1 Ig superfamily

The Ig superfamily of costimulatory molecules is formed of interactions, such as CD28 and ICOS, which enhance T cell responses. While other molecules such as PD-1 and CTLA-4 attenuate T cell activation.

The CD28-CD80/CD86 interaction is unique among costimulatory responses in that there are 2 alternate ligands for CD80/CD86, which have overlapping functions. CD28-CD80/CD86 is first molecular interactions to be engaged in the immune synapse and is absolutely required by most T cells in a cascade which provides a positive signal to the T cell and results in T cell activation and survival. CD80 and CD86 can also bind to

CTLA-4 (CD152), (with ~20 fold higher affinity than CD28) (182) which delivers an inhibitory signal to the T cells, limiting the secretion of IL-2, and thus controlling the immune response (183).

The CD28/CTLA-4;CD80/CD86 axis has a dual function in the T cell response where CD28 promotes T cell activation and survival by increasing stability of IL-2 mRNA (184) and the levels of anti-apoptotic molecules such as Bcl-$_{XL}$ (185) and CTLA-4 inhibits the T cell response by negatively regulating T cell proliferation. CTLA-4 down-regulates T cell responses by dissociation of CD28-CD80/CD86 interactions, limiting secretion of IL-2 and binding to APC resulting in the induction of indole-amine 2,3-dioxygenase (IDO) which in turn promotes the expansion of regulatory T cells (183, 186, 187).

1.4.2.2 Tumour Necrosis Factor Receptor superfamily

At present there are 19 TNF-homologous ligands identified that mediate their cellular response through 30 receptors of the TNFR superfamily (188). Members of the TNF-TNFR superfamily have been implicated in a wide range of immunological responses including T cell costimulation, chemokine production, trafficking of effector cells and down regulation of established immune responses (189, 190).

The TNF/TNFR superfamily ligand-receptor pairs consist of type I transmembrane (N-terminal extracellular) glycoprotein receptors (e.g. OX40) and type II transmembrane (C-terminal extracellular) glycoprotein ligands (e.g. OX40L). Individual polypeptide

chains of the TNF-like ligands exhibit a "jellyroll" β-sandwich structure, and form active trimeric complexes via non-covalent interactions between chains (191). The extracellular region of the receptor forms an extended structure, which contains between 2 and 6 hallmark cysteine-rich domains that form the ligand-binding site (192). The cytoplasmic domains of the TNFRs share little homology, but can be used to characterise the TNFRs into 2 groups based on the presence or absence of a 70-80 amino acid motif designated the death domain (DD).

TNFR family members that contain a DD include Fas and TNFR1 and 2. Upon ligand binding, the DD serves as a docking site for DD-containing adaptor proteins such as tumor necrosis factor receptor type 1-associated death domain (TRADD) and Fas-associated protein with death domain FADD. These adaptors can link death receptors to pro-apoptotic intracellular signalling pathways such as caspase activation (191, 192). Those TNFRs that lack a DD (e.g. CD27, 4-1BB, OX40) mediate their signal through tumour necrosis factor receptor-associated factors (TRAF), which function as scaffold and docking proteins. The TRAF-TNFR complexes initiate signal transduction pathways in the lymphocyte that result in the activation of transcription factors such as AP-1 and NF-κB, which in turn may switch on genes required for cell survival (e.g. Bcl-2) and proliferation (193). The interaction between OX40 and OX40L provides an important costimulatory signal to activated T cells mainly through T cell-APC interaction which can result in the expansion and survival of specific antigen-activated T cells. The OX40-OX40L pathway will be the focus of this thesis.

1.4.2.3 Integrins

The surface of an APC contains a number of accessory molecules which are critical for the initiation of a T cell response. They provide adhesion between the APC and T cell to stabilise the interaction and can also provide costimulation to promote T cell activation. These adhesive interactions are mediated by a group of αβ heterodimeric cell surface receptors called integrins. To date, there are 18 integrin-related α subunits and 8 β subunits, which are able to combine resulting in 24 different integrins (194).

The first integrin to be identified was LFA-1; a $\alpha_L\beta_2$ integrin heterodimer consisting of the unique α chain (CD11a) and a common β chain (CD18) that is expressed on T cells, B cells and macrophages. The ligands for LFA-1 have been identified as inter cellular adhesion molecules (ICAM)-1, -2 and -3 which are expressed on most leukocytes including APC. LFA-1 has been shown to play a critical role in the communication through the immunological synapse between the T cells and the APC. Therefore, integrins possess costimulatory as well as adhesion properties and can induce T cell activation, proliferation and IL-2 gene expression (195, 196) in addition to mediating the transendothelial migration of T cells into inflamed tissues (197).

1.4.3 Targeting costimulation in experimental models of transplantation

1.4.3.1 CD28/CTLA-4-CD80/CD86 pathway

The importance of the CD28 costimulatory pathway in allogeneic responses was first demonstrated *in vitro* by using an anti-CD28 monoclonal antibody (198, 199) or CTLA-

4-Ig fusion protein (200, 201). In rodents, blockade of the CD28-CD80/CD86 pathway by CTLA-4-Ig (a receptor Fc fusion protein), was shown to prevent acute allograft rejection, but this finding was model and strain dependent (201-204). CTLA-4-Ig also prevented the development of anti-donor antibody responses and induced long-term survival of islet, cardiac and renal transplants in rodent models (200, 205-207).

CTLA-4-Ig in combination with T cell depletion and rapamycin synergise to promote allograft survival in a full MHC mismatched mouse skin model. This combination of immunosuppressant agents acts by altering the balance of effector T cells and Treg which in turn prevent the graft against destruction by effector and memory T cells (208). Reagents designed to selectively block CD28, while allowing CTLA-4 signalling are being developed, which may alter the balance of effector T cells and Treg, without the need for other immunosuppressant agents. In a full MHC mismatch cardiac transplant mouse model, CD28 single chain Fv (scFv) fragments are able to significantly prolong allograft survival and reduce acute allograft injury (209) Poirier *et al* demonstrated blocking CD28 renal transplants in NHP increased the number of Treg in the periphery as well as in the graft, which resulted in the prevention of graft rejection and vasculopathy (210).

1.4.3.2 ICOS-ICOSL pathway

Inducible Costimulator (ICOS; CD278), a member of the Ig superfamily, is induced on activated T cells (211), while its ligand (ICOSL) is constitutively expressed on B cells and monocytes but can be upregulated by TNFα and/or LPS (212). ICOS-ICOSL interactions have a number of unique roles in the immune response including T cell

activation and differentiation (211, 213), splenic germinal centre formation (214) and Ig class switching (213). In a full-MHC mismatch mouse cardiac allograft model ICOS blockade in combination with either cyclosporine or anti-CD154 prevented chronic rejection (215, 216). In MHC mismatched grafts, blockade of ICOS-ICOSL alone had no impact on graft survival. Whereas if donors and recipients were mismatched for miHC antigens only, blockade of ICOS during T cell priming (days 0-6) accelerated rejection, while blockade during the effector/memory T cell generation phase (days 4-10) prolonged graft survival (217). This may be explained by ICOS being critical for the function of effector/memory T cells as well as with Treg (218).

These data suggest that ICOS-ICOSL blockade alone will be insufficient to induce long term allograft survival and tolerance, therefore combination with other costimulation blockade may be an important step forward if ICOS blockade is going to reach its full therapeutic potential.

1.4.3.3 CD40-CD154 pathway

CD40-CD154 pathway is the most extensively characterised interaction in the TNFR superfamily. The role of the CD40-CD154 pathway in immunity became clear when hyper-IgM syndrome was found to be a direct result of a mutation in the gene encoding CD154 (219). Initial efforts were aimed at blocking the CD40-CD154 interaction by use of monoclonal antibodies specific for CD154, and showed great promise in transplantation models in rodents (220-222) and in NHP (223-225).

However when these reagents were translated to clinical trials, serious adverse effects were observed and the trials were terminated (discussed later in Section 1.4.4). More recently, reagents which specifically target CD40 have been developed. Anti-CD40 synergises with CTLA-4-Ig to promote long term allograft survival in mouse models of skin and bone marrow transplantation. The synergy was comparable to that previously seen with anti-CD154 (226). In NHP transplant models, anti-CD40 showed significant prolongation of kidney, islet and liver allograft survival (227-229) and prevented the development of alloantibodies (230). Despite the devastating results for anti-CD154, blockade of the CD40-CD154 pathway still may contain promise in humans (231).

1.4.3.4 4-1BB-4-1BBL pathway

4-1BB (CD137) is an inducible costimulatory molecule belonging to the TNFR superfamily, and is expressed on activated $CD4^+$ and $CD8^+$ T cells and NK cells (232), while its ligand is expressed on mature DC, activated B cells and macrophages (233). In a transplantation setting, agonistic 4-1BB mAb have been shown to accelerate cardiac and skin rejection (234) whereas in a model of intestinal transplantation 4-1BB blockade prolonged allograft survival mediated by $CD8^+$ T cells, but not that mediated by $CD4^+$ T cells. This correlated to impaired priming of alloantigen-reactive $CD8^+$ T cells in the local lymph nodes (235). These data suggest targeting the 4-1BB-4-1BBL pathway may have a favourable role in modulating the immune response in more stringent CD8-mediated transplant models.

1.4.3.5 CD27-CD70 pathway

Another TNFR family member is CD27, which is expressed by activated T cells, activated B cells and NK cells. Its ligand (CD70) has a similar expression profile to CD27, but is also expressed on DC. CD70 expression is dependent on TCR stimulation and TLR stimulation (236). CD27-CD70 interactions have been implicated in T cell development, activation and T cell dependent antibody production by B cells (190). In mice, blockade of the CD27-CD70 pathway promotes extended allograft survival in fully MHC mismatched cardiac allografts in wild-type recipients (237), but induces long-term survival and prevented the development of chronic allograft vasculopathy in $CD28^{-/-}$ recipients (238). These data suggest that there is synergy between the CD28-CD80/CD86 and CD27-CD70 pathways.

1.4.3.6 OX40-OX40L pathway

OX40 (CD134) is an inducible costimulatory molecule belonging to the TNFR superfamily. This molecule is of particular interest as like other TNFR family members it is only expressed on T cells after activation. Section 1.5 describes OX40-OX40L biology in much more detail as it is the focus of this thesis.

1.4.3.7 Intergrins

Experimental transplantation models have shown that blockade of the LFA-1-ICAM interactions with anti-LFA-1 mAb can result in prolonged survival of islet (239) and cardiac (240) allografts. Integrin blockade (very late antigen-4; anti-VLA4) can also reduce the incidence and severity of artery intimal thickening in a murine cardiac allograft model which are closely associated with chronic rejection (241). Anti-VLA4 and anti-LFA-1 administered together displayed potent synergy in a murine islet model,

which resulted in significant graft prolongation compared with either of the monotherapies (242).

Although costimulation blockade is a promising strategy for the induction of tolerance, it is clear that CD28/CD154 blockade fails to address alloreactive Tm. Such Tm are likely to have a detrimental impact on tolerance induction and therefore developing reagents which target these cells would be beneficial. Indeed, Kitchens *et al* have shown that anti-VLA4 or anti-LFA-1 mAb can abrogate costimulation blockade resistant rejection mediated by Tm where anti-VLA4 blocked T cell trafficking to the graft, whilst anti-LFA-1 additionally attenuated Tm recall function (243).

1.4.4 Targeting costimulation in clinical transplantation

Targeting costimulatory molecules in either the Ig superfamily or TNFR superfamily has shown great potential in experimental models of transplantation for preventing rejection. Blockade of CD28-CD80/CD86 and CD40-CD154 interactions have clearly been the most rigorously examined in terms of their ability to induce tolerance to allografts in both rodent and NHP experimental models.

Recently, belatacept (human CTLA-4-Ig fusion protein; Nujolix®) has been approved for clinical use for the indication of prophylaxis of organ rejection in adult kidney transplant recipients (FDA in June 2011; EMA in April 2011). Belatacept is to be used

in conjunction with basiliximab induction and MMF and corticosteroids as maintenance immunosuppression (244).

The efficacy of belatacept in *de novo* kidney transplantation has already been assessed in 2 open label, randomised, multicentre phase II/III clinical trials, named BENEFIT (Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial). Both arms of the study (belatacept vs cyclosporine) resulted in similar patient and graft survival, as well as rates of infection and malignancy at 1 and 5 years (245, 246). However, at 1 year, the incidence of rejection episodes was higher in the belatacept treated patients (246). Some patients who were on high dose belatacept developed post transplantation lymphoproliferative disease (PTLD) (245-247). Belatacept has only been approved for use in adult kidney transplantation, as there was increased graft loss and mortality in liver transplant recipients (248).

In addition to the CD28-CD80/86 costimulatory pathway, interruption of the CD40-CD154 interaction also shows much promise in preventing rejection. The ability of anti-CD154 mAb to prevent renal transplant rejection was also evaluated in a Phase I-II trial, however the trial was prematurely terminated due to thromboembolic complications as a result of activated platelets expressing CD154 (249). Future plans for the development of anti-CD154 are unclear at this time, but anti-CD40 mAb are being developed which have been shown to have a similar efficacy in NHP models without the thromboembolic side-effects seen with anti-CD40L mAb (228, 250).

1.5 OX40 and OX40L Biology

1.5.1 Structure and distribution

The OX40 antigen (CD134) was originally described by Paterson *et al* as a cell surface antigen with a highly restricted distribution, being present only on activated rat $CD4^+$ T lymphocytes (251). OX40 is a type I transmembrane glycoprotein, with an extracellular domain containing 3 cysteine rich repeats (~40 amino acids each) and is predicted to be a member of the TNFR superfamily, due to sequence similarities to the low affinity nerve growth factor receptor and CD40 (252). The rat sequence of OX40 was used to clone mouse OX40 and showed an overall homology of 93% at the DNA level and 90% at the protein level between the 2 species. However the sequence homology is confined to the N-terminal portion of OX40 (containing the cysteine repeats), with little homology found in the cytoplasmic domain (253).

OX40 is not expressed on resting lymphocytes, but is transiently induced on both naïve $CD4^+$ (253) and $CD8^+$ (254) T cells after activation. Peak expression is observed 2-3 days after T cell activation, after which its expression is down-regulated. Memory T cells also do not express OX40 constitutively, but rapidly (~4hrs) upregulate OX40 after further antigen stimulation (255). This is in agreement with the proposed role of OX40-OX40L interactions i.e. in the propagation of the response, rather than in initial T cell priming.

There is a wide range of data on the kinetics of OX40 expression and the differences observed between $CD4^+$ and $CD8^+$ T cells. Differences could be related to the nature of

antigen, the longevity of the antigen persistence and the inflammatory environment. Although not essential for OX40 expression, there is a temporal link with CD28 as costimulation through this receptor has been shown to enhance the kinetics and expression levels of OX40 on T cells (256, 257). Although T cells lacking OX40 have been shown to initially proliferate, expand and produce IL-2 similarly to their $OX40^{+/+}$ counterparts, $OX40^{-/-}$ cells do not maintain sufficient expression of anti-apoptotic molecules after priming therefore markedly reducing T cell survival (256, 258). These data suggest that OX40 functions to promote effector cell survival and Tm differentiation rather than impacting initial T cell priming where CD28 and CD154 costimulation predominates. It should also be noted that OX40 has also been described on other cell types including Treg (259), activated NKT cells (260) and neutrophils (261).

The ligand for OX40 (OX40L or CD252) was first termed glycoprotein 34kDa (gp34) and was identified on human T cell leukaemia retrovirus (HTLV-1) T cells (262). The cloning of gp34 showed OX40L was also a member of the TNFR superfamily (263), and was subsequently shown to be the ligand for OX40 (264, 265). OX40L is a type II transmembrane protein with limited sequence homology to TNF-α. Although 3D modelling has suggested that both mouse and human OX40L have extended β-segments like TNF-α, OX40L has fewer amino acids in the loops facing away from the membrane resulting in a more compact structure (264). Murine and human OX40L have 68% homology at the nucleotide level and 46% at the amino acid level (264). OX40L expression was originally thought to be limited to professional APC such as B cells (266), macrophages (267), DC (268). However, the distribution of OX40L appears to

be much wider than its receptor and has now been identified on a number of other cell types including vascular endothelial cells (269), mast cells (270, 271), activated NK cells (272) as well as activated $CD4^+$ T cells (273).

1.5.2 Signalling pathways utilised by OX40-OX40L

It is currently unclear if costimulatory members of the TNFR family have any role in promoting the formation of the immune synapse, or if they directly link into TCR tyrosine phosphorylation cascade. However, both the TNFR and CD28 families of costimulatory receptors can initiate signalling pathways that are distinct from the TCR but which lead to the activation of common targets, such as the up-regulation of anti-apoptotic members of the Bcl-2 family.

TRAF-2, -3 and -5 are recruited to the cytoplasmic tail of OX40 and provide an important link between OX40 and downstream signalling pathways which induces cell survival (274, 275). Other TNFR family members can also recruit TRAFs e.g. 4-1BB (275) and CD27 (276). Recruitment of TRAF-2 and TRAF-5 by OX40 can lead to the activation of NFκB (274, 275). Weinberg *et al* developed a $CD4^+$ TCR transgenic mouse which was crossed with a dominant negative TRAF2 transgenic mouse to demonstrate the importance of TRAF2 in mediating the functional effects seen by OX40. They observed that TRAF2 was crucial for antigen and OX40 driven effector and memory T cell generation *in vivo* (277) whilst TRAF3 acts as a dominant negative mutant which competes for the recruitment of TRAF2, thereby suppressing the signal between OX40 and TRAF2 resulting in the inhibition of NFκB activation (275, 278).

OX40-OX40L signalling also results in the activation of the serine/threonine kinase protein kinase B (PKB, also known as Akt). It has been shown recently that maintenance of high PKB activity is an essential downstream signal of OX40, and is involved in the control of survival of $CD4^+$ T cells through the up-regulation of anti-apoptotic Bcl-2 family members (258). $CD4^+$ T cells that lack OX40 are unable to maintain high levels of anti-apoptotic molecules demonstrating a clear link between NFκB signalling and cell survival (279).

The importance of costimulation by OX40-OX40L interactions in T cell activation and differentiation has led to a clear association between OX40-OX40L interactions and the development of a number of diseases known to be mediated by T cells.

1.5.3 The role of OX40-OX40L in autoimmunity

A number of studies have reported the presence of $OX40^+$ cells and $OX40L^+$ cells at sites of inflammation in T cell mediated diseases which provided evidence that targeting such cells may prevent specific pathogenic responses and ameliorate disease. The potential therapeutic benefit of targeting OX40 in autoimmunity was first shown in a rat model of EAE where an OX40-specific immunotoxin, which depleted recently activated $OX40^+$ autoreactive T cells in the central nervous system (CNS), was shown to ameliorate EAE in rats when administered at the first signs of disease (280). The specific depletion of $OX40^+$ T cells in this setting allowed the pathogenic T cells to be removed while sparing the remainder of the T cell repertoire.

Myelin oligodendrocyte glycoprotein (MOG) induced EAE in $OX40^{-/-}$ recipients showed less severe clinical signs of disease and exhibited significantly decreased inflammatory infiltrates within the CNS (281). Carboni *et al* also showed that T cells infiltrating the human brain in patients with multiple sclerosis express OX40 paralleling findings from mouse models (281). Other studies have utilised methods to block OX40-OX40L interactions rather than depleting $OX40^{+}$ cells. For example, administration of an OX40-Ig fusion protein at the onset of disease resulted in a significant reduction in the severity of EAE, however, OX40-Ig treated mice relapsed with the same frequency as control treated mice (267). Nohara *et al* used an anti-OX40L mAb (RM134L) to block OX40-OX40L interactions which also resulted in amelioration of EAE by reducing the infiltration of mononuclear and $CD4^{+}$ T cells into the CNS (282). These data suggest OX40-OX40L play a key role in the migration of pathogenic T cells in EAE which ultimately cause disease.

The involvement of OX40 costimulation in autoimmunity is not restricted to EAE. In hapten-induced colitis or IL-2 knockout mice, which develop spontaneous colitis, blocking OX40-OX40L interactions using an OX40-Ig fusion protein also ameliorated disease in both models (283-285). As with activated T cells in EAE, $OX40^{+}$ T cells were observed infiltrating the lamina propria in patients with ulcerative colitis and Crohn's disease (286).

In a mouse model of arthritis (collagen induced arthritis), blocking OX40-OX40L interactions by administering either anti-OX40 or anti-OX40L mAb early in the T cell

phase of the disease ameliorates disease severity, while administration later on in disease progression had no effect (UCB, personal communication) (287). In rheumatoid arthritis patients, OX40 expression has been observed in the synovial fluid and tissue, while OX40L was found to be expressed on cells lining the synovial tissue (287). These data suggest that $OX40^+$ T cells accumulate selectively in the synovial fluid, while OX40L may be expressed on vascular endothelial cells in the synovium and may play an important role in migration of pathogenic T cells into the inflamed joint in rheumatoid arthritis patients.

OX40-OX40L interactions have also been implicated in the development of a number of other inflammatory and autoimmune diseases including type-1-diabetes (288, 289), atherosclerosis (290) and systemic lupus erythematous (SLE) (291). When taken together, these data clearly suggest that targeting OX40-OX40L interactions in autoimmunity has the potential to specifically suppress activated pathogenic T cells and therefore may be of potential therapeutic value in the treatment of autoimmune as well as in other inflammatory diseases.

1.5.4 The role of OX40-OX40L in airway disease

A number of experimental studies have investigated the role of OX40-OX40L in respiratory diseases such as viral pneumonia and asthma. DC stimulated with thymic stromal lymphopoietin (TSLP) can prime naïve $CD4^+$ T cells which can differentiate into Th2 effector T cells which secrete IL-4, IL-5, IL-9 and IL-13 triggering inflammation and the recruitment of innate immune cells including eosinophils and

neutrophils. In a model of allergic inflammation where mice are sensitised and challenged with ovalbumin (OVA), $OX40^{-/-}$ mice exhibit a significant reduction in lung inflammation, characterised with a reduction in the numbers of eosinophils in bronchoalevolar lavage (BAL) and goblet cell hyperplasia in the lungs compared to wild type controls (292, 293). In pre-sensitised mice, anti-OX40L mAb prevented Tm reactivation in the draining lymph nodes and lung tissue, resulting in reduced eosinophilia, airway hyperreactivity and Th2 cytokine production (294). While administration of OX40-Ig fusion protein reduced influenza virus induced lung inflammation without any impact on clearance of the virus or on the development of Tm responses to viral rechallenge (295).

Inflammation induced by TSLP in either the lung or the skin, resulted in Th2 infiltration and cytokine production as well as increased circulating IgE antibodies, and this inflammatory response was significantly inhibited by treatment with anti-OX40L mAb (296). A requirement for OX40-OX40L in asthma was also demonstrated in a chronic house dust mite model (currently considered the gold standard model of allergic airway inflammation that is characteristic of clinical asthma) in both mice (UCB; personal communication) and in NHP models (296). These data suggest blockade of OX40-OX40L could be anti-inflammatory in the setting of respiratory diseases, rather than immunosuppressive.

The studies described above across different diseases demonstrate that blockade of OX40-OX40L is effective at ameliorating autoimmune, allergic and inflammatory diseases without the need for global immunosuppression.

1.5.5 The role of OX40-OX40L in alloimmune responses

The OX40-OX40L interaction has also been studied in models such as graft versus host disease (GvHD) and solid organ transplantation. GvHD is a major consequence of allogeneic bone marrow transplantation, which is often lethal. GvHD occurs as a result of MHC mismatches between the bone marrow donor and the recipient and is mediated by activated T cells in the donor bone marrow. Tittle *et al* showed that there were increased numbers of $OX40^{+}CD4^{+}$ T cells in the peripheral blood and lymphohematopoietic organs (skin, spleen, lymph nodes and liver) of non-irradiated F1 rat recipients of parental donor grafts which showed signs of GVHD. $OX40^{+}CD4^{+}$ T cells in the peripheral blood were found to emerge rapidly after transplantation and before clinical signs of GVHD developed which correlates with the previously described expression pattern of OX40 on activated T cells (297). Moreover, the administration of an anti-OX40L mAb significantly reduced the lethality of acute GVHD after bone marrow transplantation, while other milder symptoms of the disease such as weight loss and alopecia were also attenuated compared to controls (298). Since activated T cells can express OX40 and OX40L, the efficacy of the anti-OX40L mAb used in these studies may be due to the clearance or modulation of the function of donor T cells in addition to blocking the engagement of OX40 on donor T cells with OX40L on recipient T cells.

In humans, a close relationship between the expression of OX40 on peripheral blood mononuclear cells and the development of chronic GVHD has also been reported (299). Elevated numbers of $OX40^{+}CD4^{+}$ T cells in the peripheral blood has been found to be indicative of the onset of chronic GVHD, and also correlated with increased severity of the disease as well as increased likelihood of resistance to immunosuppressive therapies (299).

These data suggest a role for OX40-OX40L interaction in alloimmune responses associated with GvHD, however there is scant experimental evidence to support a role for OX40 in the rejection of allografts. Indeed, anti-OX40L mAb has been demonstrated to have no impact on the rejection of full MHC mismatched islet allografts in mice (300). Similarly, blocking the OX40-OX40L pathway (using an OX40-Ig fusion protein) in a mouse model of cardiac transplantation was shown to be ineffective at prolonging allograft survival across a full MHC mismatch. However, prolonged cardiac allograft survival was observed (MST 14 days vs >100 days) when donor and recipient were mismatched at only minor histocompatability antigen loci (301). The administration of anti-OX40L mAb was shown to prolong the survival of allogeneic islets in $CD28^{-/-}$ recipient mice (300).

These data provide a clear precedent for the utilisation of the OX40-OX40L costimulatory pathway in rejection however this appears to be contingent on suboptimal or low frequency T cell responses. This is borne out by the finding that when OX40

blockade is used in combination with interruption of other costimulatory pathways, such as CD40/CD154, CD28/CD80/CD86, a more pronounced impact on skin allograft survival has been observed due to perturbation of the expansion or persistence of alloreactive effector T cells (302-304).

1.5.6 The potential of OX40-OX40L as an immunotherapeutic target

OX40-OX40L interactions promote the survival, effector function and memory differentiation of activated $CD4^+$ and $CD8^+$ T cells (255, 305, 306), whilst inhibiting Treg (307, 308). Furthermore developing reagents which block the OX40-OX40L interaction could provide a dual benefit by attenuating effector T cell responses by enhancing T cell death and in addition can promote Treg generation and/or function. Stimulation of OX40 could be exploited as part of vaccination strategies to pathogens and cancers. For example, Vetto *et al*, showed that OX40 was expressed on tumour infiltrating cells isolated from patients with head and neck carcinoma or melanoma (309).

In contrast, blocking OX40-OX40L interactions has shown promise in therapies to attenuate autoimmunity and facilitate the induction of tolerance to allografts. The benefit of this therapeutic strategy is that pathogenic effector T cell responses and Treg could be simultaneously influenced by targeting the OX40-OX40L interaction. Importantly only the activated pathogenic T cells will be inhibited, while enhancing the function of Treg, thus minimising side effects which are usually associated with global immunosuppression strategies. However, no therapies blocking either OX40 or OX40L

are currently approved for use in the clinic at this time for autoimmunity and inflammatory diseases therefore these warrant further investigation.

1.5.7 Clinical trial data

Agonistic antibodies to OX40 are currently in phase Ib clinical trials for cancer and have been shown to be safe and well tolerated (310). The primary objective of the trial is to determine the maximum tolerated dose of cyclophosphamide administered in combination with radiation and anti-OX40 given to patients with metastatic treatment resistant prostate cancer. Chemotherapy and/or radiation can induce tumour breakdown which provides a source of antigen for self-vaccination. Data from pre-clinical mouse studies have shown cyclophosphamide in combination with agonistic anti-OX40 enhanced anti-tumour immunity and resulted in regression of pre-established poorly immunogenic melanoma tumours (311). This combination of cyclophosphamide and anti-OX40 has a dual role and can amplify the $CD8^+$ T cell mediated effector response across a broad range of prostate cancer antigens as well as reducing the number of Treg, resulting in further regression of the cancer (311).

Experimental data from rodents and NHP in allergic asthma and virus induced lung inflammation, as described above, provided evidence that targeting OX40-OX40L in respiratory diseases had a beneficial outcome. A humanised OX40L mAb has been developed by Genentech and has been tested in a phase II clinical trial in the prevention of allergen-induced airway disease in adults with mild asthma. The trial was completed in January 2011, but no results have been disclosed as yet (312). The primary outcome

was the measurement of the late asthmatic response in patients treated with anti-OX40L compared to placebo. If this trial is successful the OX40-OX40L interaction will most likely be tested in other inflammatory diseases.

One possible concern with anti-OX40 or anti-OX40L reagents in the clinic would be the recent observation that OX40L is expressed on platelets (313). This was a major factor in the termination of the anti-CD154 mAb trial in which kidney allograft recipients had thromboembolic complications (249). CD154 was expressed on platelets and activation of these cells led to unexpected thromboembolic events. Therefore further analysis of platelet expression is required to ensure this does not re-occur.

In conclusion, OX40 provides a specific costimulatory signal to activated effector T cells and experimental data suggests targeting OX40-OX40L in chronic T cell mediated diseases would be a beneficial strategy.

1.7 Thesis hypothesis and aims

1.7.1 Hypothesis:

Blocking the interaction between OX40 and OX40L in solid organ transplantation will be a beneficial adjunctive therapy for the induction of tolerance.

1.7.2 Global Aim:

Members of the TNFR superfamily of costimulatory molecules are becoming increasingly recognised as key modulators of the T cell response in infection, cancer and following transplantation. However, OX40 remains a poorly defined member despite clear evidence for the importance of this interaction in a number of inflammatory situations. Defining their pattern of expression and role in the response of different T cell subsets to solid organ transplants will provide a framework to target OX40-OX40L interactions as part of immune based therapies to prevent inflammatory diseases such as autoimmunity and transplant rejection.

Therefore the global aim of the project was to define the role of OX40-OX40L interactions in T cell mediated responses in the context of transplantation. The studies were focussed on defining the utilisation of OX40-OX40L interactions by $CD8^+$ T cells and $CD4^+$ T cells (**Chapter 3 and 4**) and Treg (**Chapter 5**) responding to alloantigen in models of allogeneic skin and cardiac transplantation in the mouse. Additionally we sought to address whether blocking OX40-OX40L interactions may have an additive or synergistic effect in attenuating the response of T cells to alloantigen when combined with other immunomodulatory agents thus highlighting potential avenues for the inclusion of OX40 blockade in strategies to induce tolerance in clinical transplantation (**Chapter 6**).

Chapter 2: Materials and Methods

2.1. Mice

Mice of both sexes between 6 and 12 weeks of age (at the beginning of experiments), were provided by and housed in the Biomedical Services Unit at the John Radcliffe Hospital, Oxford, UK. All mice were housed in individually in vented cages and in a room at 22°C, with a 12 hour light-dark cycle and food and water available *ad libitum*. All studies were performed under the Animals (Scientific Procedures) Act 1986. Table 2.1 details the mice used in these studies.

Wild-type/Inbred mice (Name; Abbreviation; Haplotype)		
C57BL/6	B6	b
CBA.Ca	CBA	k
BALB/c		d
B10.S		s
BALB/cxC57BL/6	Balb/cB6F1	dxb
$Rag^{-/-}$ mice (T and B cell deficient) (Name; Abbreviation; Haplotype)		
C57BL/6 $Rag^{-/-}$	B6 $Rag^{-/-}$	b
CBA $Rag^{-/-}$	CBA $Rag^{-/-}$	k
B10.Br $Rag^{-/-}$	B10.Br $Rag^{-/-}$	k
TCR-transgenic T cells (Name; Abbreviation; Haplotype)		
TEaB6SJLCD45.1	TEa	b
DKK	-D	k
BM3 $Rag^{-/-}$	BM3	k
TCR-transgenic T cells (Name; Abbreviation; Haplotype)		
MHC Class $II^{-/-}$ $CD8^{-/-}$	MHC Class $II^{-/-}$	b

Table 2.1 – Description of the mice used in this thesis

2.1.1 Transgenic Mice

TCR transgenic mice express rearranged α and β TCR genes of known specificity and therefore contain a highly skewed T cell repertoire with 50-95% of T cells expressing both α and β chains of the transgenic TCR (314).

BM3 TCR transgenic mice (CBA background; $H2^k$) were back crossed onto a CBA $RAG^{-/-}$, and therefore only possess $CD8^+$ T cells that are reactive to MHC Class I molecule $H2^b$, via the direct pathway of allorecognition (315, 316). BM3 mice were generously provided by Professor Mellor (Institute of Molecular Medicine and Genetics, Augusta, USA). BM3 TCR can be identified by flow cytometry, by using the clonotypic antibody Ti98.

TEa TCR transgenic mice (C57BL/6 background; $H2^b$) produce $CD4^+$ T cells that are reactive to a 17-mer peptide of the BALB/c H2Eα chain presented in the context of $H2IA^b$ i.e. presented by B6 MHC Class II via the indirect pathway (317). The TEa mice were a kind gift from Dr W.Gao and Prof. T. Strom (Boston, USA). The presence of the TEa TCR can be identified by flow cytometry using monoclonal antibodies to Vα2.

DKK (-D) TCR transgenic mice (B10.BR background; $H2^k$) produce $CD4^+$ T cells that are reactive to the MHC Class II alloantigen $H2IA^s$ via the direct pathway (318). The -D mice were a kind gift from Dr B.Fazekas de St Groth (Sydney, Australia). The

presence of the -D TCR can be identified by flow cytometry using monoclonal antibodies to Vα11.

MHC Class $II^{-/-}$ mice lacked both $CD4^{+}$ and $CD8^{+}$ T cells as well as other MHC Class II^{+} cells (319).

2.1.2 Monitoring of Animal Health

Mice were monitored regularly throughout procedures and during recovery from surgery. Animals were checked for signs of adverse events such as lack of mobility and respiratory distress according to a pre-defined animal welfare score sheet. Any mice exceeding a pre-defined animal welfare score were immediately sacrificed.

2.2 Surgical Procedures

2.2.1 Pre-operative Care

Surgical anaesthesia was obtained by administration of 250µl mixture of 100µg/ml of Domitor® (Medetomidine hydrochloride; Pfizer Pharmaceuticals, UK) and 8mg/ml Ketaset® (Ketamine hydrochloride; Fort Dodge Animal Health Ltd, UK). Mice undergoing invasive surgery also received 200µl of the long acting analgesic Vetergesic® (10µg/ml) (Buprenorphine hydrochloride; Reckitt Benckinser, UK) and 120µl Metacam (250µg/ml) (Meloxicam; Boehringer Ingelheim, Germany). All anaesthesia was given subcutaneously. Donor mice for cardiac transplantation or blood

transfusion were injected with a lethal 500µl dose of Domitor/Ketaset. Skin transplant donors were killed by an approved schedule 1 method.

2.2.2 Post-operative Care

Mice were reversed from surgery using 300µl of 100µg/ml Antisedan® (atipamezol hydrochloride; Pfizer Pharmaceuticals, UK) and 500µl of room temperature saline, both given subcutaneously prior to being placed in a warming cabinet.

2.2.3 Skin transplants

Skin grafts were performed as previously described by Billingham and Medwar (320). Briefly, 1cm^2 full thickness tail skin grafts were transplanted onto a prepared graft bed on the left flank of the donor mouse, under general anaesthesia. Grafts were secured using flexible colloid (B.P. methylated, William Ramson & Son, UK) and dressed with sterile inadine gauze (Johnson & Johnson Medical, U.K.) and then bandaged using autoclave tape. The animals were reversed from anaesthetic as described above and placed in a warming cabinet at 26^{o}C for 2-4 hours before being returned to conventional housing. The gauze and bandage was removed 7-10 days after skin transplantation and assessed daily for signs of rejection. Grafts were considered rejected upon complete destruction of donor tissue.

2.2.4 Heterotopic cardiac transplants

Heterotopic cardiac transplants were performed as previously described by Corry *et al* (321). In this model, the donor ascending aorta is anastomosed end-to-side to the recipient abdominal aorta, and the pulmonary artery (PA) is sutured to the recipients inferior vena cava (IVC). Heterotopic transplantation was carried out using an operating microscope (Carl Zeiss, Germany). Operating time was ~60 minutes and overall operative mortality/technical errors were less than 10% of all transplant performed.

Briefly, donor mice were lethally anaesthetised as described in section 2.2.1. Donor mice were prepared by making a midline abdominal incision, and removing blood from the IVC, before injecting 1ml of heparin (300U/ml; CP pharmaceuticals Ltd, UK) into the IVC. The donor mouse was exsanguinated by cutting through the IVC. The thoracic cavity was exposed by cutting through the ribs, and the sternum reflected towards the head. The heart was removed by ligating the IVC, superior vena cava and the azygos vein using 7/0 braided silk (Pearsall Ltd, UK). The aorta and pulmonary artery were then separated and cut to the appropriate length. The remaining pulmonary veins were ligated before the heart was removed from the thoracic cavity and placed in ice cold saline.

Recipient mice were anaesthetised as described in section 2.2.1. A midline abdominal incision was made from the pubic symphysis to the bottom of the sternum. A self-retaining retractor was used to expose the abdominal contents, which were gently

deflected onto moist sterile gauze on top of the thoracic cavity. In order to expose the aorta and IVC, the sigmoid colon was deflected to the side using a strip of moist gauze. Using cotton buds, the abdominal aorta and IVC were dissected free from surrounding tissues in the retroperitoneum, to expose the lumber vessels which were ligated individually with 7-0 silk ties. In male recipients, testicular vessels were mobilised from the main vessels. A surgical clamp (Downs surgical Ltd, UK) was positioned at the proximal side of the bifurcation preventing blood flow in both the aorta and IVC. Another clamp was positioned just below the renal vessels.

An aortotomy was made in the recipient's abdominal aorta using a 30G needle, and then extended using microscissors superiorly to a length of equal or slightly smaller than the opening of the donor ascending aorta. The donor aorta was anastomosed end to side, using 10/0 silk (Bear Medic Cooperation, Japan). A stay suture was placed at the proximal and distal ends, to bring together the donor ascending aorta and the recipient abdominal aorta. The anastomosis was completed in a counter-clockwise direction, starting from the distal stay suture, with a continuous running stitch. The distance between the two stay sutures was completed with 4-5 sutures on each side.

A venotomy was made in the recipient's IVC with microscissors 2-3mm below the bottom of the anastomosis. This small gap allows easier access when suturing the second side. A stay suture was first placed in the distal apex bringing the donor PA and the recipient IVC together. A second stay suture was used to bring the proximal apex of the donor PA and the recipient IVC together. Stay sutures in this order (opposite to the

aorta stay sutures) allowed the same length of donor PA to be used, making the suturing more straight forward. The anastomosis was completed in a clockwise direction with a continuous running stitch. After the suture was tied to the proximal stay suture, the needle was then passed between the two donor vessels and the donor heart was flipped from the left side of the animal to the right side. The second side was completed in a similar manner as before.

Small pieces of spongostan (a haemostatic agent) (Johnson&Johnson, UK) were placed around both anastomosis. The distal clamp was removed first, to check the IVC anastomosis. After ensuring adequate haemostasis the proximal clamp was slowly removed. Within ~15 seconds, the transplanted heart filled with blood and began contracting. If there was no further bleeding, the intestines were reflected back into the abdominal cavity. The abdomen was then closed (muscle and skin layers separately) using 4/0 vicryl rapide suture (Johnson & Johnson, UK) in a continuous running stitch. On completion of the operation, the recipient was placed in a warming cabinet at 26°C for 2-4 hours before returning to conventional housing. Recipient mice were given the reversal agent, antisedan, as outlined in section 2.2.2

Mice with non-functioning grafts within four days of transplant were deemed technical failures, and mice were sacrificed and excluded from analysis (<5%). Cardiac function was monitored regularly by abdominal palpation and scored as detailed below:

0 = no palpable contraction

1 = very weak and slow cardiac contraction

2 = decreased rate or irregular rhythm

3 = decreased force of contraction, but with normal rate

4 = normal rate and rhythm

Absence of a palpable heart beat for 5 days was considered rejection (322).

2.3 Non-Surgical *In vivo* Techniques

2.3.1 Injections

Cell preparations were injected intravenously (i.v) in 200µl of phosphate buffered saline (PBS) into the tail vein without anaesthesia. Therapeutic reagents or controls were injected subcutaneously (s.c.) or i.v as appropriate without anaesthesia.

2.3.2 Generation of $CD4^+CD25^+$ regulatory T cells using the 177/DST protocol

CBA mice treated with 200µg of non-depleting anti-CD4 mAb (YTS177) i.v. on day -28 and -27. In addition on day -27, mice also received 250µl of C57BL/6 whole blood (i.v.). On Day 0, mice possess a population of $CD4^+CD25^+$ T cells capable of suppressing donor-alloantigen specific T cell responses (323, 324).

2.3.3 Blood withdrawal for donor specific transfusions

Donor mice were given terminal anaesthesia as described in section 2.2.1. Heparin (300U/ml) was taken up in the syringe, then expelled to leave only <100µl in the needle. Upto 1ml of blood was collected from either the vena cava (from cardiac

transplant donors) or directly from the heart by cardiac puncture. Blood samples were then stored on ice, until required for injection.

2.3.4 Generation of memory T cells

Memory T cells were generated *in vivo*, and mice rested for 50-100 days before use in subsequent experiments. Upon harvest, spleen and mesenteric lymph nodes (mLN) were removed from mice containing Tm and the percentage of live Tm was determined by flow cytometry. Typically >95% of the transgenic T cells co-expressed CD44 (data not shown), suggesting a memory phenotype.

TEa memory T cells - B6 $Rag^{-/-}$ mice were injected on day -1 with $1x10^6$ sorted TEa $CD4^+$ T cells. On days 0, 7, 14 mice received $1x10^7$ BALB/cB6F1 splenocytes.

-D memory T cells - B10.BR $Rag^{-/-}$ mice were injected with $1x10^6$ sorted -D $CD4^+$ T cells. The following day, the mice received $1x10^7$ B10.S splenocytes.

BM3 memory T cells - CBA $Rag^{-/-}$ mice were injected with $1x10^5$ naïve BM3 $CD8^+$ T cells (unsorted). The following day, the mice received $1x10^7$ B6 splenocytes.

2.4 Reagents

2.4.1 *In vivo*

All reagents used *in vivo* contained low or no endotoxin and were tested in house prior to use. Anti-OX40 Fab PEG (710-Fab PEG; anti-OX40) was used to block the OX40:OX40L interaction. PEGylated Fab control (A33-Fab PEG; PEG control) was

used to control for the non-specific effects of PEGylated Fab fragments. The PEGylated Fab fragments were all generated at UCB-Celltech R&D Ltd, Slough, UK. Briefly, PEGylation involves attaching one or more chains of polyethylene glycol (PEG) to a protein molecule (using maleimide chemistry). Anti-OX40 and the PEG control are Fab' fragments attached to two 40kDa PEG molecules. There are several advantages to PEGylation; reduced immunogenicity, significant improvement in circulating half life *in vivo*, improved solubility and reduced toxicity (325). The dosing regimens for anti-OX40 have previously been shown to result in a maximal pharmacological effect in animal models of autoimmune disease (UCB; unpublished data). The dosing strategies for reagents used *in vivo* are outlined in Table 2.2.

Reagent	Clone	Dosing regimen	Dose timings	Dose route
Anti-OX40	710	2.5mg/dose	2x weekly for 4 weeks	s.c
PEG control	A33	2.5mg/dose	2x weekly for 4 weeks	s.c
Anti-CD154	MR1	1mg/dose	Day 0, 7, 14	i.v
CTLA4-Ig (Abatacept)		25µg/dose	Day 0, 2, 5	i.v

Table 2.2: *In vivo* dosing regimens

2.4.2 *In vitro*

Anti-OX40, anti-CD154, anti-4-1BBL and appropriate controls were added to *in vitro* cell cultures at 10µg/ml diluted in complete media, while abatacept was used at 1µg/ml. Anti-4-1BBL (AT113-2) was a kind gift from Professor Al-Shamkani, University of Southampton.

2.5 Production of anti-4-1BBL from tissue culture supernatant

The hybridoma for anti-4-1BBL (AT113-2) was grown as spent tissue culture supernatants in complete media. The cultures were allowed to grow to exhaustion which was defined by less than 90% viability. The supernatant was centrifuged at 2000 revolutions per minute (rpm) for 5 minutes, before being stored at 4°C until purification.

2.5.1 Ammonium sulphate precipitation

Precipitation using ammonium sulphate (29.5% w/v) was performed by slowly adding the ammonium sulphate to the supernatant (295g per 1L). This mixture was stirred overnight at 4°C before being ultra-centrifuged (10,000 rpm for 20 minutes) and the supernatant discarded. The pellet was resuspended in Buffer A (11.9g Sodium dihydrogen orthophosphate and 5g sodium azide per litre of dH_20). This method of purification for immunoglobulins is based on the observation that they are precipitated by lower concentrations of ammonium sulphate than most other serum proteins. This technique provides a substantial enrichment of immunoglobulin concentration of the solution and reduces the protein load on subsequent purification steps.

2.5.2 Dialysis

Dialysis of the solution into Buffer A was performed 4 times in the next 2 days. The solution was then centrifuged at 10,000 rpm for 20 minutes. The supernatant was kept and filtered through a 22µm filter, before being loaded onto the fast protein liquid chromatography (FPLC) machine for purification.

2.5.3 Fast Protein Liquid Chromatography

The filtered supernatant was loaded onto a Hitrap-Protein G column (VWR, UK) and washed through the column with Buffer B (0.2M glycine pH 2.3). The 2ml fractions where antibody was eluted from the column were collected and optical density (280nm) was measured to calculate the amount of protein in each fraction. Total protein (mg/ml) = OD (280nm) / 1.4.

After the fractions were collected and pooled, the purified material was dialysed as before into PBS, ready for use.

2.5.4 Endotoxin Testing

All end products from the purification process were tested for endotoxin levels. The QCL-1000 kit (Lonza, UK) was used as per the manufacturer's instructions.

2.6 Preparation of specific cell populations

2.6.1 Leukocyte preparation

Spleen and/or lymph nodes were aseptically removed and a single cell suspension was generated by gentle mechanical disaggregation through a sterile 70μm nylon cell strainer (BD Biosciences, UK). Cells were washed by centrifugation for 5 minutes at 1500 rpm in PBS (Oxoid Ltd, UK) supplemented with 2% fetal calf serum (FCS, PAA Laboratories, UK). Erythrocytes were removed by hypotonic lysis using BD Pharmlyse

(BD Biosciences, UK) for 4 minutes at room temperature. Cells were washed once and then resuspended at the required concentration for further manipulation if necessary.

2.6.2 TCR-transgenic T cells

2.6.2.1 Naïve or memory BM3 RAG$^{-/-}$ CD8^{+} T cells

Single cell suspensions were prepared as previously described in section 2.6.1 from the spleens and mLN of naïve or memory BM3 RAG$^{-/-}$ mice. A 50µl aliquot of these cells were stained for CD8 and TCRβ and then analysed by flow cytometry to determine the percentage of live CD8^{+}TCRβ^{+} cells within the whole population i.e. TCR transgenic CD8^{+} cells. This percentage was then used to normalise the number of T cells to the proportion of TCR-transgenic T cells.

2.6.2.2 Naïve TEa or -D CD4^{+} T cells

Single cell suspensions were prepared as previously described in section 2.6.1 from the spleen and mLN of transgenic mice. TEa T cells were stained with CD4 and Vα2, while -D T cells were stained with CD4 and Vα11 for 1 hour at 4°C before purification based on these markers using the FACSAria (BD Biosciences). Typically, the sorted population was > 95% pure.

2.6.2.3 Memory TEa or -D CD4^{+} T cells

Single cell suspensions were prepared as previously described in section 2.6.1 from the spleen and mLN of mice containing memory T cells. A 50µl aliquot of these cells were

stained for CD4 and the respective TCR (TEa – Vα2; -D – Vα11) and then analysed by flow cytometry to determine the percentage of live $CD4^+$TCR-Tg^+ T cells within the whole population. This percentage was then used to normalise the number of T cells to the proportion of TCR-transgenic T cells.

2.6.3 T cell enrichment using Dynabeads

For experiments where large number of spleens and mLN were used for the isolation of Treg, T cells were first enriched using the following protocol. Single cell suspensions were generated from spleens and mLN as described above (section 2.6.1). Cells were re-suspended at a concentration of ~$2x10^8$/ml in PBS/2% FCS. In order to enrich for $CD4^+$ T cells, anti-B220 (RA36β2; B cells), anti-CD11b (M1/70; macrophages), anti-MHC Class II (TIB120) and anti-CD8 (YTS169) were added to the cells at a final concentration of 10μg/ml and incubated for 45-60 minutes at 4^oC. Cells were washed once and re-suspended at a concentration of ~$1x10^8$cells/ml in PBS. Sheep anti-rat coated dynabeads (Dynal A.S, Norway) were added at a ratio of one bead per cell and incubated on a rotating wheel at 4^oC for 30 minutes. Unlabelled cells were isolated by negative selection using a Dynal magnet. The resulting enriched cell population was then counted and re-suspended at the required concentration for further use.

2.6.4 Isolation of $CD25^+$ and $CD25^-$ $CD4^+$ T cells

A single cell suspension enriched for T cells was prepared as described in section 2.6.3 and enriched for $CD4^+$ T cells. After $CD4^+$ purification by Dynal beads, cells were

stained with anti-CD25-PE (ebioscience) for 30 minutes at 4°C. Cells were washed once in PBS/2% FCS before incubation with anti-PE beads (Miltenyi Biotech, Germany). Cells were washed once, and loaded onto a MACS column on a MACS magnet. $CD25^{-}CD4^{+}$ T cells were isolated by negative selection (collected from flow through), and $CD25^{+}CD4^{+}$ T cells were isolated by positive selection (those attached to the column), according to the manufacturer's instructions.

2.6.5 $CD11c^{+}$ isolation

Spleens were harvested and cell suspensions generated in section 2.6.1, and resuspended at a concentration of ~$2x10^{8}$/ml in PBS/2% FCS. In order to enrich for $CD11c^{+}$, anti-B220 (RA36β2; B cells) was incubated with the cells at a final concentration of 10μg/ml and incubated for 45-60 minutes at $4^{o}C$. Cells were washed once and re-suspended in 10% FCS/PBS. Sheep anti-rat coated Dynabeads (Dynal A.S, Norway) were added at a ratio of one bead per cell and incubated on a rotating wheel at $4^{o}C$ for 30 minutes. Residual cells incubated with anti-CD11c (Miltenyi Biotech) for 30 minutes at $4^{o}C$, before being isolated by positive selection using a Dynal magnet. The resulting enriched cell population was then counted, purity checked and re-suspended at the required concentration for further use.

2.6.6 Isolation of graft infiltrating cells from cardiac allografts

Transplanted hearts were removed from recipient mice 5 days post transplantation and graft infiltrating cells (GIC) were prepared by collagenase digestion as described by

Wagoner *et al* (326). Grafts were minced and incubated with 1mg/ml type VII collagenase solution (Sigma-Aldrich, UK) for 30 minutes at 37°C. A single cell suspension was created by mashing the collagenase digested tissue through a 40µm sieve. The suspension was centrifuged at 1500rpm for 5 minutes and resuspended in 1ml of 2%FCS/PBS buffer ready for cell surface staining.

2.7 Labelling of cells for analysis of proliferation

For carboxy fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes Inc, the Netherlands) labelling, cells were resuspended at 30 x 10^6 cells/ml in serum free media and CFSE added at 5µM final concentration. Cells were incubated at 37°C for 10 minutes and washed in ice cold RPMI before re-suspension to the required concentration.

For cell trace violet proliferating dye (Invitrogen) labelling, cells were suspended at 1x10^6 cells/ml in serum free media and dye added at 5µM final concentration. Cells were incubated at 37°C for 20 minutes and washed ice cold RPMI before re-suspension to the required concentration.

Successful labelling was confirmed by FACS before cells were used *in vitro* or *in vivo*.

2.8 Flow Cytometry

2.8.1 Cell surface staining

Single cell suspensions were prepared as described previously in section 2.4. Cells were incubated with Fc receptor block (BD Biosciences, UK) for 15 minutes at room temperature to prevent non-specific mAb binding to Fc receptors. For staining of cell surface markers, anti-mouse mAb were diluted to an appropriate concentration, added to the cells which were incubated for 40 minutes at 37°C in the dark. Following incubation, cells were washed in 2%FCS/PBS buffer and if necessary a secondary streptavidin conjugated fluorochrome was added to the cells for 30 minute incubation at 37°C in the dark. 7-AAD (7-amino-actinomycin D, BD Bioscience, UK) was included in all the mAb cocktails used in flow cytometry to allow confirmation of cell viability. After the final stage of staining, cells were washed once in 2%FCS/PBS buffer before being resuspended in 200µl 2%FCS/PBS buffer for acquisition. Cells were acquired using a FACSAria or FACS Canto and analysed using FACSDiva software (BD Biosciences, UK).

2.8.2 Intracellular staining

For intracellular staining (FoxP3 or CTLA-4), cells were fixed by incubation in fixation/permeabilisation solution (eBioscience, UK) for 90 minutes to overnight at 4°C in the dark. Cells were washed once in FACS buffer, then twice in permeabilisation buffer (eBioscience, UK). The cells were then incubated with either FoxP3-APC or CTLA-4-PE for 30 minutes incubation at 4°C. After incubation, cells were washed once in permeabilisation buffer. For Bcl-2 staining on Treg, cells were surface stained

as before (30 minutes at 4°C) and then washed in FACS buffer. Cells were then incubated in 500µl of Fix/Perm Buffer (Transcription Factor buffer Kit; BD, UK) for 1 hour at 4°C. This incubation was followed by two wash steps in wash buffer (Transcription Factor buffer Kit; BD, UK) before incubation with FoxP3-APC and Bcl-2-FITC for 30 minutes incubation at 4°C. After incubation, cells were washed once in permeabilisation buffer.

After the final stage of staining, cells were washed once in MACS buffer before being resuspended in 200µl MACS buffer for acquisition. Cells were acquired using a FACSAria and analysed using FACSDiva software (BD Biosciences, UK).

2.8.3 Enumeration of Cells

To enumerate cells, 6µM synthetic fluorescent beads (CaliBRITE beads, BD Biosciences, UK) were added at a known concentration to each tube; $1x10^5$ beads for splenocytes, $1x10^4$ for lymph node cells. The absolute cell number was calculated using the following formula:

Number of cells per tube = Number of cells acquired / number of beads acquired x number of beads added per tube

To determine the number of cells per tissue, the number of cells per tube (as above) was multiplied by the proportion of a cell suspension from a tissue that was placed in the FACS tube.

