CD4 T cell allorecognition pathways in acute and chronic allograft rejection

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This dissertation is submitted for the degree of Doctor of Philosophy

Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been submitted in whole or part for a degree at any university.

The length of this thesis does not exceed the 60,000 word limit

Summary

Solid organ transplantation is now an established and effective treatment option for end-stage organ failure. Whilst early outcomes have improved significantly over recent decades, longer-term outcomes have changed little. Despite advances in immunosuppression, most transplanted organs suffer an inevitable decline in function attributed to chronic rejection. It is evident that the alloimmune response remains incompletely characterised. Crucially, despite description several decades ago, the precise contribution that the direct (recognition of intact allogeneic MHC) and indirect (recognition of self-MHC restricted allopeptide) pathways make to allograft rejection remains incompletely understood. In this thesis, murine models of heterotopic cardiac transplantation have been utilised to analyse these pathways. The key findings of this work are as follows:

1) If able to evade NK cell killing, passenger donor CD4 T cells can make cognate, direct-pathway, interactions with recipient B cells. This interaction results in augmentation of all arms of the alloimmune response and acceleration of allograft rejection.

2) Direct-pathway CD4 T cell allorecognition is restricted to the immediate post transplantation period. Donor APCs are the major source of MHC class II for direct-pathway priming, and these are cleared rapidly by both innate and adaptive responses of the recipient, effectively limiting the longevity of direct allorecognition.

3) The duration of indirect-pathway responses against different alloantigens is variable, limited by availability of donor antigen. Expression of donor MHC class II is restricted to APCs and possibly endothelium (where expression is transient) limiting the duration of indirect-pathway allorecognition against MHC class II alloantigen. Indirect-pathway CD4 T cell responses targeted against parenchymal alloantigen are long-lived, and can provide help for generating alloantibody against different MHC alloantigens.

4) In response to continual presentation of target epitope indirect-pathway CD4 T cell responses against parenchymal expressed alloantigen are long-lived. The continual division of these cells results in greatly increased numbers of alloantigen-specific CD4 T cells in the chronic phase of the response, but despite this, memory responses are impaired.

5) Generating indirect-pathway regulatory T cells specific for parenchymal expressed alloantigen appears to be the most effective strategy to ameliorating chronic rejection.

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Abbreviations

2ME	2 - Mercaptoethanol
7-AAD	7 – Aminoactinomycin D
Ab	antibody
Ag	antigen
ADCC	antibody dependent cell-mediated cytotoxicity
ANA	anti-nuclear antibody
APC	antigen presenting cell
APC	allophycocyanin
ATRA	all-*trans* retinoic acid
AUC	area under the curve
AV	Allograft vasculopathy
BCR	B cell receptor
BM	bone marrow
BMDC	bone marrow derived dendritic cell
Breg	regulatory B cell
BSA	bovine serum albumin
CFSE	carboxyfluorescein succinimidyl ester
CMV	cytomegalovirus
DC	dendritic cell
DTH	delayed type hypersensitivity
DTR	diphtheria toxin receptor
DTx	diphtheria toxin
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
EVG	elastin van Gieson
Fc	constant-region fragment of immunoglobulin
FCS	foetal calf serum
FDC	follicular dendritic cell

FITC	flourexcein isothiocynanate
GC	germinal centre
GFP	green fluorescent protein
GM-CSF	granulocyte-monocyte colony stimulating factor
GVHD	graft-versus-host disease
HLA	human leukocyte antigen
IEL	internal elastic lamina
IFNγ	interferon gamma
Ig	immunoglobuloin
ip	intraperitoneal
iTreg	induced regulatory T cells
iv	intravenous
IVC	inferior vena cava
KIR	killer cell immunoglobulin like receptors
LLPC	long-lived plasma cell
mAb	monoclonal antibody
Mar	Marylin
MHC	major Histocompatibility Complex
MLR	mixed leukocyte reaction
Mreg	regulatory macrophage
MST	mean survival time
NK	natural killer
NKC	NK gene complex
nTreg	natural regulatory T cells
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phyocoerythrin
RA	rheumatoid arthritis
Rag	recombinase activating gene
rpm	revolutions per minute

RPMI	Roswell Park memorial institute medium
SD	standard deviation
SEM	standard error of the mean
SLO	secondary lymphoid organ
Tcm	central memory CD4 T cell
Tem	effector memory CD4 T cell
TCR	T cell receptor
TCR-/-	T cell receptor knock out
Tfr	CD4 T follicular helper regulatory cell
Tg	transgenic
TIM-1	T cell immunoglobulin and mucin-domain containing protein-1
TLR	toll-like receptor
Treg	regulatory T cell
TSDR	Treg specific demethylated region
WT	wild type

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1. The South African Surgical Society Annual Congress, Durban 2014
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Publications and Prizes

Publications

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Prizes and Awards

1. Charles Slater Fund Travel Award, University of Cambridge (2014 and 2015)
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3. Finalist for Medawar Medal, British Transplantation Society (2011, 2012 and 2014)
4. The Raymond and Beverly Sackler Scholarship (2012, 2013 and 2014)
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6. The School of Clinical Medicine 1st year PhD Research Prize (2012)
7. The Transplantation Society Mentee-Mentor Award with Mr Gavin Pettigrew (2012)
8. Biotest best basic science abstract award, European Society of Organ Transplantation (2011)

1 Introduction

1.1 Historical overview of transplantation

Transplantation is considered one of the greatest achievements in medicine over the past hundred years. The concept of replacing diseased or damaged tissues and organs can however, be traced back to the 3rd century. Several paintings depict sainted physicians Cosmos and Damien attempting to transplant an entire leg[1].

Figure 1.1 Master of Los Balbases, ca. 1495

The earliest recorded solid organ transplant took place in Vienna in 1902. Emerich Ullman performed a technically successful kidney transplant between two dogs[2]. This was followed in 1906 by the first two human kidney transplants – using pig and goat donor organs, performed by Jaboulay. Both patients died soon after transplantation with little, if any, graft function. Despite these, and other, early failed attempts the technical challenges of organ transplantation were being overcome, largely due to the pioneering suturing techniques developed by Alexis Carrel; the first surgeon awarded a Nobel prize[3].

In spite of these technical achievements it was widely recognised that 'biological factors' resulted in rapid failure of transplanted tissues leading some to suggest transplantation as not only futile but also that 'human experimentation was unwarranted and unethical'[1].

During World War II research into tissue compatibility became a priority to improve the treatment of aviator burn victims. To this end, Peter Medawar, a zoologist, was assigned the task of exploring skin allografts with plastic surgeon Thomas Gibson[4]. They observed that

allografts always failed, but importantly, that second grafts from the same donor failed more quickly; the so called 'second-set phenomenon'. Medawar and Gibson reported a detailed series of experiments using rabbit skin grafts verifying this observation, concluding that '*the mechanism by means of which foreign skin is eliminated belongs in broad outline to the category of actively acquired immune reactions*'. This observation was one of the first demonstrations of the immunological basis of rejection[4, 5]. Further advances in our understanding of the immunological basis of transplant rejection was provided by the work of Snell and colleagues, who proposed the importance of cell surface histocompatibility molecules to allograft rejection[6].

Following the war, Medawar directed his efforts towards conceiving strategies to modulate the recipient's immune response to the graft, famously coining the phrase 'acquired immunologic tolerance' to describe his vision[7]. He demonstrated in mice that intrauterine exposure to a donor strains cells resulted in a state of chimerism and permitted acceptance of subsequent donor strain skin grafts, but not third party grafts[7].

The next landmark was the first successful renal transplant performed by Merrill and Murray on December 23rd 1954 at Peter Bent Brigham Hospital in Boston, between identical twins[8]. Although performing the transplant between identical twins bypassed the immunological barrier, its publicised success renewed enthusiasm for transplantation around the world[1].

The next goal was to transplant across the genetic histocompatibility barrier. It was recognised that replicating Medawar's state of chimerism with neonatal treatment in humans was impossible. However, it was discovered that chimerism could be achieved in mice by irradiation and subsequent inoculation with bone marrow cells. Furthermore, this strategy permitted acceptance of skin allografts from the donor strain[9].