2.9 *In vitro* analysis:

2.9.1 Mixed Lymphocyte Reaction (MLR)

Cells were resuspended in complete media (RPMI 1640 supplemented with penicillin G (45U/ml), streptomycin (45µg/ml), kanamycin sulphate (90µg/ml), glutamine (2mM), $5x10^{-5}$ M/L 2-ME and 10% heat-inactivated FCS). Responder splenocytes were cultured in triplicate with irradiated (3000 rad) stimulator splenocytes or anti-CD3/CD28 synthetic beads (Invitrogen, UK) in 96-U-well plates for 1-6 days at 37°C in an atmosphere of 5% CO_2 and 95% air. Cultures were pulsed with 0.5µCi of ^{3H}-thymidine (Amersham International, UK) for the last 18 hours of culture. Plates were harvested onto glass fibre filter mats where ^{3H}-thymidine incorporation was subsequently determined via liquid scintillation counting by a Betaplate counter (Wallac, Finland). Antibodies were added at 10µg/ml, unless otherwise stated. Results are presented as counts per minute, mean ± standard deviation.

In some experiments, exogenous IL-2 (10U/ml; Peprotech) or IL-7 (1ng/ml; Peprotech) were added to appropriate wells. In certain experiments DC2.4 cells, a murine cell line (327) stimulated with 1µg/ml lipopolysaccharide (LPS) overnight before use as stimulators in mixed lymphocyte reactions.

For phenotypic analysis multiple plates were setup in order to analyse daily time-points. Supernatant was removed and frozen at -20°C for cytokine analysis, and cells were resuspended in PBS/2% fetal calf serum before staining for specific cell surface markers as described in section 2.8.1.

2.9.2 Pharmacokinetic analysis for anti-OX40 levels in serum

96-well flat bottomed plates were coated with mouse OX40-rabbit Fc (5μg/ml; RnD Systems) in 0.1M carbonate coating buffer (0.17g NaOH and 4.077g $NaHCO_3$ in 500ml deionised water; pH9.4) overnight at room temperature. Plates were washed in 0.1% Tween/PBS before 1% BSA/PBS was added to each well for 30 minutes at room temperature in order to block non-specific binding. Plates were washed in 0.1% Tween/PBS. Samples and standards were added to appropriate wells and incubated with vibration for 60 minutes at room temperature. Plates were washed in 0.1% Tween/PBS. A secondary antibody (goat anti-mouse IgG F(ab)2; diluted 1:4000; Jackson Immunoresearch) was added to each well and incubated for 30 minutes at room temperature. Plates were washed in 0.1% Tween/PBS. 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well until sufficient colour had developed. The reaction was stopped by the addition of H_2SO_4 and absorbance was read at 450nm.

2.9.3 Cytokine detection

Mesoscale discovery (MSD) Th1/Th2 multiplex kit was used to detect IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12 (total), KC and TNF-α as per the manufacturer's protocol

(MSD, USA). MSD is a multiplex assay where an array of capture antibodies against specific targets are distributed on spatially distinct electrodes (or spots) in the same well. 10µl of tissue culture supernatants with unknown quantities of cytokines were added to each well. Cytokines in the supernatants bind to capture antibodies which are immobilised on the electrode surface. A mixture of detection antibodies specific for each cytokine being analysed are labelled with an electrochemiluminescent tag and added to each well. Immediately before analysis read buffer was added to each well which provides the correct chemical environment for electrochemiluminescence. The plate is read on a MSD Sector instrument (MSD, USA) which provides a voltage across the plate which activates the tag on the detection antibodies bound to the electrode to emit light. The MSD Sector instrument measures the intensity of the emitted light thereby providing a quantitative measure of each individual cytokine which is present in the sample. Initial results to detect IFN-γ using MSD were above the standard curve so were repeated using a classical sandwich ELISA for IFN-γ (RnD systems, UK).

2.9.4 Anti-CD3 and/or CD28 Treg expansion protocol

Anti-CD3 and/or anti-CD28 (10µg/ml each) were coated onto 60mm plates and incubated overnight at room temperature. Plates were washed with PBS and then blocked with 10% FCS/PBS for 2 hours at room temperature. Plates were washed with PBS before $5x10^5$ purified $CD4^+CD25^+$ Treg were added in complete media supplemented with 1mM sodium pyruvate (Invitrogen, UK) and non-essential amino acids (Sigma, UK) and in the presence of 10ng/ml IL-2 (Peprotech, UK). Anti-OX40 or

PEG control was added at 10μg/ml on day 0. Plates were harvested on day 7 for enumeration of Treg by flow cytometry.

2.9.5 IFN-γ conditioning protocol

$5x10^5$ total $CD4^+$ T cells isolated from CBA mice were co-cultured with $5x10^4$ bone-marrow derived DC (BM-DC) in a final volume of 2ml. 5ng/ml of exogenous recombinant mouse IFN-γ was added per well. On day 7, 1ml of media was removed from each well and replaced with fresh media containing IFN-γ, BM-DC and anti-OX40 or PEG control at the same concentrations outlined as above. On day 14, cells were harvested and analysed for expression of FoxP3 by flow cytometry.

2.9.6 Suppression assay

$5x10^5$ $CD4^+CD25^-$ responder T cells (isolated from naïve CBA mice) were labelled with violet proliferation dye (Invitrogen) and incubated with purified $CD11c^+$ cells (isolated from spleens of naïve B6 mice; Miltenyi Biotech) and with varying numbers of Treg labelled with CFSE. Treg were isolated from mice which had received the 177/DST protocol and either anti-OX40 or PEG control on days -28 and -25. Proliferation of the $CD4^+CD25^-$ responder T cells was assessed by flow cytometry on day 5.

2.10 Histology

2.10.1 Immunohistochemistry on frozen sections

In experiments where BM3 $CD8^+$ T cells were adoptively transferred into $Rag^{-/-}$ mice and were given an allogeneic skin transplant the following day, skin grafts were harvested on day 10. Skin grafts were harvested and flash frozen in OCT medium (Tissue-Tek, Netherlands) until further analysis. 7μm sections were cut using a Bright cryostat. After cutting, frozen sections were air-dried overnight and fixed the next day in 100% acetone and stored at -80°C until required.

Frozen skin graft samples were defrosted at room temperature and re-hydrated in PBS and incubated with 0.1% hydrogen peroxide diluted in PBS for 5 minutes to neutralise endogenous peroxidase within each tissue. Sections were then washed 3 times in PBS before adding 10% BSA/PBS for 10 minutes to each section to block any unspecific binding prior to primary antibody staining. Sections were washed 3 times in PBS before applying primary biotinylated antibody against mouse CD3 (Ebiosciences, UK) at room temperature for 1 hour. Sections were again washed 3 times in PBS and incubated with ABC complex (Vector laboratories, USA) for 45 minutes at room temperature.

After incubation, sections were washed 3 times in PBS before the addition of diaminobenzidin (DAB; Sigma, USA) which yields a brown insoluble precipitate at the site of the primary antibody allowing the development of the reaction to be followed under the microscope. The reaction was terminated by placing the sections in running water. Lastly, all sections were counterstained with Gills haematoxylin (VWR, UK),

dehydrated in a series of ethanol gradients (70, 90 and 100%) for one minute each and cleared in xylene (VWR, UK) for 2 minutes each. Stained slides were mounted using DPX (VWR, UK) and covered with glass coverslips. For enumeration of $CD3^+$ T cells, the number of positive cells in the tissue cross section (from the middle of each skin section), was counted manually at 40X magnification in each of the 40 fields defined by a graticule.

2.10.2 Immunohistochemistry on paraffin sections

For experiments where $CD4^+$ T cells were adoptively transferred into $Rag^{-/-}$ mice, skin grafts or draining axillary lymph nodes (dLN) were harvested at day 13 and stored in formalin. Using a closed VIP tissue processor (Bayer plc, Newbury, UK), the samples were processed to paraffin wax (one skin graft per wax block) and then embedded in fresh paraffin wax. Sections of skin or lymph node were cut (4μm), fixed onto Superfrost Plus Gold slides (Thermoscientific, UK) and dried overnight at 37ºC. Sections were dewaxed in Histoclear and rehydrated using descending concentrations of ethanol. Slides were kept under running water to prevent non-specific antibody binding.

For antigen retrieval, slides were placed in Coplin jars containing citrate buffer (pH 6.1) and incubated for 27 minutes in a waterbath at 99°C before being cooled for 20 minutes at room temperature. Slides were washed in 0.05M Tris buffered saline /0.05% Tween 20 pH 7.6 before being loaded onto an automatic Lab Vision Autostainer (Lab Vision Corporation, USA) for staining. Slides of skin sections were incubated with the primary

rabbit anti-CD3 antibody at room temperature (diluted 1:100; Abcam) and dLN sections with rabbit anti-active caspase-3 antibody (diluted 1:20; Abcam). Sections were washed and then incubated with a biotinylated donkey anti-rabbit secondary antibody (diluted 1:500; Jackson Immunoresearch) and then labelled streptavidin biotin (Dako) before being incubated with the DAB for 5 minutes. The cell nuclei were lightly counterstained with haematoxylin (14 minute incubation). The sections were then dehydrated through graded ethanol, cleared in Histoclear and coverslipped before being analysed.

2.10.3 Definiens Tissue Studio

To control for biased evaluation of histological sections, automatic image analysis was performed using Definiens Tissue Studio (Definiens, Germany), which is a digital cell-based image analysis software application. Digital scans of each slide (from paraffin histology only) were taken and pre-defined regions of interest (ROI; highlighted in orange on **Figure 1**) were selected by the user to exclude the epidermis and hair follicles. From these ROI, Definiens magnified the image to 20x and the software applied a threshold in order to exclude background, non-specific staining. This rule set then classifies cells based on their relative intensity of the chosen marker to recognise and enumerate $CD3^+$ cells (from skin sections) or $caspase\text{-}3^+$ cells (from dLN). Results obtained from this software analysis were randomly compared to those obtained by pathologic inspection to ensure accuracy.

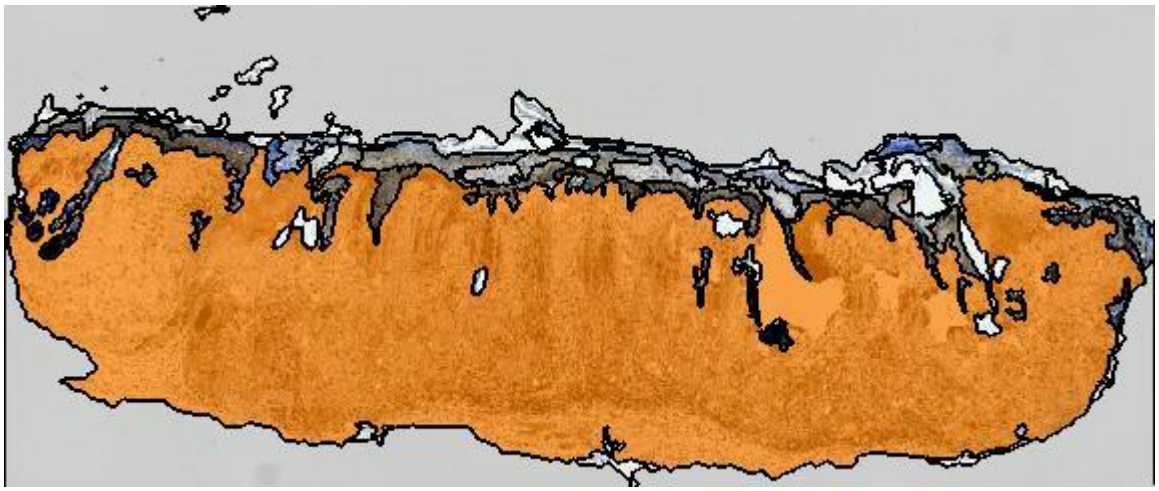


Figure 1: Defining the regions of interest for Definiens Tissue Studio analysis

2.11 Statistical analysis

All statistical analysis was performed using Graphpad version 5. Graft survival was analysed using a log-rank test. All other experiments were analysed by unpaired student *t* test. P values < 0.05 were interpreted as statistically significant. Data was presented as mean ± standard deviation unless otherwise stated.

Chapter 3: Expression and utilisation of OX40 by $CD4^+$ and $CD8^+$ T cells in response to alloantigen

3.1 Introduction

Both $CD4^+$ and $CD8^+$ T cells play a key role in transplant rejection. It is well established that $CD4^+$ T cells alone are sufficient to elicit rejection, demonstrated by the ability of $CD4^+$ T cells to restore graft rejection after reconstitution of T cell deficient mice (36, 328, 329). The importance of $CD4^+$ T cells was also demonstrated in allograft rejection using either anti-CD4 mAb or $CD4^+$ T cell deficient recipient mice (43, 330-332). Although $CD8^+$ T cell responses normally require help from $CD4^+$ T cells, in situations where the $CD4^+$ T cell response was impaired or absent, $CD8^+$ T cells have been shown to be capable of initiating rejection of cardiac (333), skin (334, 335) and islet allografts independently of $CD4^+$ T cells (333, 336).

$CD4^+$ T cells can recognise allogeneic MHC via three pathways of allorecognition; direct, indirect and semi-direct, while $CD8^+$ T cells predominately utilise the direct pathway (337), although there is also evidence that $CD8^+$ T cells can be activated through cross presentation of donor peptides presented in the context of recipient MHC class I (338).

Direct allorecognition depends on the recognition of intact donor MHC/peptide complexes, on passenger APCs, by alloreactive host T cells in the secondary lymphoid tissue (43) (Figure 1; Chapter 1). Allografts which were depleted of passenger leukocytes showed delayed rejection, thus supporting the hypothesis that acute rejection is dependent on the direct pathway (339). In contrast, the indirect pathway involves the presentation of allo-peptides (derived from donor MHC molecules) that are processed and presented in the context of recipient MHC on surface of recipient APCs (47). This pathway is thought to contribute preferentially to chronic rejection as typified by analysis of renal transplant recipients 157 months post transplantation. This cohort of 22 patients contained 13 patients with normal graft function, and 9 with on-going chronic allograft nephropathy. $CD4^+$ T cells isolated from these patients were found to respond to syngeneic peripheral blood mononuclear cells (PBMC) pulsed with allogeneic peptides but were hyporesponsive to donor PBMC suggesting that the indirect pathway of allorecognition persisted but the direct pathway waned with time after transplantation (44).

The relative contribution of these pathways in rejection was dissected using a murine skin allograft model where it was found that ~90% of alloreactive T cells responded via the direct pathway and only 10% through the indirect pathway (340).

Before T cells can elicit damage to the graft, they must have received a signal through the MHC/peptide complex and in addition a costimulatory signal. CD28-CD80/CD86 and CD40-CD154 costimulatory pathways are the most important costimulatory

interactions that lead to optimal T cell activation and proliferation. Furthermore, blockade of these pathways (alone or in combination) has been shown to prolong allograft survival and in some studies has led to long term graft survival in rodents (201, 220, 341, 342) and NHPs (224). However, blockade of CD28 and CD154 interactions rarely led to the induction tolerance of allografts which may have been due in part to the presence of CD28- and/or CD154-independent $CD8^+$ T cells (103, 343). Therefore alternative costimulatory molecules have been described (such as 4-1BB and OX40; described in more detail in Chapter 1), although their precise roles in activation and effector function of $CD4^+$ and $CD8^+$ T cells are yet to be fully elucidated. Therefore, targeting alternative costimulatory pathways that may be utilised by alloreactive $CD4^+$ and $CD8^+$ T cells may be a beneficial strategy to facilitate the induction of tolerance.

OX40 was first identified as a cell surface marker of T cell activation, identified on activated rat $CD4^+$ T cells (251). Since this identification, OX40 has been shown to be expressed on both $CD4^+$ (253) and $CD8^+$ (254, 295) T cells in experimental models as well as in patients with clinical disease; rheumatoid arthritis (287, 344, 345), GvHD (299) and breast cancer (346) suggesting that targeting OX40-OX40L could have a therapeutic benefit in T cell mediated diseases. In rheumatoid arthritis patients, OX40 expression has been detected in the synovial fluid and tissue, while OX40L was found to be expressed on cells lining the synovial tissue (287). These data suggested that $OX40^+$ T cells accumulate selectively in the synovial fluid, while OX40L is expressed on vascular endothelial cells in the synovium and may play an important role in migration of pathogenic T cells into the inflamed joint in rheumatoid arthritis patients.

Peak expression of OX40 was observed 2-3 days after T cell activation, after which OX40 expression was down-regulated, suggesting that OX40 has a delayed mode of action within the immune response. Re-expression of OX40 was rapid (~4 hours) on memory cells after further antigen stimulation (255). CD28 costimulation was not essential for OX40 expression, but could enhance the kinetics and expression levels of OX40 on T cells (256, 257). OX40L has a much wider expression profile than its receptor and is expressed on activated APCs, such as B cells (347), macrophages (267), DC (268, 348) and endothelial cells (269) from hours to days after activation.

Blockade of OX40-OX40L has been shown to ameliorate several autoimmune and inflammatory diseases. In a mouse model of collagen induced arthritis, blocking the OX40-OX40L interaction by the prophylactic administration of a neutralising anti-OX40L mAb ameliorated disease, while administration therapeutically (starting one week post collagen boost) had no impact on disease progression (287). Treatment with anti-OX40L was not able to inhibit the clonal expansion of collagen-reactive T cells in the dLN and both anti-OX40L and IgG treated animals had similar levels of proliferation (287), suggesting that blocking the OX40-OX40L interaction was not able to impact the priming of the collagen-reactive T cells.

In the field of transplantation, the role of OX40-OX40L in response to alloantigen has yet to be fully elucidated. In $CD28^{-/-}CD154^{-/-}$ double knockout mice skin allografts were rejected demonstrating productive T cell responses could still be mounted despite

the absence of CD28-CD80/CD86 and CD40-CD154 pathways (304). However blocking the OX40-OX40L interaction in these mice led to long term allograft survival suggesting that OX40-OX40L pathway is important particularly in the absence of CD28 and CD154 ligation (304). In either $CD4^{-/-}$ or $CD8^{-/-}$ mice, transient blockade of OX40, CD28 and CD154 resulted in long term skin allograft survival, while blocking only one or two of these pathways had minimal effects on graft survival (342). These data suggest that OX40-OX40L may play a role as an alternative costimulatory molecule in solid organ transplantation.

3.2 Aims and Hypothesis

There is now an expanding literature describing the role of the OX40-OX40L costimulatory pathway in $CD4^+$ and $CD8^+$ T cell responses in inflammatory situations such as cancer, infection and autoimmune disease. However, the role of OX40-OX40L in transplantation remains unclear. Therefore, the aim of this chapter was to investigate the expression of OX40 after stimulation with alloantigen and whether the blockade of the OX40-OX40L interaction plays a role in preventing allograft rejection.

We hypothesise that blocking OX40-OX40L interactions will prolong allograft survival mediated by $CD4^+$ and $CD8^+$ T cells.

3.3 Results:

3.3.1 $CD4^+$ and $CD8^+$ T cells upregulate OX40 after stimulation with alloantigen *in vitro*.

Before the role of OX40-OX40L interactions could be investigated in $CD4^+$ and $CD8^+$ T cell responses to alloantigen, we sought to assess the expression of OX40 on $CD4^+$ and $CD8^+$ T cells.

TCR-transgenic $CD4^+$ T cells (TEa; reactive to BALB/c peptide presented in the context of B6 MHC Class II) that recognise alloantigen via the indirect pathway (317), were stimulated with alloantigen (irradiated BALB/cB6F1 splenocytes) *in vitro* before being analysed for the expression of OX40 by flow cytometry. OX40 expression was upregulated after TEa T cell activation, peaking at 4 days post-stimulation (**Figure 1A and D**).

A second set of alloreactive TCR-transgenic $CD4^+$ T cells (-D) were also analysed. -D T cells, in contrast to TEa T cells, recognise $H2IA^s$ via the direct pathway of alloantigen recognition (349). -D $CD4^+$ T cells were stimulated with alloantigen (irradiated B10.S splenocytes) *in vitro* before being analysed for the expression of OX40 by flow cytometry. OX40 expression peaked between day 4 and 5 (**Figure 1B and E**). Therefore, with 2 different sets of $CD4^+$ T cells, OX40 expression was induced upon activation and was upregulated and peaked in a similar time-frame.

In order to assess whether $CD8^+$ T cells expressed OX40, alloreactive TCR-transgenic $CD8^+$ T cells (BM3) that recognise $H2K^b$ via the direct pathway were stimulated with alloantigen (irradiated B6 splenocytes) *in vitro* before being analysed for the expression of OX40 by flow cytometry. OX40 expression was found to be upregulated after T cell activation, peaking at 5 days post-stimulation (**Figure 2A**).

Expression of OX40 was also analysed on naïve polyclonal T cells which were used as whole responder splenocytes with irradiated allogeneic splenocytes, as $CD8^+$ T cells were found to be unable to survive or proliferate when cultured alone with irradiated splenocytes. To assess expression of OX40 on polyclonal T cells CBA splenocytes stimulated with allogeneic irradiated B6 splenocytes were used. Polyclonal $CD4^+$ T cells expressed OX40 from day 3, with maximal expression at day 5 (**Figure 1C and F**), which correlated with activation of the T cells. Polyclonal $CD8^+$ T cells were found to express OX40 from day 3, with maximal expression at day 6 (**Figure 2B**).

3.3.2 Anti-OX40 inhibits proliferation of -D T cells when stimulated with alloantigen *in vitro*.

To test whether OX40 interactions were utilised in $CD4^+$ T cell responses to alloantigen, an anti-OX40 Fab PEG was used which completely blocks the OX40-OX40L interaction without inducing signalling (UCB; unpublished data). -D T cells were stimulated with alloantigen *in vitro* in the presence of either anti-OX40 Fab PEG (anti-OX40) or a PEGylated Fab control (PEG control) and the proliferative response assessed by ^{3H}thymidine incorporation. Anti-OX40 was found to partially inhibit (73%

inhibition ± 13 (standard deviation (SD); $p < 0.0454$) -D T cell proliferation at 10µg/ml compared to PEG control (**Figure 3**). Therefore 10ug/ml was used in future *in vitro* experiments.

3.3.3 Anti-OX40 has no impact on the proliferative response of TEa T cells *in vitro*.

Next we wanted to investigate the impact of OX40 blockade on the proliferative responses of TEa T cells which recognise alloantigen via the indirect pathway. To this end, $5x10^4$ TEa T cells/well were stimulated *in vitro* with allogeneic irradiated splenocytes (BALB/cB6F1; $2.5x10^5$/well). A dose response curve was constructed with the addition of anti-OX40 or PEG control to cultures and the proliferative response measured. In contrast to the -D T cells, there was no impact by anti-OX40 on the proliferative response of TEa T cells at any dose tested compared to PEG control (**Figure 4A**). To ensure this was not due to the TEa response having different kinetics of proliferation compared to the -D T cells, we performed a longitudinal study to analyse the TEa T cell response. To this end, anti-OX40 or PEG control were added to the cultures at 10µg/ml and thymidine incorporation measured on days 3, 4 and 5. Again there was no impact by anti-OX40 at any time-point analysed (**Figure 4B**). These data suggest that anti-OX40 preferentially impacts $CD4^+$ T cell responses which recognise alloantigen via the direct pathway, but not the indirect pathway.

3.3.4 Anti-OX40 partially inhibits the response of BM3 transgenic $CD8^+$ T cells to alloantigen *in vitro*.

To test whether OX40 interactions were utilised in $CD8^+$ T cell responses to alloantigen, BM3 T cells were stimulated with alloantigen *in vitro* in the presence of the anti-OX40 or PEG control and the proliferative response assessed. Anti-OX40 was found to partially inhibit (74% ± 2; $p< 0.0004$) BM3 T cell proliferation at 10µg/ml compared to PEG control (**Figure 5A**).

Given that OX40 has been implicated in the effector and memory stages rather than the initiation phase of the immune response (256), the ability of anti-OX40 to inhibit proliferation was tested at various time-points following stimulation (**Figure 5B**). Blockade of OX40 was found to inhibit BM3 proliferation similarly at all time-points studied (43-49% inhibition compared to PEG control; **Figure 5B**). Co-culture of stimulator and responder cells with and without the addition of the PEG control showed that the addition of a PEG control had no impact on the allo-response *in vitro*.

We have shown that $CD8^+$ T cells did not express OX40 until 4 days post activation (**Figure 2**). We therefore tested whether anti-OX40 needed to be present coincident with $CD8^+$ T cell priming or whether anti-OX40 could still attenuate BM3 T cell proliferation when added after stimulation. Anti-OX40 added at the time of co-culture setup (day 0) resulted in a 46% ± 21 inhibition ($p< 0.0308$) of BM3 T cell proliferation compared to PEG control (**Figure 6**). Similar results were observed if anti-OX40 was added on day +1 (57% inhibition ± 13; $p< 0.0062$) or on day +2 (42% inhibition ± 8; p<

0.0368). However, anti-OX40 failed to inhibit proliferation when added to cultures on day +3 (**Figure 6**). These data are in agreement with data published by Curry *et al* who demonstrated that OX40 blockade was critically dependent on the time of addition of an OX40 immunoglobulin fusion protein (301).

3.3.5 Blockade of OX40-OX40L interactions attenuates skin allograft rejection mediated by naïve $CD4^+$ and $CD8^+$ T cells.

Since anti-OX40 inhibited the response of alloreactive -D and BM3 T cells *in vitro*, we hypothesised that blocking OX40 costimulation would attenuate the rejection of skin allografts by $CD4^+$ and $CD8^+$ T cells *in vivo*. To this end, we adoptively transferred either $1x10^5$ TEa, -D or BM3 T cells into syngeneic $Rag^{-/-}$ mice, and one day later the mice received a skin allograft. Mice were treated with PEG control or anti-OX40 from the time of transplant until day 28 post-transplant. Mice reconstituted with TEa T cells and given PEG control acutely rejected their skin allografts, whilst allograft survival was significantly prolonged in mice treated with anti-OX40 (Median survival time (MST) 49 vs. 18.5 days; $p< 0.0004$) (**Figure 7A**), despite the finding that OX40 blockade had no impact on TEa T cell responses *in vitro*.

TEa T cells respond to alloantigen via the indirect pathway, therefore we next wanted to use -D T cells which recognise alloantigen via the direct pathway as a source of effector T cells. We found that mice treated with anti-OX40 had significantly prolonged allograft survival compared to mice treated with PEG control (MST 62 vs, 22 days; $p< 0.0005$; (**Figure 7B**)).

OX40 blockade had a significant impact on graft survival mediated by $CD4^+$ T cells therefore we next wanted to investigate the role of OX40-OX40L on $CD8^+$ T cells. To this end, administration of anti-OX40 given from the time of skin transplant significantly prolonged allograft survival in $Rag^{-/-}$ mice that had received adoptive transfer with naïve BM3 T cells compared to the PEG control group (MST 59 vs.19.5 days; $p< 0.0014$; **Figure 7C**). Mice that received naïve BM3 T cells rejected skin allografts in a similar fashion whether treated with PBS or PEG control (**Figure 7C**) therefore only the PEG control was administered in subsequent experiments.

To assess whether OX40 blockade could impact polyclonal T cell responses, we adoptively transferred either naïve polyclonal $CD4^+$ or $CD8^+$ T cells into syngeneic $Rag^{-/-}$ mice. The following day mice received an $H2^{b+}$ (B6) skin allograft together with either anti-OX40 or PEG control (**Figure 8A**).

Administration of anti-OX40 given from the time of skin transplant significantly prolonged allograft survival in mice that had received naïve polyclonal $CD4^+$ T cells compared to the PEG control group (MST 97 vs.14 days; $p< 0.0006$; **Figure 8B**). Two mice out of six still had intact skin grafts at day 100. This dramatic impact of OX40 blockade on graft survival was also seen in a second strain combination when polyclonal B6 $CD4^+$ T cells were adoptively transferred into syngeneic $Rag^{-/-}$ recipients and mice received an allogeneic skin transplant (CBA) (MST 67 vs. 13 days; $p< 0.0067$; data not shown). Skin allografts transplanted to $Rag^{-/-}$ mice that had received naïve polyclonal $CD8^+$ T cells and anti-OX40 were also found to enjoy prolonged survival compared to PEG control treated mice (MST 57 vs. 17.5 days; $p< 0.0006$; **Figure 8C**).

As TEa recognised alloantigen via the indirect pathway it was likely that the polyclonal $CD4^+$ T cells elicited rejection via the direct pathway (due to far higher T cell precursor frequency), therefore we sought to develop a model that restricted allograft rejection to indirect polyclonal T cells. To this end, we adoptively transferred $1x10^6$ naïve polyclonal $CD4^+$ T cells into syngeneic $Rag^{-/-}$ recipients. One day later the mice received a MHC Class $II^{-/-}$ skin allograft and administered anti-OX40 or PEG control from the time of transplant (**Figure 9A**). The use of an MHC Class $II^{-/-}$ allogeneic skin graft removed the ability of MHC class II alloantigen to be recognised by the direct pathway and forced transferred $CD4^+$ T cells to recognise alloantigen in the context of self-MHC (indirect). Mice treated with anti-OX40 showed a significant prolongation in graft survival compared to those treated with PEG control (MST 100 vs. 16 days; $p<0.0014$; **Figure 9B**).

Taken together, these data demonstrate that the OX40-OX40L pathway plays a key role in facilitating the rejection of allografts by naïve $CD4^+$ and $CD8^+$ T cells.

3.3.6 Anti-OX40 serum levels peak at day 28, and decline by the first signs of rejection.

Interestingly, although skin allografts were not rejected whilst the anti-OX40 was administered subsequently all allografts were rejected upon cessation of therapy at 28 days post transplantation irrespective of which T cells were mediating rejection. This suggests that anti-OX40 does not result in the induction of tolerance.

We felt that this was likely to correlate with serum levels of anti-OX40 and the rate of clearance from the mouse. PEGylation has been shown to significantly prolong the *in vivo* half-life of Fab fragments (325). Therefore we measured the serum levels of anti-OX40 by ELISA at several time points to establish the pharmacokinetics of the blocking reagent.

$1x10^5$ BM3 T cells were adoptively transferred into syngeneic $Rag^{-/-}$ mice and the following day mice received an allogeneic skin transplant ($H2K^b$). Anti-OX40 or PEG control were dosed 100mg/kg s.c twice weekly for 27 days and serum was obtained at different time points post transplantation (**Figure 10**). Day 10 was chosen as we have previously shown consistent T cell priming in the dLN (axillary) at this time (350), day 28 was one day after the last dose of the regimen and the last time point analysed was when the first signs of rejection occurred, day 38 in this particular experiment. As expected the peak of the anti-OX40 levels was at day 28 (6.8mg/ml; immediately after the dosing regimen had finished). Anti-OX40 serum levels had dramatically declined by day 38 (0.05mg/ml; **Figure 10**). The half-life of anti-OX40 is 56 hours (UCB; unpublished data). These data show that anti-OX40 was cleared from the serum prior to skin allograft rejection.

3.3.7 Naïve $CD8^+$ T cells express both OX40 and OX40L, while $CD4^+$ T cells do not express OX40L after stimulation with alloantigen.

Finally, we sought to analyse the expression pattern of OX40 and OX40L during allograft rejection. Surprisingly, *in vivo*, we failed to identify any APC (within the periphery or within the graft) which expressed OX40L (data not shown). This led to the

hypothesis that OX40L may also be expressed by activated T cells, a finding previously reported on activated $CD4^+$ T cells (273).

To investigate whether either $CD4^+$ and/or $CD8^+$ T cells expressed OX40L, we co-cultured polyclonal or transgenic T cells with irradiated allogeneic stimulators and measured OX40L expression by flow cytometry. Expression of OX40L was analysed on polyclonal T cells which were used as whole responder splenocytes stimulated with irradiated allogeneic splenocytes, as they were unable to survive or proliferate when cultured alone. As previously shown, both naïve $CD4^+$ and $CD8^+$ T cells were found to express OX40 after stimulation with alloantigen *in vitro* (**Figure 1 and 2**). Surprisingly, both naïve BM3 and polyclonal $CD8^+$ T cells also expressed OX40L (**Figure 11A** and **11B**), however $CD4^+$ T cells did not express OX40L at any time-point after activation studied (**Figure 12**).

In order to validate this unexpected result we assessed the expression of both OX40 and OX40L on activated BM3 and TEa T cells in the secondary lymphoid organs of mice that had received an allogeneic skin graft. $1x10^5$ TEa or BM3 T cells were adoptively transferred into syngeneic $Rag^{-/-}$ recipients. One day later the mice received an allogeneic skin graft and were sacrificed when macroscopic changes were observed on the graft, thus pre-empting rejection. The contralateral and draining axillary lymph nodes (cLN and dLN respectively) were harvested for analysis of OX40 and OX40L (**Figure 13**) expression by flow cytometry. Confirming the *in vitro* data, OX40 was expressed on TEa T cells in the cLN and dLN (**Figure 13A**), but OX40L (**Figure 13B**)

was not expressed on T cells in any of these compartments and spleen (data not shown). In contrast, when we used a similar experimental setup to assess OX40 and OX40L on activated BM3 T cells in the dLN of mice that had received an allogeneic skin graft, we found both OX40 and OX40L were expressed by $CD8^+$ T cells (**Figure 13C**).

These results raised the possibility that costimulation through OX40-OX40L may have been provided by the ligation of OX40 by OX40L expressed by T cells rather than APC at least in the case of $CD8^+$ T cells. Indeed, Soroosh *et al* had previously demonstrated that OX40-OX40L interactions could have occurred through a T cell-T cell interaction (273). To test this hypothesis we stimulated BM3 T cells with either an immortalised allogeneic DC cell line (DC2.4) or with anti-CD3/CD28 synthetic beads. The DC2.4 cells expressed other costimulatory molecules as well OX40L (data not shown) whilst the anti-CD3/CD28 beads were devoid of costimulatory ligands. When anti-OX40 was added to the co-cultures, no impact on ^{3H}-thymidine incorporation was observed when the stimulators were DC2.4 (**Figure 14A**). However, when the BM3 T cells were stimulated with anti-CD3/CD28 beads, anti-OX40 inhibited ^{3H}-thymidine incorporation (59% inhibition ± 8 at day 4/5; **Figure 14B**) despite the absence of APC-derived costimulation.

These data suggest that OX40-OX40L costimulation was provided via T cell-T cell interactions either in *cis* or *trans*. These data provide the first demonstration of OX40-OX40L acting via a T cell-T cell interaction on $CD8^+$ T cells. Indeed, under these conditions OX40 (**Figure 15A and 15C**) and OX40L (**Figure 15B and 15D**) were expressed by BM3 T cells after stimulation with anti-CD3/CD28 beads.

In summary, we have shown that OX40 is expressed on both $CD4^+$ and $CD8^+$ T cells after stimulation with both alloantigen and anti-CD3/CD28 beads. Blockade of OX40-OX40L interaction attenuated skin allograft rejection mediated by TCR-transgenic and polyclonal T cells. We have also demonstrated that OX40-OX40L interations could costimulate $CD8^+$ T cell cell responses to alloantigen via a T cell-T cell intercation, although we did not find evidence for this type of interation during $CD4^+$ T cell responses.

3.4 Discussion

The CD28-CD80/CD86 and CD154-CD40 costimulatory pathways are believed to be the most important costimulatory interactions for T cell activation and initial proliferation. A number of studies have shown that blockade of these pathways, either alone or in combination, could prolong allograft survival in rodents (201, 220, 341, 342) and in NHP (224). Despite the experimental success of blocking these costimulatory interactions, tolerance has rarely been induced. This may be due in part to the presence of CD154 and/or CD28 independent $CD8^+$ T cells (103, 343) or memory T cells that are known to have less of a reliance on costimulation (95). Therefore, the identification and therapeutic targeting of other costimulatory pathways that may be utilised by $CD4^+$ and $CD8^+$ T cells responding to allografts may be a beneficial adjunct to such potential tolerance induction therapies.

A range of alternate costimulatory molecules have been described which includes OX40 and OX40L (305, 351, 352). Mice lacking OX40 or OX40L have been shown to have defects in $CD4^+$ T cell proliferative responses but little impact has been shown on $CD8^+$ T cells (353-356). In other systems, where mice have been engineered to over express OX40L, these mice showed expansion of $CD4^+$ T cells but not $CD8^+$ T cells (348, 357). These initial data suggested OX40-OX40L interactions had little or no impact on generation of productive $CD8^+$ T cell responses. However, other studies using $OX40^{-/-}$ antigen-specific $CD8^+$ T cells have shown OX40-OX40L interactions may impact the survival but not the function of $CD8^+$ T cells after immunisation (306). This is in line with data showing OX40-OX40L controls the survival of $CD4^+$ T cells (256, 294).

In agreement with the literature, we have shown that OX40 was expressed on activated $CD4^+$ (Figure 1) and $CD8^+$ T cells (Figure 2). These data also suggest that OX40 could act as a biomarker for T cells activated 2-3 days previously which are responding to alloantigen. Therefore providing an advantage as a biomarker compared to other conventional markers such as CD69 and CD25. Furthermore, the kinetics of expression suggests that OX40 may be more important for effector and memory T cell generation i.e. in the propagation of the response, rather than in initial T cell priming. However, the proliferation of -D and BM3 T cells was clearly diminished in the absence of OX40 signalling (Figures 3 and 5), while OX40 blockade has no impact on TEa T cells (Figure 4).

Blocking OX40 markedly impacted the ability of alloreactive $CD4^+$ and $CD8^+$ T cells to elicit rejection of skin allografts *in vivo* (Figures 7 and 8). Importantly we observed a comparable prolongation in allograft survival between transgenic and polyclonal $CD4^+$ and $CD8^+$ T cells, providing evidence that TCR transgenic T cells behave in a similar fashion to their wild-type counterparts. Anti-OX40 potently inhibited rejection mediated by both $CD4^+$ and $CD8^+$ T cells even in the presence of other costimulatory molecules, such as CD28 and CD154 which suggests that OX40 is required to propagate $CD8^+$ T cell responses to an allograft. This observation is supported by the observation that $OX40^{-/-}$ $CD8^+$ T cells became activated and expanded in a normal manner (358) but failed to maintain this expansion over time. This suggests that OX40 signalling is important for T cell survival or their ability to maintain division in the long-term.

Importantly, while anti-OX40 continued to be administered allografts remained free from rejection, however, grafts were rejected after the cessation of therapy (Figures 7, 8 and 9). It therefore appears that blockade of OX40-OX40L interactions failed to induce tolerance. Other evidence for the lack of tolerance induction is borne out of the observation that skin allografts were rejected after the anti-OX40 therapy was discontinued and the reagent cleared from the systemic circulation (Figure 10).

The failure of OX40 blockade to induce tolerance could be due to a number of factors. Firstly, not all activated $CD4^+$ and $CD8^+$ T cells expressed OX40 (Figures 1 and 2), suggesting some of the T cells are not dependent on the OX40-OX40L pathway. Numerous other costimulatory molecules within the Ig and TNFR superfamilies can make up for the lack of OX40 signalling due to the high degree of redundancy within the system, for example 4-1BB has been shown to costimulate T cell responses independently of CD28 and have a preferential role in $CD8^+$ compared to $CD4^+$ T cell responses (359), suggesting that 4-1BB is a possible candidate to provide OX40-independent costimulation to $CD8^+$ T cells.

Secondly, in this model not all T cells simultaneously encounter alloantigen following transplantation for example naïve BM3 T cells could be detected up to 50 days post transplantation despite allograft rejection (N.D Jones; personal communication). As naïve T cells did not express OX40 these cells may be able to elicit rejection upon alloantigen recognition and activation following the clearance of anti-OX40.

Interestingly, OX40 blockade was more potent when polyclonal $CD4^+$ T cells were transferred into syngeneic $Rag^{-/-}$ mice with a MHC Class $II^{-/-}$ skin graft than a wild type skin graft (Figures 8 and 9). MHC Class $II^{-/-}$ skin graft limited the response solely to the indirect pathway. The precursor frequency of alloreactive T cells which could respond to alloantigen via the indirect pathway was much lower compared to those alloreactive T cells responding to the direct pathway (340). This may explain why OX40 blockade is more potent when $CD4^+$ T cells are restricted to the indirect pathway.

Lastly, an important caveat of this adoptive transfer model is that purified naïve $CD4^+$ and $CD8^+$ T cells were transferred to $Rag^{-/-}$ knockout mice therefore these animals have no alloreactive Treg. Indeed the TCR-transgenic T cells used in these studies were devoid of T cells with regulatory phenotype (data not shown) and the polyclonal T cells were isolated as $CD4^+CD44^-CD45RB^{hi}$ and $CD8^+CD44^-$ cells to ensure Tm and Treg were excluded. Although a strict sorting strategy was used, it is possible that T cells could be converted into Treg (so called inducible Treg) by OX40 blockade, although we would suggest that this is unlikely as all the allografts were rejected following clearance of anti-OX40 (Figures 7 and 8). Treg have been shown to be critical for the maintenance of long-term graft survival following costimulatory molecule blockade in immunocompetent mice raising the possibility that tolerance would never be attainable in this model because of their absence (360).

Finally, an interesting and unexpected observation from these data was that both OX40 and OX40L were expressed by alloreactive $CD8^+$ T cells, but not $CD4^+$ T cells, following alloantigen or polyclonal stimulation (Figures 11, 12 and 15). OX40L was

originally described as a protein upregulated by the transcriptional HTLV-I gene product tax (263). From studies in the literature, OX40L has been shown to be induced, rather than be constitutively expressed, on professional APC such as B cells (347), DC (268) and macrophages (267), Langerhans cells (361) as well as other cell types including endothelial cells (269), NK cells (272) and smooth muscle cells (362). We found no evidence of OX40L on non-T cells *in vivo*, which may have been a result of differential priming and TLR triggering. This led us to investigate if OX40L was expressed by T cells.

OX40L has also been described on the surface of activated T cells (363, 364) which could then contribute to a T cell-T cell interaction (273). Certainly, this is not without precedent as there is an accumulating body of evidence that antigen experienced T cells can express accessory molecules usually associated with APC e.g. CD80, CD86, CD70 and CD40 (365-369). Bourgeois *et al* demonstrated that activated $CD8^+$ T cells express CD40, which could bind to CD154 on $CD4^+$ T cells, through a T cell–T cell interaction (368). This $CD4^+$-$CD8^+$ T cell interaction was found to support the generation of $CD8^+$ memory T cells.

To ensure the expression of OX40L we observed on $CD8^+$ T cells was not simply due to the transfer of OX40L from the stimulator splenocytes we stimulated BM3 T cells with anti-CD3/CD28 beads (Figure 15). However we could not clearly define if one cell expressed both OX40 and OX40L or whether ligand and receptor were expressed on different cells. Soroosh *et al* demonstrated OX40L was expressed by activated T cells,

but the presence of OX40 was able to cause downregulation of OX40L on the same or adjacent cells thus making identification of cells expressing both markers more complex (273). These and other data demonstrated that the expression of accessory molecules on T cells was dependent on the activation status of the T cell (370). Indeed we showed that the BM3 T cells which expressed OX40L were $CD44^+$ suggesting an antigen-experienced or memory phenotype. However, our results did not dissect which molecule, OX40 or OX40L, provided the signal that is critical for T cell survival although previous reports have suggested that OX40 delivers the signal for $CD4^+$ T cell survival, rather than OX40L (273). The expression (Figure 15) and functional (Figure 14) data suggests OX40-OX40L can interact through a T cell-T cell interaction thus providing an additional mechanism to amplify the T cell response.

In summary, we have shown that blockade of OX40-OX40L interactions prevented skin allograft rejection mediated by both naïve $CD4^+$ and $CD8^+$ T cells. A significant advantage to targeting OX40-OX40L interactions compared to other costimulatory molecules is their transient expression pattern following activation which allows specific targeting of alloreactive $CD4^+$ and $CD8^+$ T cells following transplantation without affecting the peripheral T cell repertoire.

3.5 Figures

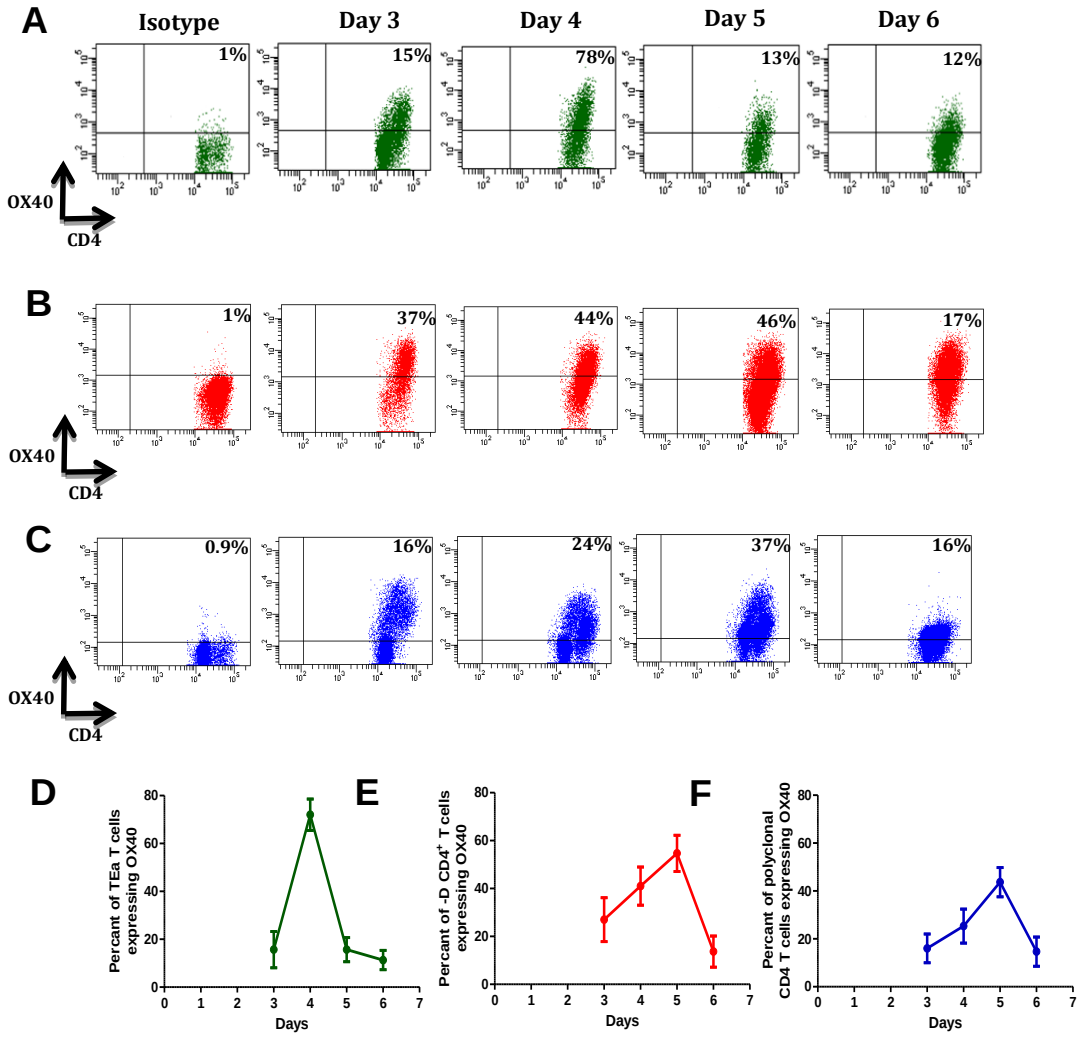


Figure 1: CD4$^+$ T cells upregulate OX40 after stimulation with alloantigen *in vitro*
2x10^5 polyclonal or 5x10^4 -D or TEa T cells were cultured *in vitro* with 4x10^5 or 2.5x10^5 allogeneic irradiated stimulator splenocytes, respectively. Expression of OX40 on TEa (A and D) and -D (B and E) T cells and polyclonal CD4$^+$ T cells (C and F) was determined by flow cytometry. Quadrant gates were set using an isotype control antibody. Results are expressed as mean % ± SD, performed in triplicate and are representative of 3 independent experiments.

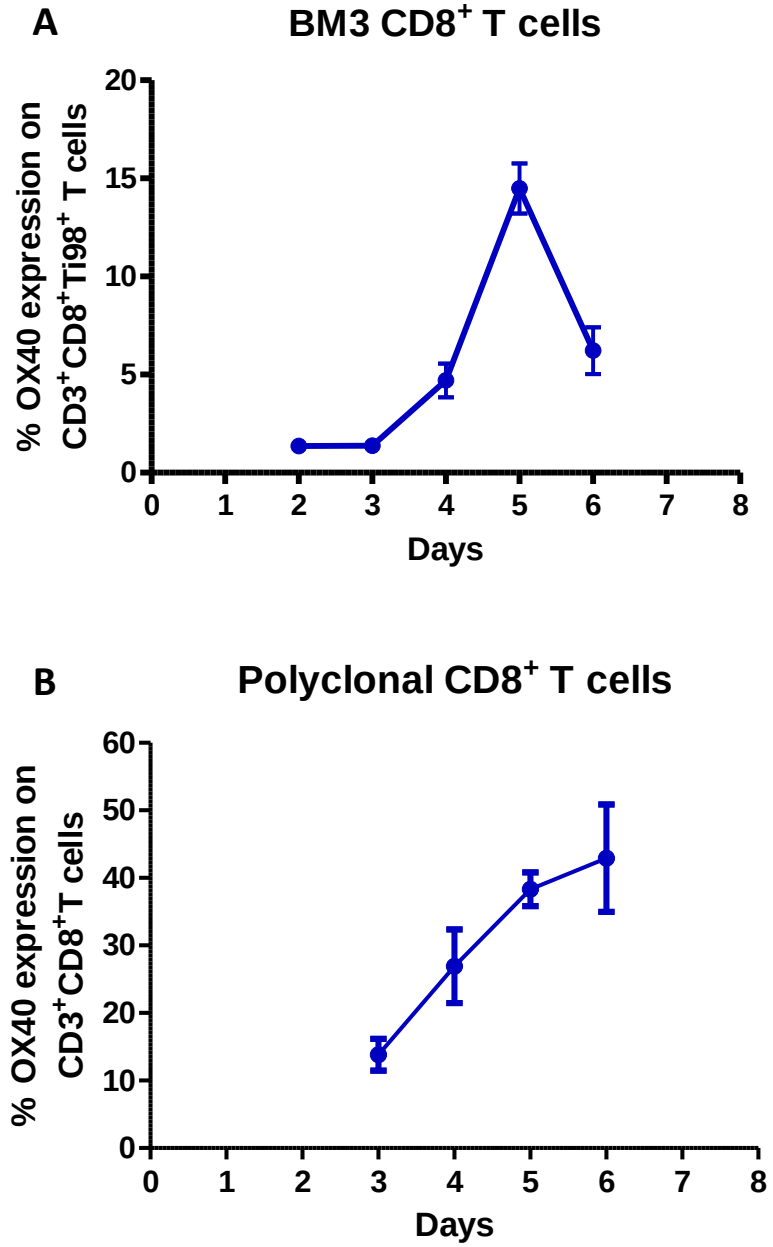


Figure 2: BM3 and polyclonal CD8$^+$ T cells express OX40 after stimulation with alloantigen *in vitro*

Expression of OX40 on BM3 (A) and polyclonal (B) CD8$^+$ T cells was analysed by flow cytometry. 2.5x10^4 BM3 or 2x10^5 polyclonal CD8$^+$ T cells were cultured with either 1x10^5 or 4x10^5 allogeneic irradiated stimulator splenocytes for up to 6 days. Quadrant gates on dot plots were set using an isotype control antibody. Results are expressed as mean % ± SD, performed in triplicate and are representative of 4 independent experiments.

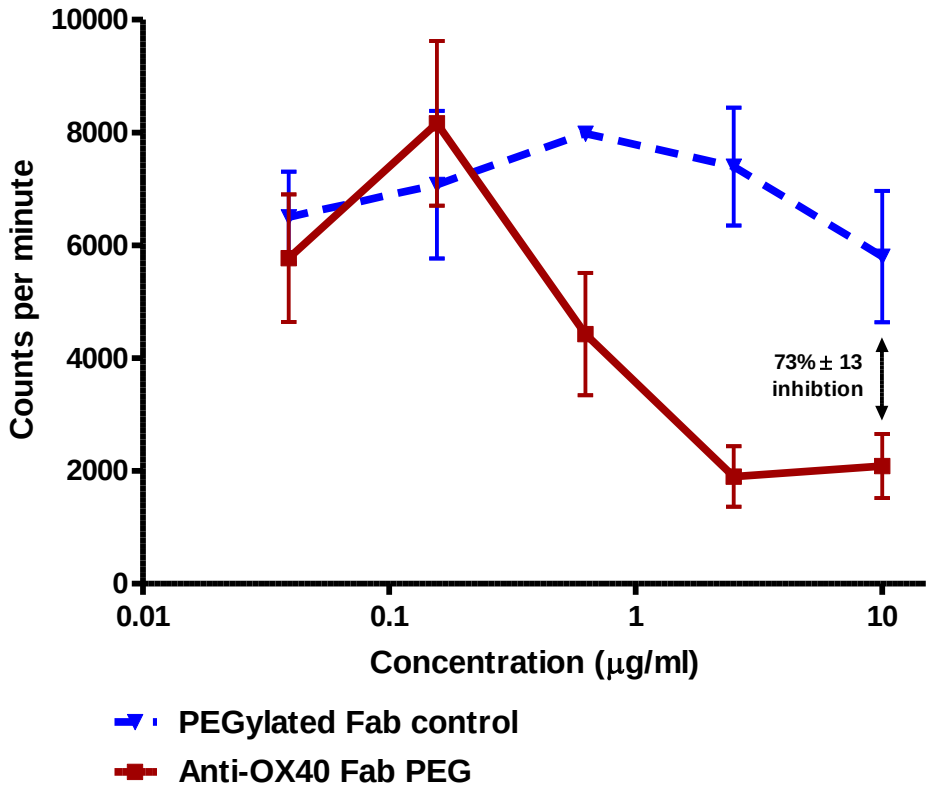


Figure 3: Anti-OX40 inhibits alloantigen-driven proliferation of -D CD4$^+$ T cells *in vitro*

$5x10^4$/well -D $CD4^+$ T cells and $2.5x10^5$/well irradiated B10.S splenocytes were cultured in a mixed lymphocyte reaction. Anti-OX40 or PEG control were added at varying concentrations (10-0.04μg/ml) to appropriate wells. Cells were pulsed with ^{3H}-thymidine on day 4, and harvested 18 hrs later. Results are expressed as mean counts per minute ± SD, performed in triplicate and are representative of 4 independent experiments.