In 1958 Murrays team adopted this strategy in human renal transplantation, subjecting recipients to total body irradiation to 'weaken' the immune system with or without donor bone marrow treatment. Only one of the twelve patients subject to this treatment (in this case without bone marrow) survived beyond a month, but this patient had good graft function for over 20 years[10]. This was the first time the genetic barrier to transplantation had been breached. Similar results were subsequently replicated in France[11, 12]. These successes questioned the requirement for true immunological tolerance and instead, emphasis shifted towards strategies aimed at prolonging graft survival without necessarily achieving tolerance.

This encouraged a search for a drug-based alternative to irradiation for suppressing recipient immunity. The first such chemical to be used was the anti-proliferative 6-mercaptopurine (6-MP), a drug being evaluated for use in oncology. It had been observed that 6-MP significantly reduced antibody responses in rabbits[13]. Subsequent experiments in dog kidney transplant recipients by Roy Calne revealed 6-MP to be effective at delaying allograft rejection albeit in experiments with only 'several' dogs with only one surviving beyond 3 weeks[14]. Early results of human transplant recipients treated with 6-MP, or its derivative azathioprine, were poor, with few surviving three months[15, 16]. This all changed in 1963 with Starzl reporting >70% 1 year survival when the corticosteroid prednisolone was added to azathioprine, a strategy rapidly adopted by transplant centres throughout the world[17].

The next major breakthrough in immunosuppression came in 1979 with introduction of cyclosporine, a calcineurin inhibitor, which, when combined with prednisolone substantially improved outcomes of kidney transplant recipients[18, 19]. The success of cyclosporine provided the opportunity and impetus for the development of clinical programmes for transplantation of other organs: liver, heart and lungs initially. In 1989 a related compound tacrolimus (FK506) was introduced as an even more potent immunosuppressant, and remains the agent of choice today[20].

In addition to the advances in immunosuppression there have been significant improvements in organ preservation, understanding of histocompatibility and organ matching, surgical technique and post-operative patient management, all of which have contributed to solid organ transplantation becoming a successful and established therapeutic option for end-stage organ failure. In the UK during the financial year 2013-14 a total of 4648 solid organ transplants were performed. However, 7005 patients remain active on the waiting list and the success of transplantation is currently limited by the availability of donor organs.

1.2 Chronic rejection

The short-term patient and allograft outcomes currently achieved are very good, with UK 1-year graft survival for kidney and liver transplantation now 94% and 92.6%, respectively (www.odt.nhs.uk). Despite significant improvements in short-term outcomes, longer term outcomes following transplantation have improved very little over the past two decades. Most allografts suffer a gradual deterioration in function that is loosely referred to as chronic rejection and is characterised by a progressive allograft vasculopathy (AV) that leads to ischaemic fibrosis and eventual graft failure[21]. This process is considered the major factor limiting long-term allograft survival; within 5 years following transplantation, chronic rejection affects up to 80% of lung allografts, 20% of kidney and 40-50% of heart allografts[22]. It is notable that rates of chronic rejection differ between organs. Several possible explanations for this have been proposed. Organs differ in the amount of lymphoid tissue contained within them and it is conceivable that the load of donor lymphoid cells may influence the development of alloimmune responses in the recipient. Organs also differ in their susceptibility to duration of cold ischaemia – perhaps related to susceptibility to damage through ischaemia/reperfusion injury – for example the heart bowel are particularly sensitive with cold ischaemia times of only several hours tolerated whilst the kidney can withstand cold ischaemic periods in excess of 24 hours[23]. It has also been observed that the liver appears somewhat resistant to alloimmunity, and that a liver allograft is often immunoprotective for other allografts transplanted simultaneously, although the basis of this has not been fully elucidated[24].

Understanding the pathogenesis of chronic rejection, together with the basis of differences between the organs, and developing novel therapies for ameliorating the, currently inevitable, progressive decline in organ function is the goal of current transplant immunological research.

1.2.1 Allograft vasculopathy

Allograft vasculopathy is a vascular disease unique to transplantation that was first described in dog cardiac allografts in the 1960s[25]. It is a diffuse, circumferential lesion of arteries. The initiating lesion is endothelial injury and dysfunction which drives an exaggerated repair response characterised by fibroelastic intimal proliferation. This leads to accumulation of smooth muscle cells, macrophages, fibroblasts and extracellular matrix within the intima that does not breach the internal elastic lamina. These lesions result in luminal stenosis and

subsequent ischaemic damage to distal parenchyma, with cell death and replacement fibrosis resulting in graft dysfunction[26-29]. These lesions contrast with atherosclerosis which is characterised by eccentric calcified fibrous plaques[30].

Although thought of as primarily an immunological lesion driven by the alloimmune response, a number of non-alloimmune factors have also been demonstrated to be important in AV pathogenesis. Thus AV is considered a multifactorial process resulting from the cumulative damaging effects of alloimmune and non-specific insults on vascular endothelial cells[26, 27]. Traditional risk factors for endothelial injury such as hyperlipidaemia, hypertension, cigarette smoke and diabetes have all been shown to accelerate AV. Similarly, non-alloimmune transplant-associated factors have also been identified as risk factors for endothelial injury and subsequent development of AV, such as the catecholamine storm accompanying brain death, ischaemia-reperfusion injury and cytomegalovirus infection[26, 27, 31].

Clearly the adaptive alloimmune response plays a central role in the pathogenesis of AV, since the number and duration of episodes of acute rejection experienced, and the degree of donor-recipient mismatch have both been shown to be independent risk factors for the development of AV[32]. Furthermore, AV only affects donor vessels, leaving the recipients vasculature in-tact. Donor specific antibody (DSA), T cells and NK cells have all been implicated to have central roles in driving the alloimmune endothelial injury and dysfunction leading to the development of the fibroelastic vascular lesions[28, 29].

1.3 The alloimmune response

Knowledge that variability in allograft rejection was genetically controlled resulted in discovery of the Major Histocompatibility Complex (MHC) (Human Leukocyte Antigens (HLA) in humans, and H-2 in mice)[33, 34]. Donor MHC molecules are the principal target of the recipients alloimmune response against an allograft.

1.3.1 Major Histocompatibility Complex

The MHC is a $4x10^6$ base pair region of DNA on chromosome 6 in humans (17 in mice) which encodes for approximately 200 genes involved with the processing and presentation of peptide antigens on the cell surface. The MHC is pivotal to the generation of an antigen specific adaptive immune response against non-self. Cell surface MHC molecules loaded with antigenic peptides form the ligand of the T cell receptor (TCR), which is restricted to the recognition of self MHC molecules[35]. Encoded within the MHC are two subsets of HLA (H-2 in mice) molecules, both members of the immunoglobulin superfamily [36].

1.3.1.1 MHC Class I

Within the MHC class I locus are three classical molecules: HLA A, B, and C (H-2D, H-2K and H-2L in mice). These are heterodimeric glycoproteins, comprising a heavy α chain (with α1, α2 and α3 domains) non-covalently linked to a constant light chain: β_2-microglobulin encoded by a non-polymorphic gene separate from the MHC locus[37]. The molecules bind peptides 8-11 amino-acids in length derived from intracellular proteins generated by the proteasome, for presentation to cytotoxic CD8 T cells[38]. Class I molecules are expressed on all nucleated mammalian cells[39]. Exogenous proteins internalised via the lysosomal system have also been found to be presented by class I molecules, a process which has been termed 'cross-priming'[40].

1.3.1.2 MHC Class II

Within the MHC class II locus are three pairs of molecules: HLA-DR, DP and DQ (there are only two mouse equivalents – H-2 I-A and I-E). These are similarly non-covalently linked heterodimeric glycoproteins, comprising an α-chain (with α1 and α2 domains) and a β-chain (with β1 and β2 domains). The peptide binding groove of class II molecules is open-ended, accommodating typically longer peptides – 12-19 amino-acids in length. The peptides presented are exogenously derived via the endosome/lysosome system, and are presented to

helper CD4 T cells[41]. MHC class II molecules are only expressed constitutively on professional antigen presenting cells (APC), such as dendritic cells (DC), macrophages and B cells.

The HLA system is the most polymorphic gene system in the human genome, with each individual expressing only a small number of alleles. MHC molecules are co-dominantly expressed so that each individual expresses six class I molecules and six class II molecules (one haplotype inherited from each parent). Early studies in transplantation identified the need to match donor and recipients based upon these cell surface glycoproteins, recognising that differences between donor and recipient at these polymorphic loci was the predominant driving force behind the alloimmune response[33, 34, 42].

Although clear that matching of MHC molecules is important, several studies identified that other antigenic determinants were important in transplantation – the so-called minor histocompatibility antigens. These were discovered in humans after a female received bone marrow from her brother, and the grafted cells were recognised as non-self, despite being a complete HLA match[43]. Further work in mice has confirmed the existence of minor histocompatibility antigens, such as the H-Y male complex of genes, but has demonstrated that the response to peptides derived from minor histocompatibility antigens is significantly weaker than to peptides derived from MHC molecules, suggesting a dominant role of the MHC in allograft rejection[44, 45].

1.4 Pathways of Allorecognition

The alloimmune response to a transplant is fundamentally different from the adaptive response against conventional antigens, posing the recipients immune system with a unique challenge. Two populations of APCs are available for recognition: those of donor origin carried with the allograft; and those of recipient origin. Both populations can be recognised by recipient T cells. This results in two distinct pathways by which allorecognition can occur (Figure 1.2). In the direct-pathway, recipient CD4 T cells recognise intact allogeneic MHC-peptide complexes expressed on donor APCs. By contrast, in the indirect-pathway, recipient APCs internalise and process a variety of donor antigen, subsequently presenting them as self-MHC restricted peptides to recipient CD4 T cells[46]. More recently, a third pathway of allorecognition has been proposed, the 'semi-direct' pathway in which it is proposed that recipient APCs can acquire intact donor MHC from donor cells, but the physiological significance of this pathway has yet to be elucidated[47].

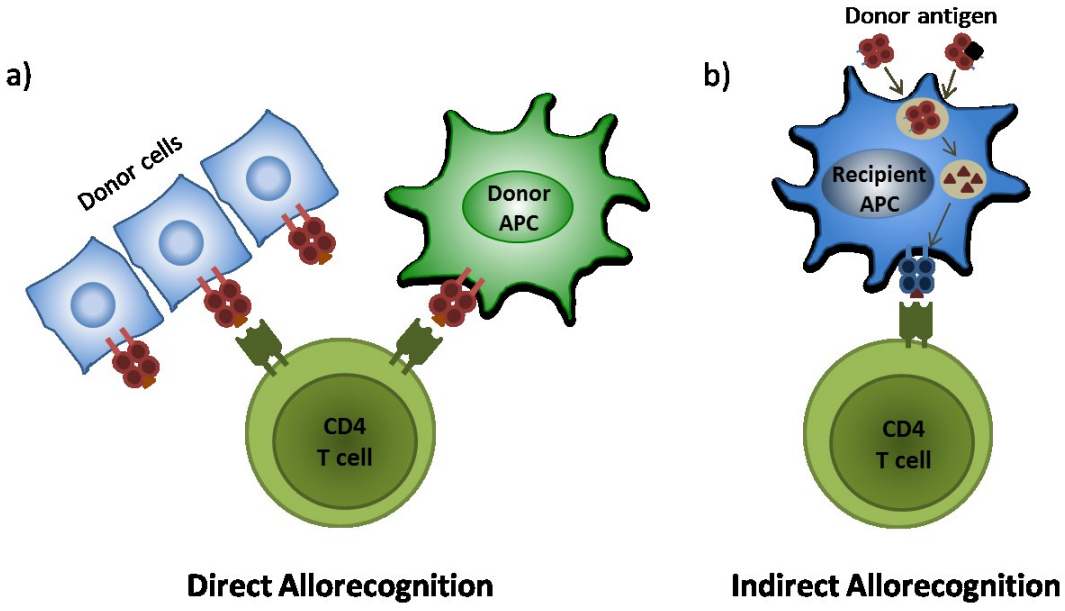


Figure 1.2 Direct and Indirect allorecognition
a) Direct allorecognition – recipient CD4 T cells experience intact MHC on donor antigen presenting cells (APCs) to recipient CD4 T cells. b) Indirect allorecognition – donor alloantigen is internalised, processed and presented as self-MHC restricted peptides by recipient APCs to recipient CD4 T cells.

Because donor APCs are thought to be rapidly eliminated after transplantation, direct-pathway responses are generally considered to be limited to the early period following transplantation. In contrast, the reliance on self-restricted recognition of recipient APCs theoretically enables indirect-pathway responses to continue indefinitely. It is thus stated, often axiomatically, that the direct-pathway is responsible for acute rejection, and the indirect for chronic rejection. However, this distinction has not been definitively confirmed[48].

1.5 Direct Allorecognition

Direct-pathway recognition of intact donor MHC presented by donor APCs was initially believed to be the predominant driving force behind allograft rejection due its observed strength. A very strong mixed lymphocyte reaction occurs when mixing donor and recipient cells, due to a high precursor frequency of recipient T cells able to recognise intact allogeneic MHC-peptide complexes – up to 1 in 10 (compared to as few as 1 in 10,000 recognising conventional antigens)[49-51]. Direct allorecognition creates a paradox when considering the stringent requirements for discriminating against non-self, imposed by self-MHC restriction[35] and there have been several theories proposed to explain the high precursor frequency of T cells with direct allospecificity. The idea of 'altered-self' was initially proposed as an explanation of how direct allorecognition occurs[52]. Consider a TCR which is specific for an antigenic peptide (X) in the context of self-MHC (A). The theory proposes that there is a high probability that there exists an allogeneic peptide (Y) which when coupled to an allogeneic MHC (B) could be recognised by that same TCR, which is supported by the observation in some studies that a significant proportion of direct-pathway T cells have a memory phenotype, implying previous priming against foreign antigen[53, 54].

Two subsequent models were proposed to explain the high precursor frequency (Figure 1.3). The 'high determinant density' model proposes that alloreactive T cells recognise amino acid polymorphisms exposed on the donor MHC molecules as antigenic, and that the peptide bound is of secondary relevance[55]. Each donor cell will express a multitude of allogenic MHC-peptide complexes, and the high density can overcome the relatively low avidity, allowing lower affinity TCR-MHC interactions to result in successful T cell activation. This has been supported by demonstrating that alloresponses can be inhibited by blocking TCR recognition sites on allo-MHC with targeted mutations suggesting they may be more important than the nature of the bound peptide[56-58]. On the other hand, the 'multiple binary complex' model suggests that the bound peptide is in fact pivotal[52]. It is suggested that allogeneic MHC is similar enough to self-MHC for direct-pathway T cells to recognise the peptide as foreign. Homologous proteins may then be recognised as foreign either because different derived peptides are presented by the allogeneic-MHC, or because identical peptides adopt a distinct conformation due to differences in the peptide binding groove[59-62]. Consequently, each alloreactive T cell clone may recognise only one, or a few, allogeneic-MHC peptide complexes, but each allogeneic-MHC will be recognised by a large number of clones, responsive to different bound peptides. This is supported by crystallographic studies which have

demonstrated that bound peptide participates in a significant number of interactions with the direct-pathway TCR[63-67].