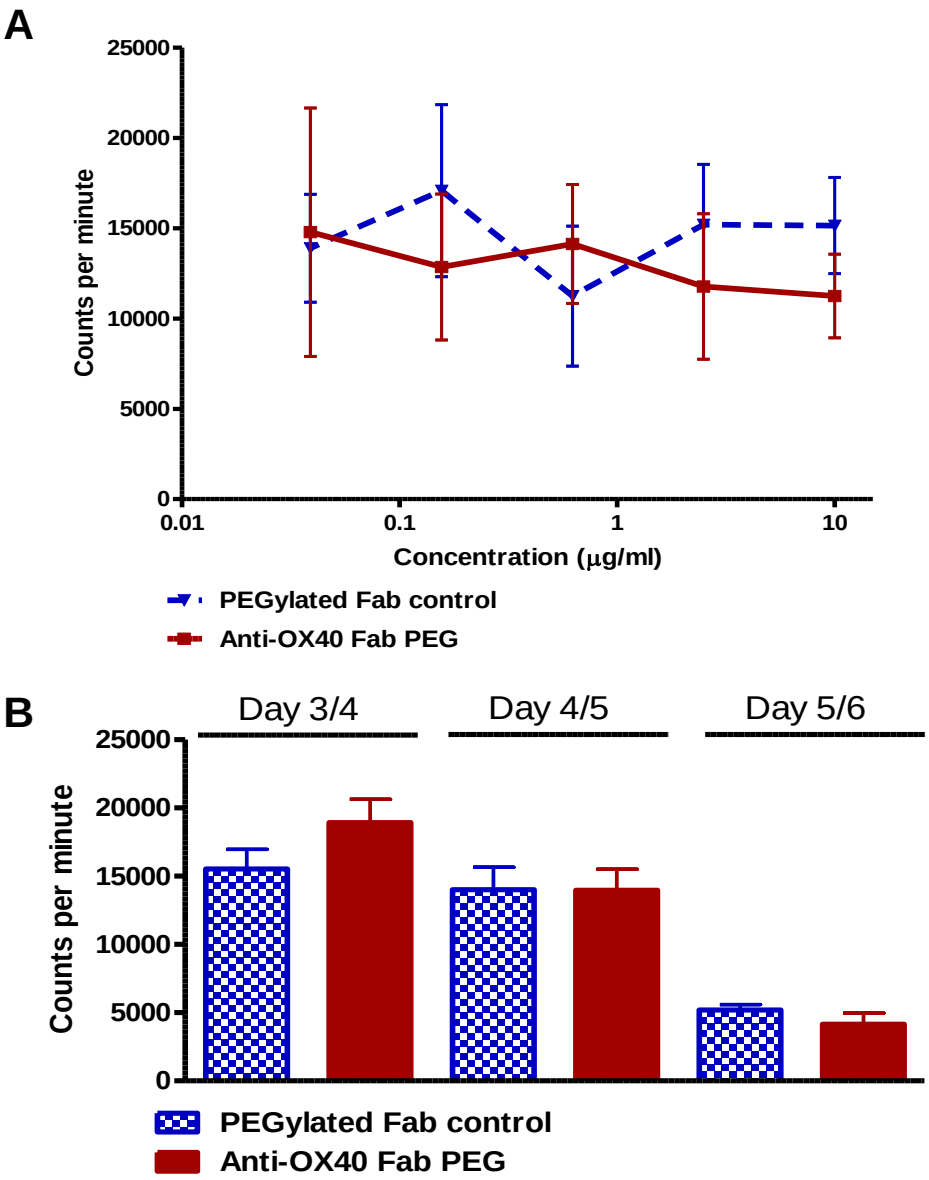


Figure 4: Anti-OX40 has no impact on the proliferation of TEa CD4$^+$ T cells following alloantigen stimulation *in vitro*

$5x10^4$/well TEa T cells and $2.5x10^5$/well irradiated allogeneic splenocytes were cultured for various lengths of time. A) Anti-OX40 or PEG control were added at varying concentrations (10-0.04µg/ml) to appropriate wells. Cells were pulsed with ^{3H}-thymidine on day 4, and harvested 18hrs later. B) Anti-OX40 or PEG control were added to the appropriate wells at 10µg/ml. Cells were pulsed with ^{3H}-thymidine on day 3, 4 or 5, and harvested 18 hrs later. Results are expressed as mean counts per minute ± SD, performed in triplicate and are representative of 4 independent experiments.

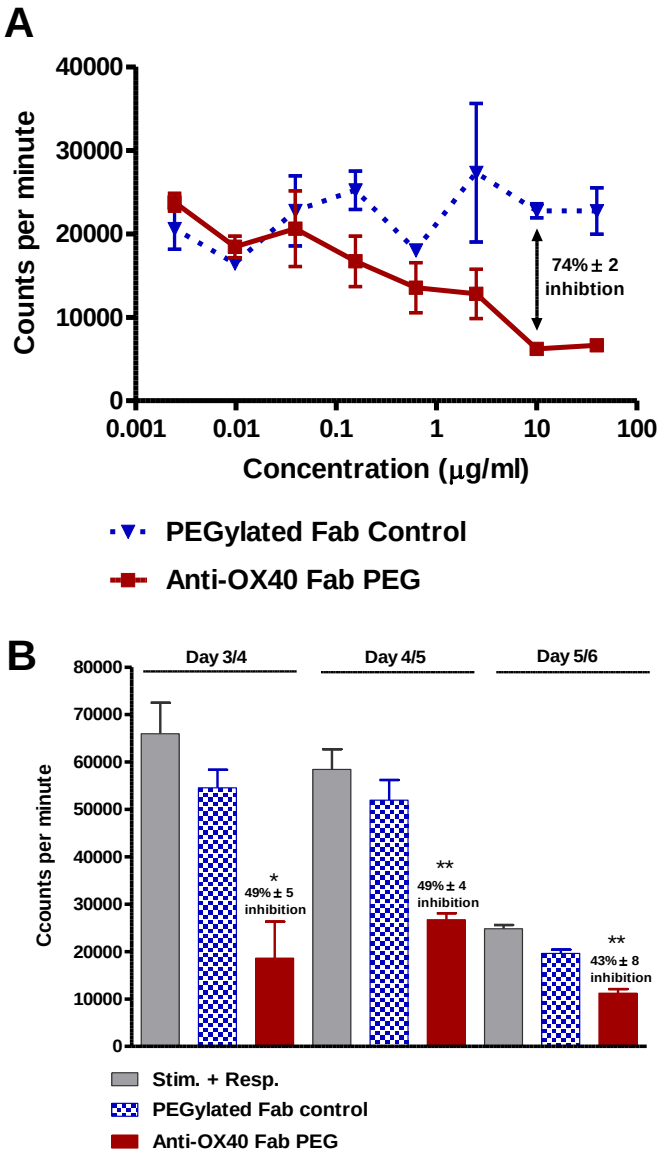


Figure 5: Anti-OX40 partially inhibits the proliferation of BM3 CD8$^+$ T cells when stimulated with alloantigen *in vitro*

2.5x10^4/well BM3 T cells and 1x10^5/well irradiated B6 splenocytes were cultured for various lengths of time. A) Anti-OX40 or PEG control were added at varying concentrations (40-0.00244µg/ml) to appropriate wells. Cells were pulsed with ^{3H}-thymidine on day 4, and harvested 18 hours later. B) Anti-OX40 or PEG control were added to the appropriate wells at 10µg/ml. Cells were pulsed with ^{3H}-thymidine on day 3, 4 or 5, and harvested 18 hours later. Results are expressed as mean counts per minute ± SD, performed in triplicate and are representative of 3 independent experiments. The unpaired student t test was used to compare differences between anti-OX40 and PEG control cultures where * represents $p<0.05$; ** represents $p<0.01$.

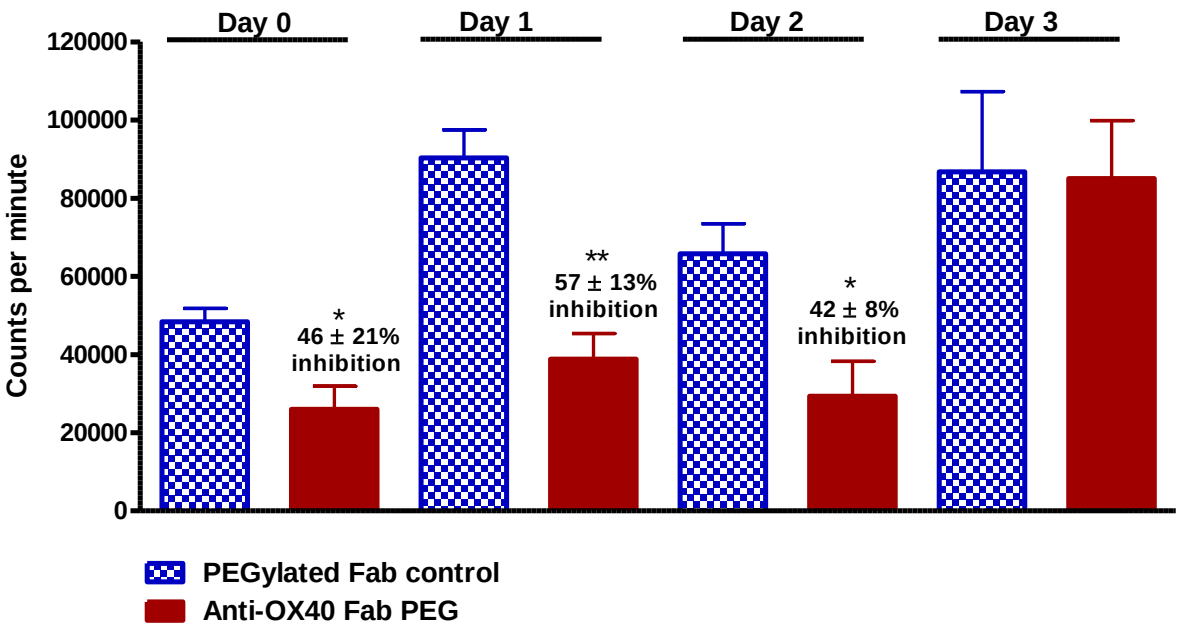


Figure 6: Blockade of OX40 before T cell activation partially blocks the proliferation of BM3 T cells

$2.5x10^4$/well BM3 T cells and $1x10^5$/well irradiated B6 splenocytes were cultured for 4 days; cells were pulsed for the last 18 hours of culture with ^{3H}-thymidine. Anti-OX40 or PEG control were added to the appropriate wells at 10µg/ml on either day 0, 1, 2 or 3 as indicated. Results are expressed as mean counts per minute ± SD, performed in triplicate and are representative of 3 independent experiments. The unpaired student t test was used to compare differences between anti-OX40 and PEG control cultures where * represents $p<0.05$; ** represents $p<0.01$.

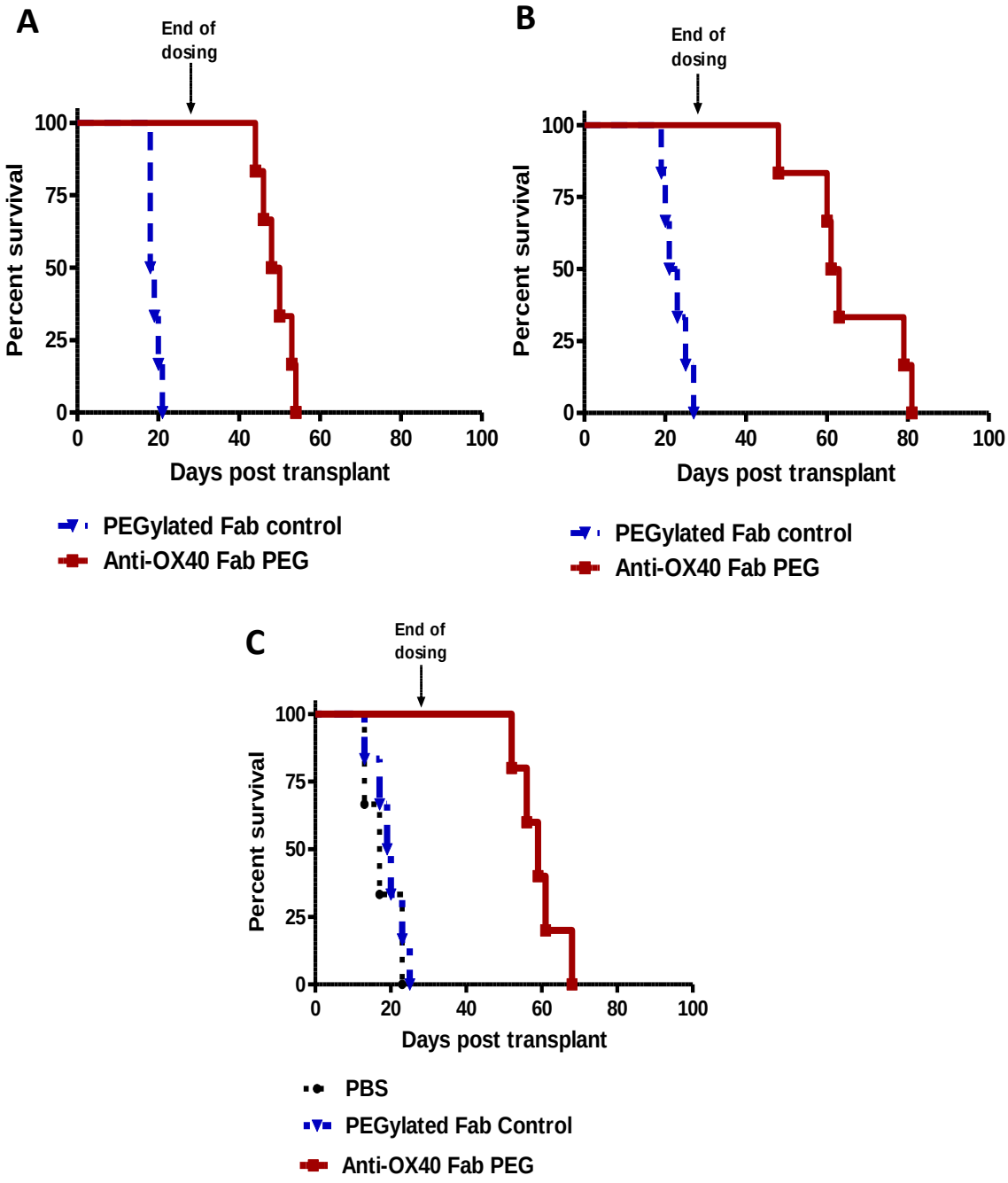


Figure 7: Blocking the OX40-OX40L pathway prevents skin allograft rejection mediated by naïve transgenic CD4$^+$ and CD8$^+$ T cells

1x10^5 naïve TEa (A), -D (B) CD4$^+$ T cells or BM3 CD8$^+$ T cells (C) were adoptively transferred into syngeneic Rag$^{-/-}$ mice. One day later, mice received an allogeneic skin graft (BALB/cB6F1, B10.S, B6 respectively) and either anti-OX40 or PEG control administered s.c at 100mg/kg twice weekly for 4 weeks. Kaplan-Meier survival curves show skin allograft rejection kinetics. (n= 6-8 mice per group).

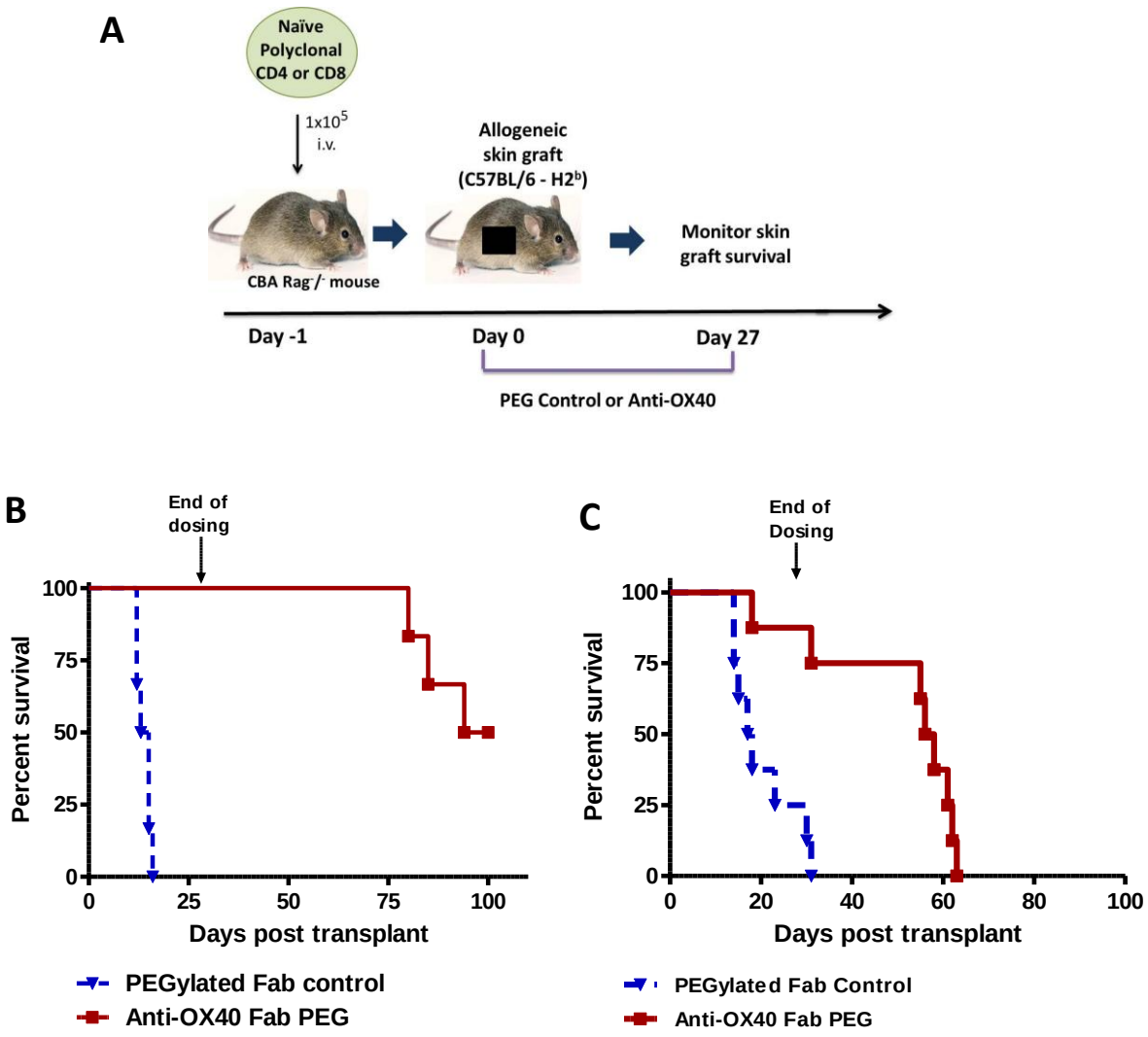


Figure 8: OX40 blockade prevents skin allograft rejection mediated by naïve polyclonal CD4$^+$ and CD8$^+$ T cells

A) 1x10^5 polyclonal, naïve CD4$^+$ or CD8$^+$ T cells were adoptively transferred into syngeneic CBA Rag$^{-/-}$ mice. One day later, mice received an allogeneic (B6; H2^b) skin graft and anti-OX40 or PEG control administered s.c at 100mg/kg twice weekly for 4 weeks. Kaplan-Meier survival curves show skin allograft rejection kinetics mediated by polyclonal naïve CD4$^+$ T cells (B) and polyclonal naïve CD8$^+$ T cells (C). (n = 6-8 mice per group).

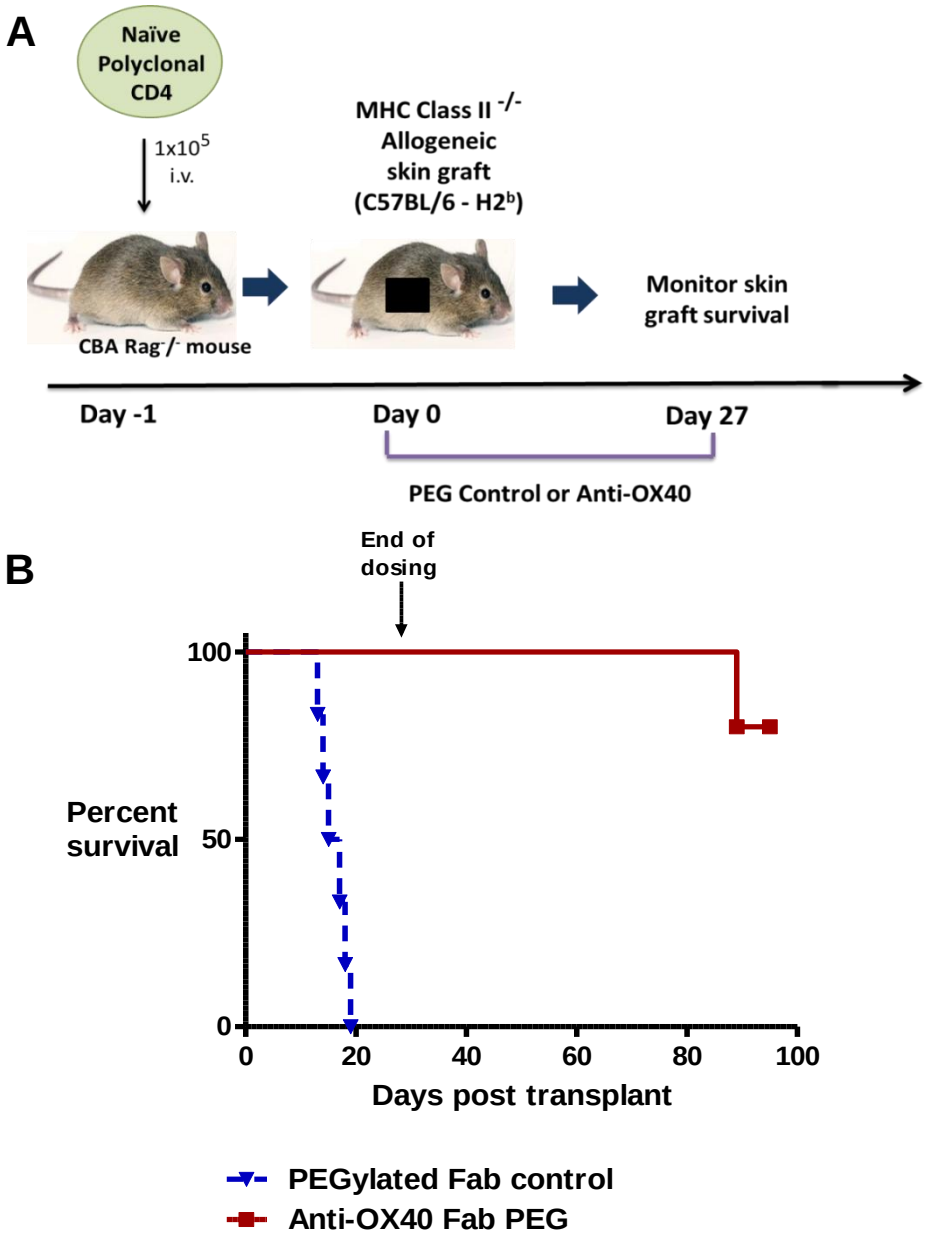


Figure 9: Anti-OX40 prevents skin allograft rejection mediated by polyclonal CD4$^+$ T cells, which can only recognise alloantigen via the indirect pathway

A) 1x10^6 naïve polyclonal CD4$^+$ T cells were adoptively transferred into syngeneic CBA Rag$^{-/-}$ mice. One day later, mice received an allogeneic skin graft deficient in MHC Class II (B6 background) and anti-OX40 or PEG control was administered s.c at 100mg/kg twice weekly for 4 weeks. B) Kaplan-Meier survival curves show skin allograft rejection kinetics mediated by polyclonal naïve CD4$^+$ T cells. (n = 6 mice per group).

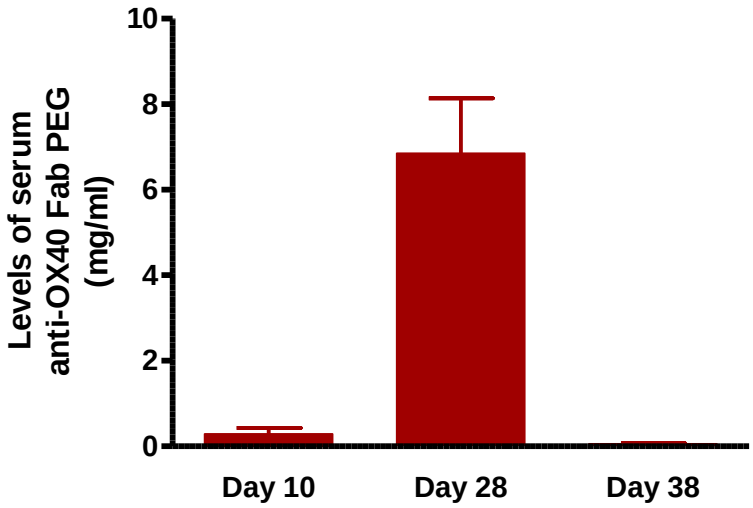


Figure 10: The serum concentration of anti-OX40 peaks in the serum on day 28 but is absent at the time of rejection

$1x10^5$ naïve BM3 $CD8^+$ T cells were adoptively transferred into syngeneic CBA $Rag^{-/-}$ mice (data pooled from both sets of mice). One day later, mice received an allogeneic ($H2^{b+}$) skin graft and anti-OX40 or PEG control were administered s.c. at 100mg/kg twice weekly for four weeks. Blood was removed by cardiac puncture on days 10, 28 and 38 (point of rejection) post transplantation. The concentration of serum anti-OX40 (710 Fab PEG) was measured by ELISA. The data is presented as mean concentration ± SD. (n = 3 mice per time-point).

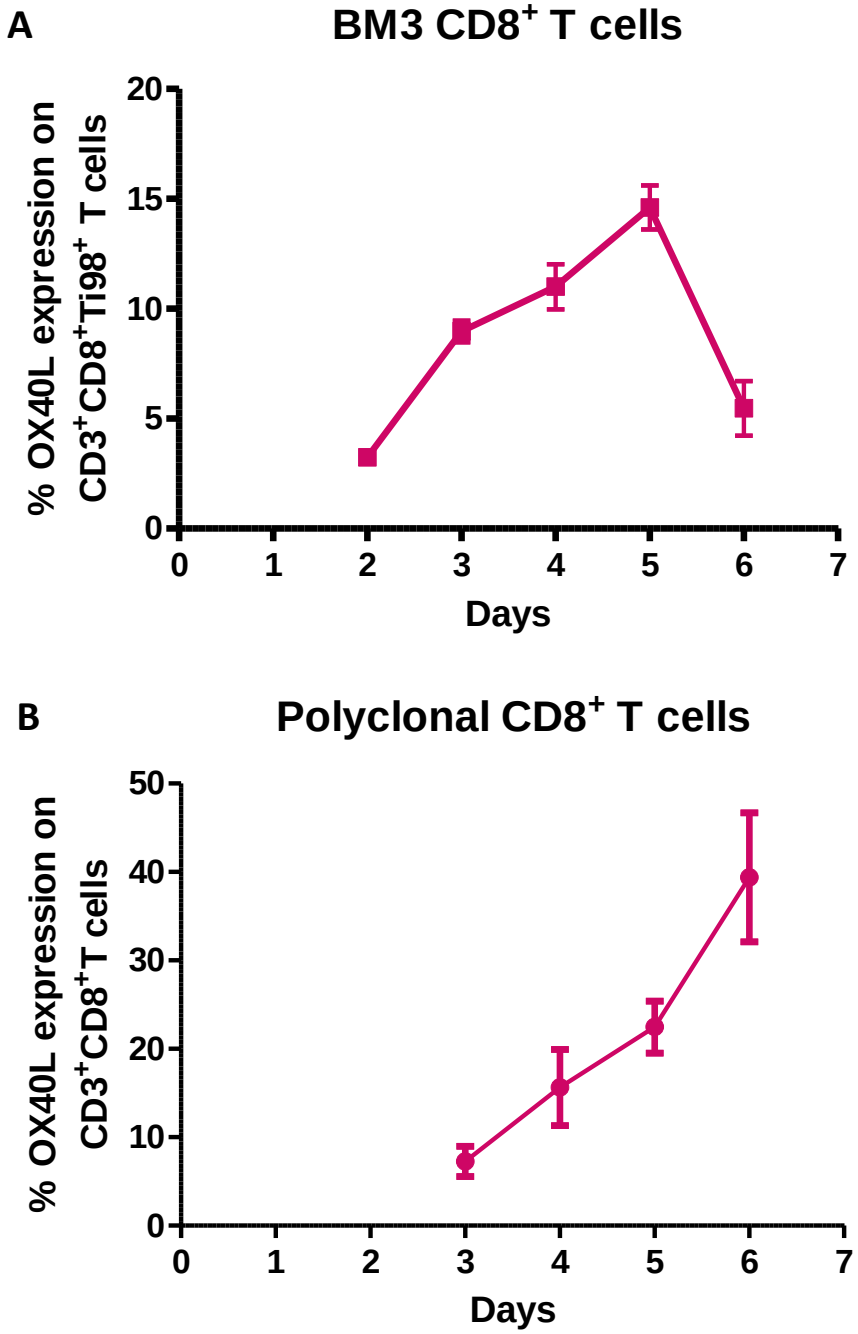


Figure 11: OX40L is expressed by BM3 and polyclonal CD8$^+$ T cells after stimulation with alloantigen *in vitro*

2.5x10^4 BM3 or 2x10^5 polyclonal CD8$^+$ T cells were cultured *in vitro* with 1x10^5 or 4x10^5 allogeneic irradiated stimulator splenocytes, respectively. Expression of OX40L on BM3 (A) and polyclonal CD8$^+$ T cells (B) was determined by flow cytometry. Results are expressed as mean % ± SD, performed in triplicate and are representative of 3 independent experiments.

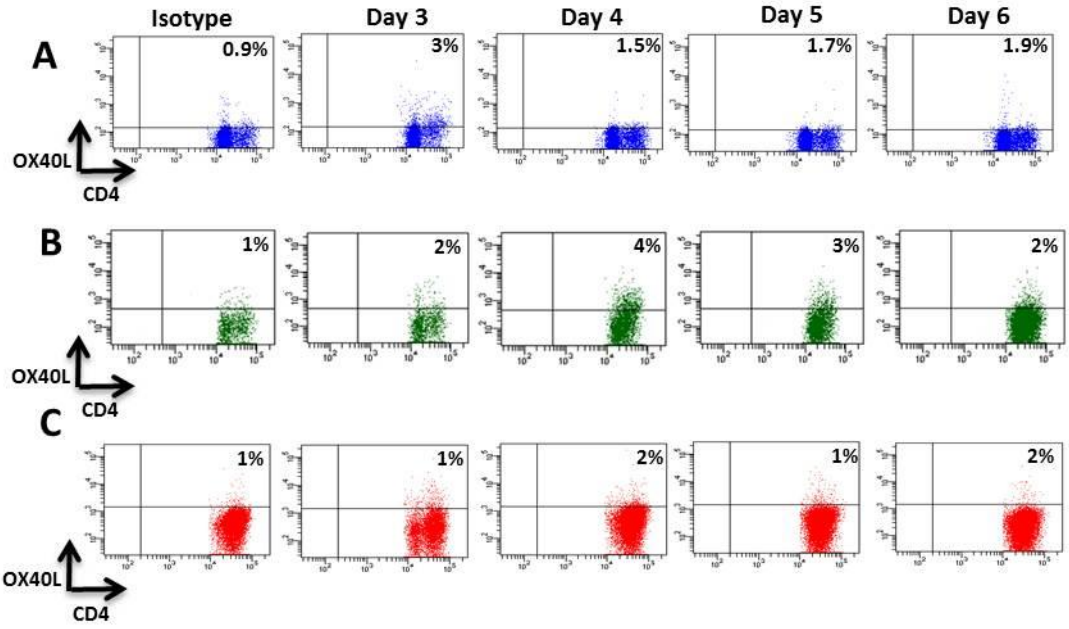


Figure 12: CD4$^+$ T cells do not express OX40L after stimulation with alloantigen *in vitro*

$2x10^5$ polyclonal or $5x10^4$ -D or TEa T cells were cultured *in vitro* with $4x10^5$ or $2.5x10^5$ allogeneic irradiated stimulator splenocytes, respectively. Expression of OX40L on polyclonal (A), TEa (B) and –D (C) CD4$^+$ T cells was determined by flow cytometry. Quadrant gates were set using an isotype control antibody. Dot-plots are representative of triplicate cultures in 3 independent experiments.

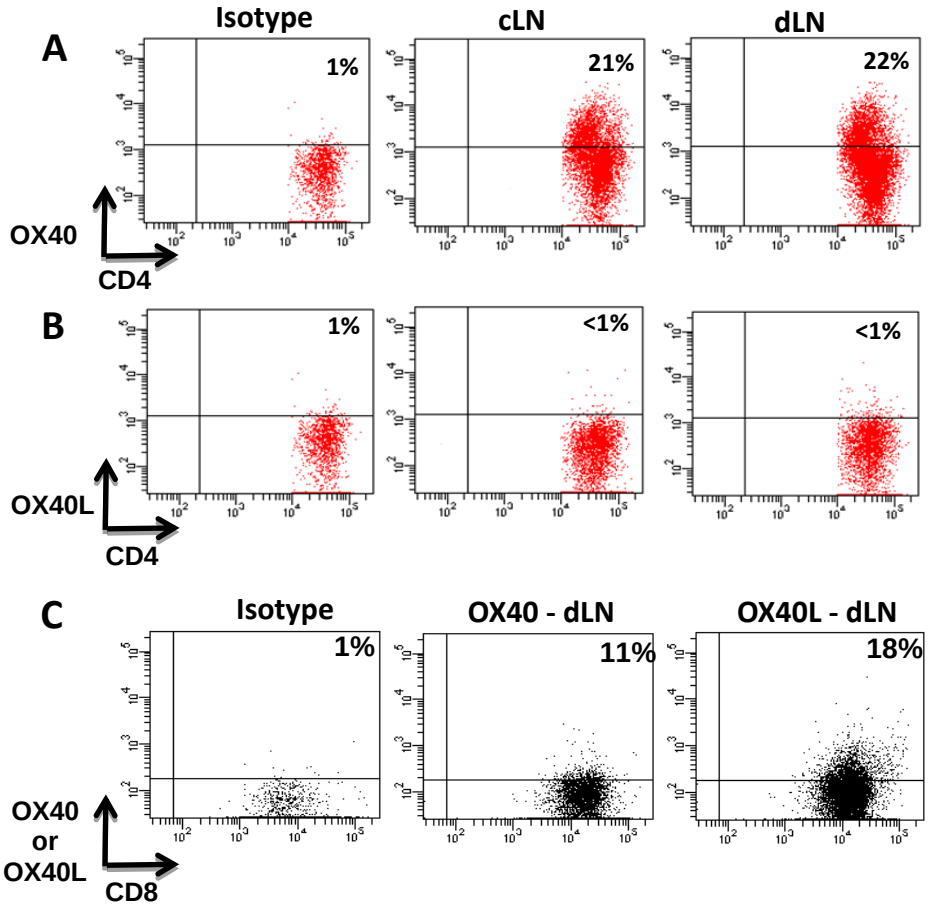


Figure 13: Both $CD4^+$ and $CD8^+$ T cells express OX40, but only BM3 $CD8^+$ T cells express OX40L after skin allografts

$1x10^5$ naïve TEa $CD4^+$ T cells were adoptively transferred into syngeneic $Rag^{-/-}$ mice. One day later, mice received an allogeneic ($H2^{b/d+}$) skin graft. Mice were sacrificed at day 15 post transplant and cLN, dLN and spleen examined for expression of OX40 (A) and OX40L (B) by flow cytometry. C) $1x10^5$ BM3 T cells were adoptively transferred into syngeneic $Rag^{-/-}$ mice which received an allogeneic skin transplant the following day. OX40 and OX40L expression on BM3 T cells was analysed in the dLN at day 10 post-transplant. (n = 4 mice per group; representative of 3 independent experiments).

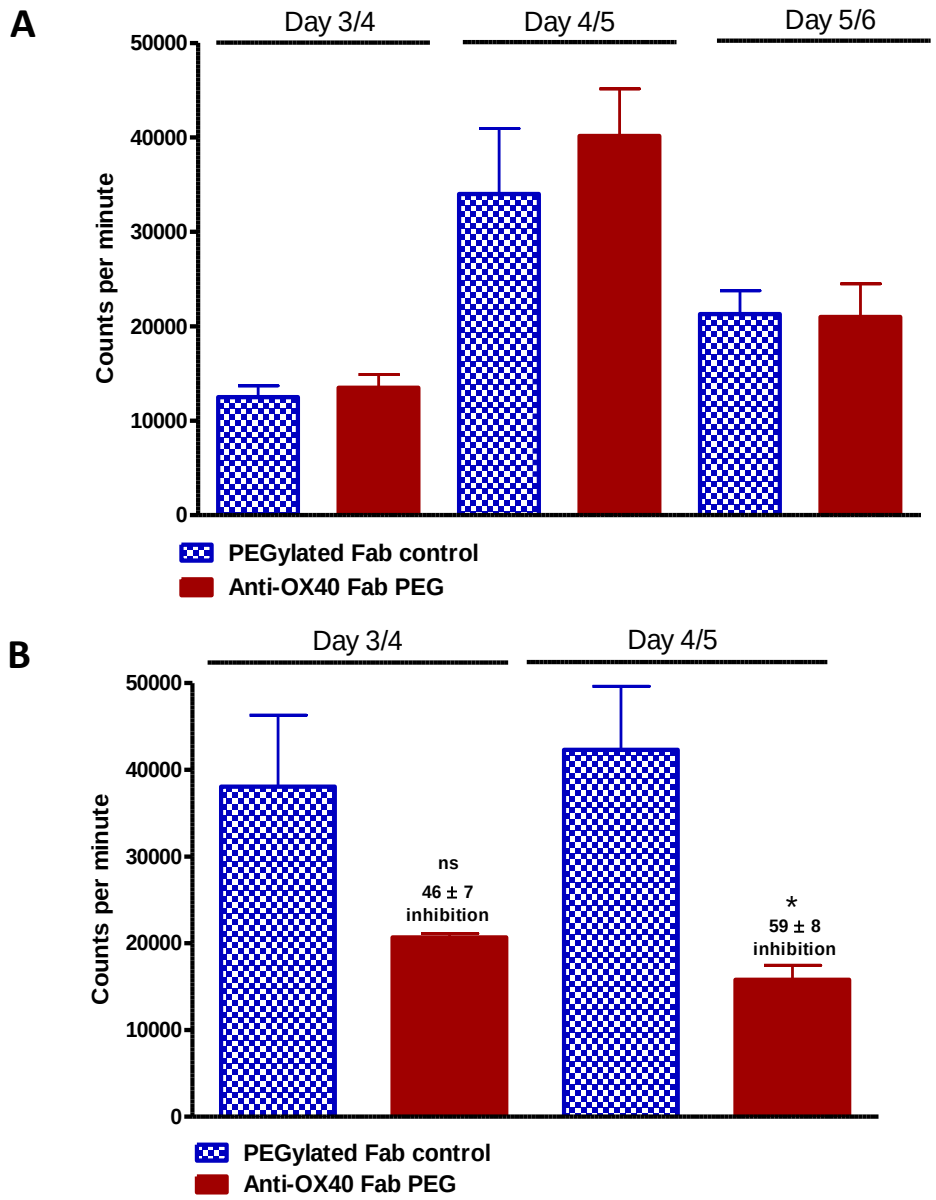


Figure 14: OX40L expressed by T cells can co-stimulate CD8$^+$ T cell proliferation
A) 2.5x10^4 BM3 T cells were cultured with LPS-matured DC2.4 cells at a ratio of 10:1 (T cell: DC). B) 1x10^5 BM3 T cells were cultured with anti-CD3/CD28 beads, at a ratio of 1:1 for 4 or 5 days. All cultures were pulsed for the last 18 hours of culture with ^{3H}-thymidine before harvest. Anti-OX40 or PEG control were added to the appropriate wells at 10µg/ml. Results are expressed as mean counts per minute ± SD, performed in triplicate and are representative of 2 independent experiments. The unpaired student t test was used to compare differences between anti-OX40 and control cultures where * represents $p<0.05$. ns represents not statistically significant.

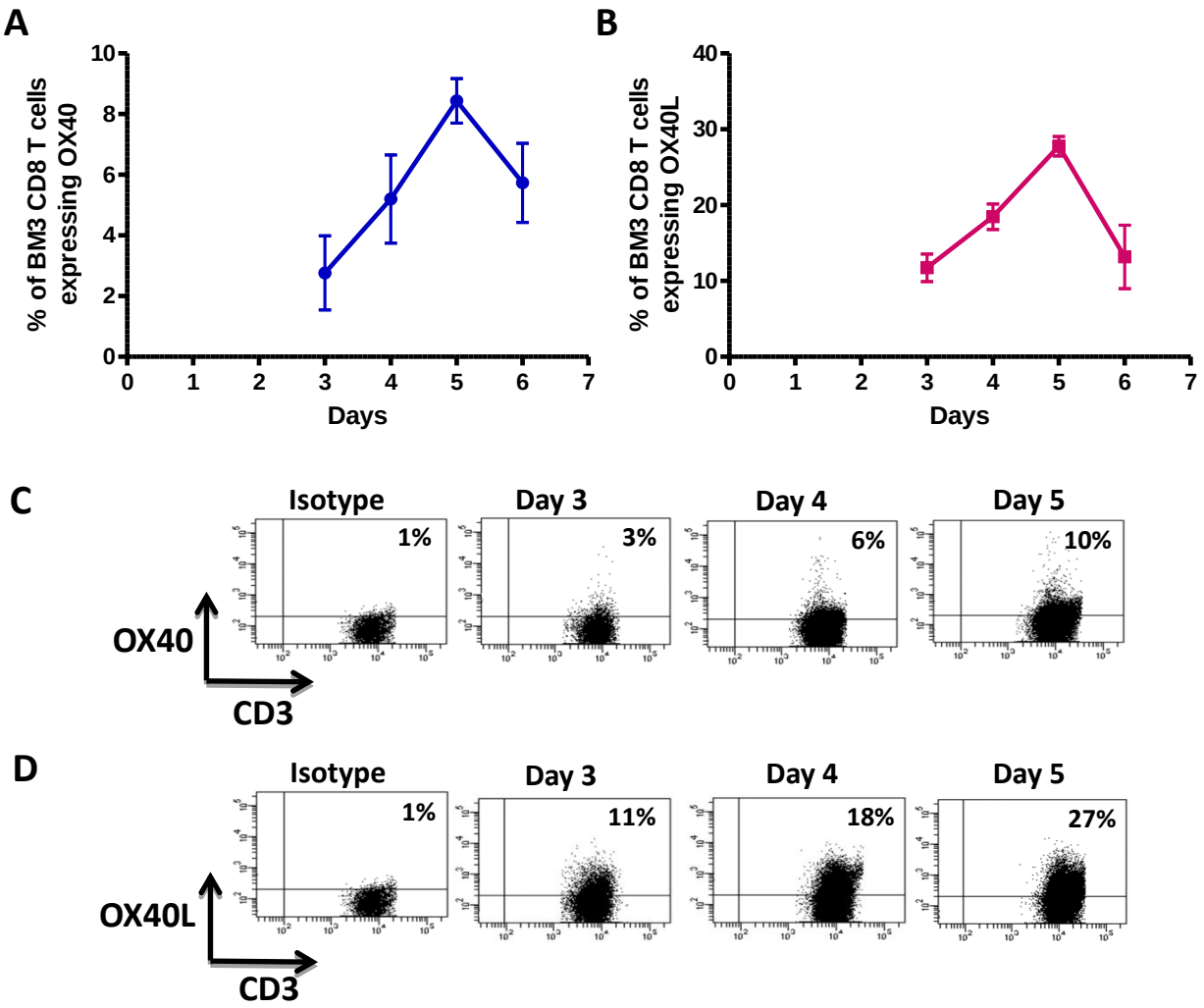


Figure 15: BM3 CD8$^+$ T cells stimulated with anti-CD3/CD28 beads express both OX40 and OX40L

1x10^5 BM3 T cells were cultured with anti-CD3/CD28 beads, at a ratio of 1:1 for various periods of time. OX40 (A and C) and OX40L (B and D) expression levels were determined on BM3 T cells by flow cytometry. Results are expressed as mean % ± SD, performed in triplicate and are representative of 2 independent experiments. Quadrant gates on dot plots were set by using an isotype control antibody.

Chapter 4: Role of OX40-OX40L in alloimmune responses

4.1 Introduction

OX40-OX40L interactions have been shown to play several roles in the immune response, however, the regulation of cell division and survival of activated T cells is the activity which is most clearly defined. This has led to the TNFR superfamily member OX40 being described as a costimulatory molecule for T cells. The first evidence for costimulatory activity of OX40 arose from the identification of OX40 in rat and human systems, where agonistic antibodies to OX40 or cross-stimulation with OX40L enhanced the proliferation of $CD4^+$ T cells *in vitro* (251, 264, 265). While other studies *in vivo* demonstrated that not only do pathogenic T cells express OX40 (344, 345) but blocking OX40-OX40L interactions ameliorated experimental models of disease such as EAE (267) and arthritis (287).

More sophisticated systems employing $OX40^{-/-}$ or $OX40L^{-/-}$ animals and neutralising antibodies specific for OX40 or OX40L have allowed a better appreciation of the prominent role for OX40-OX40L in controlling T cell responses. In studies looking specifically at $CD4^+$ T cell responses, mice deficient in OX40 or OX40L were found to have reduced primary $CD4^+$ T cell responses directed towards viruses (353) and common protein antigens (e.g. ovalbumin, key hole limpet and hen egg lysozyme) (356). Furthermore, Rogers *et al* demonstrated that OX40 deficient $CD4^+$ T cells have normal early proliferative responses, IL-2 production and clonal expansion, however, 4-

8 days after activation OX40$^{-/-}$ CD4^{+} T cells failed to maintain the expression of anti-apoptotic molecules such as Bcl-2 and Bcl-$_{XL}$ (256). As a result of reduced primary responses OX40$^{-/-}$ or OX40L$^{-/-}$ mice also had a reduction in the number of antigen specific effector T cells and consequently fewer memory T cells were generated (305, 356). In addition, in studies that have blocked OX40-OX40L interactions during CD4^{+} memory T cell responses OX40 has been shown to regulate the clonal expansion of T cells in a recall response to antigen, as well as preventing the development of clinical signs of asthma (e.g. airway hyperreactivity and eosinophils) (294).

In addition to the utilisation of OX40 during CD4^{+} T cell responses, a direct role for OX40 on the priming of CD8^{+} T cells has also been illustrated in studies using OX40$^{-/-}$ CD8^{+} T cells in response to adenovirus (358) or tumour antigens (306, 371). For example, Song *et al* showed that OX40$^{-/-}$ CD8^{+} T cells were able to undergo clonal expansion in response to peptide and irradiated splenocytes *in vitro*, however, from day 4 onwards OX40$^{-/-}$ CD8^{+} T cells showed a defect in survival as far fewer cells maintained high levels of Bcl-2 and Bcl-$_{XL}$ compared to wild-type cells (371). Moreover, OX40$^{-/-}$ CD8^{+} T cells were found to initially suppress tumour growth to a similar degree as wild-type CD8^{+} T cells, but the OX40$^{-/-}$ CD8^{+} T cells failed to provide protection over a longer period of time (371).

These and other studies have shown defects in expansion (306, 372), survival (371) and the development of the memory CD8^{+} T cell pool (373, 374). These observations were further extended by Ruby *et al* who found that an agonistic OX40 antibody significantly enhanced the generation of antigen-specific memory CD8^{+} T cells, with an apparent

preference for the generation of central memory T cells (defined by the co-expression of CD127 and CD62L) (374).

The impact of OX40-OX40L on T cell responses has also been studied in pre-clinical models using NHP where stimulating OX40 (375) or conversely blocking OX40L (296) has been shown to impact the development of memory $CD4^+$ T cell responses and inhibit antigen driven Th2 inflammation in a model of experimental asthma (296).

These data suggest that OX40-OX40L costimulation plays a key role in adaptive immunity by promoting both $CD4^+$ and $CD8^+$ T cell expansion and survival, as well as enhancing the generation of memory T cells. Although a detailed comparison of signalling components and pathways has yet to be completed. These and other studies have therefore provided the rationale to further develop reagents to target the OX40-OX40L interaction for the resolution of immune-mediated disease in humans.

4.2 Aims and Hypothesis

There is a growing body of literature demonstrating a role for OX40-OX40L in $CD4^+$ and $CD8^+$ T cell responses but the role of OX40-OX40L in transplantation remains unclear. In chapter 3 we showed that both $CD4^+$ and $CD8^+$ T cells express OX40 after activation and that blockade of OX40 significantly prolonged skin allograft survival. This suggested that OX40-OX40L interactions played a crucial role in the response of $CD4^+$ and $CD8^+$ T cells to allografts. Therefore, the aim of this chapter was to investigate the response of alloreactive T cells to allografts coincident with blockade of OX40 signalling.

To understand the role of OX40-OX40L costimulation in T cell responses to allogeneic transplants we analysed T cell activation, proliferation, migration and survival following transplantation in the presence or absence of concomitant OX40 blockade. We hypothesised that the prolonged allograft survival seen on blockade of OX40 costimulation would impact the proliferation, differentiation and/or survival of activated alloreactive $CD4^+$ and $CD8^+$ T cells.

4.3 Results:

4.3.1 Blockade of OX40-OX40L interactions does not affect initial T cell activation

Having established that blockade of OX40 attenuated $CD4^+$ and $CD8^+$ T cell proliferation to alloantigen *in vitro* (Chapter 3; Figures 3 and 5), first, we assessed whether blockade of OX40 affected initial T cell activation. The modulation of expression of CD69, CD62L, CD25 and CD44 is commonly used to determine whether naïve T cells have initiated differentiation into effector T cells following activation. In agreement with these reports we found that CD44, CD25 and CD69 were upregulated and CD62L downregulated following the activation of naïve BM3 T cells in an MLR (data not shown). Anti-OX40 had no impact on any of the markers of activation analysed compared to BM3 T cells activated in the presence of the PEG control (**Figure 1**).

4.3.2 Anti-OX40 diminishes IL-2 and IFN-γ production, but does not induce secretion of Th2-type cytokines

It has been previously suggested that stimulation through the OX40-OX40L interaction may impact the ensuing cytokine profile by skewing cytokine production towards a Th2 bias (293, 352, 376). Therefore, we sought to determine whether anti-OX40 prevented or resulted in skewed cytokine production by $CD4^+$ and $CD8^+$ T cells activated with alloantigen *in vitro*.

To this end, -D T cells were co-cultured with alloantigen and harvested at various time points for cytokine analysis. Anti-OX40 significantly inhibited IL-2 (**Figure 2A**) and IFN-γ (**Figure 2B**) production from $CD4^+$ T cells. IL-2 secretion was inhibited by 58% ± 19 at day 4 and IFN-γ secretion inhibited by 89% ± 4 at day 5.

$CD8^+$ T cells ordinarily produce a Th1-like pattern of cytokines upon activation however it had been reported that under certain conditions $CD8^+$ T cells may also secrete cytokines associated with Th2 T cells (e.g. IL-4, -5 and -9). We found that IL-2 and IFN-γ production by activated BM3 T cells was significantly inhibited by anti-OX40 (**Figure 2E and F**). IL-2 secretion was inhibited by 58% ± 5 at day 2 and 86% ± 1 at day 3 (**Figure 2E**) and IFN-γ secretion was inhibited 76% ± 14 at day 4, and 84% ± 6 at day 5 (**Figure 2F**). These data are in agreement with the data presented in Chapter 3 (Figure 5) showing that anti-OX40 inhibited the proliferation of –D and BM3 T cells. Importantly although low levels (<20pg/ml) of IL-4 and IL-5 were detected in both -D and BM3 cultures OX40 blockade did not increase the production of these cytokines (**Figures 2C, D, G, H)**. Naïve BM3 T cells or stimulators cultured alone failed to proliferate or produce detectable levels of any of the cytokines assayed at any time-point (data not shown).

4.3.3 Anti-OX40 reduces IL-2 production by BM3 T cells thereby limiting T cell growth and survival.

Next we sought to investigate whether T cell proliferation could be restored despite OX40 blockade by the addition of specific growth factors or cytokines. BM3 T cells

were co-cultured with allogeneic splenocytes in the presence of anti-OX40 or PEG control with either the addition of exogenous IL-2 or IL-7 or both cytokines. The addition of IL-2 (**Figure 3A**) partially restored the proliferation of anti-OX40 treated BM3 T cells compared to those cells treated with PEG control (19% inhibition ± 2 exogenous IL-2; $p<0.0009$). To test whether the rescue of BM3 T cell proliferation was specific to IL-2 or could be restored with other cytokines that signal via the common cytokine receptor γ chain we also added exogenous IL-7 to cultures. The addition of IL-7 had no significant impact on anti-OX40 mediated suppression (**Figure 3B**). Addition of IL-2 and IL-7 to anti-OX40 cultures resulted in proliferation of the BM3 T cells (13% inhibition ± 1 exogenous IL-2 and IL-7; **Figure 3C**) that was no different from that seen with IL-2 alone.

4.3.4 Anti-OX40 prevents accumulation of $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes following allogeneic skin transplantation.

In addition to the suppression seen *in vitro* upon OX40 blockade, in Chapter 3 we also showed that anti-OX40 suppressed skin allograft rejection mediated by either naïve $CD4^+$ or $CD8^+$ T cells. Therefore, we next sought to dissect the mechanism behind graft prolongation resulting from anti-OX40 administration. Our previous studies have shown that large numbers of primed effector/memory BM3 $CD8^+$ T cells could be detected specifically in the dLN 10 days after allogeneic skin transplantation (350). First, the impact of OX40-OX40L interactions on the clonal expansion of TEa and BM3 T cells following allogeneic skin transplantation *in vivo* was determined.

$1x10^5$ TEa, -D or BM3 T cells were adoptively transferred into syngeneic $Rag^{-/-}$ mice, and one day later such mice received either a syngeneic or allogeneic skin graft. Mice receiving an allograft were either given PEG control or anti-OX40 twice weekly until harvest. TEa and -D T cells were analysed at day 15 post transplantation, while BM3 T cells were analysed at day 10. Different time-points were analysed in these experiments as we had previously found that BM3 T cells rapidly become activated and expand following transplantation (350) whilst consistent expansion of TEa and -D T cells was only seen on day 15 post transplantation in the dLN (data not shown).

In the presence of PEG control, as expected, a significant expansion of primed effector/memory TEa T cells was found in the dLN after an allogeneic skin graft (TEa cell number 198562 ± 91555), whilst little expansion occurred in the cLN (TEa number 9628 ± 7212) (**Figure 4A**). The expansion of TEa T cells in the dLN was shown to be allo-specific as there was no expansion in the dLN after syngeneic skin graft transplantation (**Figure 4A**). In contrast, anti-OX40 treatment was found to completely prevent the accumulation of TEa T cells in the dLN (TEa cell number 23146 ± 18585; 91±10% inhibition; $p<0.0095$; **Figure 4A**) 15 days after skin grafting.