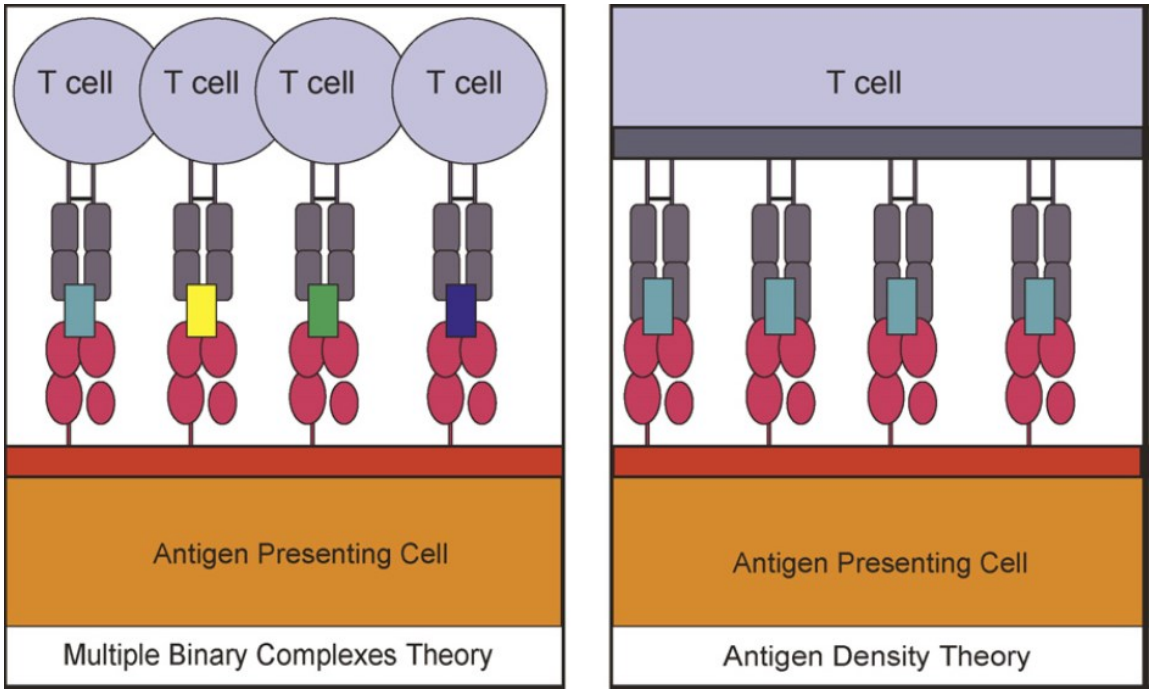


Figure 1.3 Theories of direct T cell alloreactivity
a) Peptide dependent model: T cells recognise variations in endogenous peptides presented b) Peptide independent model: T cells recognise polymorphisms of donor MHC molecules. (Reproduced from *Mol immunol, 2008; 45(3): 583-98*)

Further evidence supporting the importance of direct-pathway allorecognition to graft rejection was the observed immunogenicity of donor leukocytes carried with the allograft[42, 68]. To this end, Lechler and Batchelor demonstrated that indefinite survival of rat kidney allografts could be achieved by re-transplanting into a second host[69, 70]. Temporary 'parking' of the graft in a first immunosuppressed host led to the depletion of donor APCs before re-transplantation. This observation was reinforced when rapid rejection occurred after administration of donor APCs to the second recipient[71]. More recently Pietra *et al.* demonstrated that direct-pathway CD4 T cells were both necessary and sufficient to mediate allograft rejection in lymphocyte deficient *Rag*$^{-/-}$ animals which could reject MHC class I deficient, but not MHC class II deficient allografts when reconstituted with syngeneic CD4 T cells. Furthermore, allografts were rejected by MHC class II knock-out recipients, in which indirect allorecognition was not possible; thus highlighting the importance of donor APCs and the direct-pathway to rejection[72].

1.5.1 The contribution of direct allorecognition to rejection

These observations have resulted in many inferring that direct allorecognition is the predominantly pathway responsible for acute rejection[44, 50, 73, 74].

However, due to the likely rapid elimination of donor APCs by natural killer (NK) cells, of the innate immune system, within hours to days of transplantation, the precise contribution of direct allorecognition to rejection remains uncertain[75, 76]. In keeping with this, clinical studies have identified a decline in the frequency of T cells with direct-pathway anti-donor specificity with time after renal and cardiac transplantation[77, 78]. In these studies direct and indirect-pathway CD4 T cells are identified *in vitro,* examining peripheral blood CD4 T cell response (proliferation and cytokine production) following exposure to intact irradiated donor PBMCs or donor cytoplasmic membrane protein pulsed recipient PBMC's respectively. Although this would suggest direct-pathway responses are restricted to early following transplantation, there is evidence that direct-pathway CD4 T cells can cause pathological findings characteristic of chronic rejection in models in which a graft is not rejected acutely[79-81]. Although donor APCs have been eliminated, this does not preclude the priming of direct-pathway CD4 T cells by other donor cells within the graft[82]. The ability of donor endothelium, for example, to express MHC class II and provide adequate co-stimulation for naïve or previously primed direct-pathay CD4 T cells has not been definitively excluded[83-86]. Additionally, the recently described 'semi-direct' pathway, could also provide a route through which direct-pathway CD4 T cells could theoretically continue to be activated indefinitely[47]. Thus, the assertion that the direct-pathway is short lived and only capable of contributing to acute rejection is perhaps unfounded.

1.5.2 Direct-pathway effector functions

Once activated, direct-pathway CD4 T cells have been shown to reject an allograft both independently and in the presence of other adaptive immune cells[72, 81, 87]. These helper CD4 T cells may contribute to rejection by several mechanisms: stimulation of a delayed type hypersensitivity reaction; provision of help to cytotoxic CD8 T cells; provision of help to B cells for alloantibody production; and via a direct cytotoxic effector function.

1.5.2.1 Delayed type hypersensitivity reaction

Donor APCs prime alloreactive T cells to produce a pro-inflammatory, type-1 cytokine pattern of activation[88]. These 'Th1' CD4 T cells, by secreting cytokines, activate other innate immune cells such as macrophages which can lead to a delayed-type hypersensitivity (DTH) response. This damages the allograft by release of pro-inflammatory soluble mediators of cytotoxicity such as reactive oxygen species and tumour necrosis factor-α. The DTH response is not believed to be an acute effector mechanism and so may only contribute to chronic rejection[89-91].

1.5.2.2 Help for cytotoxic CD8 T cells

CD8 T cells conventionally receive non-cognate, linked help from CD4 T cells which simultaneously bind to an APC presenting the epitopes for both CD4 and CD8 T cell recognition, forming a three-cell cluster (Figure 1.4). It is proposed that CD4 T cell help 'licenses' the APC to make more effective MHC class I-restricted CD8 T cell presentations[92-94]. Taylor *et al.* have demonstrated *in vivo* that direct-pathway CD4 T cells can provide effective non-cognate, linked-help for direct-pathway cytotoxic CD8 T cells[87]. (In order to exert cytotoxicity to the graft, the CD8 T cell must be able to recognise allogenic MHC Class I – i.e. direct-pathway CD8 recognition). They propose a three-cell cluster requiring a *donor* APC, a direct-pathway CD4 T cell and a direct-pathway CD8 T cell. Once activated, the direct-pathway CD8 T cells can effect graft rejection upon recognition of target MHC class I alloantigen on endothelial cells or graft parenchymal cells, by either inducing apoptosis through cross linking Fas, or by releasing perforin that creates pores in the cell membrane, allowing granzymes to enter[95]. However, because donor APCs are thought to be short-lived, it follows that this linked help to CD8 T cells is restricted to early following transplantation. Nevertheless, it has been shown that direct CD8 cytotoxicity can be primed even when CD4 T cell recognition is restricted to the indirect-pathway, suggesting that the provision of CD4 T cell help for generating CD8 T cell alloimmunity is more complex than for CD8 T cell responses against conventional antigen[96].

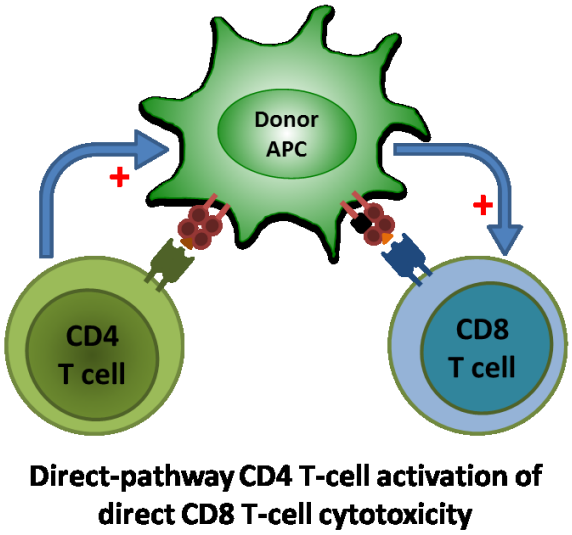


Figure 1.4 Direct-pathway CD4 T-cell activation of direct-pathway CD8 T-cell cytotoxicity
Intact donor MHC class II expressed by donor APCs is recognised by direct-pathway CD4 T cells. This licences the APC to prime direct-pathway CD8 T cells recognising intact donor MHC class I also expressed by the donor APC

1.5.2.3 Help for B cell production of alloantibody

B cell activation and generation of antibody responses against conventional antigen requires CD4 T cell help to be delivered in a cognate manner to the B cell directly, requiring the B cell to process and present its antigen in the context of MHC class II on its surface[97]. It would be predicted, therefore, that as a recipient B cell will present peptide in a self-MHC restricted fashion, only *indirect*-pathway CD4 T cells would be able to provide cognate help for an alloantibody response. Nevertheless, the ability of direct-pathway CD4 T cells to provide help for alloantibody generation has been long debated.

In 1996, two conflicting studies were published regarding the provision of help by direct-pathway CD4 T cells for alloantibody production. Kelly *et al.* demonstrated that inhibition of direct allorecognition, by blocking MHC class II alloantigen, abrogated alloantibody production, and proposed that a similar three-cell cluster for non-cognate, linked-help by direct-pathway CD4 T cells for B cells may exist as for CD8 T cells[98]. In contrast, Steele *et al.* demonstrated that MHC class II knock-out recipients, which restricts CD4 T-cell allorecognition to the direct-pathway, did not develop a class-switched alloantibody response suggesting a necessary role for recipient antigen processing[99]. These studies have been reconciled by a more recent publication. The ability of direct-pathway CD4 T-cells to provide help to B-cells for alloantibody

production has been put into doubt by work using elegant transgenic models which overcome previous criticisms including the use of non-vascularised allografts and abnormal expression of donor and recipient MHC class II. In vascularised graft models in which only T-cells with direct-pathway alloreactivity are present (with normal recipient MHC class II expression), it has been demonstrated that direct-pathway CD4 T-cells are unable to provide help for class-switched alloantibody responses, even when memory responses have developed, or the longevity of direct responses is prolonged by depletion of recipient NK cells[100]. Thus it appears unlikely that direct-pathway CD4 T cells contribute to alloantibody generation.

1.5.2.4 Direct, direct-pathway CD4 T cell cytotoxicity

The finding that a CD4 T cell reconstituted *Rag*$^{-/-}$ recipient can reject a cardiac allograft acutely implies that CD4 T cells can exert direct cytotoxicity against an allograft *in vivo*[72]. In support, three separate publications have demonstrated that monoclonal populations of direct-pathway CD4 T cells can independently, acutely reject skin grafts[81, 101, 102]. The possibility that this is due to an acute DTH response remains but seems unlikely considering the time course of the observed rejection. The precise effector mechanism employed by these 'cytotoxic' CD4 T cells is still debated. Some reports suggest cytotoxicity is dependent on FAS ligand expression[103-106], others suggest it is dependent upon perforin expression[106-108], whilst another suggests it is dependent on neither[72, 109]. Regarding vascularised cardiac allograft rejection, the role of direct-pathway CD4 T cells remains unclear. Evidence from Gill suggests that CD4 T cells can acutely reject a cardiac allograft acting alone, and that this is abrogated by using MHC class II deplete donors, implying an essential role for direct-pathway CD4 T cells[72, 110]. In contrast, Sayegh has demonstrated the ability of a transgenic population of direct-pathway CD4 T cells to reject skin but not cardiac allografts in the absence of other lymphocyte populations[102]. In this study though, a monoclonal response was being observed which may not be representative of the polyclonal response typical of direct-pathway allorecognition. These data suggest that direct CD4 cytotoxicity may not play a significant role in vascularised allograft rejection.

1.6 Indirect allorecognition

The observation that donor-leukocyte depleted allografts could be rejected with a delayed time course led to the hypothesis that, similar to conventional antigen recognition, alloantigen could be internalised, processed and presented by recipient APCs in a self-MHC restricted manner[70, 71, 111, 112]. There are several pathways by which donor antigen can be acquired: i) recipient APCs trafficking to the graft could internalise donor antigen, migrate to secondary lymphoid organs (SLO's) and activate recipient CD4 T cells[83]; ii) soluble antigen shed from the graft could be carried to the SLO's in the lymphatic or blood circulation[113]; or iii) passenger donor leukocytes trafficking to the SLO's could be endocytosed and processed by resident APCs[71, 114].

Evidence has accumulated supporting an important role for the indirect-pathway in allograft rejection. Bradley *et al.* demonstrated that in an MHC class I mismatched rat cardiac allograft model, rejection was dependent on recipient CD4 T cells that could only recognise alloantigen in a self-MHC restricted manner[115]. Fabre's group subsequently demonstrated prolonged survival of rat kidney allografts after interfering with indirect presentation by administering a monoclonal antibody against MHC class II, opsonising recipient APCs[116]. They also demonstrated accelerated graft rejection if they immunised with peptides derived from an allogeneic MHC class I hypervariable region prior to transplantation (only able to prime indirect-pathway CD4 T cells)[117]. This was similarly observed by Sayegh *et al.* who also demonstrated that donor-specific hypo-responsiveness could be induced by intra-thymic injection of allogeneic MHC-derived peptides[118, 119].

With the advent of transgenic and gene 'knock-out' technology came more definitive evidence of the importance of the indirect-pathway. Auchincloss used MHC class II knockout mice as donors, precluding the ability of donor APCs priming CD4 T cells directly[120]. Skin grafts from these donors into MHC class I disparate recipients were rejected rapidly in wild-type recipients, confirming that indirect-pathway CD4 T cells alone could drive rejection[121]. Further evidence has stemmed from TCR-transgenic mice, in which exclusive self-MHC restricted alloantigen recognition is guaranteed. Several TCR-transgenic models exist, whereby monoclonal populations of T cells express a TCR recognising peptides derived from minor antigen[122-124], MHC class I[125, 126] and MHC class II[127] presented indirectly. In all of these models, indirect-pathway CD4 T cells have been demonstrated to effect allograft rejection. Unlike the poly-specific direct-pathway response, this work suggests that indirect alloresponses can be

mediated by a limited number of T cell clones directed to a single or a few dominant determinants within the polymorphic regions on alloantigen[128-131]. It has been demonstrated that over the course of an alloresponse, new allogeneic epitopes may become dominant determinants, termed epitope spreading, which may contribute to the establishment of chronic rejection[132-135].

1.6.1 `The contribution of indirect allorecognition to rejection

The importance of the indirect-pathway to allograft rejection has become increasingly appreciated[75, 96, 135-140]. The hypothesis that it contributes only to chronic rejection stemmed from the observation that depleting donor APCs delayed acute rejection but did not prevent the development of chronic rejection[141]. The number of indirect-pathway CD4 T cells appears to increase with time after transplantation, likely due to the continuous presence of alloantigen for self-MHC restricted presentation[142]. There is also strong association between the presence of indirect-pathway T cells at late time points after human transplantation, and development of chronic rejection; further reinforcing the association between indirect-pathway allorecognition and chronic rejection[130, 143-147]. For example, a study by Baker *et al.* observed high frequencies of CD4 T cells responding to the indirect-pathway late after living donor renal transplantation, with significantly higher responsiveness in those with evidence of chronic allograft nephropathy, whilst at the same time observing direct-pathway hypo-responsiveness, implicating indirect-pathway responses in chronic rejection[148]. Further evidence of the contribution made by the indirect-pathway to human chronic rejection is provided by the correlation between donor specific antibodies and chronic rejection, since as discussed in Section 1.5.2, alloreactive B cells can only receive help from indirect-pathway CD4 T cells. Chronic antibody mediated rejection is a well-defined pathological entity, and is a marker of indirect-pathway alloreactivity[149-151]. Pathogenicity of alloantibodies is directly evidenced by deposition of the complement product C4d on allograft endothelium.

However, in addition to a role in chronic rejection, there is growing evidence that indirect-pathway allorecognition could be important early after transplantation[81]; for example, Auchincloss' demonstration of acute skin allograft rejection in the absence of direct -pathway allorecognition[121]. More recently, Brennan *et al.* described an elegant TCR-transgenic model in which directly and indirect-pathway CD4 T cells could be simultaneously tracked. Surprisingly, this work suggested that priming of the indirect-pathway is favoured, even early after transplantation[142]. The indirect-pathway cells also predominated within the effector T cell

population and perhaps more importantly, within the population with 'memory-like' phenotype, supporting a role for this pathway in acute rejection.