Following T cell priming in the dLN after allogeneic skin transplantation, T cells migrate to the spleen where they reside as long-lived effector/memory T cells. In our experiments we also found an expanded population of TEa T cells in the spleen after allogeneic skin transplantation and this re-distribution of activated TEa T cells was also

inhibited by anti-OX40 treatment at day 15 (TEa cell number 19278 ± 11296 vs. 213722 ± 18008; 92 ± 5% inhibition; $p<0.0001$; **Figure 4A**).

To confirm that the impact of anti-OX40 on the expansion and re-distribution of alloreactive $CD4^+$ T cells was not related to the TCR-transgenic T cells (TEa) used, we performed similar adoptive transfer experiments with -D T cells. Again, there was a substantial expansion of -D T cells in the dLN after an allogeneic skin graft which was markedly attenuated when mice were treated with anti-OX40 (-D cell number 70347 ± 1869 vs. 817070 vs. 222285; 99 ± 2% inhibition; $p<0.001$ **Figure 4B**).

To determine whether the impact of anti-OX40 was restricted to alloreactive $CD4^+$ T cell responses we adoptively transferred BM3 $CD8^+$ T cells into $Rag^{-/-}$ mice that received a skin allograft and anti-OX40 or PEG control. As expected, there was also a significant accumulation of primed effector/memory BM3 T cells in the dLN (BM3 cell number 43832 ± 14257), whilst little expansion had occurred in the non-draining, cLN (BM3 cell number 4068 ± 2540) in mice that had received a skin allograft and PEG control (**Figure 4C**). However, in recipients of skin allografts treated with anti-OX40 expansion/accumulation of BM3 T cells in the dLN was completely prevented (BM3 cell number dLN 6609 ± 1955; 93 ± 3% inhibition; $p<0.007$; cLN 1779 ± 767 **Figure 4C**). The expansion of BM3 T cells in the dLN of control allograft recipients was shown to be alloantigen-specific, as syngeneic skin grafts failed to evoke BM3 T cell expansion (**Figure 4C**).

4.3.5 Blockade of OX40-OX40L does not result in a long-term functional impairment of BM3 T cells.

To determine whether transient OX40 blockade permanently impaired the T cell response to skin allografts, we analysed the number of BM3 T cells in anti-OX40 or PEG control mice at different times after transplantation. Initially the T cell response was confined to the dLN (**Figure 5B**), before BM3 T cells migrated to the spleen to form a stable memory pool where they resided long-term (**Figure 5C**).

Consistent with previous data (**Figure 4**), anti-OX40 inhibited the accumulation of the BM3 T cells at day 10 post transplantation (**Figure 5B**). At this time point there was no expansion or accumulation of BM3 T cells in the spleen (**Figure 5C**). However, at a time point immediately prior to rejection and 10 days after the anti-OX40 administration was stopped (i.e. day 38) BM3 T cells were found to have accumulated specifically in the dLN, pre-empting rejection (64801 ± 18933 anti-OX40 vs. 33105 ± 8700 PEG control; **Figure 5B**). By day 100 post transplantation no difference in BM3 T cell number (in any compartment) was seen between mice that had received anti-OX40 and PEG control mice (**Figure 5**). The majority of BM3 T cells 100 days post transplantation were found to reside in the spleen (**Figure 5C**).

4.3.6 OX40 blockade has no impact on proliferation of the responding $CD4^+$ and $CD8^+$ T cells

The attenuated T cell expansion seen on administration of anti-OX40 could be due to a requirement for OX40 for optimal proliferation or that OX40 was required to maintain

the survival of activated effector cells. To distinguish between these possibilities we looked at the proliferation of alloreactive T cells (as judged by the loss of CFSE) following transplantation in the presence or absence of OX40 blockade. TEa T cells were found to have proliferated specifically in the dLN by 15 days after transplantation (**Figure 6A**). Interestingly, despite the clear inhibition of TEa expansion (**Figure 4A**), anti-OX40 failed to impact the proliferation of alloreactive T cells (**Figure 6A**). TEa T cells in mice that had received a syngeneic skin graft or in the cLN of allograft recipients maintained high levels of CFSE, consistent with the fact these cells had not proliferated or expanded by homeostatic proliferation (**Figure 6A**).

In complementary studies we also examined the impact of anti-OX40 on $CD8^+$ T cell proliferation to skin grafts. To this end, $Rag^{-/-}$ mice received $1x10^5$ CFSE labelled BM3 T cells and either a syngeneic or allogeneic skin graft and anti-OX40 or PEG control. As before, mice were analysed at 10 days post transplantation. The baseline homeostatic response of BM3 T cells was revealed by analysis of cLN where BM3 T cells from all mice had only undergone 1 to 3 rounds of cell division due to homeostatic proliferation (**Figure 6B**). The same degree of basal proliferation was also seen in both cLN and dLN following syngeneic skin transplantation (**Figure 6B and C**) as previously published (350). As with TEa T cells, despite a failure to accumulate/expand, BM3 T cells from anti-OX40 treated mice showed an identical proliferation history (as judged by the loss of CFSE) to BM3 T cells in the dLN from PEG control mice, with most cells having undergone 4 or more divisions (**Figure 6B**).

4.3.7 Anti-OX40 inhibits $CD3^+$ T cell infiltration of skin allografts.

Anti-OX40 inhibited the accumulation but not the proliferation of $CD4^+$ and $CD8^+$ T cells in the dLN following allogeneic skin transplantation. To rule out the possibility that anti-OX40 had enhanced T cell migration to other lymphoid tissues and in particular the allograft we harvested skin allografts from mice treated with anti-OX40 or PEG control at day 15 (after TEa T cell transfer; **Figures 7A, B, C**) or day 10 (after BM3 T cell transfer; **Figures 7D**). Skin allografts were analysed for the presence of $CD3^+$ T cells by immunohistochemistry as the only T cells present in these mice were the transferred TEa or BM3 T cells (**Figure 7**). We found a dramatic reduction in the number of $CD3^+$ T cells that had infiltrated skin allografts after treatment with anti-OX40 (**Figure 7B, C and D**), suggesting that anti-OX40 prevented the survival of effector $CD4^+$ and $CD8^+$ T cells rather than inducing enhanced migration of effector T cell to the skin allografts. In addition, we failed to see an increase in the number of alloreactive T cells in other lymphoid tissues as well as in the allograft at these time-points (data not shown).

4.3.8 Anti-OX40 increases the death of activated BM3 T cells.

Given that anti-OX40 similarly attenuated the response of TEa, -D and BM3 T cells to skin allografts we used the two different TCR-transgenic T cells in order to further dissect the role of OX40 in the survival of alloantigen-reactive T cells. Our *in vitro* experiments demonstrated that anti-OX40 inhibited T cell proliferation to alloantigen as measured by ^{3H}thymidine incorporation (Chapter 3 Figure 5) whilst *in vivo* there was no impact on proliferation but rather a failure for activated effector T cells to accumulate

following activation (**Figure 4C**). Therefore, we next wanted to test whether the inhibition in ^{3H}-thymidine incorporation by anti-OX40 was a result of inhibition of proliferation or whether proliferating cells failed to survive and/or expand. Flow cytometric analysis of BM3 T cells revealed that the addition of anti-OX40 significantly enhanced BM3 T cell death following stimulation with alloantigen *in vitro* as shown by an increase in the dead:live BM3 T cell ratio compared to PEG control cultures (**Figure 8**). The difference in the dead:live ratio was only observed after 4 days of culture which was in agreement with previous data showing that anti-OX40 did not affect initial T cell activation (**Figure 1**).

To confirm this finding we next looked *ex vivo* at dLN taken from mice that had received skin allografts. To this end, $1x10^5$ TEa T cells were adoptively transferred into syngeneic $Rag^{-/-}$ recipients and the following day mice received a allogeneic (BALB/cB6F1) skin allograft. Mice were either treated with PEG control or anti OX40 twice weekly, before being sacrificed on day 13 for analysis by flow cytometry. As mentioned above TEa and BM3 T cells in the dLN were analysed at different time-points due to differences in kinetics of consistent clonal expansion.

Cells from the dLN were analysed by annexin V and 7AAD viability stains to assess the proportion of apoptotic TEa T cells. Cells which were apoptotic were annexin V^+7AAD^+ (**Figure 9D**), and pre-apoptotic cells were classified as annexin V^+7AAD^- (**Figure 9C**). The number and percentage of live cells after anti-OX40 treatment was dramatically reduced after anti-OX40 treatment (**Figures 9A and B**). OX40 blockade

dramatically increased the proportion of pre-apoptotic (**Figure 9C**) but had no impact on the proportion of apoptotic TEa T cells in the dLN (**Figure 9D**), which correlated with our *in vitro* findings suggesting that OX40 signals were required for the survival of activated T cells (**Figure 8**).

Finally, to confirm that OX40 blockade increased cell death, we examined the dLN after syngeneic $Rag^{-/-}$ mice were reconstituted with TEa T cells and given an allogeneic skin graft the following day before the dLN were harvested at day 13 (same time point at data in Figure 9). We performed histological analyses of the dLN and stained sections for active caspase-3, which is a cysteine protease that has previously been shown to play a central role in apoptosis. Despite the data presented in Figures 8 and 9, anti-OX40 treatment had no impact on the number of cells expressing caspase-3 in the dLN (**Figure 10**), which may not surprising as apoptotic cells are rapidly cleared *in vivo* (377).

4.4 Discussion

In agreement with the literature, our data showed that OX40 was expressed on activated, but not naïve, $CD4^+$ and $CD8^+$ T cells (Chapter 3 Figures 1 and 2). Furthermore, the delayed kinetics of expression (OX40 was not expressed until 3-4 days after activation) was consistent with a role for OX40-OX40L in effector and memory T cell generation i.e. in the propagation of the response, rather than in initial T cell priming. This was borne out by the finding that blocking OX40 had no impact on either the activation (Figure 1) or the initial proliferation (Figure 6) of alloreactive $CD4^+$ and $CD8^+$ T cells. However, *in vitro* and *in vivo* the expansion of T cells was clearly diminished in the absence of OX40 signalling (Chapter 3 Figures 3 and 5, Chapter 4 Figure 6).

Blocking OX40 markedly impacted the ability of alloreactive $CD4^+$ and $CD8^+$ T cells to elicit rejection of skin allografts *in vivo* (Chapter 3 Figures 12 and 13). Furthermore, blockade of the OX40-OX40L pathway prevented the accumulation of alloreactive $CD4^+$ and $CD8^+$ T cells in the dLN (Figure 6). Data from our *in vitro* studies demonstrated that $CD8^+$ T cells underwent activation induced cell death in the absence of OX40 signalling (Figure 8). This finding was then confirmed *in vivo* (Figure 9). Although gene analysis of pro- and anti-apoptotic molecules was not conducted in our studies, we have shown an increased ratio of dead to live $CD8^+$ T cells in *in vitro* cultures (Figure 8) and increased proportions of pre-apoptotic $CD4^+$ T cells (Figure 9) in the dLN after treatment with anti-OX40. We did not observe differences in casapse-3 expression in the dLN, which may be due to a difference in sensitivity between flow cytometry and histology. Another explanation for the lack of difference between anti-

OX40 treatment and control could be the cell death mediated by anti-OX40 may be caspase independent. For example, lysosomal proteases such as cathespsin B are released into the cytosol which can execute cell death (378), however the mechanisms behind these proteases have not yet been defined. Therefore, OX40-OX40L interactions appeared to provide critical survival signals for effector $CD4^+$ and $CD8^+$ T cells rather than being essential for initial T cell activation and differentiation.

The significant prolongation of graft survival in the absence of OX40 was observed even in the presence of other costimulatory molecules, such as CD28 and CD154 suggesting that the OX40-OX40L interaction is an important costimulatory pathway involved in the propagation of $CD4^+$ and $CD8^+$ T cell alloimmune responses.

Our data are in agreement with previous reports where $OX40^{-/-}$ T cells were shown to become normally activated and expanded (305, 358) but failed to maintain high levels of anti-apoptotic Bcl-2 family members such as Bcl-2 and $Bcl\text{-}_{XL}$ (256, 371) thus inhibiting the ability to maintain clonal expansion over time, thereby preventing the generation or sustenance of memory T cell responses (294, 305). These data suggested that the absence of OX40 gave rise to a defect in survival or in their ability for long term division. The impaired survival of $OX40^{-/-}$ T cells could be reversed by the addition of peptide inhibitors of caspases (pan-caspase inhibitor or specific inhibitors to caspase-3 or -9) (256) suggesting caspase-mediated apoptosis appears to be a significant consequence of signalling via OX40-OX40L.

Rogers *et al* suggested that these data provide evidence that in the absence of OX40 signalling T cell death is via neglect instead of through death receptors e.g. Fas (256). Death by neglect is a result of lack of cytokines or costimulation and is a delicate balance between pro- and anti-apoptotic molecules. The absence of OX40 signalling could prevent IL-2 transcription thus inhibiting the expansion of T cells which are able to respond to alloantigen resulting in a failure to upregulate anti-apoptotic molecules such as Bcl-$_{XL}$ and Bcl-2.

On the other hand, blockade of OX40 could also have a direct impact on signalling cascades which results in an upregulation of pro-apoptotic molecules and a down-regulation of anti-apoptotic molecules. For example OX40$^{-/-}$ T cells cannot maintain PKB activation and therefore undergo cell death (258). Conversely, sustained PKB signalling driven by OX40 leads to the upregulation of anti-apoptotic molecules belonging to the Bcl-2 family, including Bcl-2, Bcl-$_{XL}$ and Bfl-1 which control T cell survival (256, 258, 379, 380). Gene analysis has revealed that survivin, which is required to maintain T cell proliferation (381), is a target of OX40 after PKB activation in antigen-activated CD4^{+} T cells. Survivin antagonises apoptosis in conjunction with aurora B (382) and blocking the progress through the cell cycle at S phase (381).

The upregulation of anti-apoptotic molecules (Bcl-$_{XL}$ and Bcl-2) to promote survival after T cell antigen recognition and OX40 costimulation is not unique to OX40 and had also been demonstrated when costimulation was provided by other Ig and TNFR family members such as CD28 (185) and 4-1BB (383, 384). For example, Li *et al* provided

direct evidence that treatment with costimulation blockade (CTLA-4-Ig and anti-CD154) enhanced the induction of apoptosis in responding T cells (385). Further experiments demonstrated that apoptotic deletion of alloreactive T cells in their model was IL-2 dependent but Fas/FasL independent (386) supporting the hypothesis of T cell death by neglect.

We were unable to distinguish whether the absence of OX40 signalling resulted in the upregulation of pro-apoptotic and downregulation of anti-apoptotic molecules directly or whether this may be the result of the lack of IL-2 secretion and signalling. However we would favour the latter, as our data provides evidence that the addition of exogenous IL-2 was able to partially restore the proliferative capacity of BM3 T cells (Figure 3).

TRAF-2, -3 and -5 are recruited to the cytoplasmic tail of OX40 and activate NFκB which provides an important link between OX40 and downstream signalling pathways which induce cell survival (274, 275). Moreover, other TNFR family members are also able to recruit TRAFs e.g. 4-1BB (275) and CD27 (276). Despite the absence of OX40-OX40L signalling, in the setting of transplantation with non-specific trauma and inflammation associated with surgery there are numerous other costimulatory ligands such as CD80/CD86 (387) and CD40 (388, 389) on donor APC that could provide survival signals to the T cells (387). However in our model with solid organ allografts, OX40 appeared to play a predominant and indispensable role in the survival of activated T cells as potential signals from other costimulatory molecules failed to prevent premature T cell apoptosis.

After i*n vivo* stimulation with donor alloantigen and anti-CD154 treatment Iwakoshi *et al* showed that alloreactive $CD8^+$ T cells rapidly acquired an activated/effector phenotype before they were eliminated in the periphery (390). Thus suggesting pre-treatment with alloantigen in the absence of CD154 interactions reduced the clonal expansion of $CD8^+$ T cells. Our data with both $CD4^+$ and $CD8^+$ T cells are in agreement with these observations and demonstrate that the inhibition of clone size in the absence of OX40 signalling is not limited to $CD4^+$ T cells which recognise alloantigen via the direct pathway but also occurred in $CD4^+$ T cells which utilised the indirect pathway as well as in $CD8^+$ T cells. These data suggest that CD28 and CD154 may be important in providing signals to initiate gene transcription of growth factors such as IL-2 which result in T cell activation and clonal expansion. While other TNFR superfamily members (including OX40) are required to sustain T cell survival allowing memory T cell responses to be generated.

In addition to anti-OX40 attenuating $CD8^+$ T cell expansion/survival we also determined if anti-OX40 affected cytokine secretion as has been previously suggested (293, 352, 376). Predictably, IL-2 and IFNγ production were markedly decreased which correlated with anti-OX40 diminishing expansion and survival. However, importantly, there was no switch to a Th2-like cytokine secretion pattern by either $CD4^+$ or $CD8^+$ T cells after blockade of OX40 (Figure 2). Other studies have shown costimulation via OX40 promoted the differentiation of naïve $CD4^+$ T cells into Th2 cells producing IL-4 *in vitro* (352, 391) suggesting signalling through OX40-OX40L could induce skewing

of the immune response towards a Th2 bias. This was also true for other costimulatory molecules as blockade of other TNFR superfamily members e.g. CD40-CD154 which have also been reported to result in immune deviation towards a Th2 cytokine bias (220, 392). OX40 blockade in our models had a significant impact on graft survival by influencing T cell survival therefore it was unlikely that there was dramatic skewing of the cytokine production. However, $CD8^+$ T cells appeared to be predisposed to producing Th1 skewed cytokines (e.g. IFN-γ) (393) which suggested that these cells may not have been as malleable as their $CD4^+$ T cell counterparts in terms of their cytokine production, which may have explained why we saw no deviation in cytokine production. Therefore, we found that the major impact of anti-OX40 blockade on T cell responses to alloantigen was on effector/memory T cell expansion/survival and not on activation, proliferation or cytokine production.

Other mechanisms which could play a role in allograft prolongation which have not been investigated in these studies include an impact by TNFR costimulatory molecules on innate immunity. For example, the blockade of CD154 has been shown to suppress innate immunity as anti-CD154 therapy reduced the early accumulation of neutrophils and monocytes, as well as reducing the late accumulation of macrophages within carotid arteries in a mouse model of arterial vessel injury (394). In addition, treatment with anti-CD154 significantly diminished the expression of the inflammatory chemokines CXCL1 and CCL5 in skin allografts compared to untreated controls (395).

Our group has demonstrated that memory $CD8^+$ T cells reject skin allografts more rapidly than naïve $CD8^+$ T cells due to the recruitment of neutrophils into the allograft (396). Early neutrophil infiltration has also been shown to correlate with number of antigen primed $CD8^+$ T cells which entered the skin after antigen challenge (397). OX40 has been reported to be expressed by neutrophils in the blood (261) and appears to delay neutrophil death *in vitro* (261), similar to the role of OX40 in promoting the survival of T cells. Neutrophils often play a key role in mediating allograft rejection and are able to migrate into inflamed tissues. After recruitment, neutrophils can interact with $OX40L^+$ cells (e.g. activated T cells (264), DC (268), endothelial cells (269, 398)) which could contribute to the survival and accumulation of neutrophils in inflamed tissues. The depletion of neutrophils has been shown to reduce cellular infiltration and thereby prevent cardiac allograft rejection (399). Therefore, anti-OX40 therapy may also aid allograft survival by impacting the survival and infiltration of neutrophils into the graft. However, following skin transplantation, neutrophils are only seen within allografts coincident with T cell infiltration. As T cell infiltration was completely blocked by anti-OX40 we were unable to examine whether anti-OX40 had any direct effect on neutrophil migration to skin allografts.

In summary, OX40 blockade enhanced allograft survival as demonstrated in Chapter 3. Our data clearly demonstrate that anti-OX40 had no impact on the initial priming or proliferation of T cells but rather impacted the expansion of activated, alloreactive $CD4^+$ and $CD8^+$ T cells in the dLN after a skin allograft. These data are in agreement with literature on the role of OX40-OX40L in other autoimmune and inflammatory diseases.

4.5 Figures

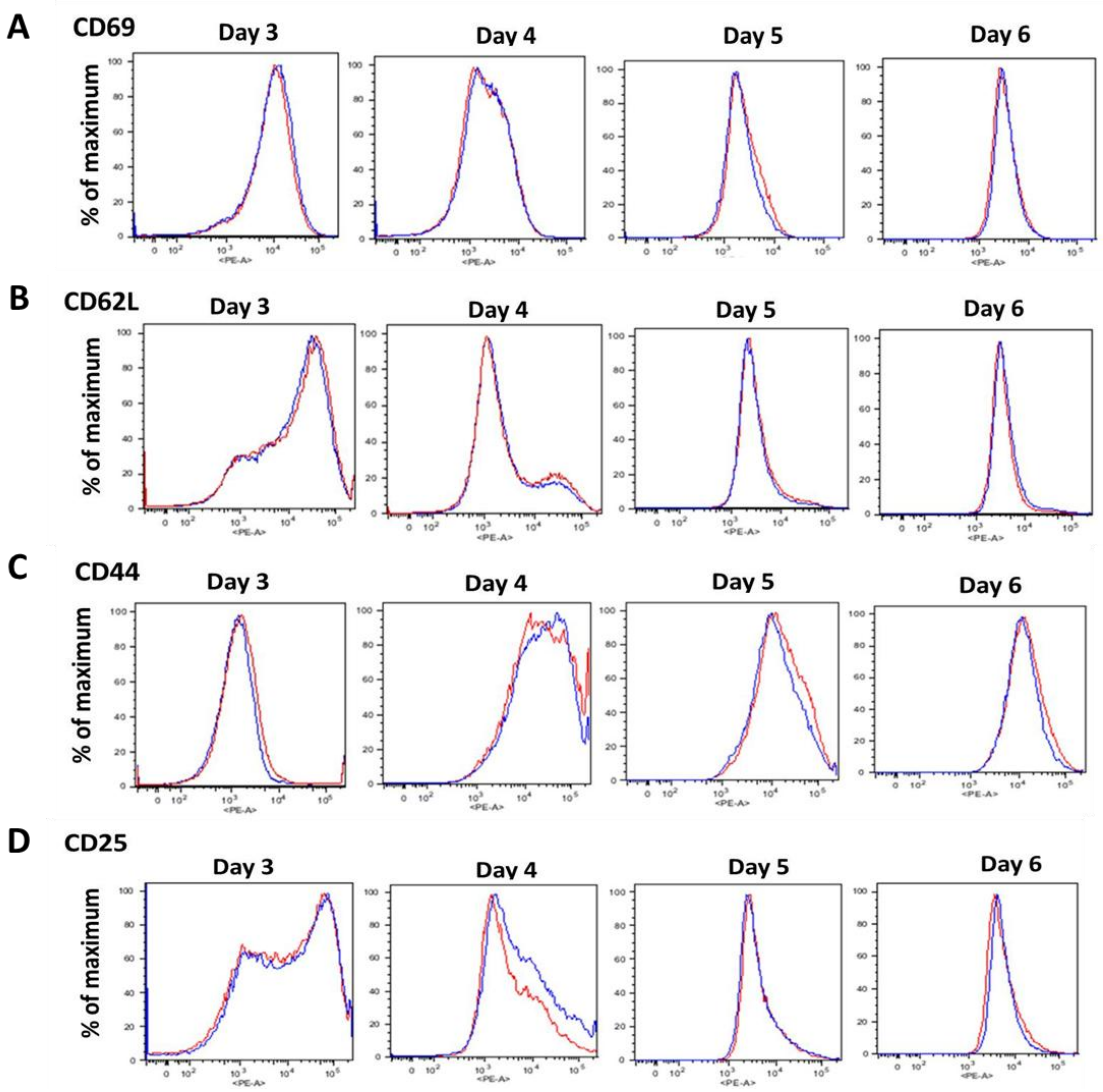


Figure 1: Blockade of OX40-OX40L has no impact on the modulation of activation markers on activated BM3 CD8$^+$ T cells

$2.5x10^4$/well BM3 T cells and $1x10^5$/well irradiated B6 splenocytes were cultured for between 3 and 6 days. BM3 T cells were harvested at various time points and analysed by flow cytometry for expression of the activation markers CD69, CD62L, CD44 and CD25. Blue lines indicate cells cultured with PEG control; red lines indicate cells cultured with anti-OX40. 3 wells analysed per time-point. Data representative of 2 independent experiments.

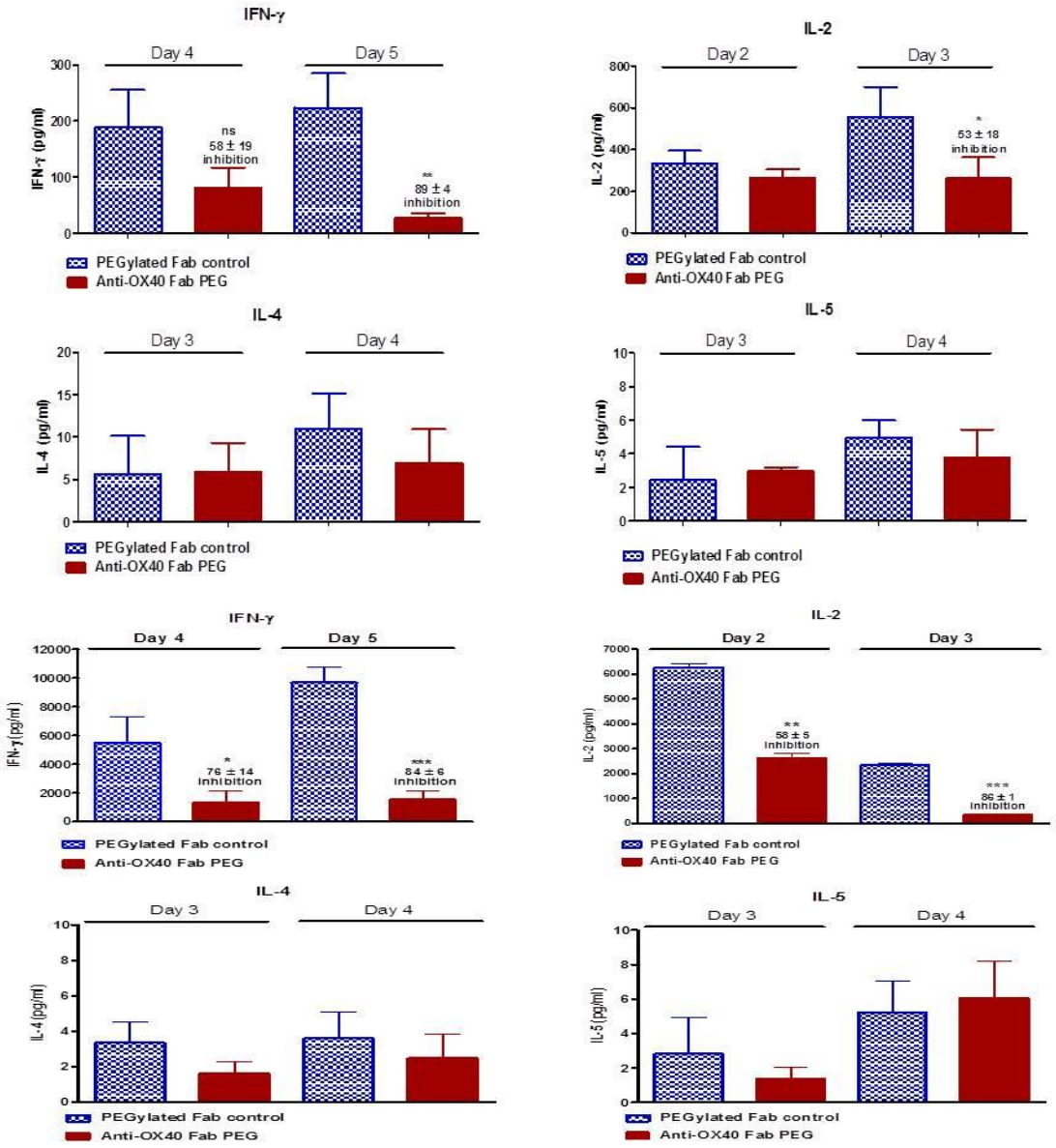


Figure 2: Anti-OX40 inhibits secretion of IL-2 and IFN-γ, but has no impact on Th2 associated cytokines

Supernatants from –D (A-D) and BM3 (E-H) MLR cultures were harvested at various time points, and cytokine analysis performed using a multiplex cytokine detection kit mesoscale discovery (MSD) or ELISA (IFN-γ). Anti-OX40 or PEG control were added to the appropriate wells at 10µg/ml. Results were expressed as mean concentration ± SD, performed in duplicate and were representative of 3 independent experiments. The unpaired student t test was used to compare differences between anti-OX40 and PEG control cultures where * represents $p<0.05$; ** represents $p<0.01$ and *** represents $p<0.001$.

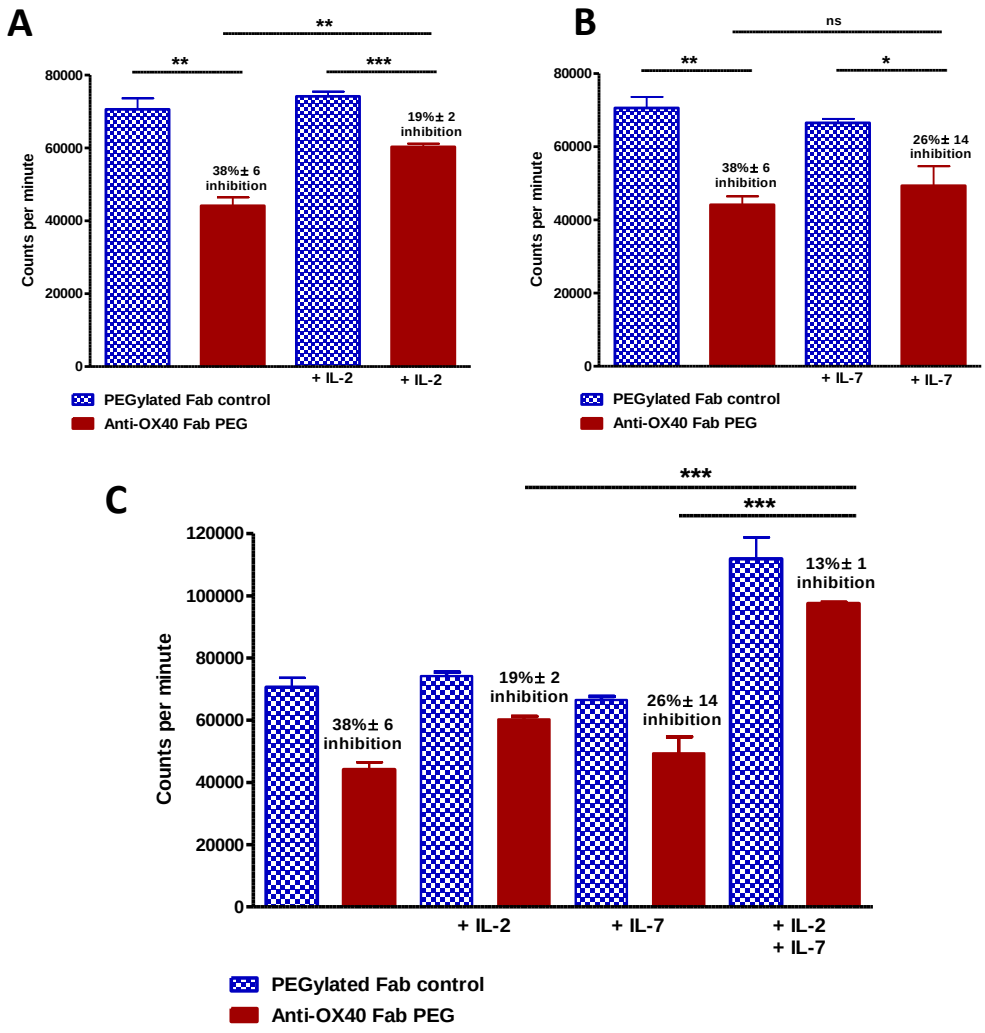


Figure 3: IL-2 partially restores the proliferative capacity of BM3 T cells in the absence of OX40 signalling

$2.5x10^4$/well BM3 T cells and $1x10^5$/well irradiated B6 splenocytes were cultured for 4 days before being pulsed for the last 18 hours of culture with ^{3H}-thymidine and harvested the following day. Anti-OX40 or PEG control were added to the appropriate wells at 10µg/ml. A) exogenous IL-2 was added at 10U/ml B) exogenous IL-7 was added at 1ng/ml C) exogenous IL-2 (10U/ml) and IL-7 (1ng/ml) was added. Results were expressed as mean counts per minute ± SD, performed in triplicate and are representative of 3 independent experiments. The unpaired student t test was used to compare differences between anti-OX40 and PEG control cultures where * represents $p<0.05$; ** represents $p<0.01$ and *** represents $p<0.001$.

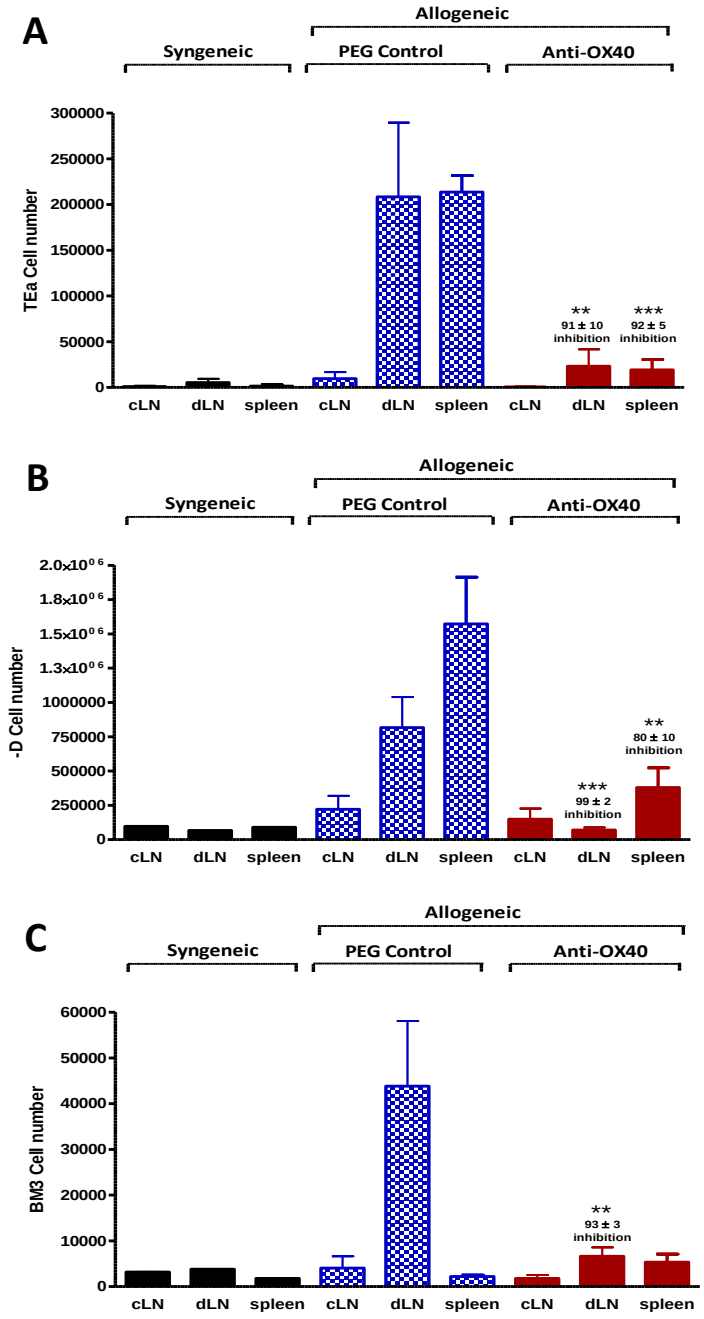


Figure 4: Blocking OX40 inhibits clonal expansion of naïve CD4$^+$ and CD8$^+$ T cells after allogeneic skin transplantation

1x10^5 naïve TEa, -D or BM3 T cells were adoptively transferred into syngeneic Rag$^{-/-}$ mice. 1 day later, mice received an allogeneic skin graft and anti-OX40 or PEG control was administered s.c at 100mg/kg. The number of TEa T cells ± SD (A), -D (B) and BM3 T cells (C) in the cLN, dLN and spleen after syngeneic or allogeneic skin transplantation was shown (n = 4 mice per group). The unpaired student t test was used to compare differences between anti-OX40 and PEG treated mice where ** represents $p<0.01$, *** represents $p<0.001$.

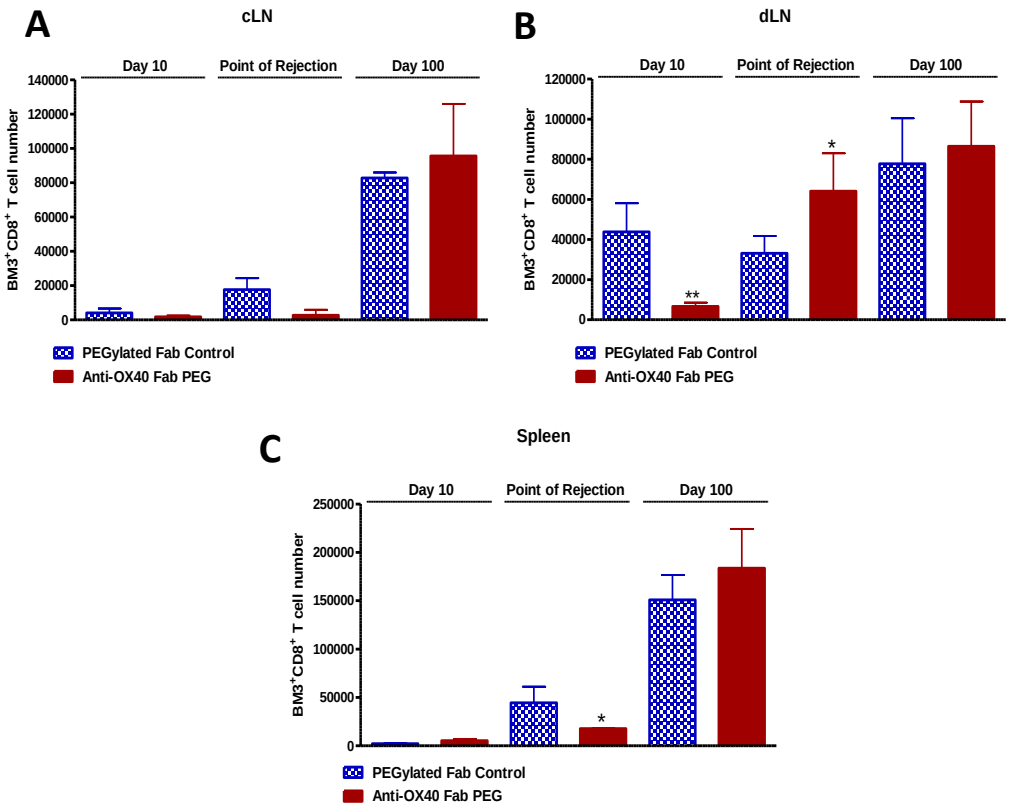


Figure 5: Anti-OX40 has no long term impact on accumulation or redistribution of BM3 T cells

$1x10^5$ naïve BM3 T cells were adoptively transferred into syngeneic CBA $Rag^{-/-}$ mice. 1 day later, mice received an allogeneic ($H2^{b+}$) skin graft and anti-OX40 or PEG control administered s.c. at 100mg/kg twice weekly for 4 weeks. Mice were harvested at various time points and the number of BM3 T cells ± SD in A) cLN, B) dLN and C) spleen determined (n = 4 mice per group). The unpaired student t test was used to compare differences between anti-OX40 and PEG control treated mice where * represents $p<0.05$; ** represents $p<0.01$.

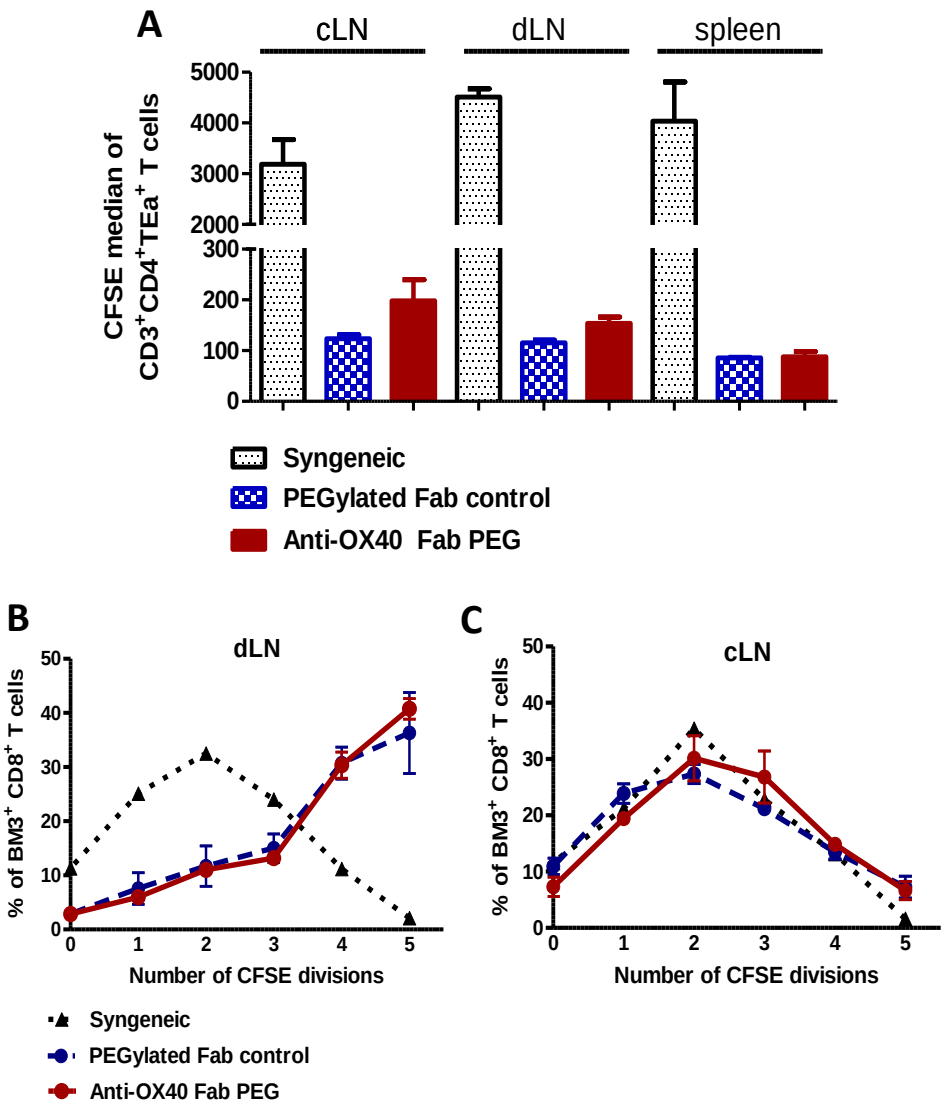


Figure 6: Anti-OX40 has no impact on the proliferation of naïve CD4$^+$ or CD8$^+$ T cells after allogeneic skin transplantation

1x10^5 naïve TEa or BM3 T cells were adoptively transferred into syngeneic Rag$^{-/-}$ mice. 1 day later, mice received an allogeneic skin graft and anti-OX40 or PEG control was administered s.c. at 100mg/kg twice weekly for 10-15days. Proliferation, measured by CFSE dilution, of A) TEa T cells B) BM3 T cells in the cLN, dLN and spleen. (n = 4 mice per group). Results were expressed as mean MFI or % ± SD, performed in triplicate and are representative of 2 independent experiments.

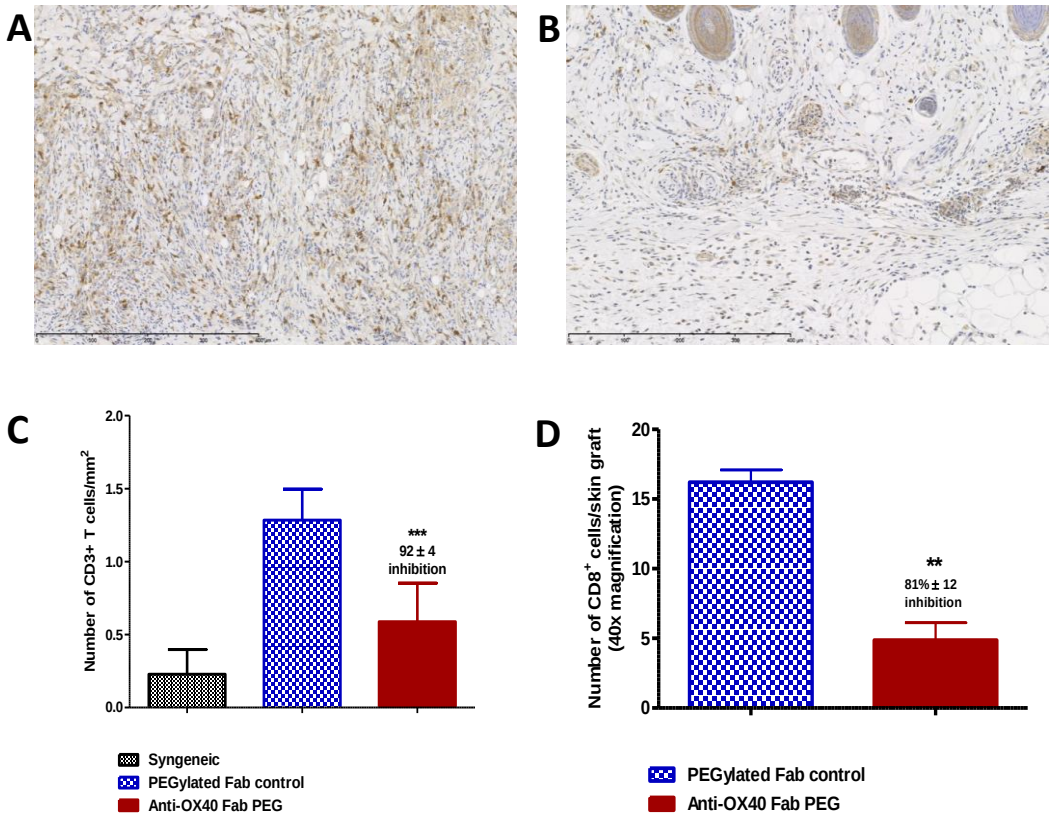


Figure 7: Blocking OX40 prevents the initial migration of $CD4^+$ and $CD8^+$ T cells into the skin graft

$1x10^5$ naïve TEa or BM3 T cells were adoptively transferred into syngeneic $Rag^{-/-}$ mice. One day later, mice received an allogeneic skin graft and either anti-OX40 or PEG control, administered s.c. at 100mg/kg. Skin grafts were analysed by immunohistochemistry for the presence of $CD3^+$ T cell infiltration. Photomicrographs were representative of 3 non-serial sections per graft taken from 4 mice per group. A) TEa T cells + PEG control B) TEa T cells + anti-OX40. Enumeration of CD3 infiltration after reconstitution with C) TEa T cells D) BM3 T cells. Results were expressed as mean number ± SD. The unpaired student t test was used to compare differences between anti-OX40 and PEG control treated mice where ** represents $p<0.01$, *** represents $p<0.001$.

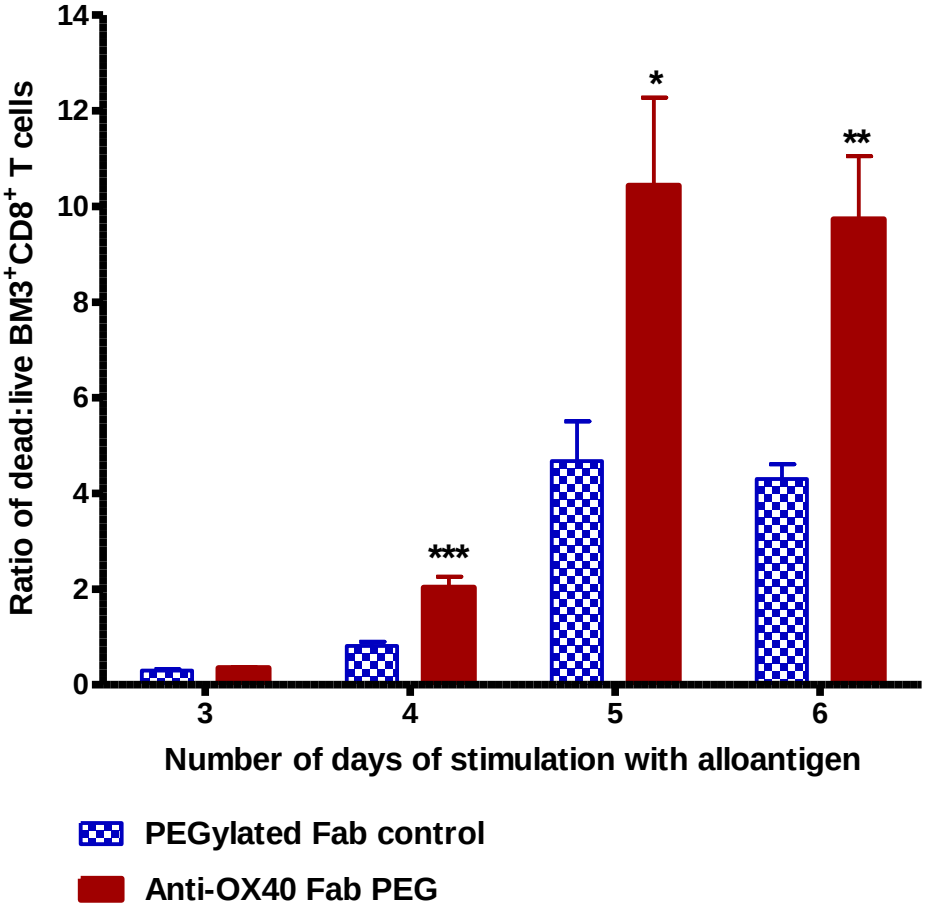


Figure 8: Anti-OX40 reduces the survival of activated BM3 T cells after stimulation with alloantigen *in vitro*

$2.5x10^4$/well BM3 T cells and $1x10^5$/well irradiated B6 splenocytes were cultured for between 2 and 6 days in the presence of either anti-OX40 or PEG control (both at 10µg/ml). Cells were analysed for phenotypic markers and viability was assessed by 7-AAD staining by flow cytometry. The ratio of dead (7-AAD$^+$ CD8$^+$ T cells) to live (7-AAD$^-$ CD8$^+$ T cells) cells was then calculated. Results were expressed as mean ± SD, performed in triplicate and are representative of 2 independent experiments. The unpaired student t test was used to compare differences between anti-OX40 and PEG control cultures where * represents $p<0.05$; ** represents $p<0.01$ and *** represents $p<0.001$.

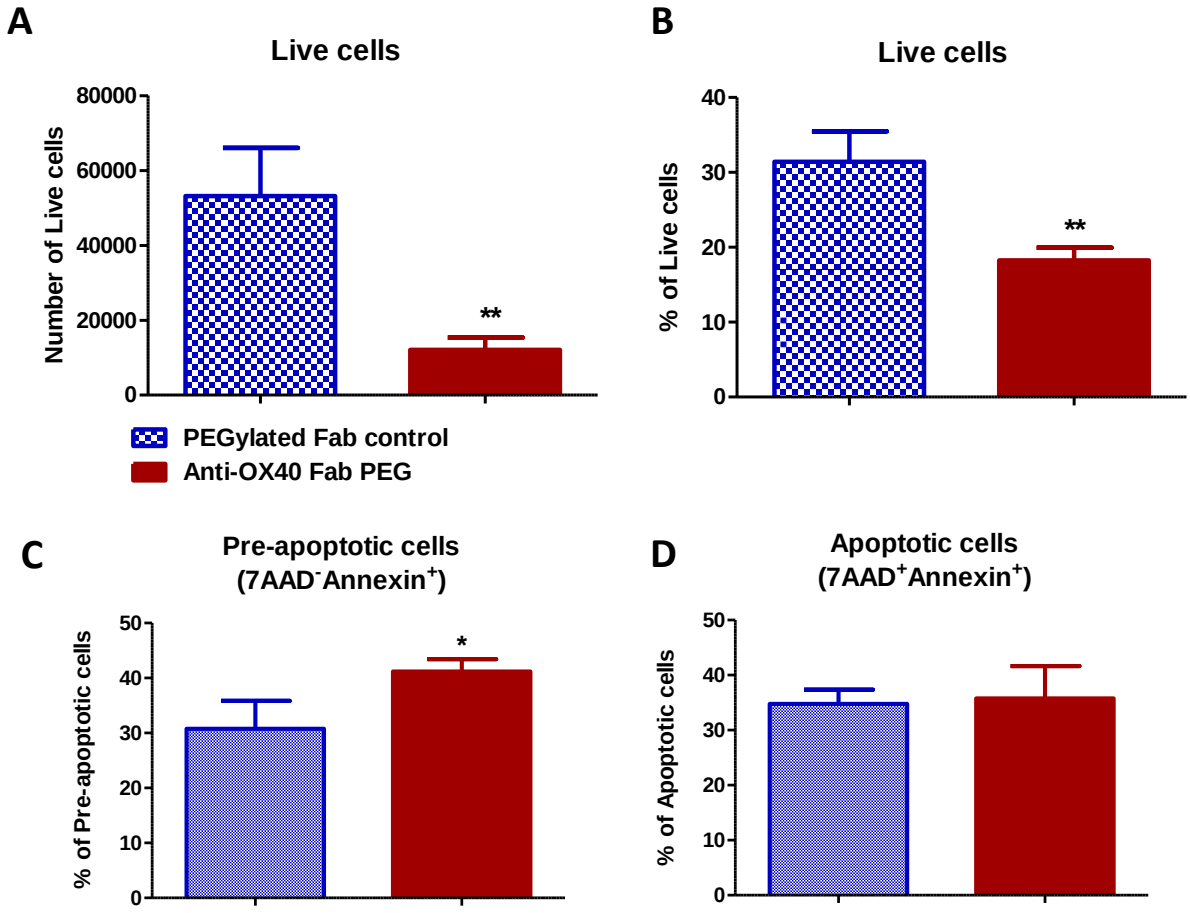


Figure 9: Anti-OX40 increases cell death in the dLN after skin allograft transplantation

$1x10^5$ naïve TEa T cells were adoptively transferred into syngeneic $Rag^{-/-}$ mice. One day later, mice received an allogeneic skin graft and either anti-OX40 or PEG control, administered s.c. at 100mg/kg and sacrficed on day 13. dLN were analysed for live (A and B), pre apoptotic (annexin V^+7AAD^-) (C) and apoptotic ($AnnexinV^+7AAD^+$) (D) $CD4^+$ T cells.