That depletion of passenger APCs prolongs allograft survival is often quoted in support of a primary role for direct allorecognition in acute rejection[69]. Gould and Auchincloss though, suggest alternative interpretations, in keeping with a concurrent importance to indirect-pathway allorecognition: i) donor APCs could function predominantly as vehicles to transport alloantigen to SLO's for indirect-pathway processing, rather than as antigen for direct-pathway allorecognition and/or ii) depletion of donor APCs, with their high expression levels of MHC class II, could reduce the availability of antigenic determinants for indirect-pathway processing[44].

1.6.2 Indirect-pathway effector functions

There is now convincing evidence that the indirect-pathway alone can reject both skin[96, 121-124, 152] and vascularised allografts[125, 126, 153]. Similar to the direct-pathway, indirect-pathway primed CD4 T cells have been demonstrated to preferentially differentiate into a pro-inflammatory type-1 cytokine-secreting phenotype and therefore can provide help to B cells and CD8 T cells[154].

1.6.2.1 Direct indirect-pathway CD4 T cell cytotoxicity

Despite not being able to directly interact with graft cells, there is increasing evidence that graft damage can be caused by indirect-pathway CD4 T cells[73, 125, 155].

One possible mechanism is the development of chronic rejection driven by indirect CD4 T cells initiating a DTH reaction within the graft – although this is not believed to be effective at rejecting vascularised allografts[156] . Several transgenic murine models have been reported in which monoclonal CD4 T cell populations restricted to the indirect-pathway have acutely rejected skin grafts, and reject cardiac allografts with a prolonged time course[157-159]. As a result, alternative hypotheses for how indirect-pathway CD4 T cells can independently effect graft rejection have been proposed. Skin grafts are known to be vascularised by recipient endothelial cells, and these are capable of presenting self-MHC restricted graft antigens that can act as ligands for indirect-pathway primed effector cells[160]. It has also been speculated that the process of recipient endothelialisation can occur in vascularised allografts although the extent and significance are unclear[73, 161]. A further mechanism postulates that graft damage

can be effected by indirectly primed T cells that have translocated into a graft and recognise their ligands on graft infiltrating recipient APCs, leading to bystander killing of graft cells in a manner analogous to DTH[83, 162-164] (Figure 1.5).

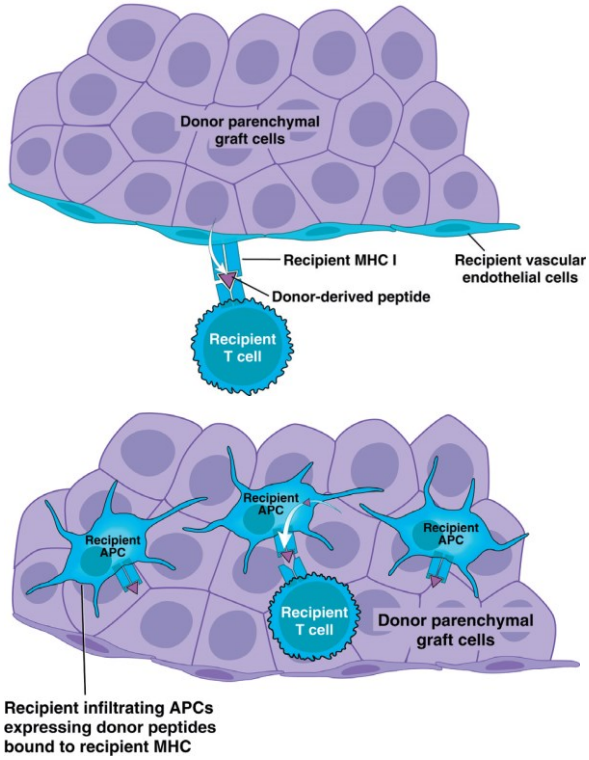


Figure 1.5 Postulated mechanisms by which graft damage can be effected by primed indirect-pathway CD4 T-cells

a) Recipient endothelialisation of allografts, with subsequent processing of donor antigen and presentation in the context of recipient MHC, and b) graft infiltrating recipient APCs processing and presenting donor antigens, both provide a potential target to indirect-pathway CD4 T cells. (Reproduced from *Am J Transplantation 2003; 3:525-533*)

1.6.2.2 Help for B cell production of alloantibody

In the case of T-dependent antigens, B cells, in most circumstances, require a cognate interaction with antigen specific CD4 T-cells in order to differentiate into antibody-secreting plasma cells (Figure 1.6)[97]. Because indirect-pathway CD4 T-cells are self-MHC restricted, unlike direct-pathway CD4 T-cells, they alone possess the ability to provide cognate help to B cells for alloantibody production[87, 99, 100, 140, 165]. B cells recognise intact alloantigen which is subsequently internalised, processed and presented as self-MHC restricted peptide to indirect-pathway CD4 T cells in order to receive the necessary help to drive humoural alloimmunity. Pettigrew *et al.* demonstrated that although immunisation with allogeneic MHC derived

peptides could prime an indirect CD4 T-cell response, a destructive humoral response could only be elicited with the transfer of additional intact allogeneic MHC molecules, suggesting the requirement for B cell ligation of intact antigen in order to receive help from indirect-pathway CD4 T cells[140].

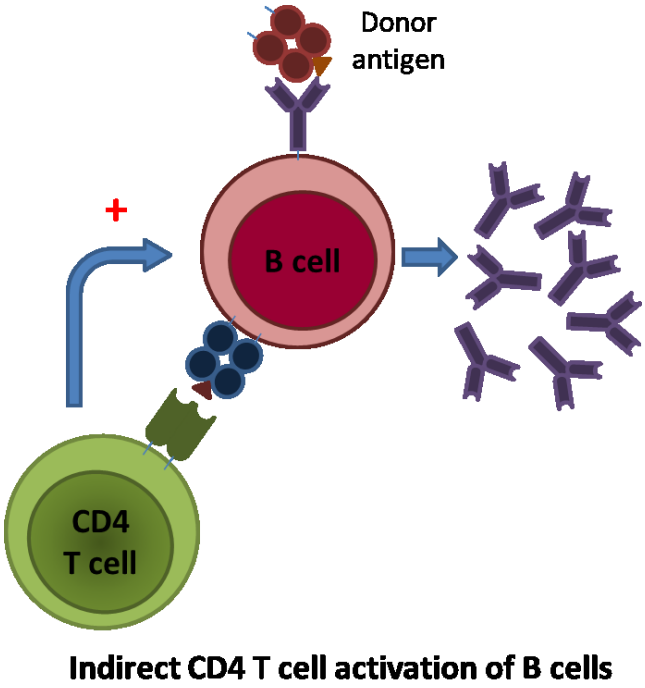


Figure 1.6 Indirect-pathway CD4 T-cell activation of humoral alloimmunity.
A B cell specific for alloantigen binds to, and internalises its target alloantigen, which is processed and presented in a self-MHC restricted manner to indirect-pathway CD4 T cells, which in turn provide help to the B cell for continued maturation of the alloantibody response.

1.6.2.3 Help for CD8 T cell cytotoxicity

Indirect-pathway CD8 T cells recognising allogeneic peptides restricted to self MHC can theoretically be activated by indirect CD4 T cells by a classical 3-cell cluster with a recipient APC[160]. Benichou confirmed the ability of self-MHC restricted indirect CD8 T cells to effect acute rejection of a single MHC class I mismatched skin graft after immunisation with peptides derived from the allogeneic MHC class I[166]. This was confirmed by Heeger who demonstrated that a transgenic population of CD8 T cells recognising allogeneic antigen by the indirect-pathway could contribute to the rejection of skin grafts, but only when recipient endothelial cells could present the antigen[160, 167]. This suggests that, certainly for skin grafts, indirect-pathway CD8 T cells may play a role in rejection due to graft revascularisation. However, a

question still remains as to how relevant this population of CD8 T cells is in clinical transplantation of vascularised organs due to the uncertainty surrounding whether the ligand for these cells is present in these grafts. Heeger *et al* demonstrated in one model of cardiac transplantation, that indirect-pathway CD8 T cells were merely non-pathogenic bystanders due to the absence of a cognate antigen on the allograft (due to endothelium being predominantly of donor origin)[168]. Even priming these CD8 T cells had no impact on the kinetics of rejection.

Direct-pathway alloreactive CD8 T cells, by contrast, are able to bind to graft cells and effect graft rejection. The ability of indirect-pathway CD4 T cells to prime these cells was first suggested by Lee *et al.* who demonstrated that murine skin grafts could be rejected by CD8 T cell cytotoxicity in a model in which CD4 T cell recognition was restricted to the indirect-pathway[96, 121].They demonstrated that direct-pathway CD8 T cells were recognising intact donor MHC class I on donor cells, and that the helper CD4 T cells were recognising self-restricted alloantigen on recipient APCs by the indirect-pathway proposing a 4-cell cluster model with unlinked CD4 T cell help for the direct-pathway CD8 T cells. This model has faced criticism as, devoid of physical linkage, it seems to be inherently unstable and potentially uncontrolled, raising the potential for bystander CD8 T cell activation by self-MHC restricted CD4 T cells presented with unrelated peptides (Figure 1.7).

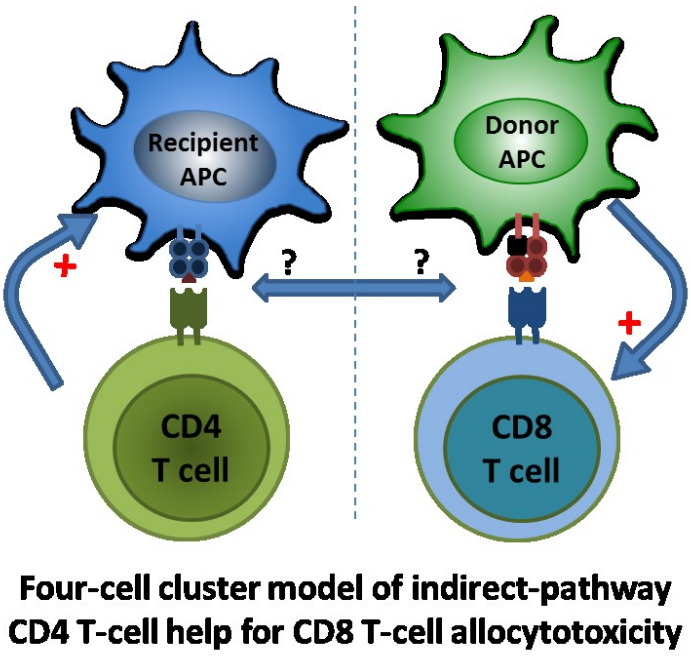


Figure 1.7 Four-cell cluster model of indirect-pathway CD4 T-cell help for CD8 T cell allo-cytotoxicity
Indirect-pathway CD4 T cell recognising allopeptide presented by a recipient APC provide 'unlinked' help to a direct CD8 T cell that recognises intact alloantigen on the surface of a donor APC.

One reconciliatory theory postulates that physical linkage of the 4 cells could be achieved if the recipient APC was a B cell. The B cell receptor could recognise its epitope on the donor APC, simultaneously presenting processed antigen in a self-MHC class II restricted fashion to an indirect-pathway CD4 T cell. The donor APC could also provide the epitope for a direct-pathway CD8 T cell, thus bringing the two T cells in close proximity, allowing for more controlled unlinked help to be provided, thus preventing non-specific CD8 T cell activation[96].

This debate has led to the proposition of an alternative explanation - a third pathway of allorecognition, termed the 'semi-direct' pathway, which may reconcile the above findings[47].

1.7 The 'semi-direct' pathway of allorecognition?

The observation that DCs are able to acquire intact MHC molecules from other cells *in vitro* and *in vivo,* 'cross-dressing', has led to the suggestion that allogeneic MHC molecules acquired by recipient APCs could permit on-going presentation of antigen to direct-pathway T cells – a process now described as the semi-direct-pathway[47, 169-171]. This model would then permit the recipient APC to simultaneously present processed alloantigen in the context of self MHC class II to an indirect-pathway CD4 T cell, and intact allogeneic MHC class I to a direct-pathway CD8 T cell, thus reconciling the requirement for linked-help in a three-cell cluster (Figure 1.8)[172].

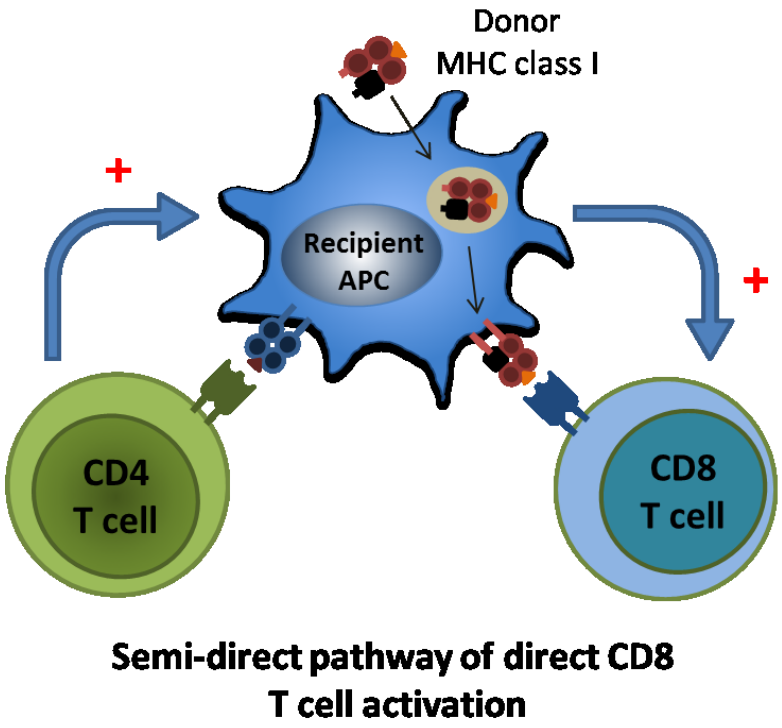


Figure 1.8 Semi-direct-pathway of direct-pathway CD8 T cell activation
Recipient APCs acquire intact donor MHC class I and present this intact on their surface, co-localising intact donor MHC class I and recipient MHC class II to a single APC, enabling the provision of linked-help from indirect-pathway CD4 T cells for generating cytotoxic CD8 T cell alloimmunity.

Lechler's group have provided convincing evidence for the intercellular transfer of MHC molecules, demonstrating that transfer can occur both by cell-to-cell contact through capture of membrane fragments - trogocytosis, and through exosome transfer[173]. They have additionally demonstrated the ability of these acquired MHC molecules to activate T cells, confirming the functional ability of the acquired molecules. Although most evidence for this

process is based on *in vitro* studies, there is increasing *in vivo* evidence for the existence of MHC transfer. Lechler's group has shown acquisition of MHC upon adoptive transfer of DCs lacking both MHC class I and II molecules[174]. This was followed by Brown *et al.* demonstrating APCs expressing both donor and recipient MHC class II molecules after cardiac and kidney transplantation in their murine model[175]. Previous work from our laboratory has also provided evidence supporting the potential for the semi-direct-pathway to have clinical relevance[176]. Following *in vitro* co-culture of BALB/c and C57BL/6 DCs, some C57BL/6 DCs were demonstrated to present acquired BALB/c MHC class I H-2D^{d} both as processed allopeptide and intact unprocessed antigen. Transfer of these DCs into recipients of BALB/c cardiac allografts provoked direct-pathway CD8 T cell cytotoxicity that was dependent on indirect-pathway recipient CD4 T cells.

The relevance of this trogocytosis of alloantigen is, however, still unclear, because to be functionally important presentation of intact donor alloantigen by recipient APCs must be more effective for triggering cytotoxic alloimmunity than conventional encounter on donor APCs, and this has not been shown. Thus, despite increasing evidence supporting MHC transfer *in vivo*, the role of the semi-direct-pathway in the alloimmune response remains uncertain[165, 177, 178].