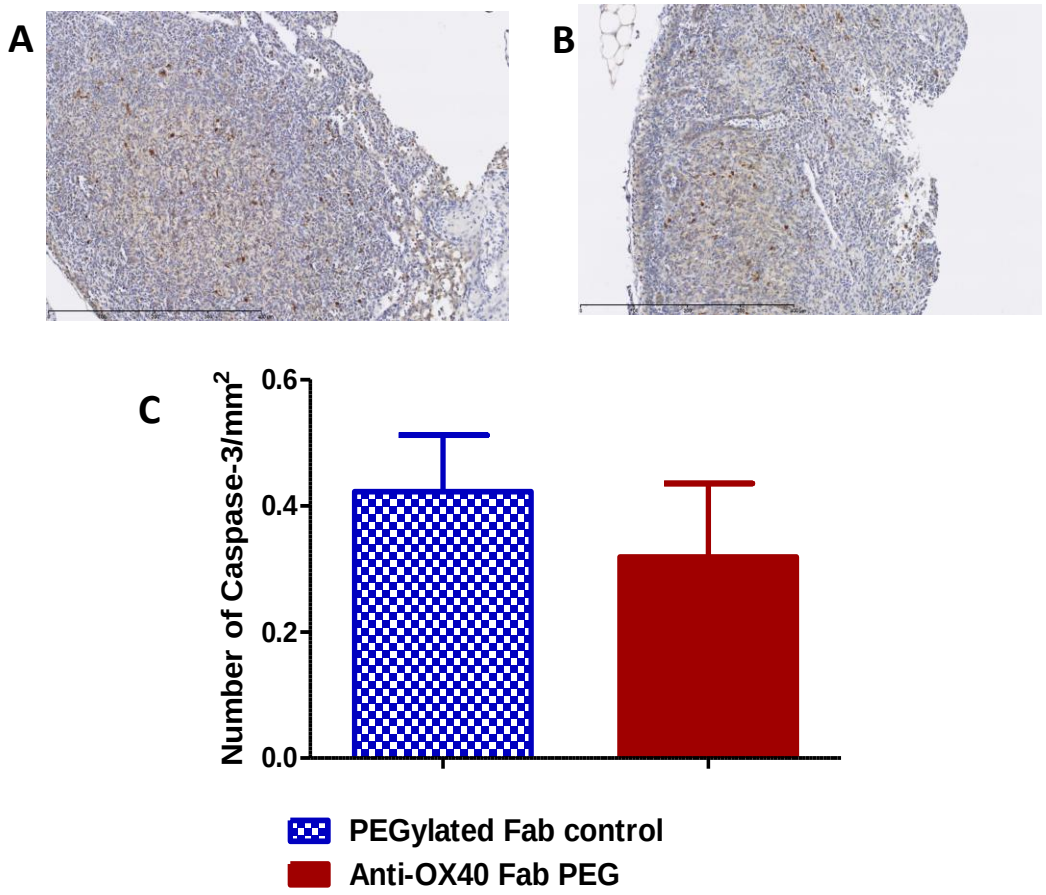


Figure 10: Anti-OX40 has no impact on caspase-3 expression in the dLN of mice after allogeneic skin transplantation

$1x10^5$ naïve TEa T cells were adoptively transferred into syngeneic $Rag^{-/-}$ mice. One day later, mice received an allogeneic skin graft and either anti-OX40 or PEG control, administered s.c. at 100mg/kg and sacrficed on day 13. Histological analyses of dLN were performed for expression of caspase-3; PEG control (A) anti-OX40 (B), enumeration of caspase-3 expression/mm^2 of dLN (C).

Chapter 5: Role of OX40-OX40L in Regulatory T cells

5.1 Introduction

Interest in suppressor cells was revived by Sakaguchi *et al* who demonstrated that $CD4^+$ T cells that constitutively express CD25 (IL-2Rα chain) derive from the thymus and that depletion of this T cell subset in naïve mice results in multi-organ autoimmune disease when subsequently transferred into immunodeficient recipients (153, 400). This subset of $CD4^+$ T cells was later termed regulatory T cells (Treg).

The discovery of a master regulator or transcription factor that was selectively expressed by Treg (forkhead/winged helix family transcription factor (FoxP3) (154, 155)) has contributed to defining Treg as a distinct T cell population. $FoxP3^{-/-}$ causes lethal X-linked fatal autoimmune diseases mediated by $CD4^+$ T cells in mice (154, 164) and man (163, 166) as a result of a lack of Treg. This lethality is dependent on $CD4^+$ T cells, as demonstrated by the depletion of $CD4^+$, but not $CD8^+$ T cells which abrogates disease in experimental studies (401).

$CD4^+CD25^+FoxP3^+$ Treg can develop either in the thymus (termed natural Treg; nTreg) or arise following activation in the periphery (termed inducible or adaptive Treg; iTreg). Specific signals, including IL-2, TGF-β (402) and retinoic acid (403), have been shown to be critical for the induction and/or maintenance of Treg and it is generally accepted

that Treg are able to control autoreactive T cells as well as suppressing immune responses against foreign antigens in various contexts; infection (404), malignancy (405) and transplantation (406).

In the field of transplantation, active regulation of immune responses has been shown to be critical for the induction and maintenance of tolerance to donor antigens (407-409). For example, studies from our laboratory have demonstrated that wild-type CBA mice treated with a donor specific transfusion (DST) combined with anti-CD4 mAb (177/DST protocol) 28 days before transplantation accept cardiac allografts indefinitely without further intervention (410, 411). DST in this context acts to provide a large bolus of alloantigen-bearing cells and alloantigen which can be processed and presented by recipient APC (412) providing TCR-mediated stimulation and activation of alloantigen-reactive T cells, whilst the anti-CD4 mAb impacts $CD4^+$ T cell activation.

Importantly this protocol involves the delivery of DST by the intravenous route thereby challenging the immune system with donor alloantigen in the absence of inflammation. This 177/DST protocol is believed to both expand pre-existing nTreg (413) and in addition promote the conversion of naïve $CD4^+$ T cells into iTreg (413, 414). In addition, 28 days after administration of 177/DST in wild-type CBA mice, Treg isolated from spleen and mLN were able to prevent skin allograft (B6) rejection when co-transferred with naïve effector T cells into syngeneic $Rag^{-/-}$ mice (324).

Phenotypic analysis has shown that Treg constitutively express a number of costimulatory molecules including CTLA-4 (158, 159), GITR (156, 157) and OX40 (259, 415). Signalling via molecules such as GITR abrogates the suppressive capacity of Treg (157, 416) and abolishes Treg mediated skin allograft survival (156) raising the question of whether OX40 signalling also inhibits Treg activity or whether OX40-OX40L interactions may be important for the survival of recently activated Treg akin to that shown for conventional T cells (Chapters 3 and 4).

Costimulation blockade can impact both the effector T cells and Treg in an alloimmune response. Mice deficient in CD28 or its ligands CD80 and CD86 display a profound reduction in the number of thymic and peripheral Treg (417, 418). Furthermore, it has been reported that $CD28^{-/-}$ non-obese diabetic (NOD) mice display exacerbated autoimmune diabetes compared to wild-type NOD controls (417). These studies suggest that costimulation may be important for the selection of Treg and/or the peripheral survival of Treg.

$OX40^{-/-}$ mice have a reduced frequency of $CD4^{+}CD25^{+}$ Treg in very young mice (16-18 days old), but this is corrected in adulthood (12-13 weeks) (259) suggesting that OX40-OX40L is dispensable for the development of nTreg. Furthermore, $OX40^{-/-}$FoxP3-GFP knock-in mice were found to have no defects in the number and proportion of peripheral $FoxP3^{+}$ Treg (308), indicating that OX40-OX40L interactions play no role in the development of Treg. However, OX40 signalling and a low dose of antigen (moth

cytochrome C) has been shown to suppress the differentiation of naïve TCR-transgenic $CD4^+$ T cells into $FoxP3^+$ Treg (307).

OX40-OX40L interactions may also impact the development of other immunoregulatory populations as an agonistic OX40L mAb has been reported to inhibit the generation of IL-10 producing Tr1 cells induced by the immunosuppressive agents dexamethasone and vitamin D3 from either naïve or memory $CD4^+$ T cells (419). These data collectively provide evidence that signalling through OX40-OX40L expressed by recently activated $CD4^+$ T cells suppress antigen driven induction of immunoregulatory cells.

In addition to affecting the generation of iTreg, ligation of OX40-OX40L has also been reported to block the suppressive capacity of Treg in *in vitro* culture systems which contain both nTreg and iTreg. Two mechanisms appear to be involved in this suppression. A direct effect of OX40 signalling on Treg function which was found to abrogate the suppressive capacity of Treg (308, 415) *in vitro*, and was comparable to the loss of function observed with an agonistic anti-GITR mAb (415). The ability of OX40 signalling to inhibit Treg suppression has also been demonstrated in a number of autoimmune and inflammatory diseases such as GvHD (415), colitis (259, 420) and allograft rejection (308). In the case of transplantation, costimulation via OX40 had no impact on the survival or proliferation of Treg but rather inhibited FoxP3 gene expression (308). Additionally, OX40 can provide costimulation to activated effector T cells which has been shown to render them resistant to suppression (421).

5.2 Aims and Hypothesis

Immunoregulatory cells, in particular $FoxP3^{+}CD4^{+}$ Treg, are an important feature of the immune response to allogeneic transplants. Experimental evidence suggests that in a range of immune diseases, including transplantation, that OX40-OX40L interactions may play an opposing role on effector T cells and Treg. In Chapters 3 and 4 we have shown that blockade of OX40 signalling prevents allograft rejection mediated by $CD4^{+}$ and $CD8^{+}$ T cells as activated alloantigen-reactive T cells fail to induce genes important for survival. Therefore, we next sought to investigate the role of OX40-OX40L interactions in both nTreg and iTreg that arise following conversion from naïve $CD4^{+}$ T cells. Our hypothesis was that blockade of OX40 would enhance the ability of Treg to suppress T cell responses to allografts.

5.3 Results:

5.3.1 $CD4^+Foxp3^+$ Treg constitutively express OX40, but not OX40L.

Firstly the expression of OX40 and OX40L was analysed on $CD4^+CD25^+FoxP3^+$ T cells (Treg). To this end, spleens were harvested from both naïve wild-type CBA and B6 mice and the expression of OX40 and OX40L determined on Treg by flow cytometry. We compared two different wild-type strains as these were used in later experiments. Irrespective of the background strain, Treg expressed OX40 (**Figure 1A**) but not OX40L (**Figure 1B**).

5.3.2 A small prolongation in cardiac allograft survival is observed after anti-CD4/DST in the absence of OX40 signals.

In our laboratory we have developed protocols that involve the administration of a non-depleting anti-CD4 mAb (YTS177; 177) and a DST which leads to indefinite cardiac allograft survival in immunocompetent recipients (B6 to CBA) and generates alloreactive Treg (422). However, the same 177/DST protocol fails to induce long term survival in more stringent strain combinations such as BALB/c to B6 recipients (N.D. Jones, personal communication). Although this protocol has been shown to generate donor-reactive Treg in B6 recipients it is likely that their number/function is simply not sufficient to control rejection in this high-responder strain combination.

Therefore, we used this model to determine whether OX40 blockade during Treg induction by the 177/DST protocol would lead to increased survival of a subsequent cardiac allograft. To this end, B6 mice received 177/DST with or without OX40

blockade (on days -28 and -25) and a donor-type (BALB/c) cardiac allograft on day 0 (**Figure 2A**). Mice which received OX40 blockade in addition to the 177/DST showed a small but significant prolongation in cardiac allograft survival compared to PEG control treated mice (MST 19.5 vs. 16 days; $p<0.014$; **Figure 2B**). BALB/c cardiac allografts are normally rejected within 9 days in untreated recipients in this strain combination (data not shown).

5.3.3 OX40 blockade enhances Treg potency *in vitro*

Next, we wanted to ask whether the Treg generated by 177/DST in the absence of OX40 signalling had enhanced activity compared to Treg generated in the presence of OX40. To address this question, alloreactive Treg were generated *in vivo* following administration of 177/DST in the presence or absence of anti-OX40 (days -28 and -25). $CD4^+CD25^+$ Treg were isolated on day 0 and tested for their relative ability to suppress an alloreactive T cell response *in vitro*.

$1x10^5$ responders ($CD4^+CD25^-$) from naïve CBA mice, labelled with a cell trace violet dye to assess proliferation, were cultured with varying numbers of CFSE-labelled Treg isolated from mice treated with 177/DST with or without OX40 blockade together with $1x10^4$ $CD11c^+$ stimulator cells. Culture wells with responders and stimulators only underwent significant proliferation (**Figure 3**). Addition of Treg, purified from 177/DST/PEG control treated mice, at a 1:1 ratio markedly inhibited the proliferation of responding $CD4^+$ T cells (75% inhibition ± 3) (**Figure 3**). With decreasing numbers of Treg isolated from PEG control treated mice (i.e. increased ratio of naive to Treg)

proliferation was restored to similar levels to that seen with no Treg (**Figure 3**). Importantly, Treg generated under OX40 blockade showed a greater suppressive capacity than their PEG control counterparts, which was revealed following the titration of decreased numbers of Treg into MLR cultures (at a 1:1 ratio 89% inhibition ± 2 vs. 75% ± 3) (**Figure 3**).

5.3.4 Anti-OX40 enhances the ability of Treg generated by 177/DST to prevent skin allograft rejection.

Previous work from our laboratory has shown that the 177/DST protocol generates alloreactive Treg which prevent rejection of skin allografts mediated by a defined number of naïve T cells (324, 423). The data presented above suggests that if OX40 interactions were blocked during Treg generation by 177/DST the resultant Treg were more suppressive *in vitro*. Therefore, we next sought to confirm that this was also the case *in vivo*. To test whether blockade of OX40 at the time of Treg generation ultimately enhances Treg potency, CBA mice were treated with 177/DST and either anti-OX40 or PEG control on days -28 and -25. Treg were isolated from such mice and adoptively transferred into syngeneic $Rag^{-/-}$ mice together with syngeneic naïve T cells ($CD4^{+}CD25^{-}$). The following day mice received an allogeneic skin transplant (**Figure 4**).

Immunodeficient mice reconstituted with naïve T cells acutely rejected B6 skin allografts (MST 17 days; **Figure 5A**). At a ratio of 1:1 ($1x10^5$/population) naïve to 177/DST generated Treg, skin allografts survived indefinitely (MST >100 days; **Figure**

5A). Mice treated with either 177 or DST alone acutely rejected their skin grafts demonstrating that both 177 and DST were required to generate alloantigen-reactive Treg in this model (data not shown) (324).

Although 177/DST generated Treg were able to control allograft rejection at a 1:1 ratio with naïve T cells, when the number of naïve T cells was increased relative to Treg, differences in graft survival were observed. At a ratio of 4:1 (naïve:Treg), blockade of OX40 at the time of 177/DST resulted in the generation of Treg that were able to prevent skin allograft rejection whereas Treg generated by 177/DST in the presence of the PEG control failed to prevent allograft rejection (MST >100 days vs. 16.5 days; $p<0.0003$; **Figure 5B**). These data suggest that blockade of OX40 interactions increased the potency of alloreactive $CD4^+CD25^+$ Treg generated by treatment with 177/DST.

5.3.5 OX40 blockade at the time of Treg generation does not result in increased suppression of a BM3 T cell response to alloantigen

We have shown that Treg generated in the absence of OX40 signalling were more effective than those generated in the presence of OX40-OX40L interactions in their ability to regulate the response of alloreactive T cells both *in vitro* (Figure 3) and *in vivo* (Figure 5), albeit in situations where purified Treg were used at artificial ratios. Therefore, we next sought to ask whether Treg generated using the 177/DST protocol in wild-type mice, in the presence or absence of OX40 signalling, demonstrated a difference in their ability to control T cell responses to alloantigen *in situ*. CFSE

labelled $H2K^b$-reactive TCR-transgenic CD8 T cells (BM3) were transferred to naïve syngeneic mice or to mice that that had received 177/DST with either anti-OX40 or PEG control 28 days previously. Mice received an injection of allogeneic splenocytes (B6; $H2K^{b+}$) the following day and spleens were harvested for analysis by flow cytometry 3 days after splenocyte challenge (**Figure 6A**).

As expected, the transfer of BM3 T cells and allogeneic splenocyte challenge resulted in a massive expansion of BM3 T cells in the spleen (133427 cells ± 25690) compared to mice that received BM3 with no challenge (10872 cells ± 5071; $p<0.0001$; **Figure 6B**). The presence of 177/DST generated alloreactive Treg was found to prevent BM3 T cell expansion (23896 ± 8665 vs. 133427 ± 25690; $p<0.0009$; **Figure 6B).** Treg generated in the presence of anti-OX40 had a similar suppressive capacity compared to Treg generated in PEG control mice (**Figure 6B**). Enumeration of Treg in the spleens of animals showed that there was also no difference in the number of Treg (**Figure 6C**), however there was an increase in the percentage of splenocytes that were Treg (**Figure 6D**). This percentage increase in Treg may reflect an increase in alloreactive Treg however this could not be confirmed in these experiments.

5.3.6 OX40 blockade has no impact on the conversion of naïve $CD4^+$ T cells into Treg

Although there was no gross difference between the number of Treg generated in the presence or absence of OX40 this could be a result of lack of sensitivity in this model due to a potentially low precursor frequency of alloreactive Treg and the lack of

reagents that would aid identification of alloreactive Treg. Therefore, other *in vitro* and *in vivo* assays were employed to attempt to determine whether anti-OX40 enhanced the generation of iTreg from naïve $CD4^+$ T cells or aided the expansion of pre-existing nTreg, either of which would result in a relative increase in potency.

Previously our laboratory has shown that the culture of $CD4^+$ T cells with allogeneic immature DC in the presence of 5ng/ml IFN-γ leads to induction of Treg that were able to suppress skin allograft rejection (424, 425). To determine whether anti-OX40 promotes the generation of Treg *in vitro* this IFN-γ conditioning protocol was used with and without OX40 blockade (**Figure 7A**). Anti-OX40 was found to have no impact on the number of $FoxP3^+$ Treg generated (17726 ± 4710 vs. 23467 ± 13114 cells per well; **Figure 7B**), however anti-OX40 did significantly impact the percentage of Treg within the cultures compared to PEG control cultures (58% ± 5 vs. 35% ± 11; $p<0.0001$; **Figure 7C**).

In Chapter 4 we showed that anti-OX40 had no impact on the proliferation of T cells but significantly undermined the survival of effector T cells. This observation led us to examine the number of effector ($CD4^+FoxP3^-$) T cells after stimulation with alloantigen and IFN-γ. Anti-OX40 was found to significantly decrease the number of effector T cells (**Figure 7D**) through preferential cell death (assessed by 7AAD viability stain) of the effector T cell whilst sparing the Treg population (**Figure 7E**) (425). Therefore, these data suggest that OX40 blockade does not enhance conversion of naïve $CD4^+$ T

cells into Treg under these *in vitro* conditions but may alter the effector to Treg balance through the inability of effector T cells to sustain expansion in the absence of OX40.

Conversion of naïve T cells into Treg likely requires a number of growth factors and cytokines, which may not be accurately recapitulated *in vitro*, so next we sought to determine whether OX40 blockade aids the conversion of naïve T cells to Treg *in vivo*. Treg generated by 177/DST in CBA mice routinely result in the long term survival of skin and cardiac allografts (324, 423, 426). Although this protocol fails to induce significant cardiac allograft prolongation across a full MHC barrier in B6 recipients 50% of mice permanently accept BALB/cB6 F1 cardiac allografts (N.D. Jones personal communication) suggesting that Treg are generated by 177/DST in B6 mice. In fact this has been previously shown by our laboratory (413) confirming that B6 mice are appropriate to investigate impact of anti-OX40 on the generation of Treg by 177/DST.

To examine whether anti-OX40 promotes the development of iTreg, $1x10^6$ alloreactive, TCR-transgenic $CD4^+$ T cells (TEa) or $3x10^6$ $GFP^-CD45.2^+CD4^+$ T cells (isolated from FoxP3-GFP knock-in mice) were transferred into syngeneic CD45.1 congenic B6 mice on day -29. The following day mice were pre-treated with 177/DST (days -28 and -27) with and without OX40 blockade (days -28 and -25; **Figure 8A and 8C**). Using T cells negative for GFP or TCR transgenic T cells ensured that the starting population was almost devoid of $Foxp3^+$ Treg (purity > 98%; data not shown). Spleens were then harvested on day 0 to look for the presence of either TEa^+FoxP3^+ T cells or $CD45.2^+FoxP3^+$ T cells.

Unfortunately, regardless of the starting population, 177/DST did not induce Treg with or without the addition of anti-OX40 (**Figures 8B and 8D**). Following adoptive transfer of GFP^- T cells, a small number (~250) of GFP^+ (therefore $FoxP3^+$) T cells were recovered (data not shown), however as this was 12,000 fold less than the input population we would refrain from interpreting this as genuine Treg induction.

5.3.7 Anti-OX40 does not promote the expansion of nTreg *in vitro* and *in vivo*

The data presented in Figure 8 suggested that the induction of alloreactive Treg is a relatively minor contributor to the alloreactive Treg pool generated by 177/DST, therefore we sought to examine the role of OX40-OX40L on the expansion of pre-existing alloreactive nTreg following exposure to alloantigen. The manipulation or expansion of pre-existing, already differentiated Treg is an important and clinically relevant issue in the treatment of T cell mediated diseases.

To this end, $5x10^5$ nTreg ($CD4^+CD25^+$) were stimulated with plate-bound anti-CD3/anti-CD28 and 10ng/ml exogenous IL-2 *in vitro* (427) which resulted in an approximate 15 fold increase in Treg numbers by day 7 (**Figure 9**). Stimulation of nTreg with both anti-CD3 and anti-CD28 in the absence of OX40 signalling did not result in an increase in Treg numbers compared to PEG control treated cultures (**Figure 9A**). As previously shown, Treg do not express OX40L (Figure 1), therefore recombinant OX40L (10μg/ml) was immobilised on the plate in addition to anti-CD3

and anti-CD28 in order to provide sufficient OX40L to allow the interaction between OX40 and its ligand. However, there was no difference in the number of expanded nTreg between anti-OX40 and PEG control treated cultures (**Figure 9A**).

From these data, the question arose whether signalling via CD28 was providing a stimulus which was masking any effect caused by OX40 blockade. Therefore we next compared stimulating nTreg with anti-CD3 and anti-CD28 with anti-CD3 only. There was no significant difference between the two stimuli (**Figure 9B**), and again the addition of plate-bound OX40L failed to reveal a difference between anti-OX40 and PEG control (**Figure 9B**), suggesting that OX40-OX40L interactions play no role in the expansion of Treg.

In order to confirm this finding, Treg expansion was also examined *in vivo*. $1x10^6$ GFP^+CD4^+ Treg (isolated from FoxP3-GFP knock in mice) were transferred into CD45.1 congenic B6 mice. Mice were then treated with 177/DST with and without OX40 blockade (**Figure 10A**). On day 0, spleens were harvested for the numerical analysis of $CD4^+CD45.2^+$FoxP3-GFP^+ T cells. Despite only a small number of $CD4^+CD45.2^+FoxP3^+$ T cells being recovered, anti-OX40 failed to result in increased expansion of nTreg *in vivo* (**Figure 10B and 10C**). The expansion observed in this experiment was lower than expected however this may have been due to lack of space as immunocompetent (B6) mice were used as recipients.

5.3.8 Anti-OX40 has no impact on markers of Treg which are involved in suppression

Thus far we have provided evidence that OX40 blockade does not increase the precursor frequency of alloreactive Treg generated by 177/DST suggesting that the increased potency of such Treg may be attributable to differences in function upon reactivation of Treg on a per cell basis. Initially the expression of molecules involved in the suppressive activity of Treg (CD25, CTLA-4, CD73, CD39, GITR and FoxP3) were compared between $CD4^{+}FoxP3^{+}$ Treg from naïve mice or mice treated with 177/DST with and without blockade of OX40. As expected all Treg were found to express the aforementioned molecules however there was no difference in expression of the molecules between the different cohorts of Treg (**Figure 11**).

5.3.9 OX40 blockade preferentially provides Treg with a survival advantage compared to Treg generated in the presence of OX40 signalling

OX40 blockade significantly enhanced cell death in effector T cells (Chapter 4) which led us to investigate whether OX40 blockade could impact the survival of Treg which resulted in an increase in potency. To address this, the number of live Treg (that had been generated by 177/DST in the presence or absence of OX40-OX40L interactions) present after 5 days of culture with syngeneic responder ($CD4^{+}CD25^{-}$) T cells and allogeneic $CD11c^{+}$ splenocytes was determined. 177/DST Treg generated in the absence of OX40 signalling had a significant survival advantage both in terms of the number (at 1:1 ratio Treg number 34527 ± 3368 vs. 5934 ± 384) (**Figure 12A**) and percentage of cells (at 1:1 ratio % Treg 46.4% ± 0.8 vs. 12.3 ± 1.1) (**Figure 12B**)

compared to Treg generated in PEG control treated mice. This survival advantage was evident at all the ratios of responders to Treg following reactivation with allogeneic splenocytes *in vitro*.

5.3.10 Anti-OX40 significantly increases the anti-apoptotic molecule Bcl-2

We have shown that Treg which are generated *in vivo* in the absence of OX40 signalling are more potent than Treg isolated from control treated mice (Figures 3 and 5) and suggested that this was due to an increase in the ability of Treg to survive following reactivation (Figure 12). To confirm this observation we examined the expression levels of the anti-apoptotic molecule Bcl-2 on Treg. To this end, Treg were generated *in vivo* using the 177/DST protocol in the presence or absence of OX40 blockade. Treg were isolated from the spleen and mLN from these mice 28 days later, and Treg were co-cultured with syngeneic responders and allogeneic $CD11c^+$ stimulators for up to 5 days. Treg were analysed by flow cytometry for their expression of Bcl-2. Cultures with Treg generated in the absence of OX40 signalling were found to have a significantly higher number of $Bcl\text{-}2^+$ Treg at days 4 ($Bcl\text{-}2^+$ Treg cell number 2410 ± 255 vs. 844 ± 559; $p< 0.0116$; **Figure 13**) and 5 ($Bcl\text{-}2^+$ Treg cell number 7943 ± 594 vs. 3466 ± 627; $p< 0.0009$; **Figure 13**). These data suggest that the increased potency of 177/DST Treg generated in the absence of OX40 signalling may be due to the increased survival of Treg following re-activation.

5.3.11 Anti-OX40 does not increase the number of 177/DST generated Treg in the spleen or allograft after allogeneic cardiac transplantation.

Treg generated/expanded in the presence of OX40 blockade proved to be more effective at preventing T cell responses to alloantigen *in vitro* and *in vivo*. The data in the previous section suggested that this may be due to such Treg surviving better than control Treg following re-activation by alloantigen. To examine this *in vivo*, wild-type CBA mice received 177/DST with and without OX40 blockade (days -28 and -25) and on day 0 mice received a donor-type cardiac allograft (B6). Spleens and cardiac allografts were harvested on day 5 to analyse the number and relative proportion of Treg amongst live leukocytes (**Figure 14A**). Previous work from our laboratory has shown that only alloreactive T cell infiltrate allografts after allogeneic cardiac transplantation (428) therefore the analysis of intragraft Treg (assumed to be mostly alloreactive) may provide a clearer picture of how alloreactive Treg generated in the absence of OX40 interactions may respond upon re-exposure to alloantigen *in vivo*.

Treatment with anti-OX40 at the time of 177/DST failed to alter the number or proportion of $CD4^+$ (**Figures 14B and C**) and $CD8^+$ (**Figures 14D and E**) in both the spleen and cardiac allograft compared to control mice. In addition, OX40 blockade was found to have no effect on Treg numbers in the spleen (**Figure 14F**) 5 days after transplantation, although there was a trend towards Treg being present at increased numbers (observed in 2 independent experiments). Importantly, there was no difference in the number of Treg infiltrating cardiac allografts at this time between anti-OX40 and PEG control treated of mice (**Figure 14G**).

5.4 Discussion

Our previous data have clearly demonstrated a role for OX40-OX40L in allograft rejection. Anti-OX40 affects T cell responses to alloantigen by increasing effector T cell survival by reducing the clone size, which results in abbreviation of the immune response. These data raise the question whether anti-OX40 can impact Treg function as Treg constitutively express OX40.

In this chapter we demonstrate the exposure of Treg to OX40 blockade and alloantigen generates more potent Treg, which are capable of suppressing naïve T cell responses. A number of costimulatory molecules which are required for optimal naïve T cell responses are shared by Treg for example CD28. CD28 signalling is required for the optimal suppressive function of Treg (417). However, not all costimulatory molecules promote Treg-mediated suppression, for example, signalling through GITR (a TNFR superfamily member) which is expressed on Treg (like OX40) has been shown to result in a loss of Treg function (157, 416). Importantly blockade of OX40 and GITR has a similar impact on effector T cells and results in a delay in disease onset or attenuated severity in models of diabetes (288, 429). These data have led to the question as to whether OX40 has a diametric role on effector T cells and Treg and whether blockade of this interaction could be beneficial by diminishing alloreactive effector T cell responses whilst promoting alloreactive Treg number/function.

In our studies, OX40 blockade affected the ability of Treg generated *in vivo* under the cover of anti-CD4 and DST to regulate allo-specific immune responses by increasing the potency of Treg (Figures 3 and 5). In addition, there are also reports in the literature which show OX40-OX40L can impact Treg function (308) while signalling via OX40-OX40L in effector T cells results in enhanced survival and generation of productive memory T cell responses (255, 305, 306). These data suggest OX40-OX40L has a dichotomous role on effector/memory T cells and Treg. Burocchi *et al* confirmed a diametric role for OX40-OX40L in a tumour model, whereby signalling via OX40 inhibited Treg mediated suppression and enhanced effector T cell activation (430). *In vitro* data suggested that signalling through OX40-OX40L resulted in a reduction in FoxP3 expression (308). Our data is consistent with these data thereby showing OX40 signalling enhances the survival of effector/memory T cells while also preventing the survival of Treg in response to alloantigen.

Previous work in our laboratory has shown 177/DST generates Treg that have a greater capacity to regulate and thereby prevent skin allograft rejection compared to naïve Treg on a per cell basis (324). Importantly 177/DST has been shown to generate Treg from naïve $CD4^+$ T cells (413, 414) and in addition expand pre-existing nTreg (413). Both the generation of iTreg and the expansion of alloreactive nTreg could explain why the Treg generated in the absence of OX40 signalling were more potent at the time of transplantation (i.e. day 0).

To dissect how OX40 blockade was able to increase Treg potency, firstly *in vitro* and *in vivo* models were used to examine the role of OX40-OX40L on conversion of naïve $CD4^+$ T cells into Treg and secondly on the expansion of nTreg following activation. The generation of improved methodologies to generate iTreg or expand alloreactive nTreg could prove beneficial in clinical transplantation (431, 432).

There is conclusive evidence that $FoxP3^-$ T cells can be converted into $FoxP3^+$ T cells which have suppressive function in the periphery (433, 434) confirming conversion is a valid biological phenomenon. So *et al* demonstrated that OX40 signalling could antagonise FoxP3 induction (307) suggesting that OX40 signalling may impact the induction of FoxP3 expression and therefore iTreg generation. Data such as these provide a clear rationale for blocking OX40 signalling in order to promote iTreg generation. However we found no evidence that the administration of 177/DST resulted in the generation of alloreactive iTreg (Figures 7 and 8). This was in contrast to previous data from our laboratory using the same protocol (413).

There are a number of reasons why we failed to detect 177/DST generated iTreg in our experiments. For example, memory T cells arise as a result of pathogenic infection and may be attributed to differences in cleanliness within animal facilities. The results previously described by our group were performed in different animal facilities so the health status of the mice may have been different to the mice used in our studies. Xiao *et al* demonstrated that OX40 signalling resulted in the expansion of IFN-γ producing memory T cells which inhibited the induction of Treg in a cell-contact independent

fashion (435). Whilst this may be a reason for differences between our data and others, an important point to note is that the number of iTreg generated by Francis *et al* was very small (~1500) (413) so these differences could be attributed to differences in the gating strategy used for flow cytometry analyses. Gao *et al* showed significant conversion of naïve $CD4^+$ T cells into Treg within 4 days by using a combination of rapamycin and anti-CD154 (436) therefore we may have missed the peak of the conversion by looking 28 days after anti-CD4/DST. However, we cannot rule out that OX40 had no impact on conversion of naïve $CD4^+$ T cells into iTreg but this does not provide a complete explanation for the increase in Treg potency we observed at day 0. Conversion of naïve $CD4^+$ into iTreg appears to be a minor component of the 177/DST protocol and from our data we can only conclude that anti-OX40 had no impact on iTreg generation and could not alter the precursor frequency Treg pool when assayed on day 0.

In our studies, 177/DST showed a small increase in alloreactive nTreg, however no difference was observed with the addition of anti-OX40 suggesting pre-existing alloreactive nTreg do not have enhanced expansion and survival *in vivo* after 177/DST. This was then confirmed by *in vitro* analyses with polyclonal stimulation of nTreg and again anti-OX40 failed to expand nTreg. However, Ruby *et al* demonstrated that OX40 signalling was required for the expansion of the Treg population in naïve wild-type mice (437).

These data suggest that nTreg expansion is context dependent as mixed results have been reported. Our data in models of allograft rejection demonstrated OX40 blockade increased the survival and therefore potency of Treg. However it appears that under certain circumstances OX40 signalling is required for the sustained expansion of Treg (420). The co-transfer of effector T cells and wild-type Treg prevented the development of T cell induced colitis, however $OX40^{-/-}$ Treg failed to abrogate colitis and failed to accumulate in the colon and lymphoid organs during mucosal inflammation (420). In addition, the requirement of OX40-OX40L was elegantly demonstrated in a tumour model, where cyclophosphamide (CTX) and agonistic OX40 were administered to tumour-bearing mice in addition to either GFP^- or GFP^+ $CD4^+$ T cells. CTX and OX40 signalling preferentially enhanced the expansion of nTreg in the tumour dLN, while failed to induce conversion of naïve $CD4^+$ T cells into iTreg (311). These data highlight the fact that the role of OX40 on Treg is context dependent.

When taken together our data failed to show that OX40 blockade altered the precursor frequency of alloreactive Treg after 177/DST. However, a clear impact of OX40 blockade on the long-term function of alloreactive Treg was revealed following restimulation in an *in vitro* suppression assay (Figures 12 and 13). Treg from 177/DST/anti-OX40 treated mice proved to be more potent than Treg generated in the presence of OX40 signalling due to improved survival with many more live Treg surviving throughout the culture period and showed increased expression of the anti-apoptotic molecule Bcl-2.

Although the generation of alloreactive iTreg or expansion of nTreg is not altered in the absence of OX40 signalling, the increase in potency observed is clearly context dependent. The finding that alloreactive Treg formed following alloantigen exposure (+anti-CD4) in the absence of OX40 had superior survival and suppression of alloantigen responses *in vitro* prompted us to determine whether the same was true *in vivo*.

One of the problems with this approach is that alloreactive Treg cannot be distinguished from Treg of other specificities *in vivo*. To avoid this issue we used a model where the graft infiltrating cells are limited to T cells which are actively responding to alloantigen (428), although it should be noted that this has only been shown for conventional T cells rather than Treg which may express a different chemokine receptor expression pattern.

However, there were no differences between the number of graft infiltrating Treg between anti-OX40 and PEG control treated mice at 5 days post transplantation (Figure 14). This could be due to the limited number of APC in the graft at this time-point which may fail to activate all peripheral alloreactive Treg cells. In this model, 177/DST results in long-term allograft (B6) survival in CBA recipients, therefore a later time-point could be analysed. This would allow for more T cells (including Treg) to become activated by APC presenting alloantigen which may reveal subtle differences between anti-OX40 and PEG control. As this model results in long term survival, the addition of anti-OX40 could not positively impact allograft survival but may reduce the degree of vasculopathy which would indicate OX40 blockade could improve the regulation of T

cell responses. In future studies, time-point and histological analyses may reveal differences between anti-OX40 and PEG control treated mice.

Enhanced Treg survival may be only part of the story and OX40 blockade may have other effects on Treg function which were not examined in our studies which could also increase Treg potency. Therefore further studies would be required to dissect the role of OX40-OX40L on molecules that have been associated with Treg function such as IL-10 and TGF-β. Analysis of Treg isolated from tumour sites demonstrated that tumour resident Treg exhibited a reduction in IL-10 production in the presence of OX40 signalling (430) which correlated with a decrease in the transcription factor interferon regulatory factor 1 (Irf1). This has also been found to be associated with IL-10 production from Treg isolated from the lamina propria (438). These data suggest further analyses of molecules such as TGF-β, IL-10 and CTLA-4 could provide useful dissection on the mechanisms by which OX40-OX40L impact Treg and their suppressive capacity.

OX40 signalling has been described to alter Treg positioning and homing. Following administration of agonistic OX40, Treg exhibited an increase in the expression of CCR8 and CD103 and a decrease in CCR4 (430). However these findings have not been replicated in disease models for example as $OX40^{-/-}$ Treg express comparable levels of CD103 to wild-type Treg and in a T cell transfer induced colitis model $OX40^{-/-}$ Treg were able to accumulate in the mLN and lamina propria (439), thus making the interpretation of the transcriptome data more complex. These analyses interrogate

patterns of expression at a specific time of OX40 signalling and not on the long term impact of OX40 signalling.

One important caveat of the data summarised here from the literature and our own work is that OX40 is either triggered or blocked at the time of exposure to alloantigen or activation. In our studies, OX40 was only blocked during the initial exposure to alloantigen (i.e. at the time of 177/DST). From previous work we have shown that the anti-OX40, anti-CD4 and alloantigen are cleared from the circulation rapidly which suggests OX40 blockade had a long term impact on Treg. To establish whether this increase in potency and survival of Treg observed after anti-OX40 is reversible or persists long term, epigenetic studies would be required which unfortunately were not performed as part of our studies. For these studies, Treg could be generated *in vivo* by the 177/DST protocol in the presence or absence of OX40 signalling and isolated on day 0, as in our studies. Treg would be restimulated with alloantigen *in vitro* and the surviving Treg (presumably alloantigen-specific) could be analysed for epigenetic changes or by microarray. These analyses could determine whether these changes are short or long term and if other molecules/signals are integrated into OX40-OX40L signalling.

In summary, our data demonstrate that OX40 blockade significantly increased the potency of alloreactive Treg by promoting their survival following re-activation resulting in enhanced regulation of allo-specific T cell responses. Furthermore, these dramatic results combined with data from Chapter 3 and 4 highlight the differential

impact of OX40 signalling on effector/memory T cells and Treg and further suggest that OX40 blockade may be a beneficial adjunct to strategies to promote the induction of tolerance to allografts.

5.5 Figures

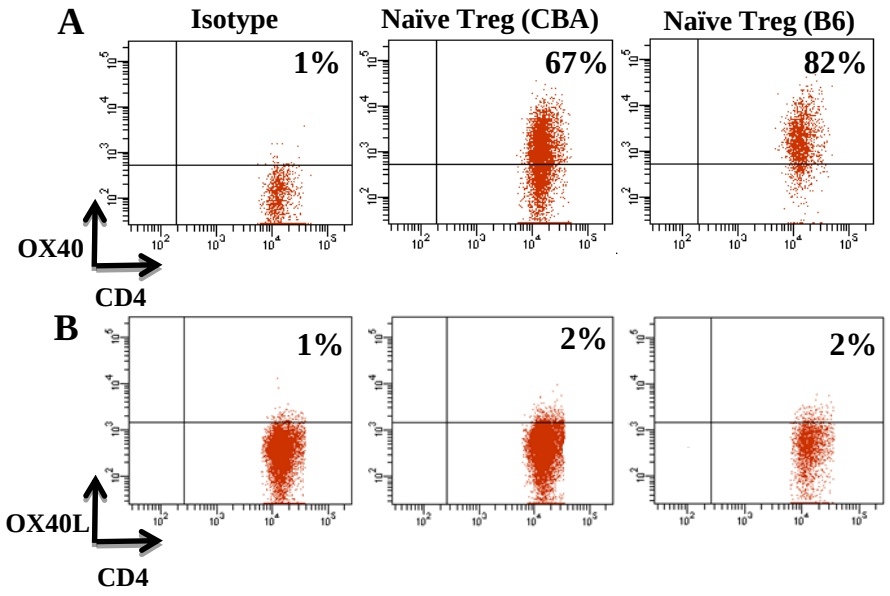


Figure 1: Regulatory T cells constitutively express OX40, but not OX40L
Naïve wild type CBA or B6 mice were harvested and the spleens analysed for expression of OX40 (A) or OX40L (B) on Treg ($CD4^+FoxP3^+$). Isotypes were used to set the quadrant gates and dot plots are representative of 3 independent experiments (n = 4 mice per group).

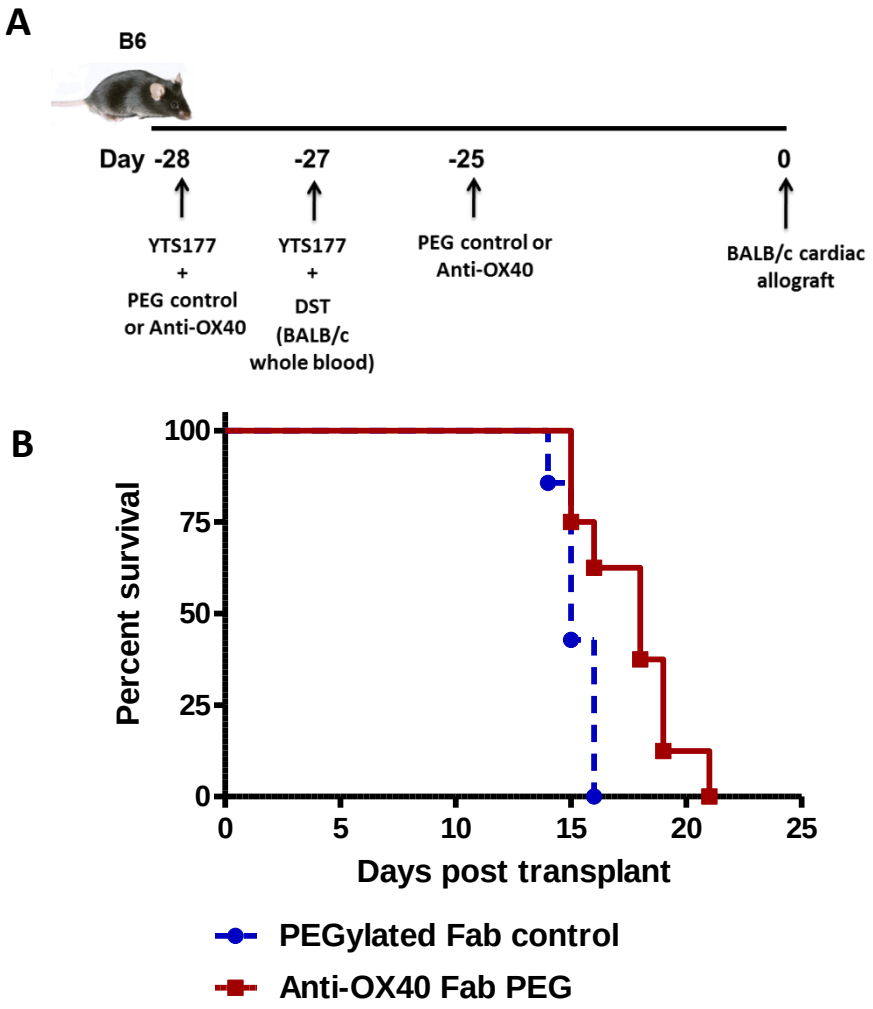


Figure 2: 177/DST and OX40 blockade at the time of Treg induction causes a small prolongation of cardiac allograft survival

A) B6 ($H2^b$) mice were pre-treated with 200µg non-depleting anti-CD4 mAb (YTS177) and 250µl DST of whole BALB/c blood on days -28 and -27. Anti-OX40 or PEG control was given 100mg/kg subcutaneously on days -28 and -25 only. On day 0, mice received a vascularised heterotopic BALB/c cardiac allograft and graft survival was monitored. B) Kaplan-Meier survival curves show cardiac allograft survival. (n = 6-8 mice per group).

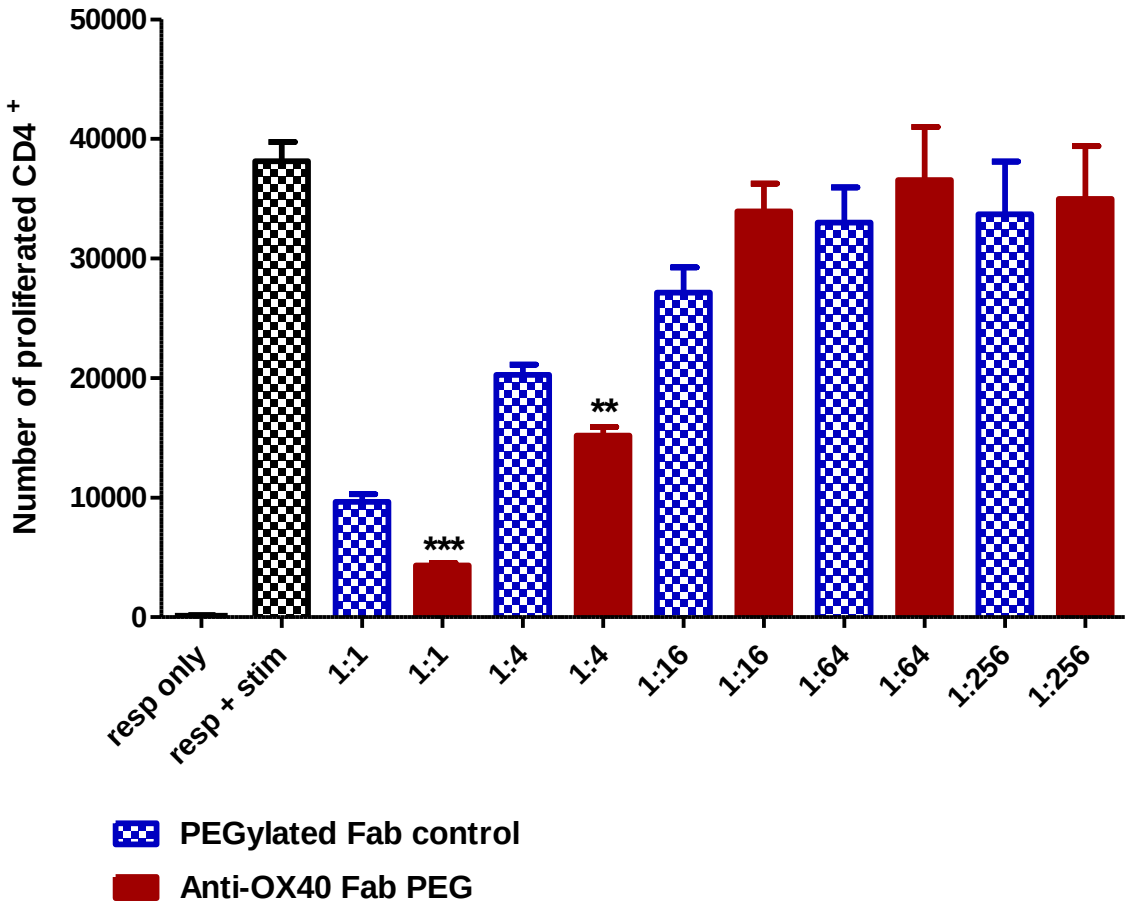


Figure 3: Treg generated in the absence of OX40 signalling have a greater suppressive capacity in an *in vitro* suppression assay

Mice were administered 200μg of non-depleting anti-CD4 mAb and DST on days -28 and -27. Anti-OX40 or PEG control were administered at 100mg/kg subcutaneously on days -28 and -25. Treg ($CD4^+CD25^+$) were purified on day 0 from mice treated with 177/DST and labelled with CFSE. Naïve responder T cells ($CD4^+CD25^-$) were purified from naïve CBA mice and labelled with a violet fluorescent dye. Stimulator $CD11c^+$ cells were purified from B6 splenocytes. Responder and stimulator cells ($1x10^5$ per population) were co-cultured and in appropriate wells Treg were added at varying ratios to the responder cells. Cultures were analysed after 6 days of culture by flow cytometry. Results are expressed as mean ± SD, performed in triplicate (n = 6 mice per group (pooled)) and representative of 2 independent experiments). ** represents $p<0.01$ *** represents $p<0.0001$

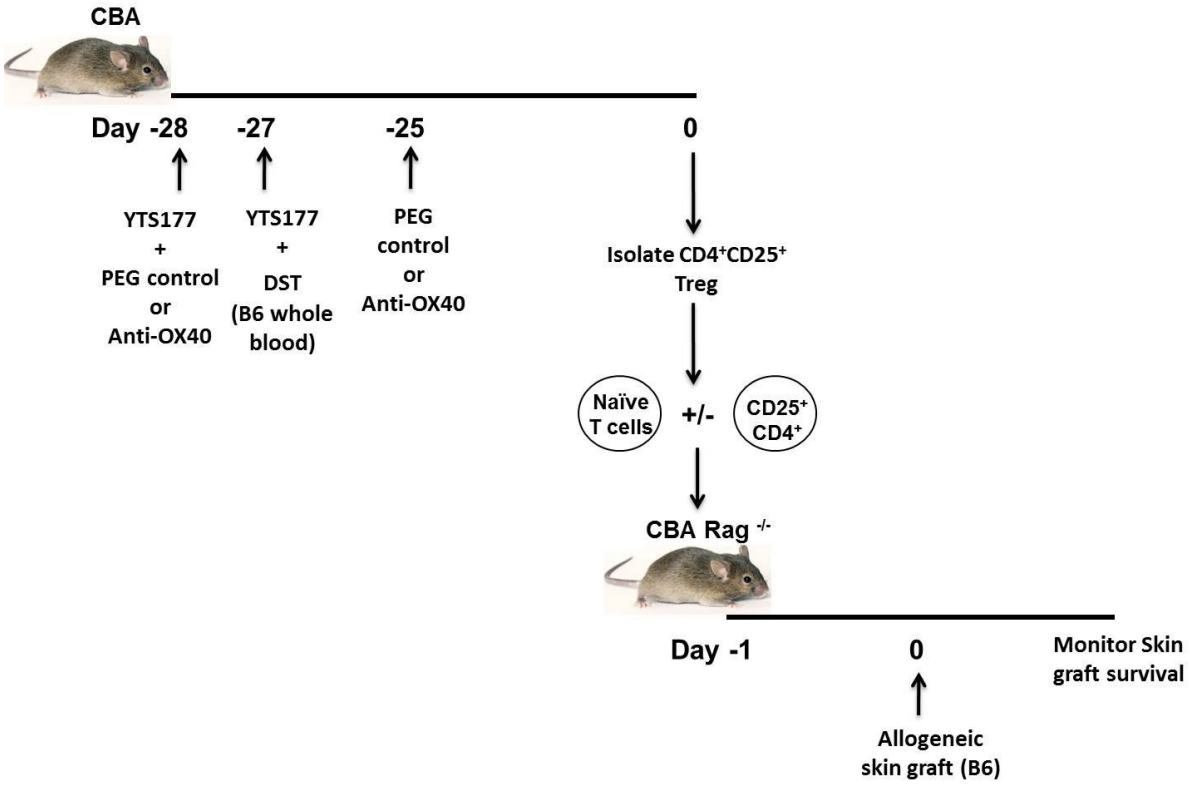


Figure 4: Protocol for the generation of Treg

CBA ($H2^k$) mice were pre-treated with 200µg non-depleting anti-CD4 mAb (YTS177) and 250µl DST of whole B6 blood on days -28 and -27. Anti-OX40 or PEG control was given 100mg/kg subcutaneously on days -28 and -25 only. Spleen and mLN were harvested and Treg ($CD4^+CD25^+$) were purified on day 0. $CD4^+CD25^-$ T cells (naïve T cells) were also purified from unmanipulated CBA mice. After purification, Treg and naïve T cells were adoptively transferred into syngeneic $Rag^{-/-}$ recipient mice at varying ratios. The following day mice received a skin allograft ($H2^b$). Skin graft survival was monitored and rejection classed as complete loss of viable donor tissue.

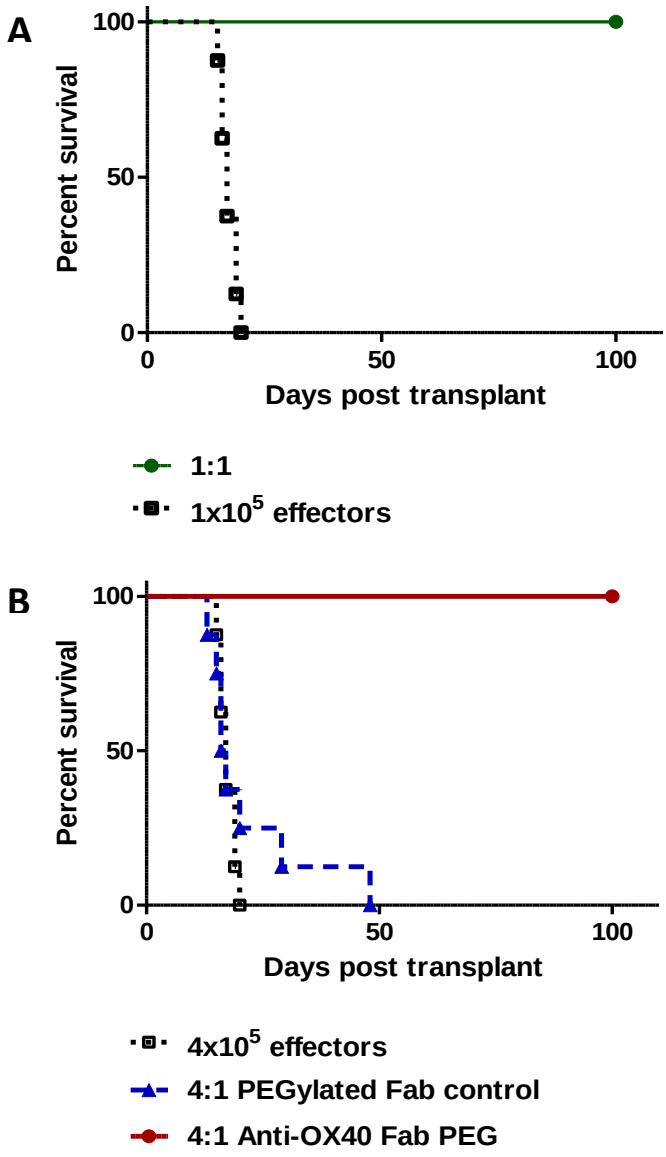


Figure 5: Anti-OX40 enhances the generation of alloantigen experienced Treg *in vivo*

Using the protocol described in Figure 3, Treg and naïve T cells were adoptively transferred at varying ratios into syngeneic $Rag^{-/-}$ recipients. Kaplan-Meier survival curves show skin allograft rejection kinetics. A) Donor graft survival after reconstitution with $1x10^5$ naïve T cells (effectors) and $1x10^5$ Treg and (1:1 ratio). B) Donor graft survival after reconstitution with $4x10^5$ naïve T cells (effectors) and $1x10^5$ Treg (4:1 ratio). In both A) and B) a group mice was included that received effector T cells alone. (n = 4-8 mice per group and data pooled between 2 independent experiments).

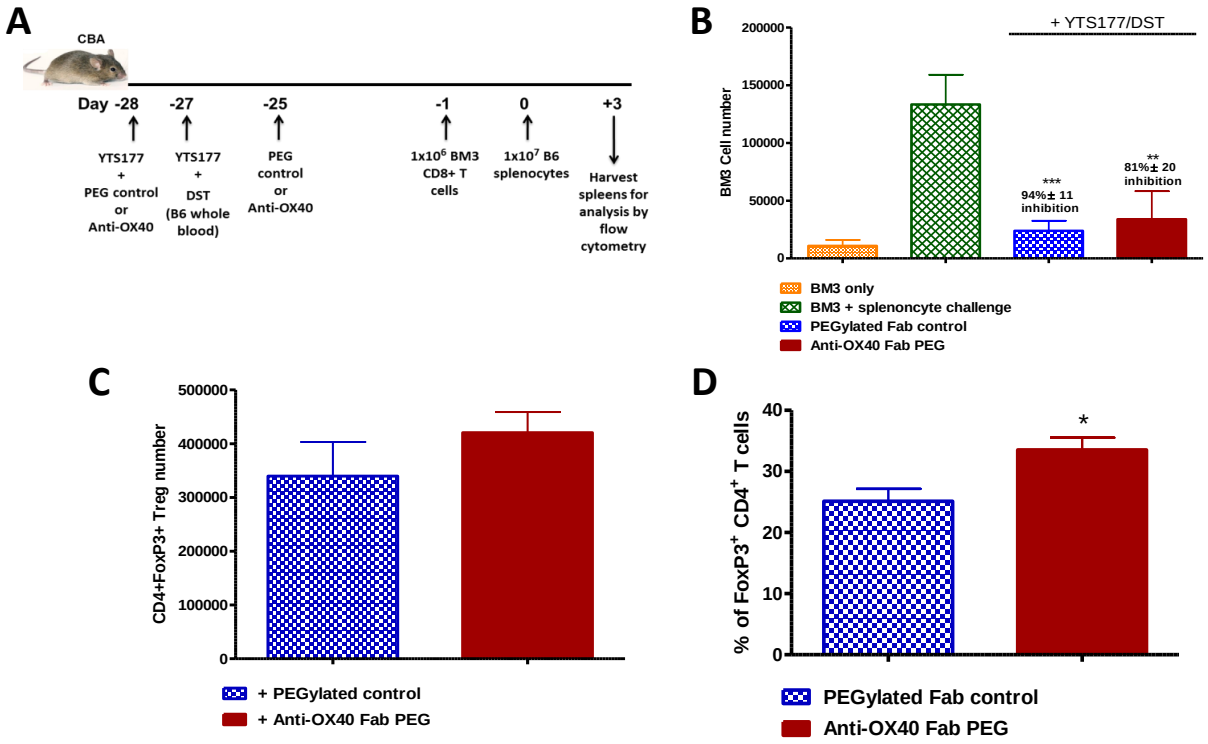
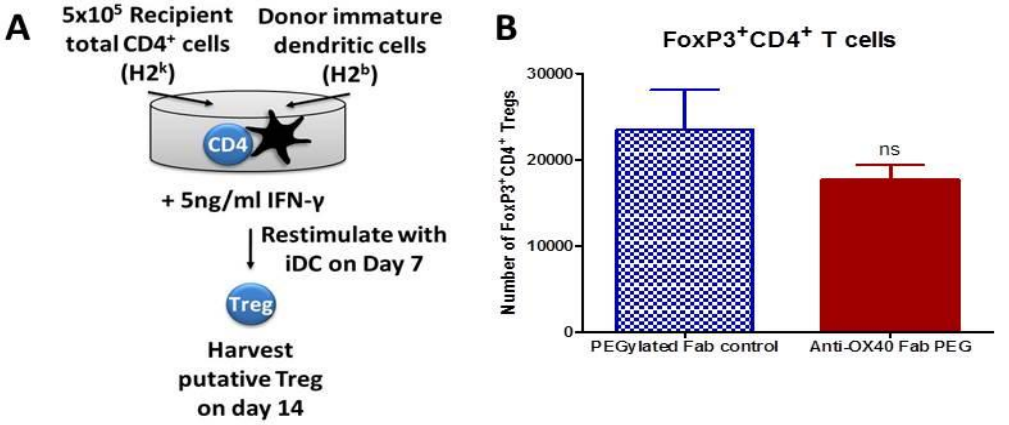
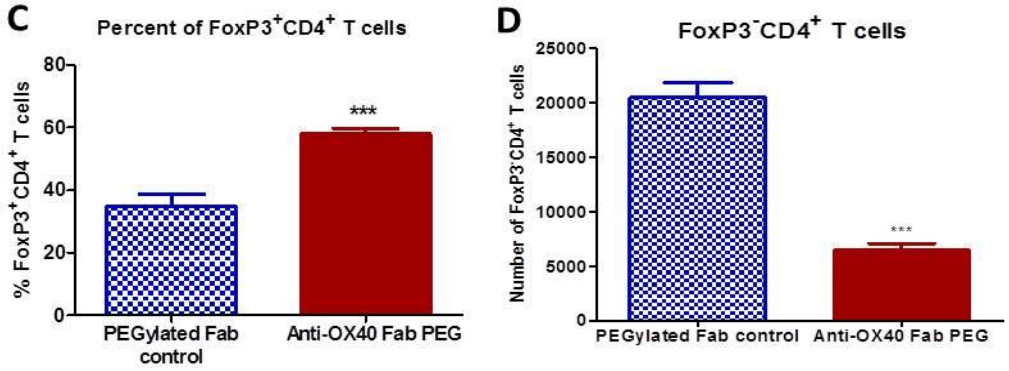


Figure 6: Anti-OX40 has no impact on number of Treg, but does increase the percentage of Treg in the spleen following YTS177/DST treatment *in vivo*

CBA ($H2^k$) mice were pre-treated with 200µg non-depleting anti-CD4 mAb (YTS177) and 250µl donor specific transfusion (DST) of whole B6 blood on days -28 and -27. Anti-OX40 or PEG control was given 100mg/kg subcutaneously on days -28 and -25 only. On day -1 all mice received $1x10^6$ CFSE labelled BM3 $CD8^+$ T cells. One day later mice were given an allogeneic splenocyte challenge ($1x10^7$ B6 splenocytes). Spleens were harvested on day +3 for analysis by flow cytometry of BM3 T cell number (B), Treg number (C), and % Treg (D). Results are expressed as mean ± SD, performed in triplicate (n = 4 mice per group and representative of 4 independent experiments). * represents $p<0.05$, ** represents $p<0.01$, *** represents $p<0.0001$.



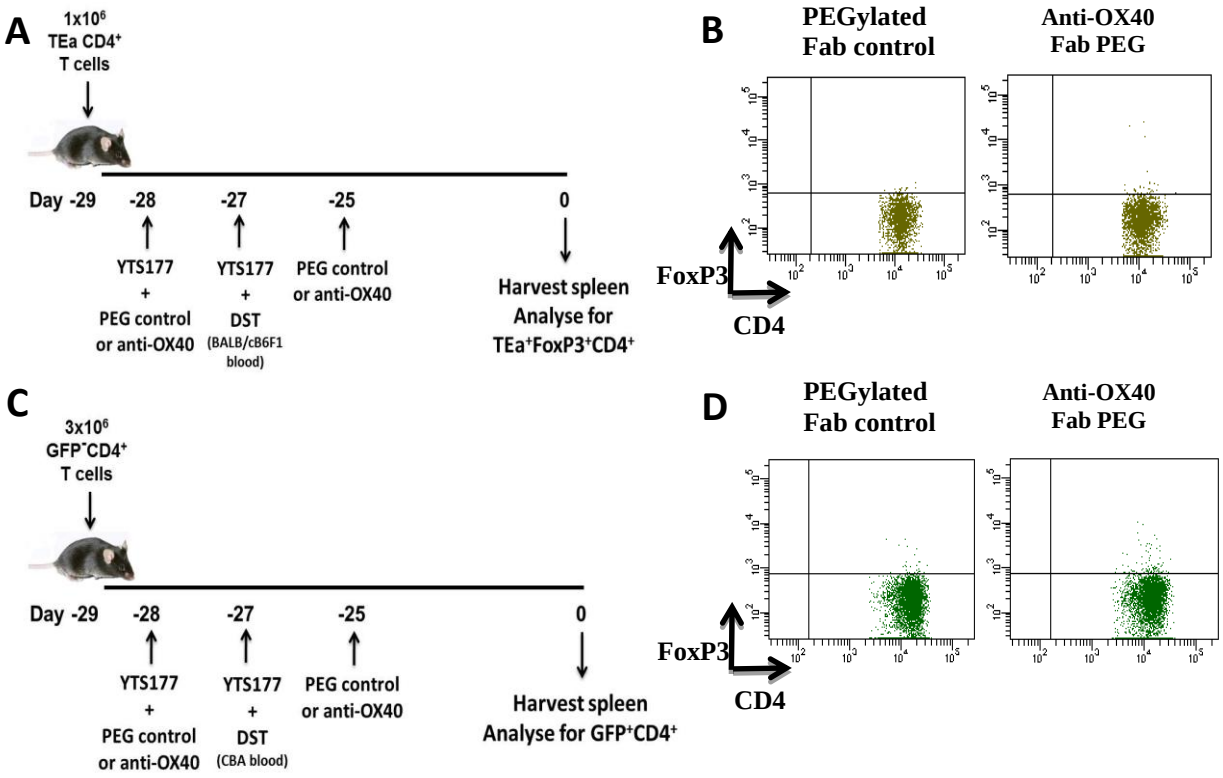


E

Treatment group	% of 7-AAD staining in $CD4^+FoxP3^-$ T cells (mean ± SD)	Range
PEG control	17% ± 6	11-28%
Anti-OX40	35% ± 12	29-45%

Figure 7: Anti-OX40 does not aid the generation/expansion of Treg but decreases the survival of effector T cells thereby increasing the ratio of regulatory to effector T cells

A) Purified $CD4^+$ T cells (isolated from CBA mice; $H2^k$) were co-cultured with immature DC (B6; $H2^b$) and exogenous IFN-γ (5ng/ml). Anti-OX40 or PEG control was added to the appropriate wells at 10μg/ml. Cultures were then re-stimulated under the same conditions on day 7 and the number and proportion of conventional and regulatory T cells determined on day 14 of culture. The number (B) and percentage (C) of $CD4^+FoxP3^+$ Treg and number of $CD4^+FoxP3^-$ effector T cells (D) are shown. E) Percentage of dead ($7\text{-}AAD^+$) $CD4^+FoxP3^-$ effector T cells. Results are expressed as mean ± SD, performed in triplicate, n = 4 mice per group and representative of 2 independent experiments). *** represents $p<0.0001$, ns represents not-statistically significant.



E

Input population	Treatment	Number of FoxP3⁺ (Mean ± SD)
TEa	PEG control	1250 ± 838
TEa	Anti-OX40	1881 ± 1077
GFP⁻	PEG control	147 ± 67
GFP⁻	Anti-OX40	344 ± 180

Figure 8: 177/DST failed to induce Treg from naïve CD4$^+$ T cells *in vivo*

Either 1x10^6 CD4$^+$ TCR transgenic (TEa) T cells or 3x10^6 Foxp3-GFP$^-$CD4$^+$ T cells were adoptively transferred into B6 recipients. One day later mice were given 200µg non-depleting anti-CD4 mAb and DST on days -28 and -27. Anti-OX40 or PEG control were administered at 100mg/kg subcutaneously on days -28 and -25. Spleens were harvested on day 0 and analysed for the presence of either TEa or Foxp3-KI CD4$^+$FoxP3$^+$ Treg. Isotypes for FoxP3 were used to set quadrant gates. (n = 4-6 mice per group).

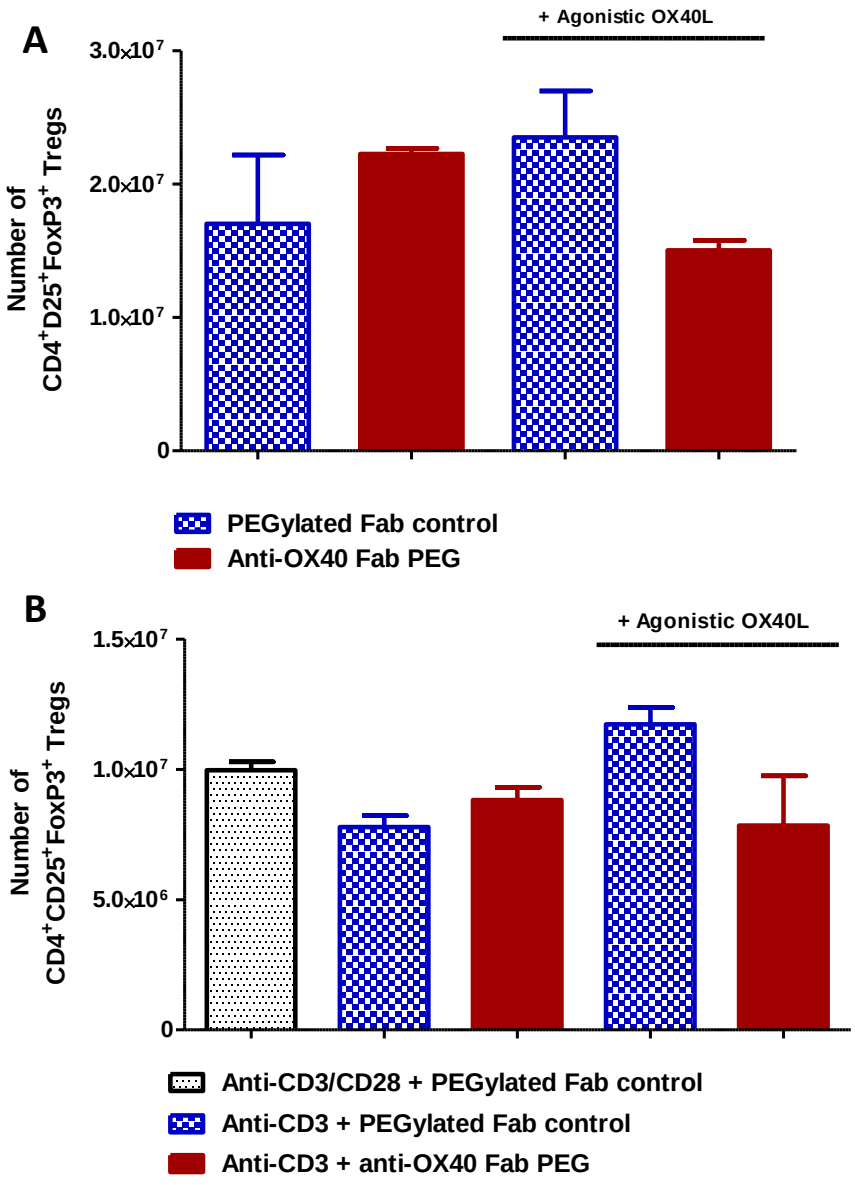


Figure 9: Blockade of OX40 has no impact on the expansion of Treg *in vitro*
$CD4^+CD25^+$ Treg were isolated and purified from wild type CBA spleens and co-cultured with plate bound anti-CD3 and anti-CD28 (A) or anti-CD3 only (B) for 7 days. Appropriate wells either received PEG control or anti-OX40 (10µg/ml). Agonistic OX40L (10µg/ml) was also plate bound in order to provide a stimulus for ligation of the OX40-OX40L pathway in specific wells. Results are expressed as mean ± SD, performed in triplicate and representative of 3 independent experiments.

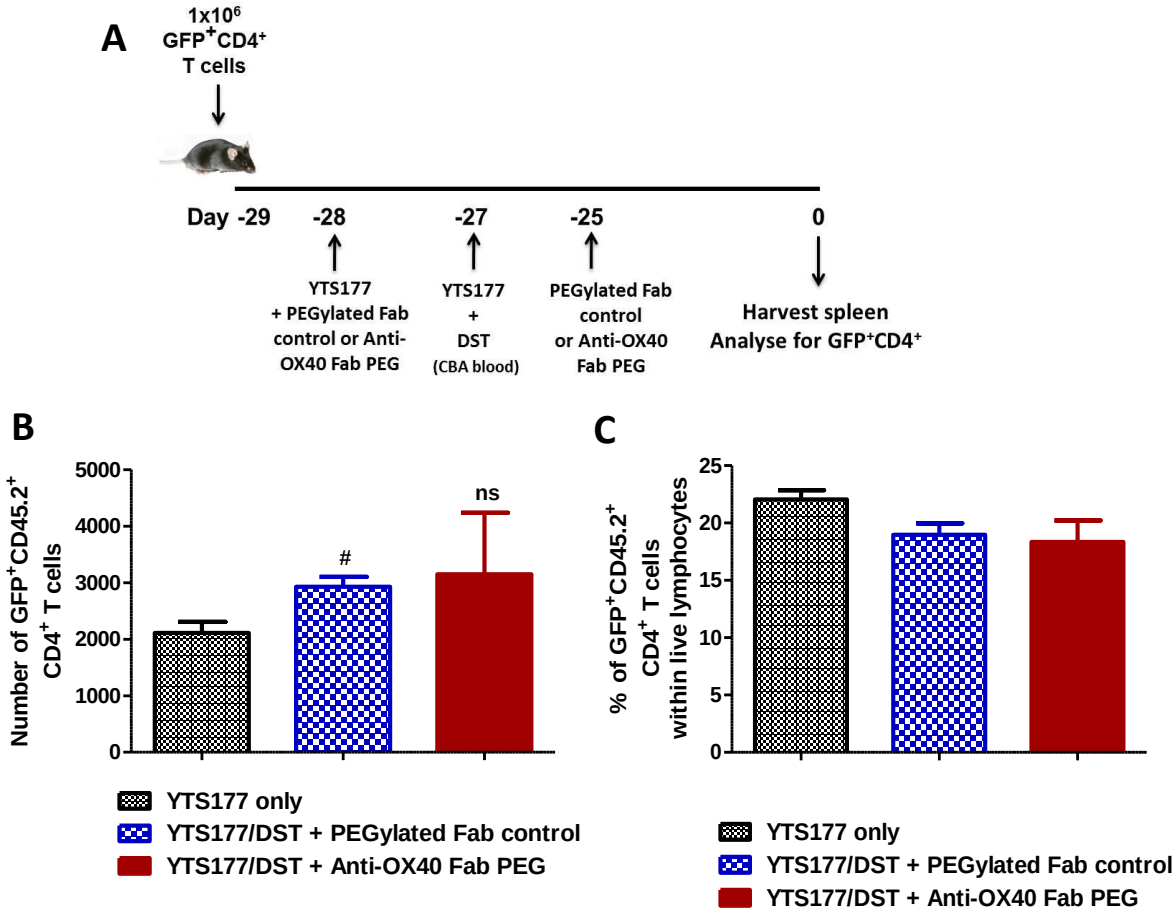


Figure 10: OX40 blockade does not facilitate alloantigen-induced clonal expansion of Treg *in vivo*

GFP^+CD4^+ T cells were adoptively transferred into B6 wild type recipients. One day later mice were given 200µg non-depleting anti-CD4 mAb and DST on days -28 and -27. Anti-OX40 or PEG control were administered at 100mg/kg subcutaneously on days -28 and -25 (A). Spleens were harvested on day 0 and quantified for $CD4^+FoxP3^+$ T cells; number (B) and percentage (C). Isotypes for FoxP3 were used to set quadrant gates. (n = 4-6 mice per group). Results are expressed as mean number of % ± SD, n = 4-6 mice per group # represents $p<0.01$ compared to 177 only.

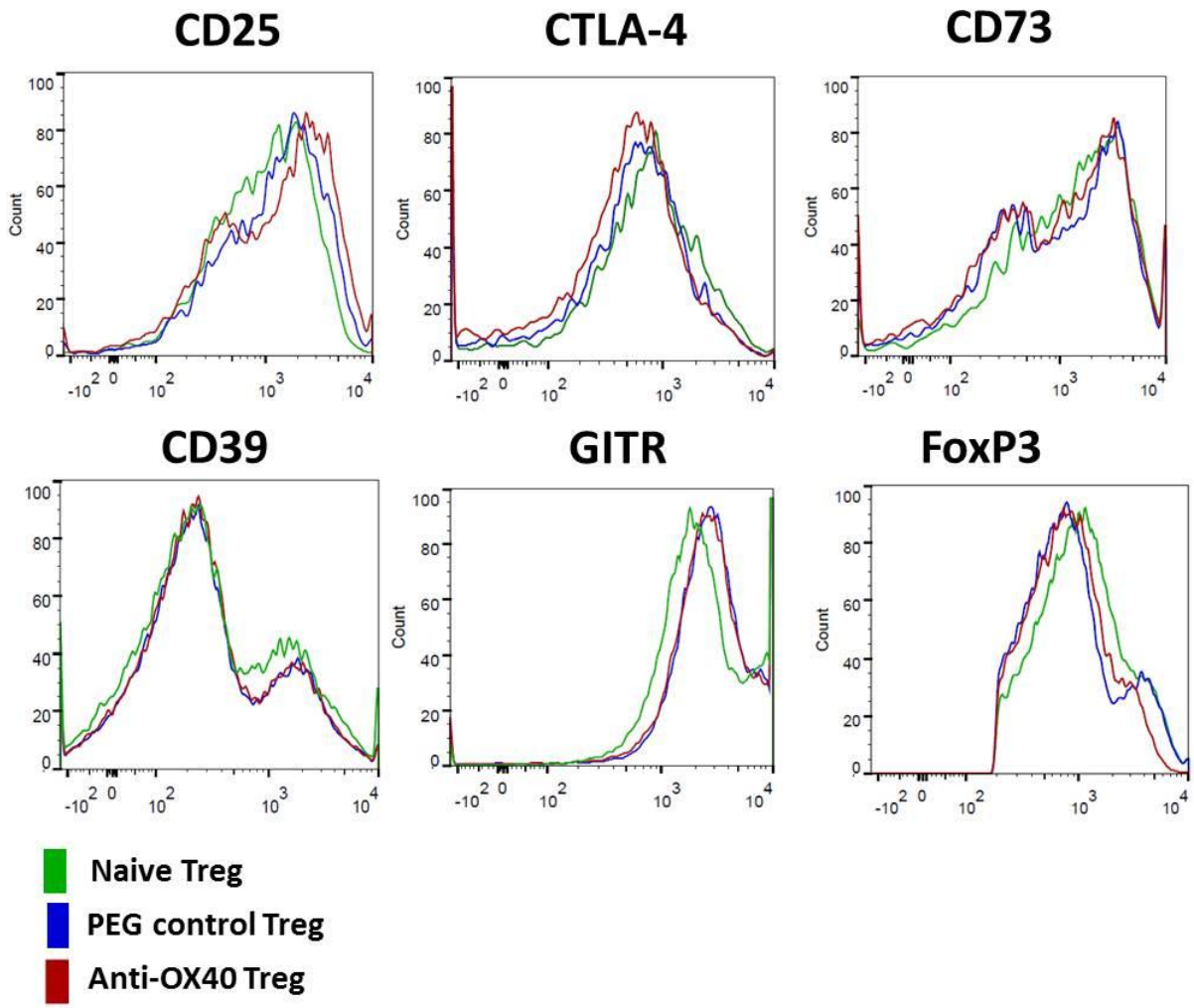


Figure 11: OX40 blockade has no impact on markers which correlate with Treg function

Treg ($CD4^+CD25^+$) were purified from naïve mice or mice treated with 177/DST with and without OX40 blockade. Treg were analysed by flow cytometry without further stimulation. Common markers of potency were analysed CD25, CTLA-4, CD73, CD39, GITR and FoxP3. n = 6 mice per group (pooled); and representative of 2 independent experiments.

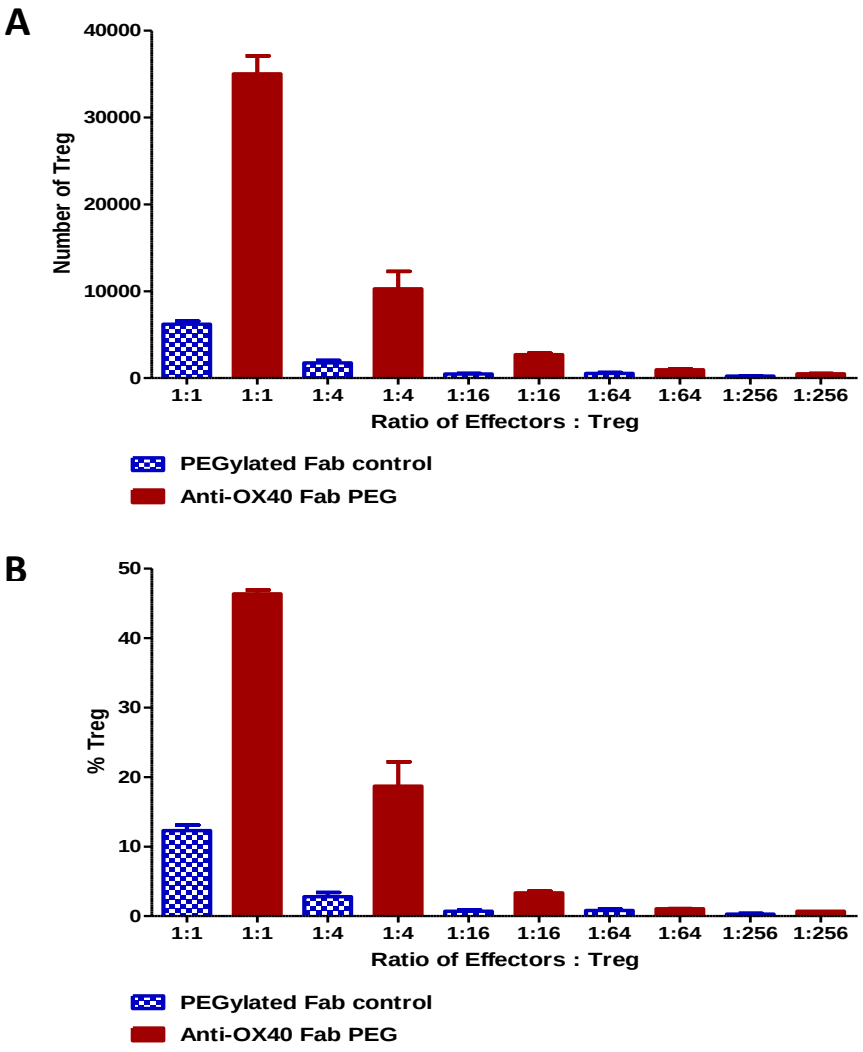


Figure 12: Blockade of OX40 increases the survival of Treg

Mice were administered 200µg of non-depleting anti-CD4 mAb and DST on days -28 and -27. Anti-OX40 or PEG control were administered at 100mg/kg subcutaneously on days -28 and -25. Treg ($CD4^+CD25^+$) were purified on day 0 from mice treated with the 177/DST protocol and labelled with CFSE. Naïve responder T cells ($CD4^+CD25^-$) were purified from naïve CBA mice and labelled with a violet fluorescent dye. Stimulator $CD11c^+$ cells were purified from spleens of B6 mice. Responder and stimulator cells ($1x10^5$ per population) were co-cultured and in appropriate wells. Treg were added at varying ratios to the responder cells. Cultures were analysed at day 6 by flow cytometry. Results are expressed as mean number or % ± SD, n = 6 mice per group (pooled) and representative of 2 independent experiments.

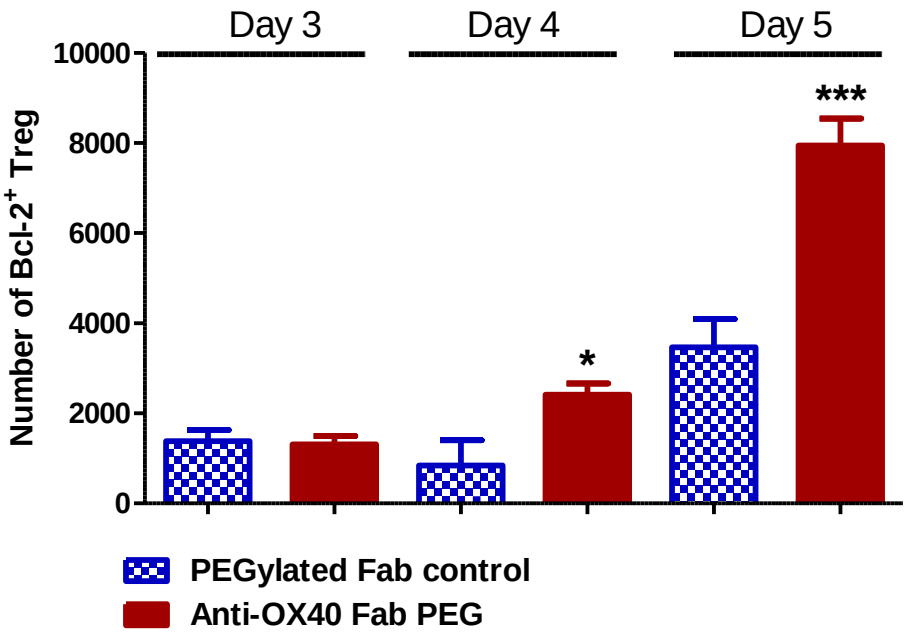


Figure 13: Anti-OX40 significantly increases the expression of Bcl-2 in Treg
Mice were administered 200µg of non-depleting anti-CD4 mAb and DST on days -28 and -27. Anti-OX40 or PEG control were administered at 100mg/kg s.c. on days -28 and -25. Treg ($CD4^+CD25^+$) were purified on day 0 from mice treated with the 177/DST protocol and labelled with CFSE. Responder T cells ($CD4^+CD25^-$) were purified from naïve CBA mice and labelled with a violet fluorescent dye. Stimulator $CD11c^+$ cells were purified from spleens of naïve B6 mice. Responder and stimulator cells ($1x10^5$ per population) were co-cultured and in appropriate wells Treg were added at varying ratios to the responder cells. Cultures were analysed for Bcl-2 expression by Treg at days 3-5 by flow cytometry. Results are expressed as mean number ± SD, n = 6 mice per group (pooled) * represents $p<0.05$, ** represents $p<0.001$.

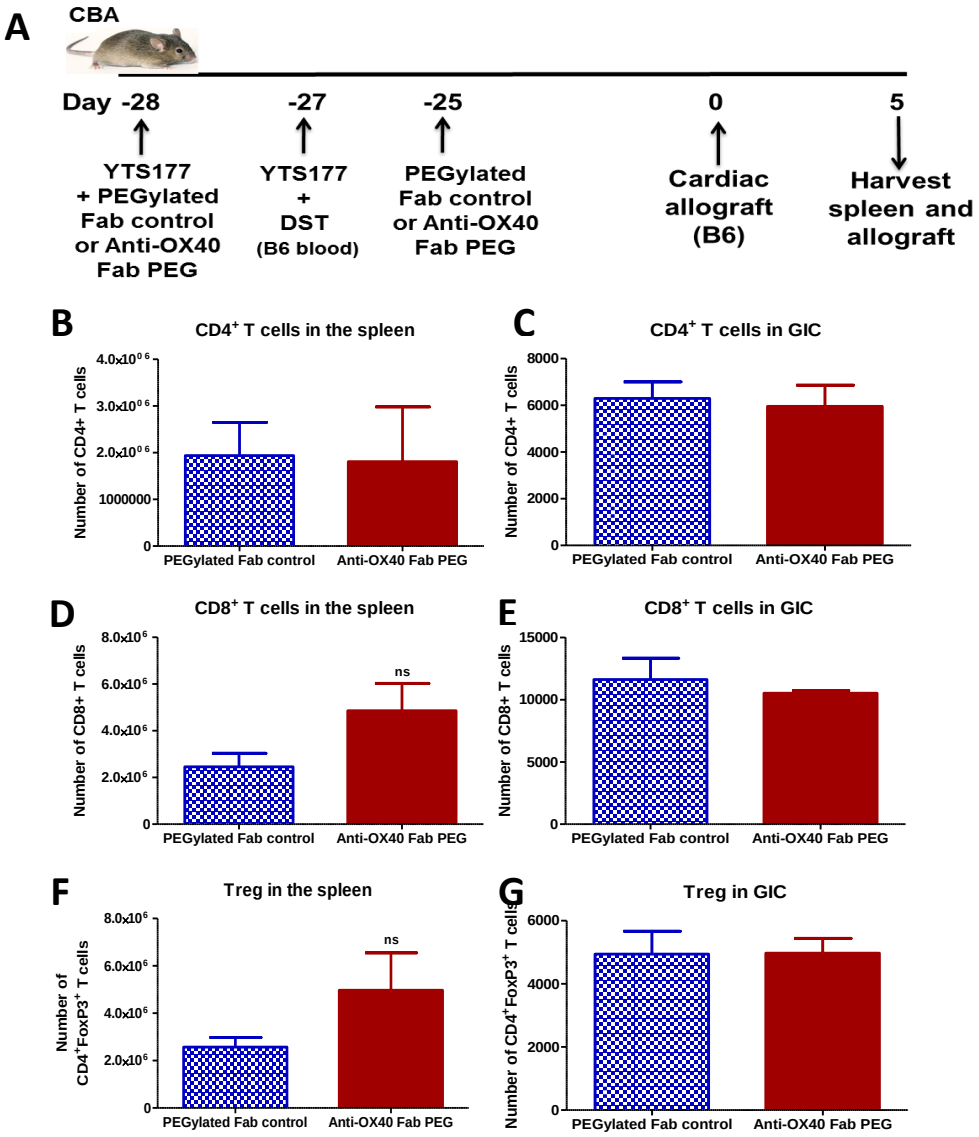


Figure 14: Anti-OX40 has no impact on the number of $CD4^+CD25^+FoxP3^+$ T cells 5 days after a cardiac allograft

CBA mice received 200µg of non-depleting anti-CD4 mAb and DST (B6) on days -28 and -27. Anti-OX40 or PEG control were administered at 100mg/kg subcutaneously on days -28 and -25. Recipient mice were given a vascularised heterotopic cardiac allograft (B6) on day 0. $CD4^+$ (B and C), $CD8^+$ (D and E) T cells and $CD4^+FoxP3^+$ Treg (F and G) present in spleen and graft infiltrating cells were analysed on day +5 by flow cytometry. Results are expressed as mean ± SD, n = 4 mice per group and representative of 2 independent experiments.

Chapter 6: OX40 blockade as an adjunctive therapy to pre-existing immunosuppressive strategies

6.1 Introduction

Memory T cells (Tm) are now accepted as being a barrier to tolerance induction, as a number of strategies (e.g. CD28 and/or CD154 blockade) that are successful at preventing allograft rejection by naïve T cells (using mouse models in pathogen free conditions) (103, 201, 221) fail to induce tolerance in rodents which have been pre-sensitised with donor alloantigen (101, 104). The success of tolerance induction strategies is also strain-dependent in mouse models (440). Mice on a B6 background were able to generate CTL responses and IFN-γ producing cells despite CD28 and/or CD154 blockade, while mice on a C3H background failed to mount these responses (440).

Although data from NHP studies is more complex it is clear that tolerance induction is more difficult to achieve in higher animals (223-225, 441) which may be explained in part by the increased complexity of the higher vertebrate immune system and the increased diversity of pre-existing immunity due to exposure to environmental antigens (442). These data highlight the limitations of current protocols and demonstrate the need for therapeutic strategies which can target alternative pathways. The failure of

costimulation blockade to induce tolerance in the aforementioned models has been shown to be mediated by Tm and/or a subset of CD28/CD154 independent $CD8^+$ T cells. For example, asialo $GM1^+CD8^+$ T cells were found to be critical in costimulation blockade resistance in a mouse model of skin allograft rejection (443). Therefore blockade of other costimulatory molecules such as OX40-OX40L has recently been investigated in models of CD28/CD154 independent rejection (304).

It is now clear that Tm participate in the response to allografts and are not restricted to patients that have received prior sensitisation with alloantigen in the form of a previous transplant, blood transfusion or pregnancy. Indeed, it has been shown that a subset of pre-existent Tm, generated as a result of previous encounter with either infectious or environmental antigens, cross-react to alloantigen (a process termed heterologous immunity) (101). Adams *et al* first demonstrated this phenomenon by showing that mice that were repeatedly infected with virus became resistant to tolerance induction due to the generation of virus-reactive Tm which could cross-react with donor alloantigen (101). These and other reports (444) suggest that even in non-allo-sensitised patients alloreactive Tm are likely to be present and may prove disruptive to attempts to induce tolerance to allogeneic tissue transplants.

In NHP models of kidney allografts, the number of pre-existing donor-reactive Tm has been found to determine the outcome of allograft survival after conditioning with total body irradiation, ATG and cyclosporine (105). When low numbers of donor-reactive Tm were present, cynomologus monkeys exhibited long-term allograft survival and

tolerance was induced. However, when higher numbers of donor-reactive Tm were present in the peripheral blood, the monkeys rejected their allografts acutely despite therapy (105). In addition, in clinical transplantation even where rejection is suppressed by the use of conventional immunosuppressive drugs the presence of alloreactive Tm prior to transplantation has been shown to correlate with an increased incidence of acute rejection (98).

These data highlight the fact that the presence of high numbers of donor-reactive Tm prior to transplantation is detrimental to transplant survival regardless of whether this is induced by costimulatory molecule blockade or conventional immunosuppressive agents. Therefore, the development of drugs which could inhibit Tm as well as other cells in the immune repertoire would clearly be beneficial in preventing allograft rejection.

Memory T have reduced costimulatory requirements (95), high proliferative capacity and in addition are able to home to non-lymphoid tissues (445) as they express unique homing receptors which allows them to readily accumulate in inflammatory sites such as the allograft (446, 447). Moreover, Tm have been shown to be resistant to current immunosuppressive strategies and costimulation blockade due to their reduced activation threshold, enhanced proliferative capacity and rapid cytokine secretion (448) compared to naïve T cells.

Given the finding that Tm can elicit rejection in the absence of CD28/CD154 it is important that other alternative pathways that may provide a costimulatory signal to Tm are explored. The inducible nature of other costimulatory molecules such as 4-1BB, OX40 and ICOS suggests that these alternative costimulatory molecules may play a role in propagating effector/memory T cell responses even if Tm do not require CD28/CD154 costimulation during T cell priming.

OX40 signalling has been shown to be required to sustain memory T cell responses. Gaspal *et al* elegantly demonstrated that OX40 signalling in concert with CD30 signals were required for productive secondary antibody responses (449). $CD30^{-/-}OX40^{-/-}$ T cells had similar proliferation compared to wild-type controls but these double deficient T cells failed to survive *in vivo (449).*

In models of transplantation, blockade of the OX40-OX40L pathway alone (using anti-OX40L mAb) failed to promote the survival of full MHC-mismatched skin allografts (304), however OX40 blockade markedly prolonged skin allograft survival in $CD28^{-/-}$ $CD154^{-/-}$ mice. In contrast, 4-1BB, CD70 or ICOS blockade failed to promote survival of skin allografts even in $CD28^{-/-}CD154^{-/-}$ mice suggesting that certain aspects of immunity may be uniquely controlled by OX40 (304). In addition, blockade of the CD28-CD80/CD86 pathway and OX40-OX40L induced long term cardiac allograft survival (302). These data show that rejection mediated by CD28/CD154 independent T cells can be prevented by coincident blockade of the OX40-OX40L pathway and suggests that adjunctive therapies to interrupt multiple costimulatory pathways may be

necessary to achieve long term allograft survival in more stringent models and perhaps in man.

6.2 Aims and hypothesis

The T cell repertoire that elicits allograft rejection consists of both naïve and memory T cells, therefore identifying pathways which simultaneously target all potentially damaging T cell subsets will be beneficial for allograft survival. In chapter 3 and 4 we showed that OX40 blockade can impact both naïve $CD4^+$ and $CD8^+$ T cells, and in chapter 5 we demonstrated that blockade of OX40-OX40L may enhance Treg survival following alloantigen-mediated activation. However, whether anti-OX40 suppresses or enhances alloantigen-reactive Tm responses to an allograft remains unclear.

The aim of this chapter was to investigate the role of OX40-OX40L interactions in the response of Tm to alloantigen *in vitro* and *in vivo*. Furthermore, the potential of anti-OX40 to synergise with other therapeutic agents to produce long term allograft survival was determined.

6.3 Results

6.3.1 Generation and characterisation memory T cells

Naïve wild-type mice have a small proportion of memory T cells (**Figure 1A**), which are insufficient in number to perform *in vivo* experiments, therefore our laboratory has developed protocols to generate greater numbers of Tm *in vivo*. To this end, Tm were generated by injecting $1x10^6$ naïve alloreactive T cells (BM3, -D, or TEa) into syngeneic $Rag^{-/-}$ mice and challenging such mice with an allogeneic splenocyte injection the following day. Polyclonal memory T cells were generated by challenging naïve wild-type mice with allogeneic splenocytes. Mice were then left (50-100 days) to allow for alloantigen clearance and the generation of quiescent Tm before the spleen and mLN were harvested from these mice. The resultant population was dramatically enriched for Tm (**Figure 1B**) and typical numbers of Tm isolated were $4x10^5$-$2x10^6$/mouse. To confirm that these cells were in fact Tm, the CD44 expression (**Figure 1C**) and IFN-γ production was analysed (**Figure 1D**). As expected, all Tm expressed high levels of CD44 (**Figure 1C**) and secreted IFN-γ more rapidly and to a greater level compared to naïve T cells (**Figure 1D**).

6.3.2 Memory $CD4^+$ and $CD8^+$ T cells upregulate OX40 after stimulation with alloantigen *in vitro.*

First we examined the expression of OX40 and OX40L on memory $CD4^+$ and $CD8^+$ T cells in response to alloantigen. BM3 ($2.5x10^4$/well) and TEa ($5x10^4$/well) Tm were stimulated with alloantigen (irradiated B6 or BALB/cB6F1 splenocytes; $1x10^5$/well or $2.5x10^5$/well respectively) *in vitro* before being analysed for the expression of OX40 and OX40L by flow cytometry. OX40 expression was upregulated on day 1 on both

BM3 and TEa T cells, peaking between days 2-3 post stimulation (**Figure 2A and C**). OX40L was only expressed after stimulation with alloantigen on BM3 T cells, not TEa T cells (**Figure 2B and D**).

Next we sought to analyse the expression of OX40 and OX40L on polyclonal Tm ($CD3^+CD44^+CD62L^-$) which were used as whole splenocytes stimulated with allogeneic splenocytes (**Figure 2E and F**). Memory T cells were analysed as a whole population, rather than sub-dividing into $CD4^+$ and $CD8^+$ T cells due to a shortage of numbers. Tm from spleens and mLN of CBA mice ($2x10^5$/well) were stimulated with allogeneic B6 splenocytes ($4x10^5$/well). Polyclonal Tm expressed OX40 from day 2 (**Figure 2E**), but did not express OX40L (**Figure 2F**).

6.3.3 Anti-OX40 does not attenuate memory $CD4^+$ and $CD8^+$ T cell responses *in vitro*

In order to test the importance of OX40-OX40L interactions in memory T cell responses, anti-OX40 was added to MLR cultures and proliferation measured. Memory $CD4^+$ TEa or -D T cells (both $CD4^+$ Tm; $5x10^4$/well) were cultured with allogeneic irradiated splenocytes ($2.5x10^5$/well). Anti-OX40 or PEG control were added at 10μg/ml to appropriate wells and the proliferative response of Tm was assessed. Anti-OX40 failed to inhibit the proliferative response of either TEa (**Figure 3A**) or -D (**Figure 3B**) Tm.

Memory $CD8^+$ T cell responses were also analysed where alloreactive BM3 Tm ($2.5x10^4$/well) were stimulated with allogeneic irradiated splenocytes ($1x10^5$/well) in the presence of either anti-OX40 or PEG control. Again anti-OX40 had no impact on BM3 Tm responses at any time point tested (**Figure 4A**). A dose-response curve was also generated which showed that anti-OX40 did not suppress the proliferative response of BM3 Tm at any concentration tested (**Figure 4B**).

6.3.4 Anti-OX40 significantly attenuates skin allograft rejection mediated by Tm

Despite having observed that OX40 blockade had no impact on Tm proliferation *in vitro*, we sought to determine whether Tm utilised OX40-OX40L interactions to facilitate skin allograft rejection *in vivo*. To this end, $CD4^+$ Tm ($5x10^4$ TEa or $1x10^5$ -D; generated as detailed in section 6.3.1) were adoptively transferred into syngeneic $Rag^{-/-}$ mice which received an allogeneic skin graft (BALB/cB6F1 or B10.S respectively) the following day. Anti-OX40 or PEG control were administered subcutaneously twice weekly for 4 weeks. Mice reconstituted with $1x10^5$ TEa Tm and treated with PEG control rejected their skin allografts acutely, while treatment with anti-OX40 was found to markedly prolong skin allograft survival (MST 89.5 vs. 18 days; $p<0.0007$; **Figure 5A**). Furthermore, treatment of mice reconstituted with -D Tm with anti-OX40 also resulted in significantly prolonged allograft survival compared to mice treated with PEG control (MST 63 vs. 30 days; $p<0.0005$; **Figure 5B**).

Given that anti-OX40 clearly attenuated $CD4^+$ Tm-mediated allograft rejection, next we sought to investigate the role of OX40-OX40L in $CD8^+$ Tm responses to allogeneic skin grafts. To this end, syngeneic $Rag^{-/-}$ mice received adoptive transfer with either $1x10^5$ or $5x10^4$ BM3 Tm and treatment with either anti-OX40 or PEG control. Surprisingly, administration of anti-OX40 had no impact on allograft survival ($1x10^5$ MST 13 vs. 13 days (**Figure 6A**); $5x10^4$ MST 16 vs. 15 days (**Figure 6B**)). However, when the number of transferred BM3 Tm was reduced to $2.5x10^4$, anti-OX40 significantly prolonged allograft survival (MST 62 vs. 23 days; $p<0.0246$; **Figure 6C**). Further decreasing the number of transferred BM3 Tm ($1x10^4$) resulted in anti-OX40 prolonging skin allograft survival but graft survival was not improved compared to transfer of $2.5x10^4$, with all allografts eventually being rejected (MST 70 vs. 42 days; $p<0.0246$; **Figure 6D**).

6.3.5 Anti-OX40 prolongs skin allograft survival where rejection is mediated by either polyclonal $CD4^+$ or $CD8^+$ memory T cells

To confirm that OX40 blockade could prolong skin allograft survival mediated by Tm, we adoptively transferred $1x10^5$ $CD4^+$ or $CD8^+$ polyclonal Tm (generated as in section 6.3.1; $H2^k$) into syngeneic $Rag^{-/-}$ recipients. The following day mice received an allogeneic skin transplant and either anti-OX40 or PEG control. Mice reconstituted with polyclonal $CD4^+$ Tm and treatment with PEG control rejected skin allografts acutely. However, skin allograft survival was significantly prolonged when transplanted to mice that also received anti-OX40 (MST 51.5 vs. 15.5 days; $p<0.0005$; **Figure 7A**). Similar results were observed with mice that received polyclonal $CD8^+$ Tm (anti-OX40 = MST 61 vs. PEG control = 25 days; $p<0.0027$; **Figure 7B**). This

effect by anti-OX40 was strain independent as significant skin allograft prolongation was observed after adoptive transfer of polyclonal $CD4^+$ Tm (isolated from B6 mice) into syngeneic $Rag^{-/-}$ recipients (anti-OX40 MST = 47 days vs. PEG control = 21.5 days; $p<0.0027$; data not shown).

6.3.6 Anti-OX40 inhibits accumulation of memory $CD4^+$ T cells in secondary lymphoid organs and the allograft

Thus far we had demonstrated that OX40-OX40L played a critical role in skin allograft rejection mediated by both $CD4^+$ and $CD8^+$ Tm. In order to determine whether the effect of OX40 blockade on Tm was similar to naïve T cells (i.e. premature deletion and failure to maintain clonal expansion), the response of TEa $CD4^+$ Tm to a skin allograft (transferred to $Rag^{-/-}$ mice as above) was tracked *in vivo* during treatment with either anti-OX40 or PEG control. Mice were sacrificed 15 days post-transplantation and the dLN, cLN and spleen was harvested (time-point previously shown to be the peak of clonal expansion in the dLN (data not shown)).

Expansion of TEa Tm in the dLN after transplantation was evident in mice treated with PEG control however in contrast in anti-OX40 treated mice the expansion of TEa Tm cells was clearly diminished (794248 ± 91555 vs. 186037 ± 49822, respectively; $p< 0.0267$; **Figure 8A**). The expansion of TEa Tm cells was observed after allogeneic but not syngeneic skin transplantation and the response was limited to the dLN as there was little/no expansion observed in the cLN (**Figure 8A**).

Next we sought to investigate the impact of OX40 blockade on the proliferative capacity of the Tm after allogeneic skin transplantation. We measured proliferation by labelling the input Tm population with CFSE. Similar to the data presented with naïve T cells (chapter 4) OX40 blockade had no impact on the proliferation of memory T cells after an allogeneic skin graft (**Figure 8B**). Mice which received a syngeneic skin graft failed to undergo extensive cell division demonstrating that alloantigen was required to initiate proliferation and clonal expansion.

Given that certain Tm subsets may have different trafficking patterns compared to naïve T cells, we next determined whether OX40 blockade had enhanced or prevented migration of the Tm into the skin allograft. To this end, skin grafts were analysed for CD3 expression 15 days after transplantation to syngeneic $Rag^{-/-}$ mice reconstituted with TEa Tm. Mice were either treated with anti-OX40 or PEG control. Anti-OX40 did not enhance the migration of Tm from periphery into the skin allograft, indeed anti-OX40 markedly diminished Tm infiltration in line with its impact on Tm survival (**Figure 8C, D, E**).

Taken together the data presented in this thesis on the effect of OX40 blockade on naïve T cells (chapter 3 and 4) and Tm (Figures 5, 6, 7 and 8) to allografts suggests that the OX40 signals are required to sustain both naïve and memory T cell responses to allografts through supporting effector T cell survival.

6.3.7 OX40 blockade alone does not suppress skin or cardiac allograft rejection

Thus far we have observed significant prolongation of allograft survival by anti-OX40 where rejection was mediated by limited numbers of naïve and memory $CD4^+$ and $CD8^+$ T cells. In addition, anti-OX40 appeared to result in Treg that show enhanced survival following re-activation and exhibited increased potency in their ability to control skin allograft rejection. With this in mind we next tested whether OX40 blockade could attenuate rejection in the face of a complete immune repertoire. To this end, CBA recipient mice received either a fully MHC-mismatched skin (**Figure 9A**) or cardiac (**Figure 9B**) allograft (B6), and received either anti-OX40 or PEG control subcutaneously twice weekly for 4 weeks or until rejection. Anti-OX40 was found to have no impact on skin (**Figure 9A**) or cardiac (**Figure 9B**) allograft survival compared to PEG control treated mice.

6.3.8 Anti-OX40 had no impact on graft survival across a minor histocompatibility antigen mismatch barrier

The experiments performed used models where allograft and recipient were mismatched across all MHC, therefore we next tested whether OX40 blockade could impact the kinetics of rejection where donor and recipient were only disparate across miHC mismatches. CBA ($H2^k$) mice received a skin graft from B10.Br ($H2^k$) and were treated with anti-OX40. Such mice were found to acutely reject their skin grafts with the same tempo as those treated with PEG control (MST 13 days vs. 12 days; **Figure 10A**).

Finally, we tested whether anti-OX40 could suppress rejection across a sex-mismatch (male donors to female recipients). However, anti-OX40 treated mice rejected sex-mismatched skin grafts with a similar tempo to PEG control treated mice (MST 20 days vs. 17.5 days; **Figure 10B**).

6.3.9 Anti-OX40 in combination with abatacept has a synergistic effect on the proliferation of $CD4^+$ T cells

Despite OX40 blockade having a significant impact on rejection mediated by naïve and memory $CD4^+$ and $CD8^+$ T cells where the number of cells responding to the allograft was limited we found that there was no impact on the kinetics of rejection in a T cell replete mouse even across a minor antigen, sex-mismatch. These data suggest that although anti-OX40 has immunosuppressive potential other costimulatory molecules may be able to substitute for the lack of OX40 signalling in some circumstances.

These observations led us to investigate if blockade of other costimulatory molecules in combination with OX40 blockade could completely inhibit T cell responses to alloantigen. To this end, we utilised naïve TEa T cells stimulated with allogeneic (BALB/cB6F1) splenocytes *in vitro* and the addition of reagents to block various costimulatory pathways (anti-OX40, anti-4-1BBL, anti-CD154 and abatacept (CTLA-4-Ig)) either alone or together. Blockade by anti-OX40, anti-CD154, anti-4-1BBL or abatacept alone had no impact on the proliferative response by TEa T cells (**Figure 11**). Abatacept failed to inhibit TEa proliferative responses as a sub-optimal concentration was used in these studies. A sub-optimal concentration was used to allow any additive

or synergistic effect by anti-OX40 to be revealed. Only the combination of anti-OX40 and abatacept was able to inhibit the proliferation of the TEa T cells (42% ± 12; **Figure 11**).

6.3.10 Anti-OX40 synergises with anti-CD154 or abatacept to further the inhibition of proliferation of naïve BM3 T cells

We next sought to examine the response of BM3 T cells with combinations of costimulation blockade. BM3 T cells were stimulated *in vitro* with allogeneic (B6) splenocytes in the presence of costimulation blockade. Both anti-OX40 and abatacept alone inhibited the BM3 T cell response by 36% ± 2 and 60% ± 10, respectively (**Figure 12A**). In contrast, blockade of 4-1BB-4-1BBL and CD154-CD40 interactions alone had no impact on proliferation (**Figure 12A**). However, addition of anti-OX40 to cultures together with either anti-CD154 (**Figure 12C**) or abatacept (**Figure 12D**) was found to enhance the inhibition of proliferation, although combining the three reagents together did not inhibit proliferation further (**Figure 13**). In contrast, anti-4-1BBL did not demonstrate an additive inhibitory effect when combined with blockade of any other costimulatory molecule (**Figures 12B and 13A, B, D**). Finally, the combined blockade of OX40, CD28, 4-1BBL and CD154 was no more effective at suppressing BM3 T cell proliferation than anti-OX40 combined with either anti-CD154 or abatacept (**Figure 13D**).

6.3.11 Costimulation blockade has no impact on memory T cell responses *in vitro*

Memory T cells have a much lower requirement for costimulation compared to naïve T cells (95, 450). Memory BM3 T cells were stimulated with allogeneic splenocytes *in vitro*, and anti-OX40, anti-4-1BBL, anti-CD154 and abatacept (CTLA-4-Ig) were added alone or in combination and the proliferative response measured. Memory BM3 T cell responses could not be inhibited by any monotherapy or combination of costimulation blockade (**Figure 14**).

6.3.12 Anti-OX40 in combination with blockade of CD154 and CD28 synergise to further inhibit the proliferation response of whole splenocytes *in vitro*

To assess whether costimulatory molecule blockade could impact a more complex T cell response to alloantigen, potentially consisting of both naïve and memory, $CD4^+$ and $CD8^+$ T cells, whole CBA splenocytes were stimulated with allogeneic (B6) irradiated splenocytes in the presence of blockade of costimulatory pathways. Both anti-CD154 and abatacept were found to inhibit the T cell response to alloantigen when added alone (**Figure 15**). The combination of anti-OX40, anti-CD154 and abatacept further inhibited the proliferative response (**Figure 15**) suggesting that combinations of agents that block distinct costimulatory molecules could synergise to facilitate allograft survival following transplantation.

6.3.13 Anti-OX40 and anti-CD154 do not synergise to prolong allograft survival

Based on the *in vitro* data presented in Figures 11-14, we sought to determine if combined blockade of several costimulatory molecules could attenuate alloreactive T cell responses *in vivo* and result in prolonged allograft survival. It has been previously shown that tolerance induction can be achieved in CBA recipients of B6 skin allografts using co-receptor (anti-CD4 and anti-CD8) and CD154 blockade (anti-CD154; MR1; data not shown) but not by anti-CD154 alone. Therefore, we sought to ask whether anti-OX40 blockade in combination with anti-CD154 would be sufficient to induce tolerance to skin allografts in this setting. CD154 is predominantly involved in initial T cell activation and priming, while OX40 is important later in the immune response, thus providing a good rationale to test this combination. However, anti-OX40 in combination with anti-CD154 was found to have no impact on skin allograft survival (**Figure 16A**). In addition, in a stringent model of cardiac allograft rejection (BALB/c to B6) anti-OX40 did not enhance allograft survival above and beyond that seen with anti-CD154 alone (MST 57 vs. 56 days, respectively; **Figure 16B**).

6.3.14 Anti-OX40 and abatacept did not synergise to prolong cardiac allograft survival

Abatacept (CTLA-4-Ig) demonstrated potent inhibitory effects on T cell responses (Figures 12 and 14) and was found to show a degree of synergy with anti-OX40 *in vitro* (Figures 12, 13, 14) therefore we next investigated whether anti-OX40 could synergise with abatacept *in vivo*. CBA wild-type recipient mice were transplanted with a cardiac allograft (B6) on day 0, and then received a sub-optimal dosing regimen of abatacept

(25μg i.v on days 0, 2 and 5; dosing regimen provided by Dr Thomas Chan (personal communication)) with and without co-administration of anti-OX40 (100mg/kg s.c twice weekly for four weeks). Mice treated with abatacept and anti-OX40 showed the same inhibition of graft survival as abatacept only treated mice (MST 52 days vs. 52 days, respectively; **Figure 17**).

6.4 Discussion

Alloimmune responses are complex and require orchestration of a number of different cell types in order to elicit rejection. Memory T cells are key components of the immune response and are vital for protective immunity against invading pathogens, but also pose a substantial barrier to allograft survival (447, 451). For example, Heeger *et al* showed that alloreactive Tm were present in patients prior to kidney transplantation and that increased number of such cells were associated with an increased incidence of acute rejection (98). Furthermore, the BENEFIT clinical trial, which tested the efficacy of belatacept (CTLA-4-Ig) in kidney transplant recipients, found increased rates of acute rejection in the belatacept treated group compared to controls (246). These acute rejections have been suggested to have been mediated by the presence of a small potent population of alloreactive Tm (452). This is in agreement with experimental findings that have shown that Tm have a reduced reliance on costimulation and are therefore more resistant to costimulation blockade (95, 104, 453, 454).

Overall the results of the experiments presented in this chapter were mixed as the *in vivo* data (Figures 5-7) showed that anti-OX40 markedly attenuated Tm-mediated allograft rejection in contrast to the lack of effect of anti-OX40 on Tm responses to alloantigen *in vitro* (Figures 3-4). *In vitro* assays often do not correlate to *in vivo* data, making interpretation of the data complex. There are a number of critical differences between the response generated *in vitro* and *in vivo*. For example, the APC which present antigen to the responding T cells are fundamentally different; *in vivo* alloantigen is presented by skin DC in our experiments which are likely to be highly activated,

while *in vitro* quiescent whole splenocytes were used as APC. In addition, the timing of analyses may explain the differences between *in vitro* and *in vivo* analyses. Although differences between anti-OX40 and PEG control cultures were also not observed *in vitro* when the proliferative response of Tm was measured at later time-points (day 4-6; data not shown).

Importantly the *in vivo* data showed skin allograft rejection mediated by naïve and memory T cells was attenuated by anti-OX40 and was reproducible whether TCR-transgenic or polyclonal $CD4^+$ and $CD8^+$ naïve and memory T cells were used. OX40 blockade on Tm survival following re-activation was shown to be defective but detailed analysis on these Tm at the molecular level was not performed. The literature supports these data, for example Gramaglia *et al* observed similar numbers and proliferation of effector $CD4^+$ T cells in the primary response in the absence of OX40 signalling, but these cells failed to survive long term therefore resulting in reduced numbers of Tm in $OX40^{-/-}$ mice (305).

We have shown adoptive transfer of Tm into $Rag^{-/-}$ mice and OX40 blockade resulted in prolonged allograft survival, which was due to defective survival of Tm in the dLN. Our data contrasts that by Vu *et al*, who demonstrated that OX40 blockade did not prolong skin allograft rejection mediated by Tm (303). In addition, OX40 blockade had no impact on homeostatic proliferation of Tm, but inhibited their ability of produce IFN-γ *in vitro* thus inhibiting the effector function of Tm. Interestingly, when OX40

blockade was combined with CD28/CD154 blockade skin allografts survived long term (303).

A key difference between our studies and those of Vu *et al*, was the method for Tm generation. In our studies, Tm were generated by reconstituting $Rag^{-/-}$ mice with $1x10^5$ TCR-Tg cells and challenging these $Rag^{-/-}$ mice or wild-type mice with allogeneic splenocytes 1 day later. These mice were then rested for 50-100 days to allow the T cells to become quiescent which generates mostly T_{EM} ($CD44^+CD62L^{lo}$) rather than T_{CM} (N.D. Jones unpublished data). Vu *et al* generated Tm either by homeostatic proliferation where recipient $Rag^{-/-}$ mice were injected with $2x10^6$ naïve T cells (isolated from wild-type) mice or by priming with donor alloantigen (i.e. skin graft) before allowing the cells to become quiescent for 28-42 days. These methods of Tm generation also generated predominantly T_{EM}, although detailed analyses were not performed (303). Therefore the number of Tm which can mediate allograft rejection differs between these studies.

The model used in our adoptive transfer studies is a contrived system in that a finite number of T cells were transferred into $Rag^{-/-}$ mice and the experiments performed in the absence of naturally occurring Treg. However, although anti-OX40 attenuated rejection in such restricted conditions, when anti-OX40 was administered to skin or cardiac transplant recipients no increase in allograft survival was seen in immunocompetent mice (Figure 9). There are a number of reasons which could explain these differences.

TCR affinity for alloantigen may affect the way which a T cell can respond and its requirement for OX40. In a $Rag^{-/-}$ mouse, the absolute number of T cells adoptively transferred with a high affinity for alloantigen will be significantly lower than in wild-type mice. Therefore wild-type mice have an increased precursor frequency of high affinity OX40-independent T cells. This increased number of OX40-independent T cells maybe above the threshold for rejection, hence why anti-OX40 cannot impact the rejection in wild-type mice. Therefore TCR-antigen affinity may play a key role in determining the use of OX40 in allowing T cells to respond and expand in response to alloantigen. Other costimulatory molecules, such as 4-1BB, may be critical for initial proliferation and clonal expansion of high-affinity T cells.

Secondly, antigen-specific precursor frequency is another critical factor which can determine the outcome of the response (455, 456). Ford *et al* demonstrated antigen-specific $CD8^+$ T cell precursor frequency controlled the degree of proliferation and expansion of donor-reactive T cells in costimulation-resistant allograft rejection (457). Other studies have shown high frequency $CD4^+$ T cell populations were able to mount productive effector T cell responses to help donor specific B cells and $CD8^+$ T cells although their proliferation and cytokine production was inhibited (458). As we have shown anti-OX40 has no impact on proliferation of effector T cells but inhibits their survival, therefore T cells with a low precursor frequency which have normal proliferation are more likely to be affected by anti-OX40 rather than those high-frequency T cells which exhibit less proliferation but increased effector function. Our

data is in agreement with this hypothesis as anti-OX40 failed to impact allograft rejection mediated by larger numbers of BM3 T cells, while anti-OX40 could prolong allograft survival mediated by lower numbers of BM3 T cells (Figure 6).

Thirdly, the adoptive transfer systems that were used were contrived in that only one subset of T cells (i.e $CD4^+$ or $CD8^+$) could mediate skin allograft rejection. Previous unpublished data from our laboratory has provided evidence suggesting that when both $CD4^+$ and $CD8^+$ T cell are present, significantly lower numbers of TCR-Tg^+ T cells are required for the rejection of cardiac or skin allografts compared to when a single alloreactive $CD4^+$ or $CD8^+$ T cell population is transferred (N.D. Jones unpublished data). In addition, Treg can regulate either $CD4^+$ or $CD8^+$ T cell responses, but fail to when both populations are present (N.D. Jones unpublished data). Future experiments would address the impact of OX40 when both $CD4^+$ and $CD8^+$ T cells were present in $Rag^{-/-}$ mice. These data suggest that the $CD4^+$ and $CD8^+$ T cells responding in concert to an allograft in wild-type mice may overcome any costimulatory requirement for OX40-OX40L interactions.

The most likely explanation for the differences between the impact of OX40 on allograft rejection in $Rag^{-/-}$ and wild-type mice is the difference in absolute number of T cells capable of responding to alloantigen. Surprisingly though, when the precursor frequency of graft-reactive T cells was lowered by transplanting across multiple minor mismatches or sex-mismatch in wild-type mice, anti-OX40 remained unable to impact skin allograft survival (Figure 10).

It should be noted however that these data are not in agreement with those reported by Curry *et al*, who demonstrated attenuation of cardiac allograft rejection across a miHC mismatch by the administration of OX40 immunoglobulin (Ig) fusion protein (301). Clearly, this difference could be explained by the differences in the immune responses to skin and cardiac allografts (333). As already discussed, the cellular requirements to reject a cardiac and skin allograft are very different; 6000 fold less cells have been shown to be required to reject a skin allograft compared to a cardiac allograft (333) suggesting that skin allografts are more sensitive to rejection which may have impacted on our ability to see any beneficial effects of anti-OX40 in wild-type mice. In addition, there are likely to be differences between the health status of the mice used in our studies and those of Curry *et al.* Memory T cells develop in response to pathogen infection and therefore varying degrees of cleanliness will alter the precursor frequency of memory T cells in the mice.

Thus far although we have provided evidence that anti-OX40 can attenuate allograft rejection mediated by naive and memory T cells under certain circumstances. However, the same effect is not seen in immunocompetent mice (Figure 9 and (301)) which suggests precursor frequency may be important. In addition, there may be a degree of redundancy amongst costimulatory molecules and that multiple costimulatory molecules may need to be blocked to impact allograft rejection in this setting. Indeed, this has been shown by other groups, for example, Yuan *et al* demonstrated that a combination of anti-OX40 and CTLA-4-Ig resulted in long term survival of rat cardiac

allografts across a full MHC mismatch, while anti-OX40 alone was ineffective (302). We addressed the combination of anti-OX40 and anti-CD154 or abatacept *in vitro* and showed an additive effect compared to either reagent alone but *in vivo* no additional benefit was observed.

CD28/CD154 blockade in a skin allograft model demonstrated that these molecules are not absolutely required for rejection in certain circumstances (304). T cell mediated rejection which occurs independently of CD28/CD154 blockade is believed to be predominately mediated by activated $CD8^+$ T cells, Tm and NK cells (443, 459). Identification of alternative pathways which could be targeted to prolong allograft survival either alone or in combination with CD28/CD154 blockade are therefore being explored such as OX40 (302, 304).

Importantly the success of OX40 blockade is reliant on the expression of OX40 being upregulated after T cell activation as OX40 expression does not occur until after several rounds of cell division (304). Therefore it is likely that anti-OX40 would be a more successful therapy in CD28/CD154 independent responses. Blockade of CD28/CD154 and OX40 on T cells dependent on CD28 and CD154 will not upregulate OX40 and therefore anti-OX40 will fail to synergise with the other reagents.

CD28/CD154 independent T cells could be primed independently of these costimulation molecules and therefore have a high affinity for antigen which suggests targeting other

costimulatory molecules such as OX40 may prove successful in targeting CD28/CD154 independent rejection.

The OX40-OX40L pathway has previously been shown to be involved in CD28 and CD154 costimulation independent T cell responses. For example, Demirci *et al* clearly demonstrated a role for OX40-OX40L in CD28/CD154 independent skin allograft rejection as administration of anti-OX40L to CD28/CD154 double deficient mice allowed skin allografts to survive indefinitely (304). These data were confirmed in wild-type rodents, where OX40 blockade synergised with CD28/CD154 blockade to prevent both skin and cardiac allograft rejection (302, 304). In addition anti-OX40 significantly prolonged islet allograft survival in $CD28^{-/-}$ mice however this was not generally applicable to all TNFR superfamily members as anti-CD70 failed to promote islet allograft survival in this model (300). Again, anti-OX40 had no impact on the survival of allogeneic islets when CD28 signalling was left intact.

In our studies, OX40 blockade synergised with either abatacept or anti-CD154 in vitro, but the triple combination had no advantage on inhibiting the proliferation response. In addition, anti-OX40 had no impact on cardiac allograft survival when administered either alone (Figure 9), or in combination with abatacept (CTLA-4Ig; Figure 17). Importantly, abatacept was used *in vivo* at a sub-optimal dose in order to allow us to see a further prolongation of allograft survival. In addition, anti-OX40 combined with anti-CD154 did not prolong skin or cardiac allograft survival over and above that seen with anti-CD154 alone (Figure 16) suggesting that OX40 blockade in combination with other

costimulation blockade was unable to overcome the magnitude and complexity of a fully allogeneic immune response. We did not perform all the costimulation blockade combinations *in vivo* as we used the *in vitro* data as a screening tool to identify possible synergistic interactions to reduce the number of mice used in these studies.

In conclusion, we have shown OX40 blockade can attenuate skin allograft survival mediated by Tm in addition to naive T cells under conditions where the number of responding T cells was limited. Therefore, the impact of anti-OX40 on T cell mediated immunity to allografts appears to be conditional as blockade of the OX40-OX40L pathway alone was insufficient to prolong skin or cardiac allograft survival across a full MHC or even a miHC antigen barrier, even when combined with blockade of CD28 or CD154 molecules. These data suggest that after blockade of these costimulatory molecules, a residual population of cells remains which is untouched by the forms of costimulation blockade used in these studies.

6.5 Figures

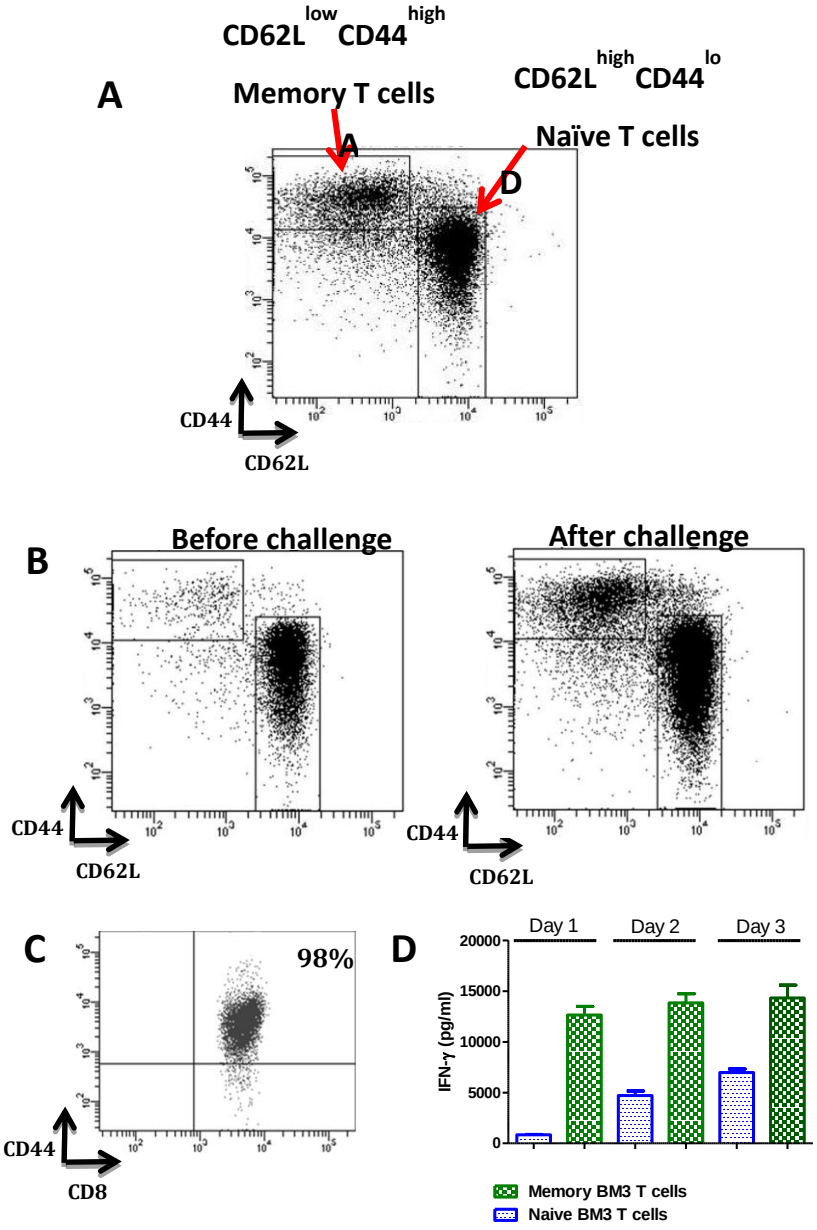


Figure 1: Characterisation of memory T cells generated by alloantigen challenge *in vivo*

Wild-type mice contain a small population of memory T cells (A), but the numbers were insufficient for subsequent experiments. To generate TCR-Tg specific memory T cells $1x10^6$ TCR-Tg naïve T cells were adoptively transferred into syngeneic $Rag^{-/-}$ recipients and the following day mice received $1x10^7$ allogeneic splenocyte challenge. Mice were left for 50-100 days before Tm were isolated and confirmed for a memory phenotype (B and C), and IFN-γ production measured (D).

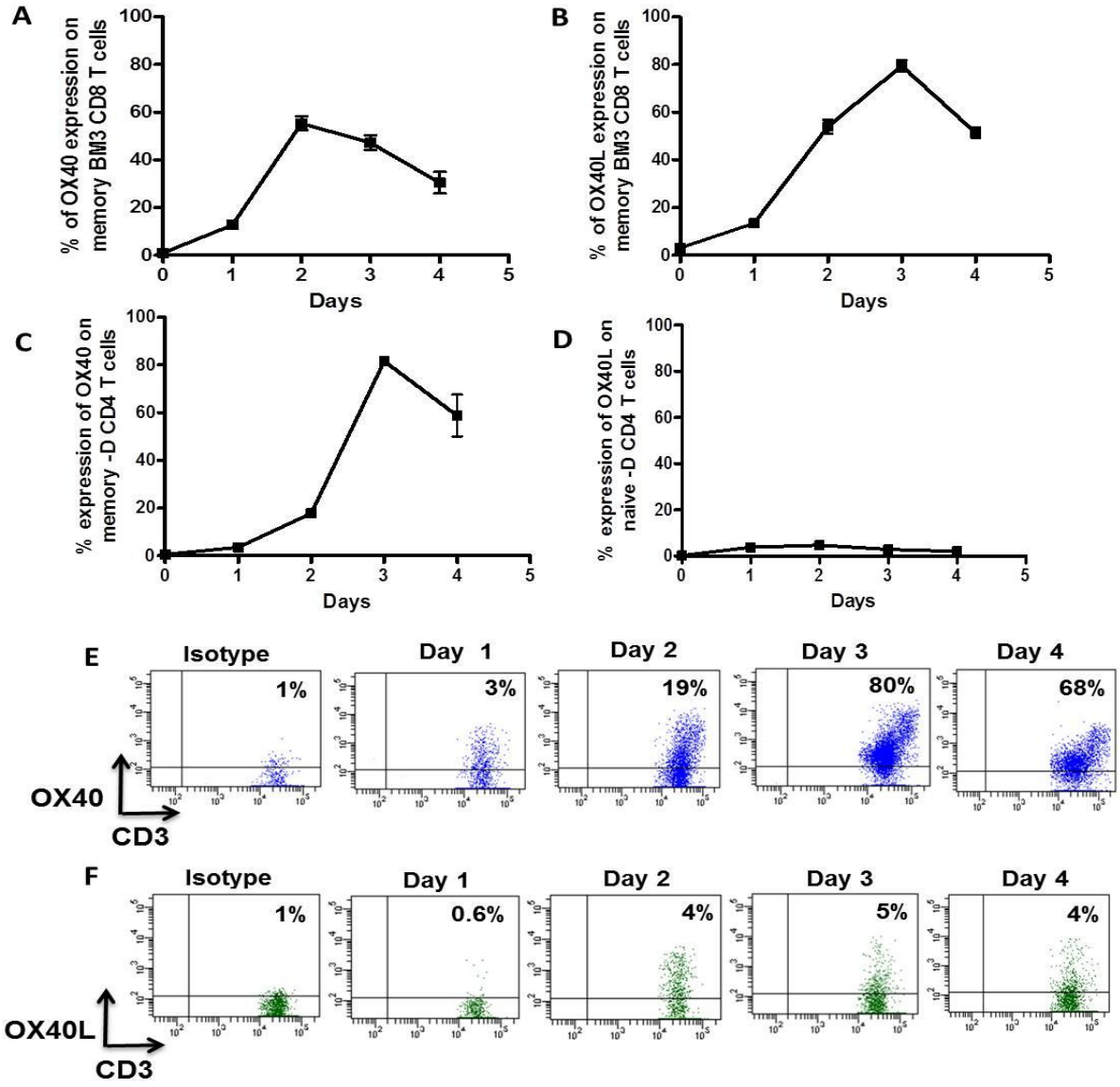


Figure 2: Memory CD4$^+$ and CD8$^+$ T cells express OX40, but only memory BM3 T cells express OX40L

$2.5x10^4$ BM3 or $5x10^4$ -D Tm cells were cultured *in vitro* with $1x10^5$ or $2.5x10^5$ allogeneic irradiated stimulator splenocytes, respectively. In addition $2x10^5$ polyclonal Tm cells were cultured with $4x10^5$ irradiated allogeneic splenocytes. Expression of OX40 on BM3 (A) and -D (C) Tm and polyclonal CD4$^+$ Tm (E) was determined by flow cytometry. Expression of OX40L on BM3 (B) and -D (D) memory T cells and polyclonal CD4$^+$ memory T cells (F) was determined by flow cytometry. Results were expressed as mean % ± SD, performed in triplicate and are representative of 3 independent experiments. Quadrant gates were set using an isotype control antibody.

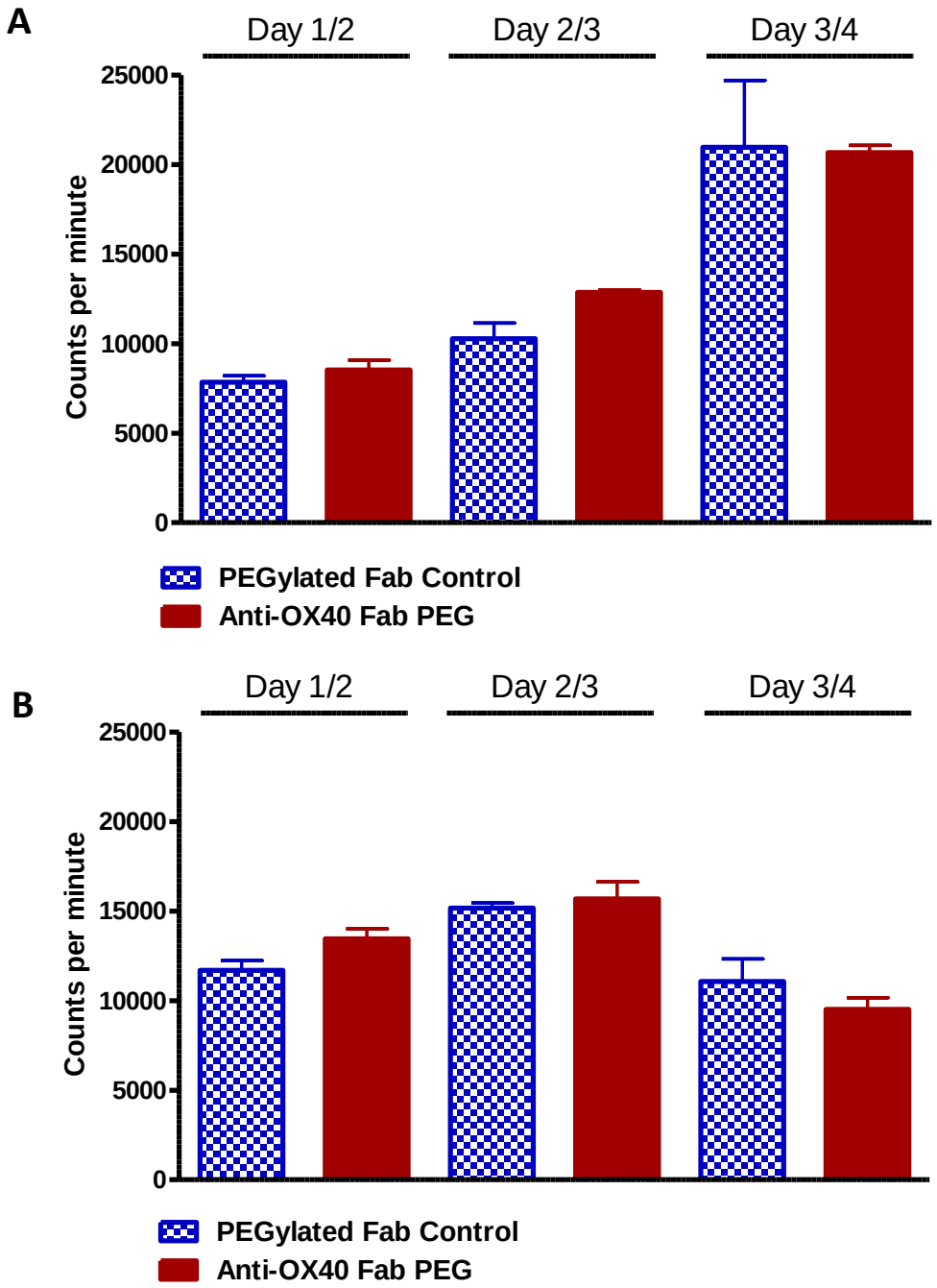


Figure 3: Anti-OX40 has no impact on memory CD4$^+$ T cell responses *in vitro*
$5x10^4$/well memory TEa or -D T cells and $2.5x10^5$/well irradiated allogeneic splenocytes were cultured for various lengths of time. Anti-OX40 or PEG control were added at 10µg/ml to appropriate wells. Cells were pulsed with ^{3H}-thymidine on day 1, 2 or 3, and harvested 18 hrs later. A) TEa Tm B) -D Tm. Results are expressed as mean counts per minute ± SD, performed in triplicate and are representative of 2 independent experiments.

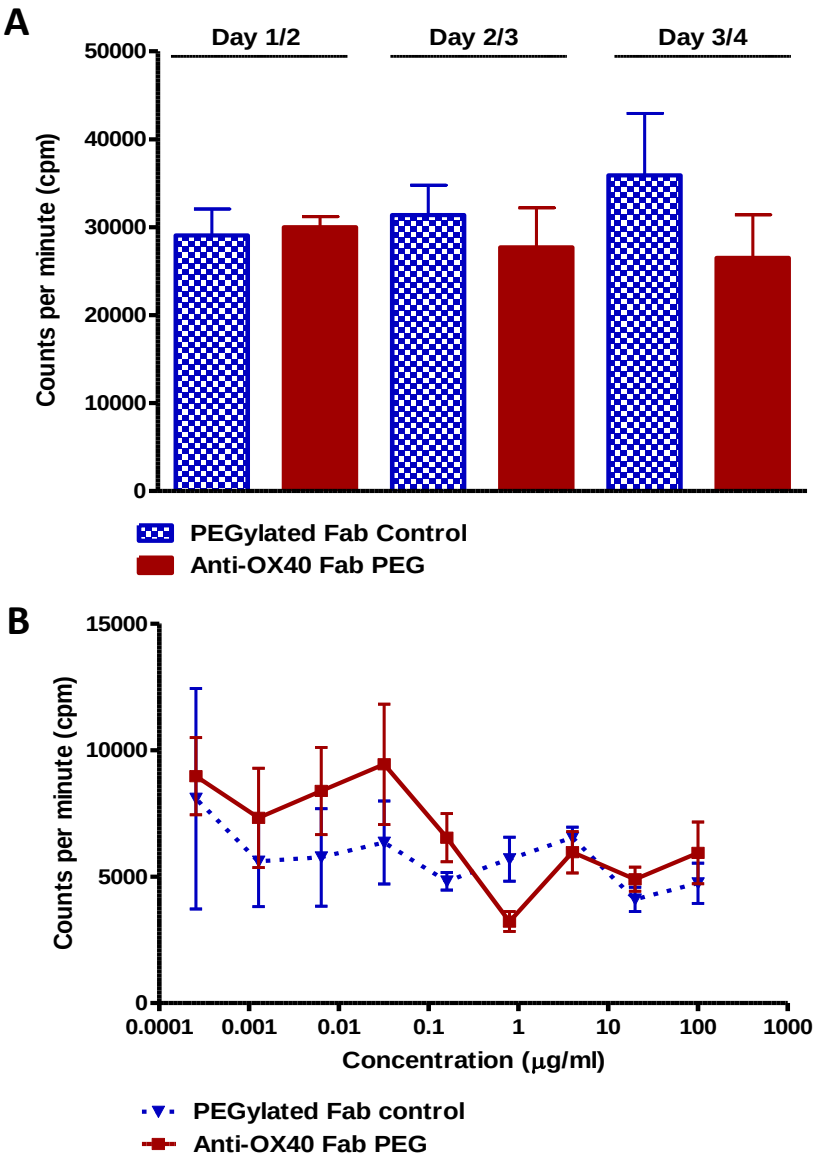


Figure 4: OX40 blockade has no impact on CD8$^+$ memory T cell responses *in vitro*
2.5x10^4/well memory BM3 T cells and 1x10^5/well irradiated B6 splenocytes were cultured for various lengths of time. A) Anti-OX40 or PEG control were added to the appropriate wells at 10µg/ml. Cells were pulsed on day 1, 2 or 3 and harvested 18 hrs later. B) Anti-OX40 or PEG control were added at varying concentrations (100-0.000256µg/ml) to appropriate wells. Cells were pulsed with ^{3H}-thymidine on day 2, and harvested 18 hrs later. Results are expressed as mean counts per minute ± SD, performed in triplicate and are representative of 3 independent experiments.

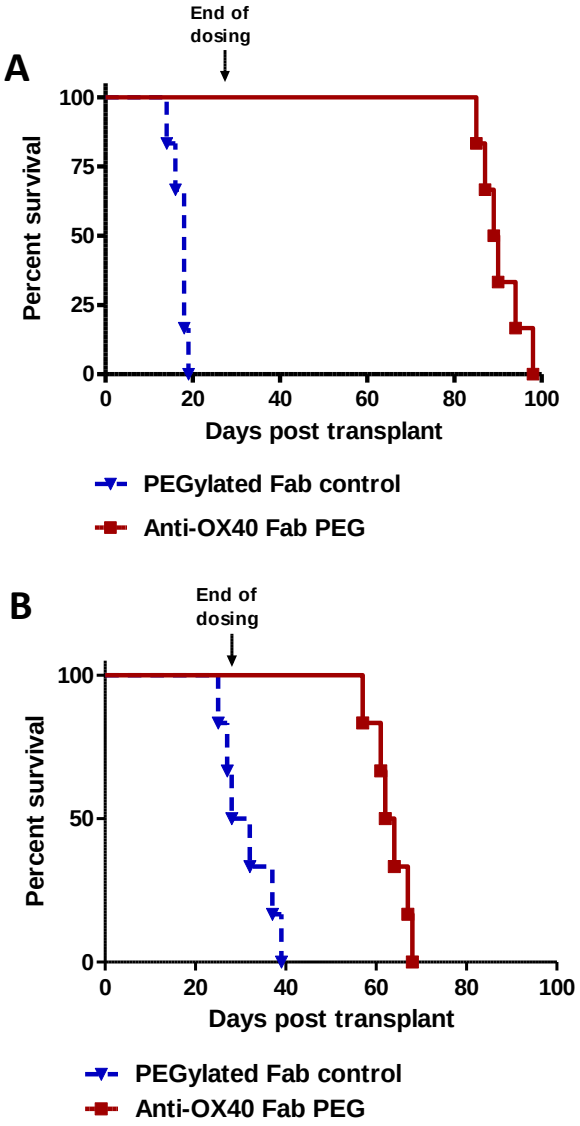


Figure 5: Anti-OX40 attenuates skin allograft rejection mediated by memory CD4$^+$ T cells

$5x10^4$ memory TEa (A) or $1x10^5$ memory -D (B) CD4$^+$ T cells were adoptively transferred into syngeneic Rag$^{-/-}$ mice. One day later, mice received an allogeneic skin graft (BALB/cB6F1 or B10.S respectively) and either anti-OX40 or PEG control administered s.c at 100mg/kg twice weekly for 4 weeks. Kaplan-Meier survival curves show skin allograft rejection kinetics. (n= 7-8 mice per group).

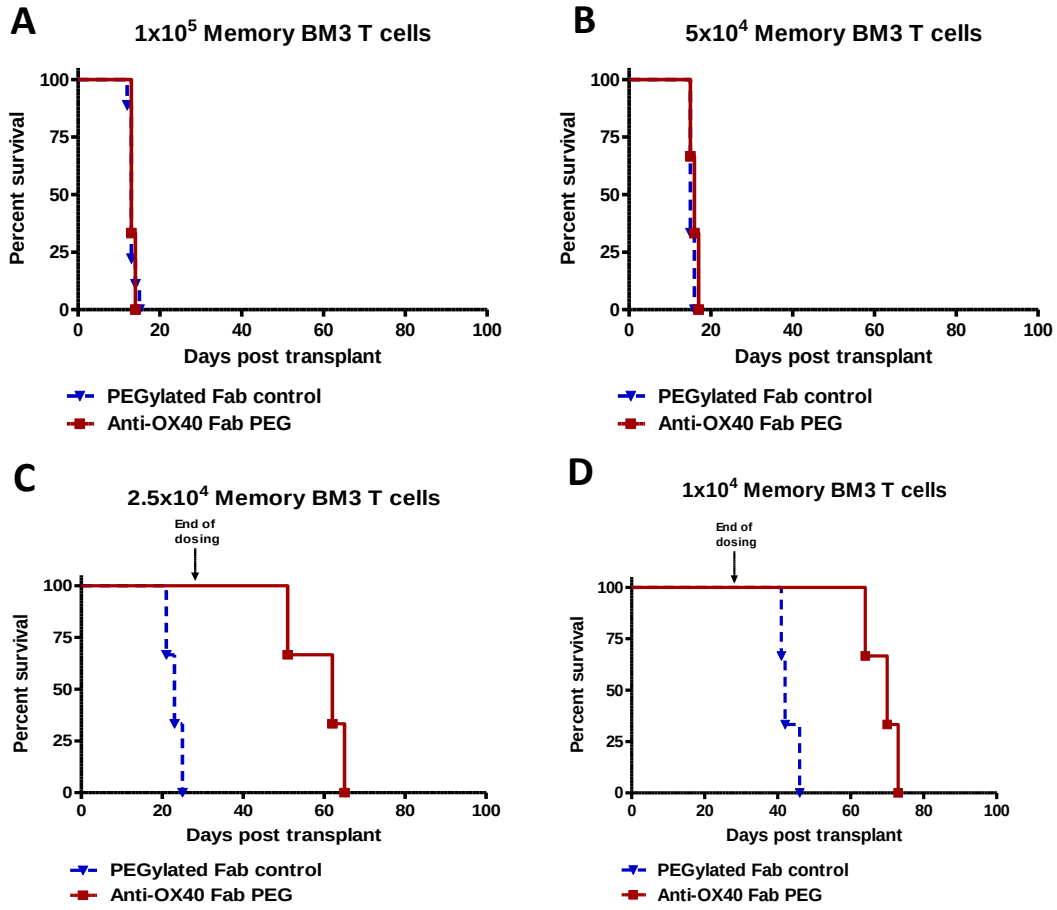


Figure 6: Anti-OX40 attenuates skin allograft rejection mediated by BM3 Tm in manner that is dependent on T cell number

$1x10^5$ (A), $5x10^4$ (B), $2.5x10^4$ (C) or $1x10^4$ (D) BM3 Tm were adoptively transferred into syngeneic $Rag^{-/-}$ mice. One day later, mice received a skin allograft (B6) and either anti-OX40 or PEG control administered s.c. at 100mg/kg twice weekly for 4 weeks. Kaplan-Meier survival curves show skin allograft rejection kinetics. (n= 4-6 mice per group).

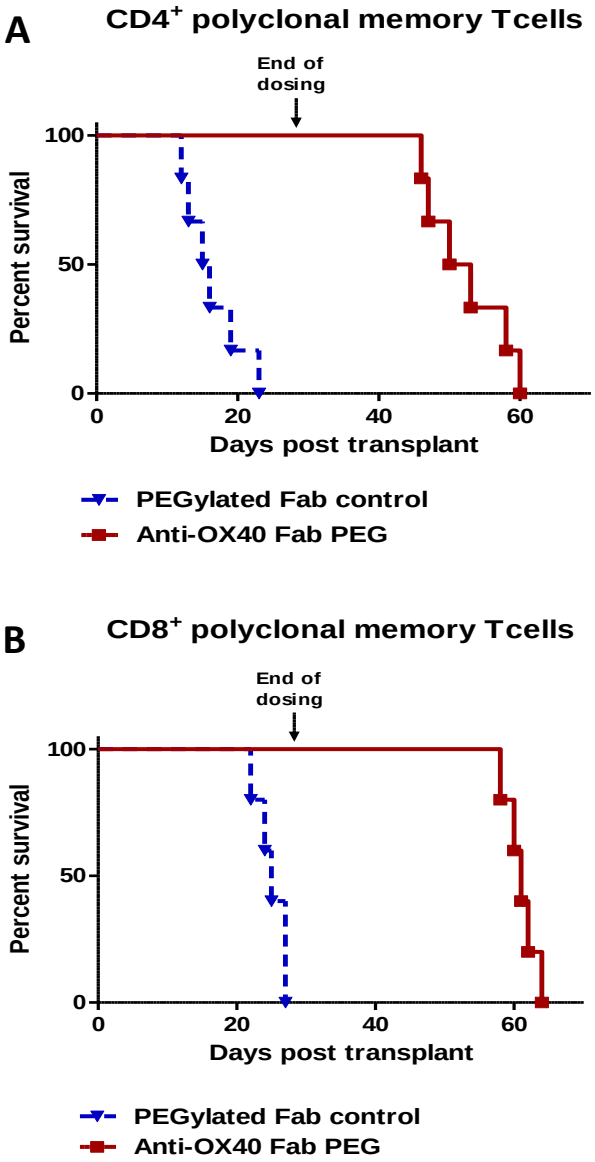


Figure 7: OX40 blockade attenuates skin allograft rejection mediated by polyclonal memory T cells

$1x10^5$ polyclonal, $CD4^+$ or $CD8^+$ TTm (generated in vivo by allogeneic splenocyte challenge in wild-type CBA mice) were adoptively transferred into syngeneic CBA $Rag^{-/-}$ mice. One day later, mice received an allogeneic (B6; $H2^b$) skin graft and anti-OX40 or PEG control administered s.c at 100mg/kg twice weekly for 4 weeks. Kaplan-Meier survival curves show skin allograft rejection kinetics mediated by polyclonal $CD4^+$ Tm (A) or polyclonal $CD8^+$ Tm (B). (n = 6-8 mice per group).

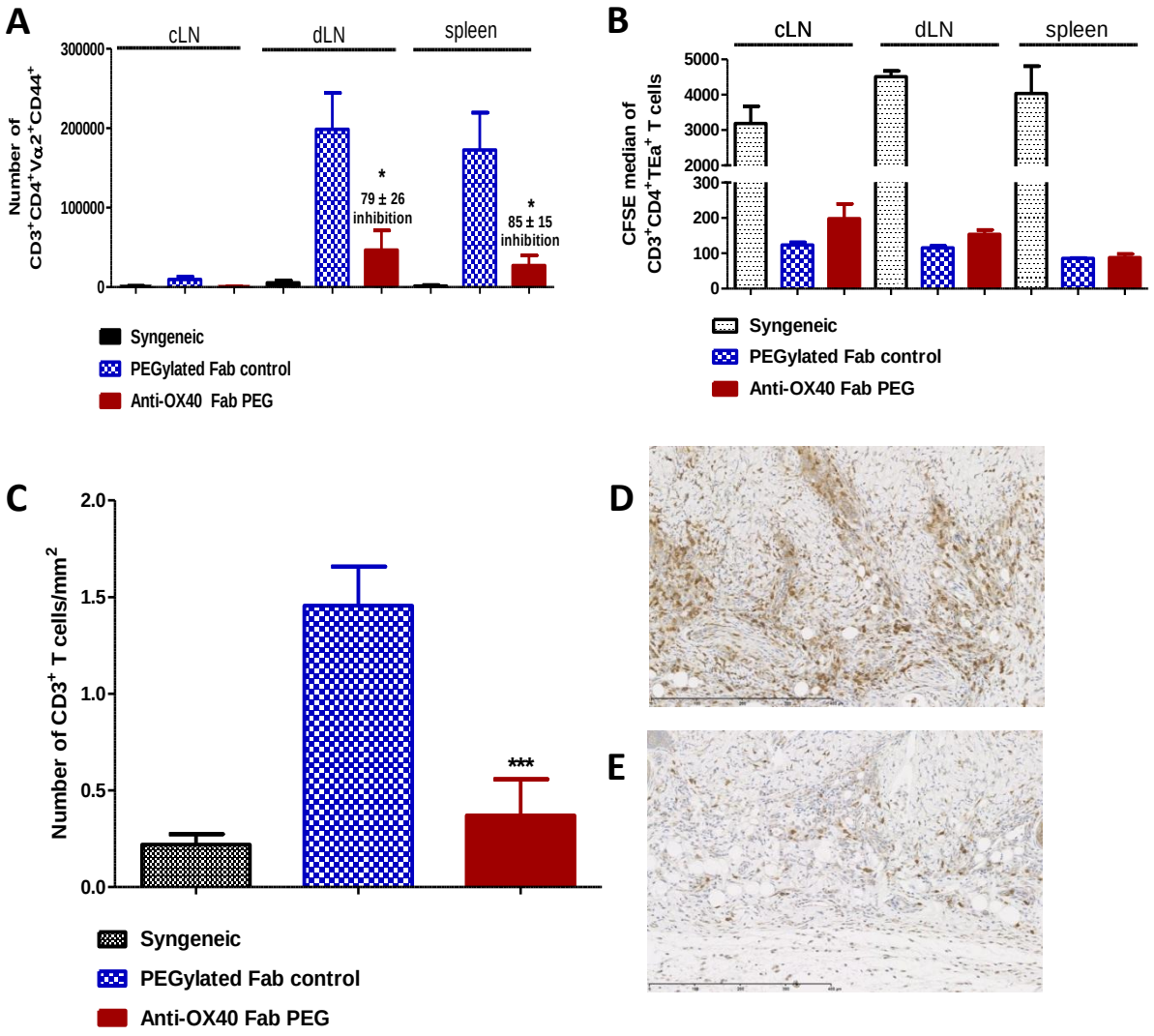


Figure 8: Anti-OX40 prevents clonal expansion of memory T cells after allogeneic transplantation

$1x10^5$ TEa Tm were adoptively transferred into syngeneic $Rag^{-/-}$ mice which received an allogeneic (BALB/cB6F1) skin graft the following day. Mice received either anti-OX40 or PEG control s.c. twice weekly for 15 days. Spleen, dLN and cLN were harvested at day 15 for analysis of TEa number (A), proliferation (B) and CD3 infiltration of skin allografts (C, D, E). Results were expressed as mean number ± SD. n = 4-6 mice per group, unpaired student t test was used to compare differences between anti-OX40 and PEG treated mice where * represents $p<0.05$ and *** represents $p<0.0001$.

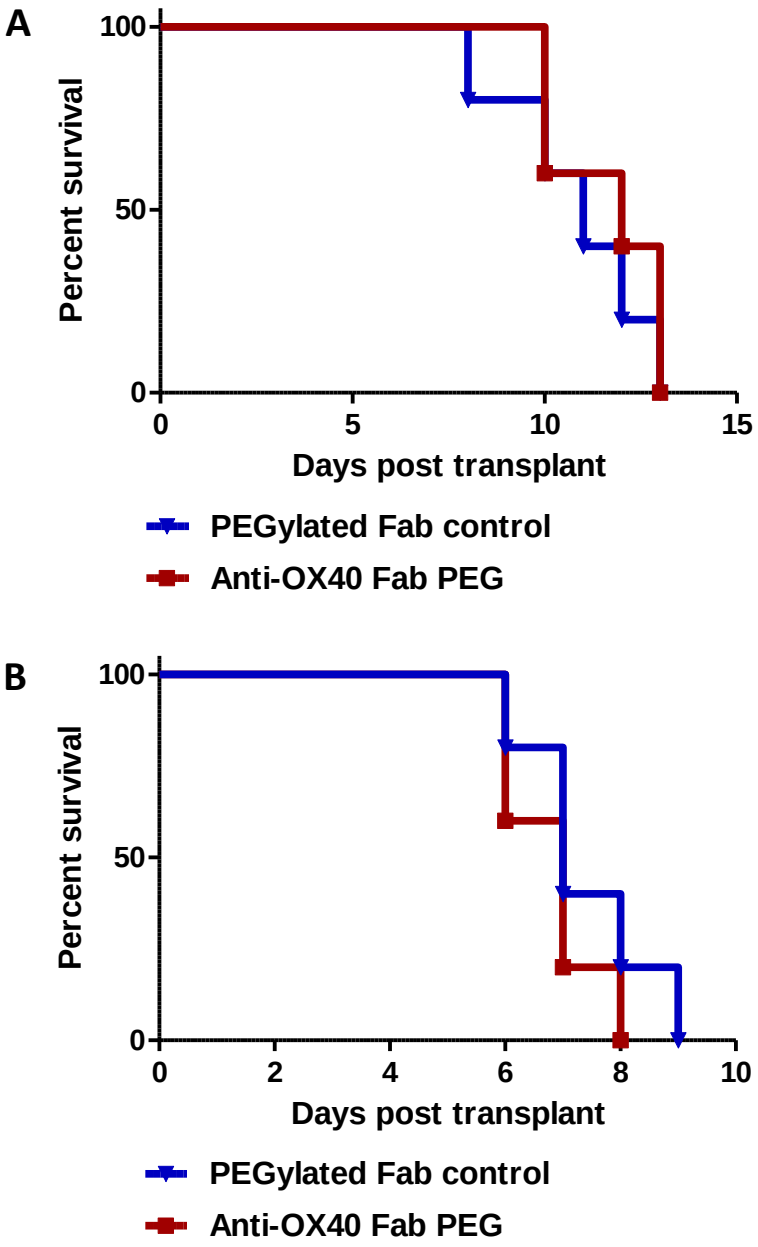


Figure 9: Anti-OX40 does not extend skin or cardiac allograft survival
CBA mice received either a skin (A) or cardiac (B) allograft (B6) on day 0 together with either anti-OX40 or PEG control administered s.c. at 100mg/kg twice weekly for 4 weeks (or until rejection). Kaplan-Meier survival curves show skin (A) and cardiac (B) allograft rejection kinetics. (n= 5 mice per group).

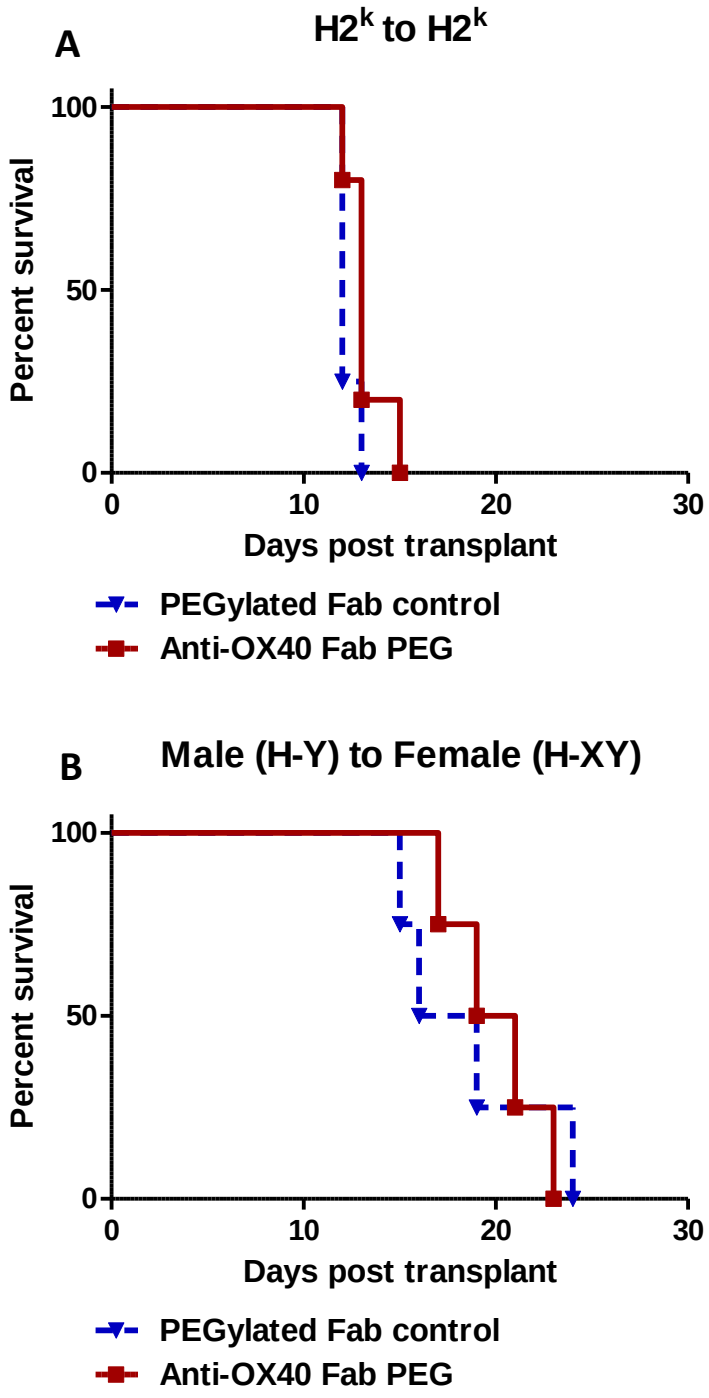


Figure 10: Anti-OX40 has no impact on minor mismatched allograft survival
(A) Wild-type recipient CBA mice received a minor mismatched skin allograft (B10.Br) on day 0. (B) Wild type female CBA received a sex-mismatched skin allograft (male donor skin). Anti-OX40 or PEG control was administered s.c. at 100mg/kg twice weekly for 4 weeks. Kaplan-Meier survival curves show skin allograft rejection kinetics. (n= 4-5 mice per group).

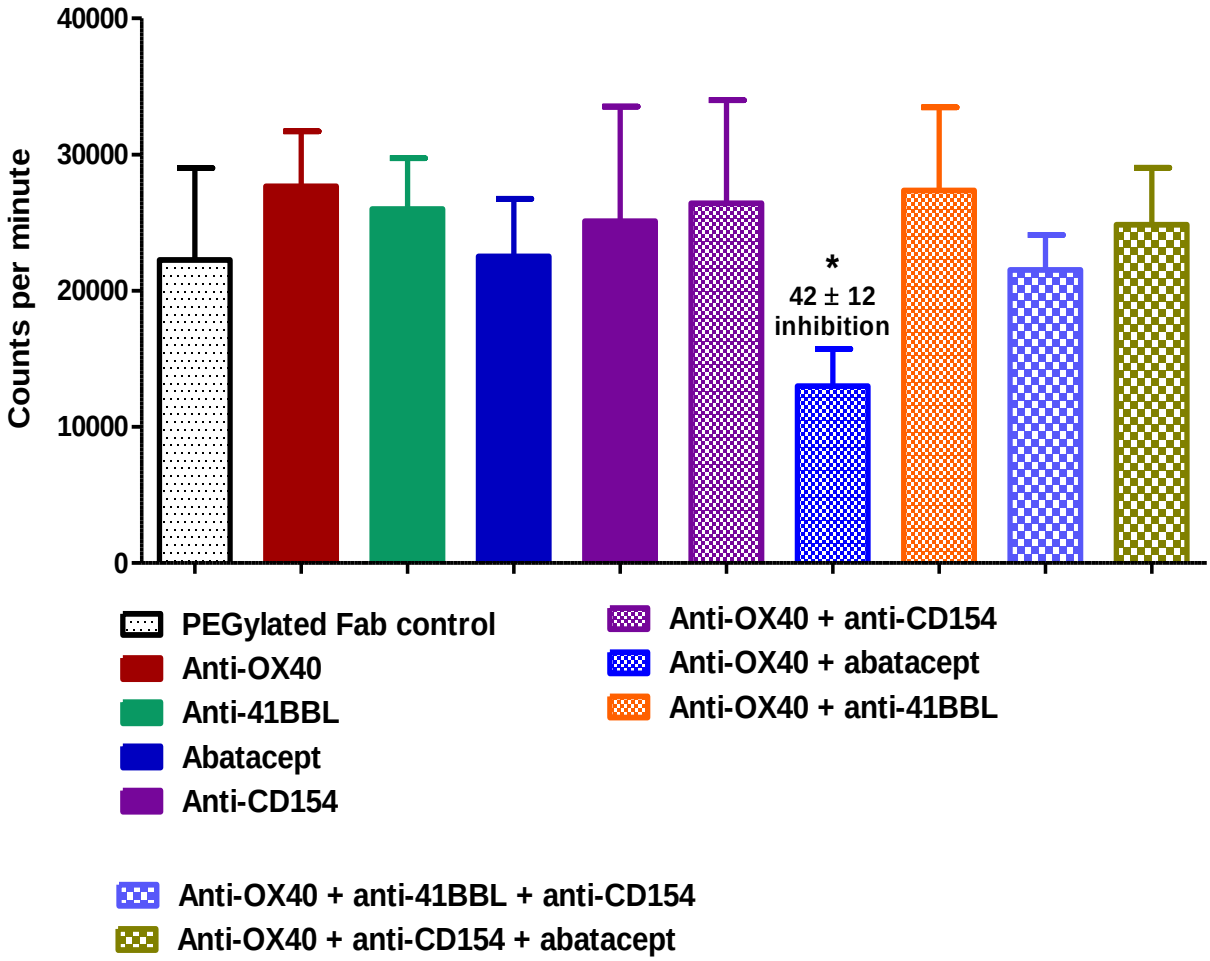


Figure 11: Anti-OX40 and either anti-CD154 or abatacept leads to enhanced suppression of proliferation *in vitro*

$2.5x10^4$/well naïve BM3 T cells and $1x10^5$/well irradiated B6 splenocytes were cultured, pulsed with ^{3H}-thymidine on day 4 and harvested 18 hrs later. Blocking reagents were added at 10μg/ml (except for abatacept at 1μg/ml). Results are expressed as mean counts per minute ± SD, cultures were performed in triplicate and are representative of 2 independent experiments. To determine the statistical significance between groups (using unpaired student t test) monotherapies were compared to control, double therapies were compared to monotherapies, triple therapies to double therapies, and finally 4-treatments to triple therapies. * represents $p<0.05$.

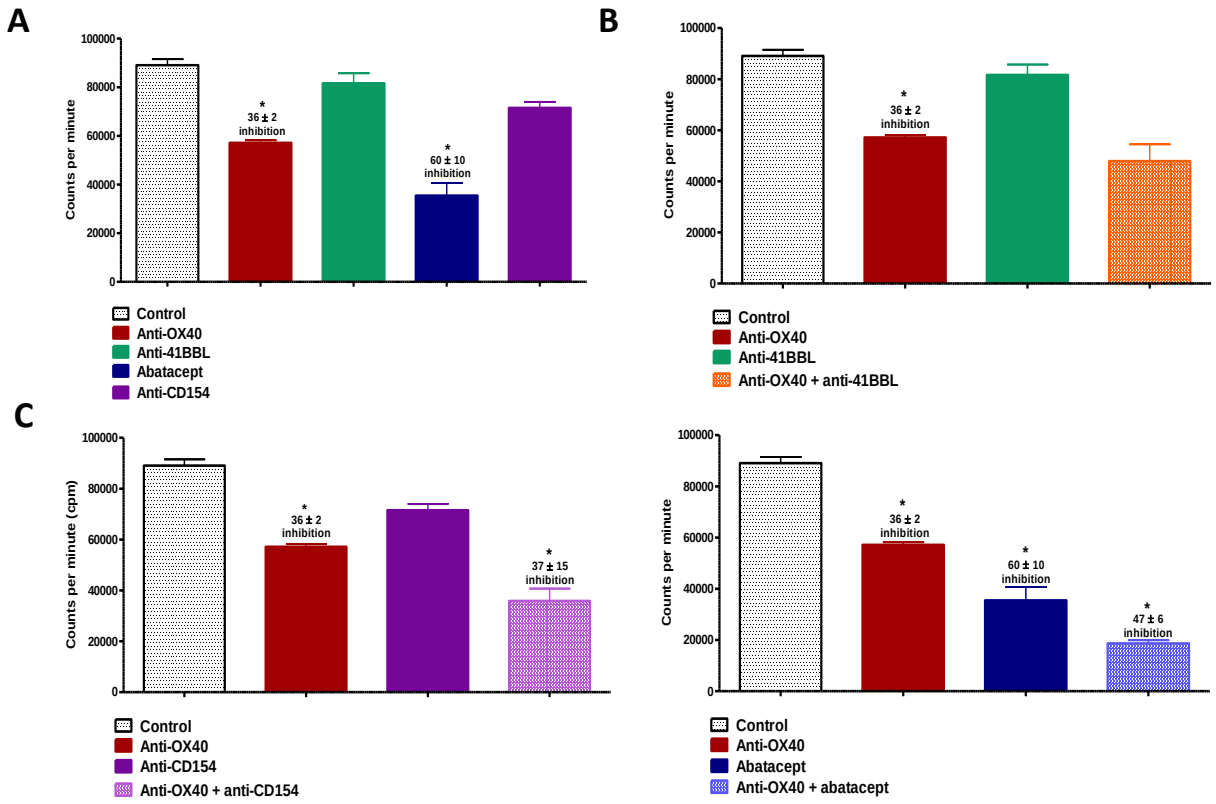


Figure 12: Anti-OX40 and either anti-CD154 or abatacept leads to enhanced suppression of proliferation *in vitro*

$2.5x10^4$/well naïve BM3 T cells and $1x10^5$/well irradiated B6 splenocytes were cultured, pulsed with ^{3H}-thymidine on day 4 and harvested 18 hrs later. Blocking reagents were added at 10μg/ml (except for abatacept at 1μg/ml). Results are expressed as mean counts per minute ± SD, cultures were performed in triplicate and are representative of 2 independent experiments. To determine the statistical significance between groups (using unpaired student t test) monotherapies were compared to control, double therapies were compared to monotherapies. * represents $p<0.05$

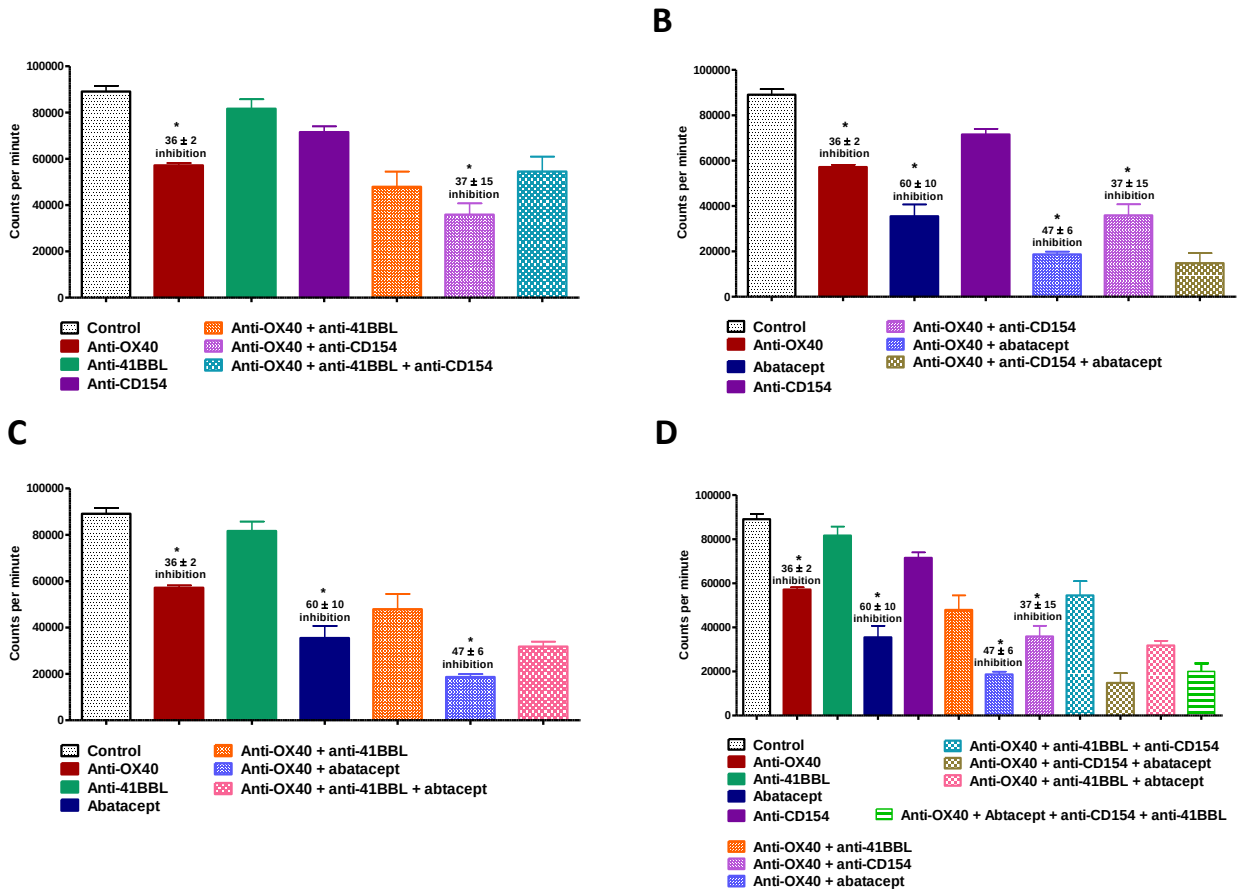


Figure 13: Anti-OX40 and anti-CD154 or abatacept do not synergise to further inhibit proliferation *in vitro*

$2.5x10^4$/well naïve BM3 T cells and $1x10^5$/well irradiated B6 splenocytes were cultured, pulsed with ^{3H}-thymidine on day 4 and harvested 18 hrs later. Blocking reagents were added at 10µg/ml (except for abatacept at 1µg/ml). Results are expressed as mean counts per minute ± SD, cultures were performed in triplicate and are representative of 2 independent experiments. To determine the statistical significance between groups (using unpaired student t test) monotherapies were compared to control, double therapies were compared to monotherapies, triple therapies compared to double therapies and finally 4-treatments to triple therapies. * represents $p<0.05$

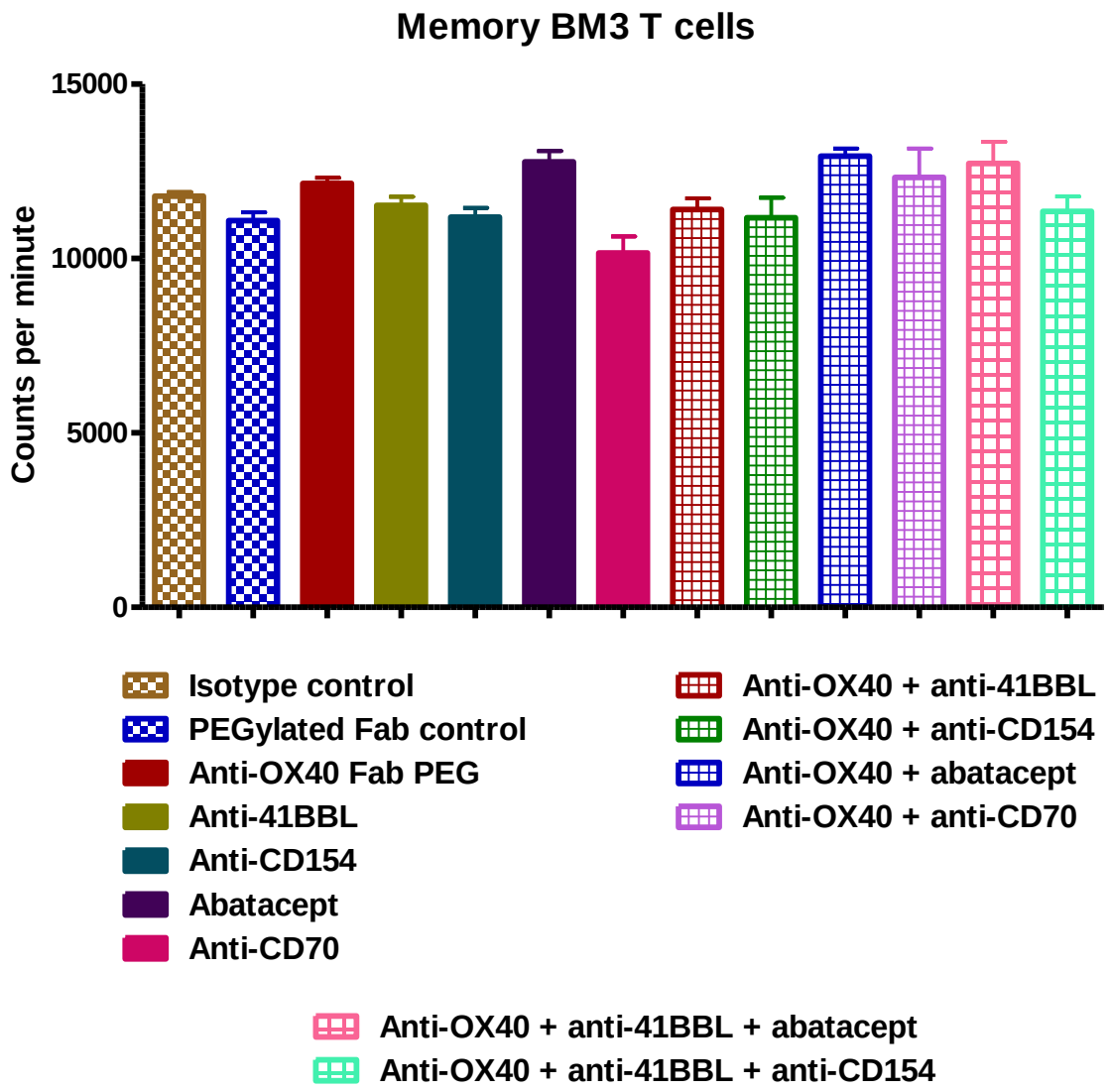


Figure 14: Costimulatory molecule blockade has no impact on the proliferation of memory BM3 T cells

$2.5x10^4$/well memory BM3 T cells and $1x10^5$/well irradiated B6 splenocytes were cultured, pulsed with ^{3H}-thymidine on day 2 and harvested 18 hrs later. Blocking reagents were added at 10μg/ml (except for abatacept at 1μ/ml). Results are expressed as mean counts per minute ± SD, cultures were performed in triplicate and are representative of 2 independent experiments.

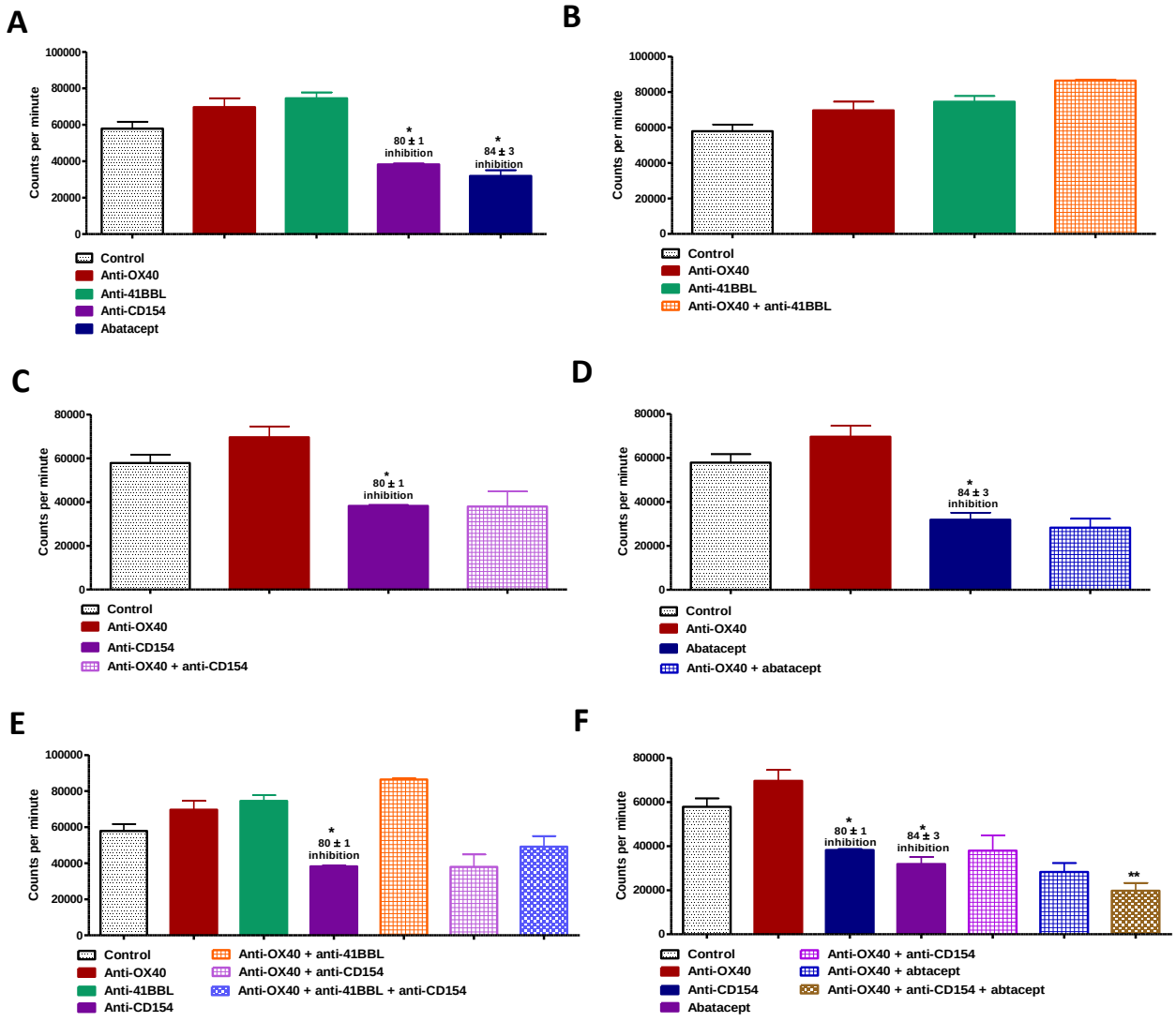


Figure 15: Anti-OX40 fails to synergise with other costimulation blockade to inhibit proliferative responses of whole wild-type splenocytes *in vitro*

$2x10^5$ polyclonal T cells (isolated from CBA mice) were cultured with $4x10^5$ irradiated allogeneic (B6) splenocytes, pulsed with ^{3H}-thymidine on day 2 and harvested 18 hrs. Blocking reagents were added at 10μg/ml (except for abatacept at 1μ/ml). Results are expressed as mean counts per minute ± SD, cultures were performed in triplicate and are representative of 2 independent experiments. To determine the statistical significance between groups (using unpaired student t test) monotherapies were compared to control, double therapies were compared to monotherapies, triple therapies to double therapies, and finally 4-treatments to triple therapies. * represents $p<0.05$

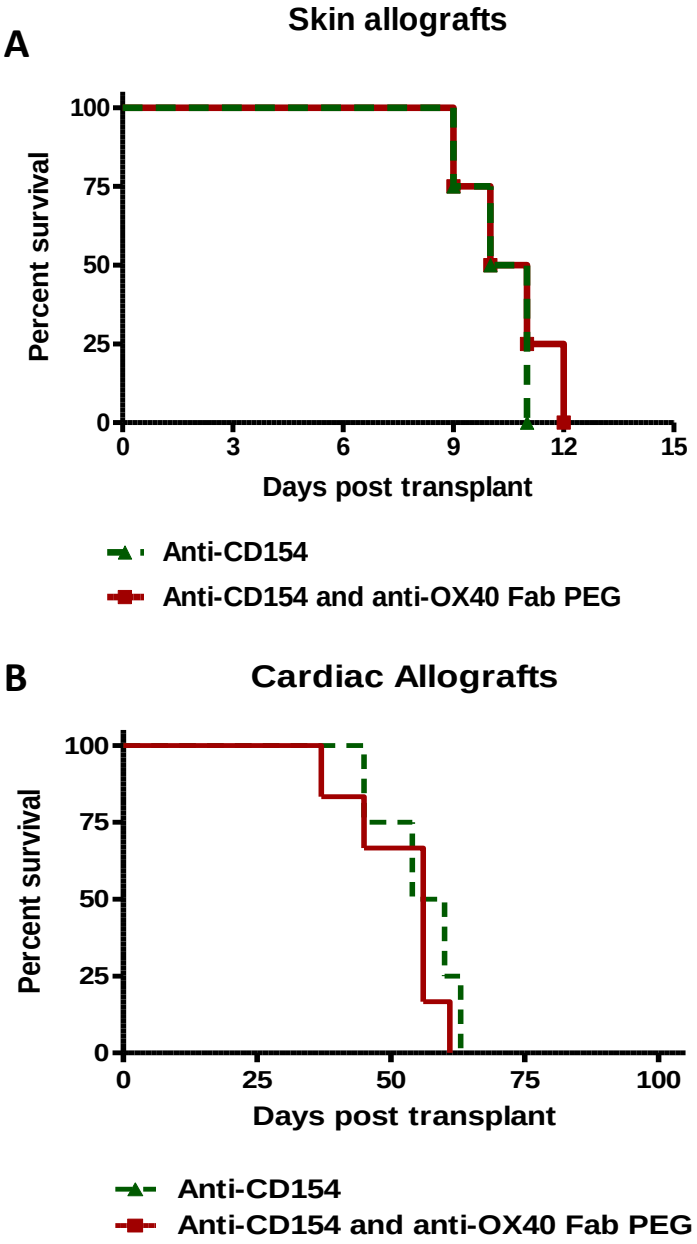


Figure 16: Anti-OX40 and anti-CD154 do not synergise to prevent allograft rejection

A) Wild-type recipient CBA mice received a skin allograft (B6) on day 0. B) Wild-type recipient B6 mice received a cardiac allograft (BALB/c) on day 0. Anti-OX40 or PEG control was administered s.c. at 100mg/kg twice weekly for 4 weeks (or until rejection). Anti-CD154 (MR1) was given i.v. at 1mg/dose on days 0, 7 and 14. Kaplan-Meier survival curves show skin (A) and cardiac (B) allograft rejection kinetics. (n= 4-5 mice per group).

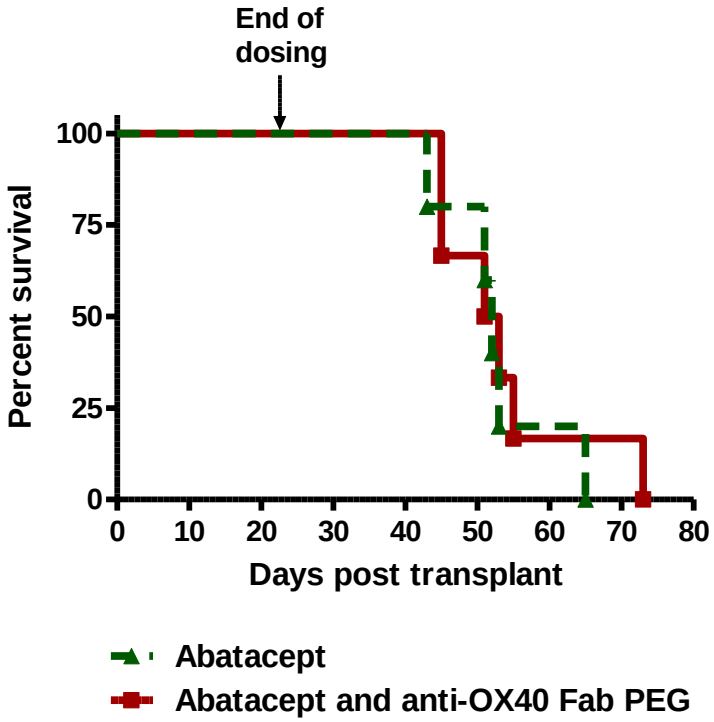


Figure 17: Anti-OX40 and abatacept do not synergise to prevent cardiac allograft rejection

Wild-type recipient CBA mice received a cardiac allograft (B6) on day 0. Anti-OX40 or PEG control were administered s.c. at 100mg/kg twice weekly for 4 weeks (or until rejection). Abatacept (CTLA-4-Ig) was given i.v. at 25μg/dose on days 0, 2 and 5. Kaplan-Meier survival curves show cardiac allograft rejection kinetics. (n= 5-6 mice per group).

Chapter 7: Discussion

7.1 Summary of data

This thesis has attempted to reveal a role for OX40-OX40L in immune responses directed towards alloantigen by dissecting the role of this costimulatory interaction on specific T cell populations which are involved in allograft rejection and the induction of tolerance. The role of OX40-OX40L in immune responses will be discussed later in this chapter, but is summarised in Figure 1A. Our findings of blocking OX40 are summarised in Figure 1B.

7.1.1 Chapter 3 – Expression and utilisation of OX40 by $CD4^+$ and $CD8^+$ T cells in response to alloantigen

The main objective of this chapter was to determine whether OX40 and/or OX40L was expressed by naïve $CD4^+$ and $CD8^+$ T cells and then investigate whether OX40-OX40L interactions were important in allograft rejection. We observed that both $CD4^+$ and $CD8^+$ T cells expressed OX40 after T cell activation. From these findings, we next sought to block the OX40-OX40L interaction to determine the impact on responses to alloantigen. To this end, we transferred a small number of alloreactive, TCR-transgenic T cells into syngeneic $Rag^{-/-}$ mice which were then given an allogeneic skin transplant the following day. OX40 blockade was able to attenuate skin allograft rejection mediated by either naïve $CD4^+$ or $CD8^+$ T cells. This result was confirmed by using naïve polyclonal T cells, which yielded comparable results. Surprisingly, OX40L was also expressed by $CD8^+$ T cells, but not $CD4^+$ T cells. These results led us to investigate

whether OX40-OX40L could interact via a T cell-T cell dependent manner. Using anti-CD3/CD28 synthetic beads, we demonstrated that blocking OX40 inhibited proliferation *in vitro* suggesting that OX40 costimulation could be generated following interaction with OX40L (in *cis* or *trans*) on activated T cells as well as the previously reported OX40L on DC and lymphoid tissue inducer cells (LTi).

7.1.2 Chapter 4 – Role of OX40-OX40L in alloimmune responses

The aim of chapter 4 was to provide an insight into the mechanisms involved in the suppression of skin allograft rejection by anti-OX40. The impact of OX40 signalling on the initial activation, proliferation, clonal expansion, migration and survival of alloreactive T cells was determined. *In vitro*, in an MLR, anti-OX40 did not alter the modulation of cell surface markers following T cell activation suggesting that OX40-OX40L interactions were not involved in the initial priming of T cells. However, OX40 blockade reduced proliferation and $CD4^+$ and $CD8^+$ T cell production of IL-2 and IFN-γ but importantly did not skew the cytokine production towards a Th2 response, as previously demonstrated (293, 352, 376).

In vivo, mice treated with anti-OX40 had a dramatic reduction in the number of T cells in the dLN following allogeneic skin transplantation suggesting that OX40 blockade prevented the clonal expansion and accumulation of T cells. However anti-OX40 had no impact on the proliferation of T cells as CFSE division profiles were comparable between treatment groups. Therefore, OX40 may play a role in the propagation of T cell responses to skin allografts rather than in initial priming. We hypothesised that

OX40-OX40L interactions were required for the survival of effector T cells. Indeed, this proved to be the case as both *in vitro* and *in vivo* anti-OX40 increased cell death amongst the responding T cell population which was in agreement with other published data (256). Therefore, OX40-mediated costimulation prevented activation induced cell death in part by promoting IL-2 production which may provide a potential survival signal.

7.1.3 Chapter 5 – Role of OX40-OX40L on regulatory T cells

The expression of some costimulatory molecules is shared between Treg and effector T cells, although such costimulatory signals may have a similar or diametrically opposed functions. Our data demonstrated a clear role for OX40-OX40L in promoting the survival of effector T cells therefore the question remained whether OX40 blockade could alter the potency or function of $CD4^+FoxP3^+$ Treg.

Treg were shown to constitutively express OX40, but not OX40L. Using a well-established protocol of anti-CD4 and a DST to generate alloreactive Treg we found that Treg generated in the absence of OX40 signalling were more potent at inhibiting allo-specific $CD4^+$ T cell responses than Treg generated where OX40 was available. However, OX40 blockade failed to aid the conversion of naïve $CD4^+$ T cells into Treg and similarly failed to enhance the expansion of nTreg. However, Treg generated in the absence of OX40 *in vitro* enjoyed extended survival upon re-activation compared to their counterparts formed in the presence of OX40 (as indicated by an increase in number of Treg and expression of Bcl-2). We therefore propose that such Treg would

maintain their suppressive function for longer than those formed in the presence of OX40 signalling. These data confirm that OX40-OX40L have diametrically opposed actions on Treg and effector/memory T cells.

7.1.4 Chapter 6 - OX40 blockade as an adjunctive therapy to pre-existing immunosuppressive strategies.

In chapter 6, our aim was to determine whether OX40-OX40L interactions are required for optimal Tm responses (which are a known barrier to tolerance induction) as well as assessing whether OX40 blockade could promote allograft survival in immunocompetent mice alone or in combination with reagents that block other costimulatory pathways.

Similar to our results with naïve T cells, OX40 blockade significantly attenuated skin allograft rejection mediated by memory $CD4^+$ and $CD8^+$ T cells. The impact of OX40 blockade on naïve and memory T cell responses appeared to be the same i.e. anti-OX40 prevented Tm clonal expansion but had no impact on proliferation. Disappointingly, anti-OX40 did not significantly impact skin or cardiac allograft survival when used either alone or in combination with other costimulation blockade such as anti-CD154 and CTLA-4Ig in wild-type mice.

Our data demonstrate that OX40-OX40L interaction plays a crucial role in the costimulatory cascade of T cell activation and blockade of this interaction could have a therapeutic benefit in preventing allograft rejection or autoimmunity by inhibiting

pathogenic effector/memory T cells whilst enhancing the suppressive capacity of Treg. However, T cell requirements for OX40-OX40L interactions were found to be conditional and limited to manipulated experimental models, as in immunocompetent mice anti-OX40 was not able to attenuate skin allograft rejection alone or in combination with other costimulatory blockade.

7.2 Challenges in the translation of costimulation blockade to NHP and man

There is a growing body of evidence in rodent models to suggest that blocking costimulation can lead to tolerance induction (221, 341), however, data from NHP studies suggest that tolerance induction is more difficult to achieve in higher animals including humans (223-225, 441). NHP have a more complex immune system compared to small rodents and have a higher diversity of pre-existing immunity as a result of exposure to environmental antigens (105, 442). Although outbred laboratory animals have a wider diversity of exposure to such antigens, they are still less immunologically educated compared to humans. Despite these differences, large animal models remain the best way to gain knowledge before initiating a safe and ethically robust clinical trial.

Belatacept (human CTLA-4-Ig fusion protein; Nujolix®) is the only reagent that targets costimulation that is currently approved for clinical use in the indication of prophylaxis of organ rejection in adult kidney transplant recipients. Belatacept is an effective primary immunosuppressant that results in enhanced renal function at 1 and 5 years (245, 246) compared to cyclosporine treatment thus removing the requirement for

calcineurin inhibitors. However at 1 year, the incidence of acute rejection was higher in those patients treated with belatacept compared to the control arm (246). The rates of infection and malignancy were comparable between both arms, while the metabolic and cardiovascular risk profiles were better in the belatacept treated patients (460). The specific causes of the observed increased rates of rejection are unknown at the moment, but there are two cellular subsets which could provide explanation. Memory T cells are more resistant to costimulation blockade, therefore belatacept may fail to suppress a subset of alloreactive T cells, i.e. memory T cells (452). In addition, belatacept treated patients had markedly increased numbers of Treg in the graft during episodes of acute rejection (461).

These data provide evidence that targeting costimulatory molecules can have a positive outcome on graft survival but also highlight the need to develop alternative reagents which can suppress all cellular subsets involved in allograft rejection.

7.3 Role of OX40-OX40L in immune responses

7.3.1 Effector T cell responses

Our data suggest that signals via OX40-OX40L are received after initial T cell priming, which then aid the survival of activated T cells rather than enhance/modulate cell division or the migration of T cells following allograft transplantation. In keeping with these data, $OX40^{-/-}$ $CD4^{+}$ T cells have been shown to be unable to maintain high levels of anti-apoptotic molecules such as Bcl-2 and $Bcl\text{-}_{XL}$ (256, 258) that also suggest that OX40 signals promote the survival of effector T cells and the generation of Tm. These

data also concur with published data whereby administration of agonistic OX40 enhanced primary T cell expansion and survival which in turn increased the frequency of memory T cells (305, 374, 462).

7.3.2 Memory T cell responses

The presence of Tm prior to transplantation has been associated with an increased risk of acute rejection (98) therefore developing strategies to prevent the deleterious effects by Tm would be advantageous to the clinic. A number of alternative costimulatory molecules (such as 4-1BB, OX40, ICOS) are inducible in nature with delayed expression patterns compared to other costimulatory molecules such as CD28, which suggests these molecules may play a particular role in the effector/memory T cell phase rather than in the initial T cell activation. For example, signalling through 4-1BB has been shown to be critical for the survival and effector function of memory $CD8^+$ T cells (463).

$OX40L^{-/-}$ mice have a significantly smaller population of memory $CD4^+$ T cells compared to wild-type mice (357, 464). Furthermore, our data suggests that blockade of OX40-OX40L interactions can attenuate skin allograft rejection mediated by memory as well as naïve alloreactive $CD4^+$ and $CD8^+$ T cells. In keeping with our work, Vu *et al* demonstrated that skin allograft rejection mediated by Tm was resistant to CD28/CD154 blockade however blockade of CD28/CD154 in combination with anti-OX40 induced long term skin allograft survival. Interestingly, blockade of 4-1BB or

ICOS in combination with anti-CD28/CD154 completely failed to prevent skin allograft rejection (303).

Although Tm have been associated with preventing tolerance induction (101), some Tm are more malleable than others and can be tolerised in specific scenarios, for example, memory $CD8^+$ T cells specific for influenza virus can be tolerised by injection of soluble antigen (465). Although we and others have suggested that anti-OX40 can attenuate memory T cell responses to allografts the success of this strategy may depend on the type of Tm as it has been reported that there are differential effects of OX40 signalling in central and effector Tm. Central Tm did not require OX40 signalling for proliferation and cytokine production after *in vitro* recall responses, while effector Tm generated in the absence of OX40 signalling had dramatically reduced levels of IFN-γ, IL-4 and IL-13 after re-stimulation *in vitro* compared to wild-type effector Tm (464) thus suggesting these Tm subsets have different costimulation requirements. In our studies we did not distinguish between central and effector memory but this could be further investigated in future studies.

Memory T cells have been described as having a lower dependence on costimulation than naïve T cells which may make them less susceptible to therapeutic intervention. For example, naïve T cells stimulated with anti-CD3 alone proliferated slightly but failed to produce IL-2. In contrast Tm can proliferate and produce high levels of IL-2 in response to anti-CD3 only. When naïve T cells were stimulated with increasing numbers of APC, proliferation and IL-2 production was comparable to that of Tm

stimulated with anti-CD3 alone. The addition of accessory cells or APC has no impact on the proliferation of Tm, but did increase IL-2 production by two fold (454).

The requirement for costimulation by Tm may also depend on the type of environment within which Tm are re-activated, for example, in a model of nematode infection, blocking CD28 signalling during secondary infection failed to inhibit the response (466). In contrast, in a model of lung inflammation, in mice pre-sensitised to ovalbumin, blockade of OX40 at the time of antigen challenge prevented the accumulation of Tm in the lung and dLN (294). These data suggest that activation of Tm could be CD28 independent but may depend on alternative molecules such as OX40, rather than being entirely costimulation independent.

7.3.3 Th differentiation

In addition to providing survival signals, OX40 signalling has also been implicated in the differentiation of effector T cells, although no alterations in cytokine production were seen in our experiments following stimulation of $CD4^+$ or $CD8^+$ T cells with allogeneic splenocytes in the presence of anti-OX40. This may have been due to the dominant response to alloantigen being Th1, while in other scenarios Th2 responses are predominant. For example, Akiba *et al* provided the first *in vivo* evidence that OX40 was critical for Th2 responses. To this end, in a model of parasite infection (*L. major*) OX40 blockade inhibited IgG1 and IgE (Th2-associated) antibody production and inhibited Th2 associated cytokine production (IL-4 and IL-13) (376). Furthermore, using OX40L transgenic mice which elicited excessive interaction between OX40 and

OX40L in a strain of mice which are usually resistant to L. major resulted in enhanced Th2 responses (467). DC activated by TSLP mediated polarisation of T cells towards a Th2 lineage which may provide an initiation step in the development of allergic inflammation. Microarray analyses showed that OX40L is strongly induced by TSLP, and that blockade of OX40L prevented TSLP-activated DC polarisation of naïve T cells (468) and prevented TSLP-induced skin and lung inflammation by reducing cellular infiltrates (296).

Other T cell subsets, such as Th17 and Th9 have been recently described and have been implicated in a number of autoimmune diseases, such as rheumatoid arthritis (469), and allergic inflammation (89, 470) as well as allograft rejection (87, 302). For example, the ligation of OX40 inhibited conversion of naïve $CD4^+$ T cells into Th17 cells suggesting that blocking OX40 may increase the conversion of naive T cells to Th17 cells and intensify disease severity in models primarily driven by Th17 cells (89). Recently OX40 signalling has been shown to potently induce Th9 cells (89), that induce airway inflammation, demonstrating a role for Th9 cells *in vivo* (89). Further work is required to dissect the role of OX40 signalling on Th17 and Th9 cells and to determine if blockade of OX40 could modulate a Th17 or Th9 driven immune response.

7.3.4 Regulatory T cells

OX40 is a potent costimulator of effector and memory T cell responses but in addition we have shown OX40 signalling can negatively regulate Treg responses to alloantigen. We and others have shown that nTreg and iTreg constitutively express high levels of

OX40 but not OX40L (308). However, $OX40^{-/-}$ and wild-type mice have comparable numbers of Treg suggesting that OX40 is not required for nTreg genesis (308).

In our hands, we were unable to provide evidence that anti-OX40 aided the conversion of naïve $CD4^+$ T cells to Treg or altered the number of alloreactive nTreg in the Treg pool *in vivo*. Importantly, our analyses were 27 days post alloantigen stimulation therefore small changes in Treg expansion may have been missed. However, *in vitro* it has been shown that the activation of naïve T cells with anti-CD3 and syngeneic APC readily induces iTreg under polarising conditions (TGF-β and IL-2) *in vitro* and that this induction was inhibited in the presence of OX40-OX40L signalling (89). Activation of the AKT-mTOR axis has been shown to inhibit iTreg generation from naïve $CD4^+$ T cells (471) therefore as OX40 signals via this axis OX40 signalling may disturb the generation of iTreg (258).

Despite no increase in the precursor frequency being observed in our studies, OX40 blockade increased the potency of Treg 27 days after stimulation which was due to increased survival of Treg upon reactivation. This appears to be context dependent as Griseri *et al* demonstrated Treg stimulated with anti-CD3, APC and IL-2 in the absence of OX40 signalling decreased the total number of $FoxP3^+$ Treg and showed an increase in the proportion of Treg expressing annexin V (420). While Vu *et al* showed OX40 signalling had no impact on the survival or proliferation of Treg, but instead inhibited the expression of FoxP3 gene transcripts (308). These data suggest further studies are required to determine the molecular pathways involved in OX40 signalling which can

interact with pro- and anti-apoptotic molecules as well as other molecules associated with Treg function.

Others have shown an agonistic OX40 mAb abolished the suppressive capacity of Treg due to altered expression of FoxP3 gene expression (308), however we saw no alteration in FoxP3 levels. Epigenetic studies would be required to determine the effect of OX40 blockade on other markers associated with Treg and whether these changes were temporary or irreversible.

Interestingly, Malmstrom *et al* showed that DC can lose expression of OX40L after exposure to Treg in a model of IBD (284). This may provide one explanation of how Treg induced a suppressive effect as the deprivation of OX40L on the DC could reduce the ability of the DC to support the survival of antigen-activated T cells. However it remains unclear as to whether Treg lose their suppressive function when OX40 signalling is restored or whether this increase in potency is irreversible.

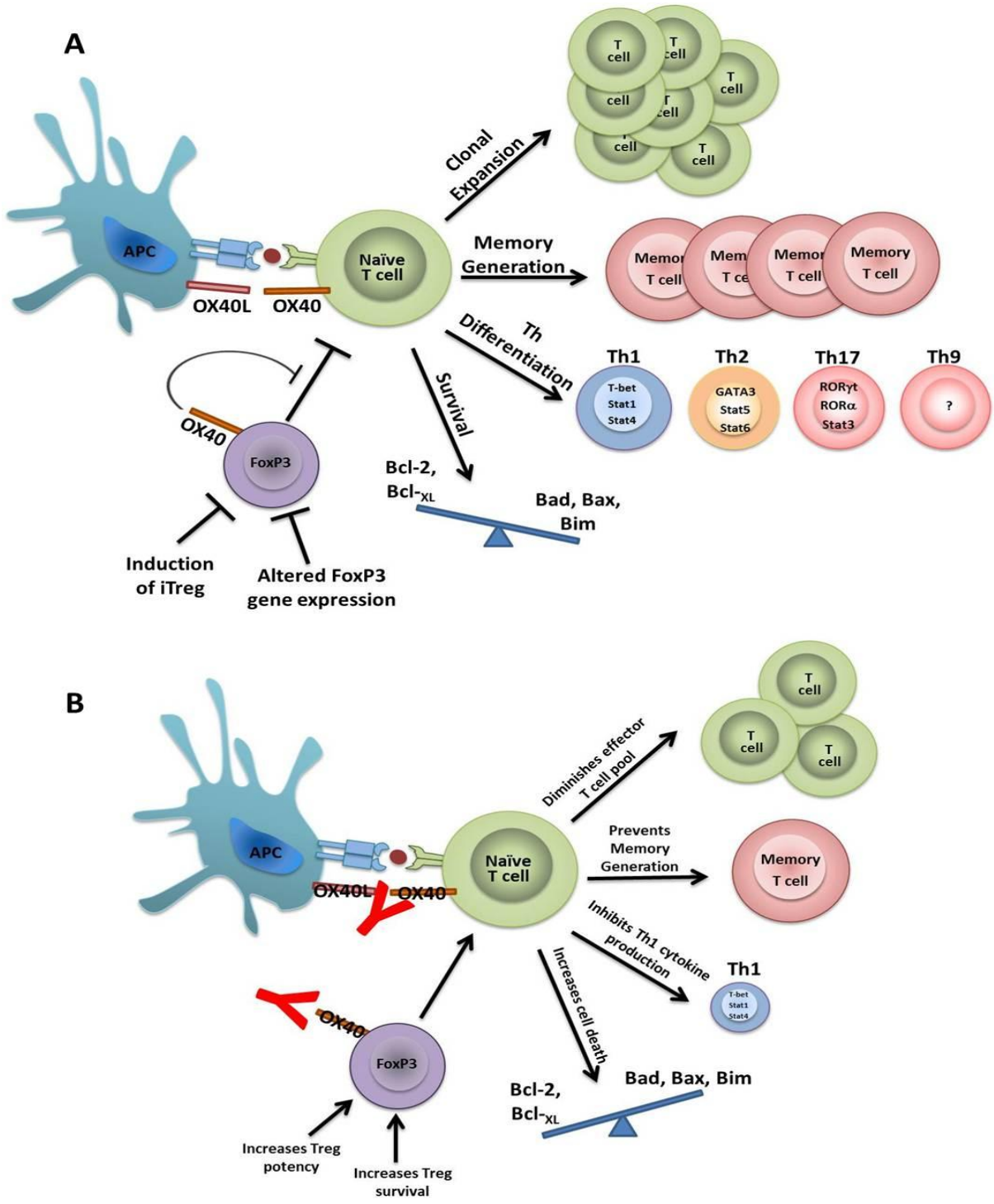


Figure 1: The role of OX40-OX40L in the development of an immune response against alloantigen. A) Summary of the role of OX40-OX40L signalling in the immune response B) Summary of our findings when anti-OX40 (depicted as red Y) is used to block immune responses

7.4 Impact of OX40-OX40L interactions on other cell types

OX40 has been described as a potent costimulatory molecule and is tightly associated with T cell activation. $OX40^+$ T cells have been observed in patients with a range of autoimmune and inflammatory diseases (285, 287) making the OX40-OX40L interaction an attractive target for the treatment of T cell mediated diseases (472). However, in addition to T cells and APC, other cells have been shown to express OX40 and OX40L respectively and therefore developing reagents to target OX40-OX40L could also impact these cell types especially if a depletion approach was used.

7.4.1 Neutrophils

Even in the absence of inflammation, human peripheral blood neutrophils have been shown to express OX40 (261). Ligation of OX40 on neutrophils by OX40L expressed by APC has been shown to result in the enhanced survival of the neutrophils which correlates with a reduction in caspase-3 activation and expression of the pro-apoptotic molecules Bax and Bid (261). It is therefore likely that therapeutic strategies involved in blocking or depleting $OX40^+$ cells would also impact neutrophils, which are an important first line of defence for the eradication and control of bacterial and fungal infection (although dispensable for anti-viral responses) (295).

7.4.2 Mast cells

Mast cells are critical to pathogen clearance, inflammation and allergy and express OX40L (270, 473). Interestingly, Nakae *et al* found that OX40L was constitutively expressed by mast cells isolated from BALB/c mice but not from C57BL/6 mice (271). Co-culture of T cells with OX40L expressing mast cells, previously stimulated with IgE and antigen, enhanced T cell activation (271). In addition, Treg suppress the degranulation of mast cells through OX40-OX40L. For example, in a model of acute anaphylaxis, it was shown that both Treg depleted and $OX40^{-/-}$ mice had increased levels of circulating histamine which suggests that Treg control allergic responses via OX40-OX40L (473). Activated mast cells secrete the cytokine TSLP, so in concert with OX40L expression, mast cells may be able to recruit effector/memory T cells to sites of inflammation and propagate productive immune responses.

7.4.3 Lymphoid Tissue Inducer Cells

LTi are $CD4^{+}CD3^{-}$ and function to organise the development of lymphoid tissues. Adult, but not embryonic, LTi have been shown to constitutively express OX40L and CD30 (another TNFR superfamily member) in both mouse and human (474, 475). $ROR\gamma t^{-/-}$ mice which lacked LTi failed to evoke memory antibody responses, although primary responses were normal, suggestive of a failure to generate productive Tm responses (476). Therefore these data suggest OX40L blockade inhibits Tm responses.

7.4.4 Platelets

Platelets are crucial in pro-inflammatory environments such as in atherosclerosis as they recruit circulating leukocyte such as T cells and DC to the lesion thereby promoting an immune response. Genetic associations between OX40L and the susceptibility to atherosclerosis have been observed (290) which has given rise to the idea that therapeutic targeting of OX40-OX40L may attenuate the development or progression of atherosclerosis.

Blocking OX40L in low density lipoprotein receptor$^{-/-}$ mice led to a 50% reduction in the formation of atherosclerotic lesions via suppression of Th2 mediated inflammation (477), thereby confirming that OX40-OX40L plays an important role in atherosclerosis. Importantly, OX40L was expressed on platelets of healthy controls as well as at elevated levels in patients with acute coronary syndrome (ACS) (313). The increased expression of OX40L on platelets from patients with ACS also showed a positive correlation to matrix metalloproteinase (MMP) -3 and -9 which play a role in plaque disruption resulting in ACS, while no correlation was observed on platelets from healthy controls (313).

These data highlight a significant concern for translating agonistic or antagonistic reagents targeting OX40 or OX40L towards the clinic as a Phase I-II trial evaluating anti-CD154 for the prevention of renal transplant rejection had to be terminated due to thromboembolic complications as a result of activated platelets expressing CD154 (249). Therefore great care must be taken in targeting OX40-OX40L to prevent the

development of adverse effects associated with expression of OX40L on platelets. In our studies, the reagent used to block OX40 was a Fab PEG and was therefore unable to costimulate thus the expression of OX40L on platelets was not determined as it would have had no impact in our studies.

No single pathway is known to regulate one cell type exclusively. This was highlighted by a Phase I clinical trial testing the efficacy of CD28 superagonist (TGN1412) in healthy volunteers (478). Pre-clinical data suggested TGN1412 preferentially targeted activated Treg and was proposed to restore tolerance. However, when administered to healthy volunteers it caused a massive cytokine storm which required intensive medical interventions (478). Why these adverse events were not observed in rodents and NHP models is still unknown. These data highlight the risk of administering reagents which modulate T cell responses and are not specific. Therefore targeting OX40-OX40L may impact only the antigen-specific T cells without disrupting other T cell repertoires which is critical in the establishment of tolerance. Therefore the efficacy of OX40 blockade may be dependent on several factors such as the number of pre-existing Tm which may impede tolerance induction. Thereby suggesting targeting OX40-OX40L may not only be a promising target for tolerance induction but in addition it may provide a selective approach to dampen alloimmune responses which would result in less global immunosuppression required and a reduction in unwanted side effects in the clinic.

7.5 Where does OX40 fit into current clinical application and are therapeutic strategies targeting OX40-OX40L worth pursuing?

Current immunosuppressive agents are non-specific and therefore there is an urgent unmet clinical need to develop strategies that have a higher specificity for disease-causing antigen specific T cells. Due to the lack of specificity, current regimens result in global immunosuppression which increases the risk of infections (31) and malignancies (32) following long-term use.

OX40 targeted immunotoxins may be successful in depleting pathogenic T cells resulting in amelioration of disease. For example, administration of an OX40 immunotoxin was found to significantly decrease the number of antigen-reactive T cells in the spinal cord, resulting in amelioration of EAE in mice (280). Using a depletion approach may also be advantageous as this would facilitate depletion of a pool of autoreactive T cells instead of simply blocking their function and would require administration only during clinical relapses. However OX40 is expressed on a number of other cells types (as discussed above), not only effector and memory Tm and the impact of depletion of these cells would need to be critically evaluated before such treatments were evaluated in the clinical setting. With this in mind, we would suggest that blockade of OX40-OX40L interactions seems to be a more likely avenue for therapeutic intervention in autoimmune or inflammatory diseases.

The question remains as to where costimulation blockade fits into current clinical practices and how do current immunosuppressive agents used to prevent allograft rejection disrupt this form of therapy? Certainly, in a mouse model, blockade of CD28 and/or CD154 induced tolerance to alloantigen but when this regimen was combined with cyclosporine, tolerance induction was prevented (221). However, in a NHP model where CTLA-4-Ig prolonged allograft survival but did not induce tolerance, cyclosporine had an additive effect on graft survival (479). These data suggest that the success of costimulation blockade combined with cyclosporine is dependent on whether the strategy is immunosuppressive or tolerogenic.

Rapamycin and anti-CD154 synergise to convert naïve $CD4^+$ T cells into apoptosis-resistant Treg which are capable of attenuating skin allograft rejection, whilst cyclosporine prevented the conversion of naïve $CD4^+$ T cells into Treg by anti-CD154 mAb (436). Therefore, calcineurin inhibitors may be detrimental to the development/maintenance of Treg by inhibiting IL-2 production which is required for their homeostasis and function (480). Further studies would be required to determine the outcome of anti-OX40 in combination with other immunosuppressive agents. However, if current regimens inhibit T cell activation (e.g. cyclosporine) then the upregulation of OX40 would be unlikely and therefore any therapeutic effect by anti-OX40 would be masked.

For use in transplantation, it is envisaged that OX40 blockade would be combined with other induction and maintenance agents. As OX40 appears to play a more important

role in the propagation rather than initiation of immune responses, blockade of OX40 in conjunction with targeting other costimulatory molecules such as CD28 and CD154, which are required in initial T cell priming, could prove beneficial. For example, a proportion of $CD8^+$ T cells have been found to be CD28/CD154 independent which suggests they depend on alternative costimulatory pathways. Therefore targeting OX40 may inhibit this small subset of T cells which has been previously shown to prevent tolerance induction (103, 343).

However, in contrast, we did not see any additive or synergistic effect on skin or cardiac allograft survival when OX40 blockade was combined with CTLA-4-Ig (to prevent CD28 signalling) or anti-CD154. This may be as a result of abatacept (CTLA-4-Ig) and anti-CD154 preventing T cell activation, which in turn prevented OX40 upregulation on the T cell surface thereby removing any therapeutic effect of anti-OX40. Extensive studies would be required to demonstrate any deleterious or synergistic role for OX40 blockade with current immunosuppressive agents.

The need to combine anti-OX40 with other immunosuppressive agents may limit its use in transplantation. However blockade of OX40-OX40L has proven successful in mouse models of autoimmune diseases (282, 287) and allergic inflammation (294, 295) thereby providing other disease areas which could benefit from blockade of OX40. In particular there is a growing body of evidence that OX40 signalling affects Th2 and more recently Th9 differentiation so targeting OX40 in allergic inflammation may be successful.

$OX40^{-/-}$ mice which had been sensitised and challenged with ovalbumin had a significant impairment in the ability to generate Th2 responses characterised by IL-4, IL-5, IL-9 and IgE production compared to wild-type mice (89, 292). This failure to generate Th2 responses led to a reduction in eosinophilia in the bronchoalveolar lavage (BAL), mucus production, goblet cell hyperplasia which are all features of lung inflammation and abolished airway hyperreactivity (292). The chronic house dust mite model is considered a gold standard model for allergic airway inflammation due to the cellular infiltration, which is characteristic of clinical asthma. Sensitisation and challenge (5 weeks later in the absence of adjuvant) with house dust mite provides a model with chronic pathology. Blockade of OX40 at the time of sensitisation with house dust mite significantly inhibited the infiltration of eosinophils, neutrophils and T cells in both the BAL and lung tissue which reduced lung inflammation (UCB personal communication). Importantly, OX40 blockade could also inhibit memory T cell re-activation in pre-sensitised mice which reduced eosinophilia, airway hyperreactivity and therefore lung inflammation (294), therefore providing evidence that blocking OX40 may be beneficial in the treatment of asthma.

Given the accumulating data in mouse models, a humanised blocking mAb targeting against OX40L has been developed by Genetech, and is currently undergoing investigation in a Phase II trial. This trial was powered to examine the efficacy of blocking OX40L in allergen induced airway obstruction in adults with mild asthma. The study was completed in January 2011, but results are still to be published (312).

In summary, our data has shown that OX40 blockade can significantly attenuate skin allograft rejection mediated by naïve and memory $CD4^+$ and $CD8^+$ T cells by inhibiting effector T cell survival. In addition anti-OX40 increased the potency of Treg by increasing their survival. These data are in agreement with the literature, but OX40 blockade was not able to impact allograft survival in immunocompetent mice. These data suggest that whilst OX40 blockade has therapeutic potential further studies are required to address whether anti-OX40 could be used in combination with other immuosuppressive agents to attenuate allograft rejection. OX40 blockade has also proven successful in animal models of autoimmune and inflammatory diseases which provides rationale for translating this research into NHP models and ultimately man.

7.6 Conclusions

It is evident from data published following experimental and clinical trials that more effective and specific immunosuppressants are needed to further reduce the significant morbidity amongst transplant recipients due to infections, malignancies and graft loss as a result of chronic rejection. The blockade of OX40-OX40L provides an attractive target due to the diametric role on effector/memory T cells and Treg. The immunobiology behind OX40-OX40L has been extensively studied in this thesis and has proven to be a successful therapeutic in limited scenarios where single T cell populations mediate allograft rejection. However, anti-OX40 failed to prevent allograft rejection in immunocompetent mice which warrants further investigation to fully assess whether anti-OX40 may be useful as part of a tolerance induction strategy.

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