1.8 NK cell allorecognition

Natural killer (NK) cells are effector cells of the innate immune system and have the ability to kill virus-infected or tumour cells without prior antigen priming, secrete cytokines, and regulate both innate and adaptive immune responses[179]. Despite not expressing clonal antigen receptors, NK cells are able to discriminate between cells of self and non-self. NK cells express a range of germ-line encoded inhibitory and stimulatory receptors found to be encoded within the NK gene complex (NKC)[179, 180]. Inhibitory killer cell immunoglobulin-like receptors (KIR) bind to classical MHC class I molecules, preventing the lysis of autologous cells in humans, leading to the concept of 'missing-self' activating NK cells[181, 182]. (The Ly49 receptor family are functional homologues in mice[183]). Additionally, NK cells also express a series of stimulatory receptors such as the evolutionary conserved CD94, NKG2 and NKR-P1 groups that bind to molecules associated with infection or cellular distress. Activation of NK cells has been demonstrated to require more than just absence of self MHC class I, but instead the balance of signals through inhibitory and stimulatory receptors regulate NK cell activation[184].

1.8.1 The role of NK cells in allograft rejection

Early studies suggested that NK cells may not have a role in solid organ rejection since adaptive immune deficient SCID and *Rag*$^{-/-}$ mice were unable to reject allografts[185]. However, it is simplistic to argue that this is evidence for absence of NK alloreactivity, since it is recognised that NK cell effects can require adaptive immunity, for example antibody production to drive antibody-dependent cell-mediated cytotoxicity. In keeping with this, more recently it was observed that if NK cells in *Rag*$^{-/-}$ mice were activated by administration of IL-15 they were able to acutely reject skin allografts, highlighting that the role of NK cells in transplantation is more complex than initially recognised[186].

1.8.1.1 NK cells as effectors in allograft rejection

A variety of mechanisms are thought to contribute to NK cell activation – all of which are present following transplantation: i) detection of missing, or altered-self MHC molecules; ii)

engagement of the Fc portion of IgG antibodies as opsonins; iii) detection of altered molecules on stressed cells; or iv) an inflammatory environment with cytokines such as IL-12, IL-15 and IFNγ[187, 188]. NK cells have been demonstrated to be potent effector cells that can mediate rejection of both bone-marrow and xenogeneic solid organ allografts[189-191]. Furthermore, NK cell infiltration into allogeneic allografts is frequently observed in models of both acute and chronic rejection, suggesting that NK cells have a role in mediating allograft injury[192, 193]. For example, advanced AV lesions developed in cardiac allografts transplanted from parental to F_1 hybrid mice, a transplant system known to lack specific anti-donor T cell reactivity but retain anti-donor NK cell responses. NK cell depletion significantly abrogated the development of these lesions, suggesting that NK cells activated by missing-self MHC class I on donor endothelium can contribute to the development of AV[28]. In support, NK cells have been observed as infiltrates in AV lesions in human transplant recipients[194].

NK cells have several effector mechanisms all of which have been observed in experimental transplant models, and by which they could mediate allograft injury. NK cells can produce pro-inflammatory cytokines such as IFNγ and TNFα that can induce DTH or cause direct tissue damage; NK cells can produce granzymes which induce target cell cytolysis; NK cells express Fas ligand which would enable them to induce Fas mediated killing; and NK cells produce chemokines such as monokine-induced by gamma-interferon that recruit activated T cells[190, 195-197].

In human clinical transplantation, evidence for a role of NK cell alloimmunity in allograft rejection was provided by a study examining the impact of KIR-ligand matching between donor and recipients[198]. In a cohort of patients with minimal HLA mismatch (to minimise the impact of adaptive alloimmunity), KIR-ligand mismatch (recipient NK cells lacking receptors recognising donor MHC class I as 'self') was identified as an independent predictor of poor graft outcome suggesting NK cell alloreactivity to play an important role in allograft rejection. More convincing evidence for a role of NK cells in allograft rejection has subsequently been provided with microarray analyses reproducibly demonstrating NK cell gene transcripts to be upregulated in renal transplant biopsies in the context of antibody mediated rejection, implicating NK cells as effectors[199-201].

Thus, there is compelling evidence that NK cells can contribute to allograft injury and rejection, acting in conjunction with adaptive alloimmune responses.

1.8.1.2 NK cells in the maintenance of tolerance

There is however, a growing body of evidence suggesting that NK cells can also contribute to the induction of allograft tolerance. In a murine study of cardiac transplantation, depletion of NK cell subsets resulted in significantly accelerated rejection kinetics suggesting a role for NK cells in suppressing immune responses[202]. Similar findings have also been observed in bone-marrow transplantation, whereby NK cells have been demonstrated to regulate the development of graft-versus-host disease (GVHD)[203, 204]. In both of these studies, regulation of GVHD development was found to be due to NK cell deletion of alloreactive donor T cells. Several mechanisms have been proposed for how NK cells regulate alloreactive T cells. There is some evidence that antigenically stimulated and activated T cells upregulate ligands for the stimulatory NK cell receptor NKG2D which target them for direct NK cell cytolysis[205]. Activated T cells are also known to upregulate expression of the death receptor Fas, the ligand of which (Fas ligand) is expressed on activated NK cells. In support of this as a potential regulatory mechanism, NK cells were unable to prevent GVHD and control the expansion of T cells from Fas-deficient donors[203]. A more recent suggestion has been the possibility that regulatory subsets of NK cells produce regulatory cytokines such as IL-10 and regulate T cells through this mechanism[206].

An alternative mechanism of NK cell mediated tolerance identified in solid organ transplantation involves deletion of donor DCs. In a skin transplant model, rejection ensued in recipients deplete of NK cells[207]. NK cells were responsible for rapid deletion of passenger donor DCs which limited their survival and dissemination within the recipient. This was found to alter the location and extent of T cell priming, dampening the adaptive alloimmune response and promoting a tolerogenic environment[208].

Taken together, it is clear that NK cells have the potential to influence outcomes following transplantation. However, current understanding of NK cells and their impact on alloimmunity is incomplete, hindering development of approaches to harness or control their behaviour.

1.9 Regulatory immune cells

It is now widely accepted that there exist regulatory populations of leukocytes able to promote tolerogenic immune responses. Harnessing these cells in clinical transplantation to prolong allograft survival has become the focus of much on-going research[209]. The drive for introducing regulatory cellular immunotherapy to transplantation is to remove the requirement for lifelong treatment with immunosuppressive drugs and their complications such as toxicity, infection and malignancy, as well as ameliorating the inevitable development of chronic rejection. Several populations of regulatory immune cells have been discovered.

1.9.1 Regulatory T cells

The existence of a population of immune cells with suppressor properties was first demonstrated in 1971 by Gershon and Kondo[210]. Their experiments suggested a role for thymocytes in generating tolerance, and that tolerance could be transferred to other hosts by transfer of spleen cells. Adoptively transferrable suppression of transplant rejection became a consistent finding, but the cells responsible were not yet characterised and some scepticism remained[211]. Subsequently Hall *et al.* provided strong evidence in a rat cardiac allograft model, that $CD25^+$ CD4 T cells were responsible for mediating transplant tolerance[212, 213]. This was extended to demonstrate that transferred tolerance was 'infectious' in that transferred regulatory T cells could recruit non-regulatory CD4 T cells in a recipient to become regulatory[214]. Subsequent work demonstrated that these cells could also control autoimmunity and GVHD[215]. The next major step forward was discovery of the master transcription factor for regulatory CD4 T cells: Forkhead box P3 (*FOXP3*) in 2003, deficiency of which results in the severe autoimmune disease scurfy in mice, or immunodysfunction polyendocrynopathy and enteropathy, x-lined syndrome in humans[216-218].

Further work has confirmed that regulatory CD4 T cells (Treg) can be reliably defined by expression of high levels of CD25 (the IL-2 receptor α-chain) and the transcription factor *FOXP3*, and that these cells play a role in limiting immune responses such as those observed in autoimmunity such as type 1 diabetes and chronic inflammatory conditions such as inflammatory bowel disease, inhibit GVHD and prevent or delay allograft rejection in animal models[178, 219]. Two populations of Treg have been characterised. Natural Treg (nTreg),

represent 5-10% of the peripheral CD4 T cell population (natural, nTreg) and are selected in the thymus to maintain peripheral tolerance against self-antigen. Induced Treg (iTreg) develop in response to antigenic encounters in a tolerogenic environment in the periphery (induced, iTreg)[209, 220]. It has been proposed that nTreg, which are positively selected with a moderate to high affinity for self-antigens in the thymus have a role in regulating against autoimmunty, whilst iTreg derived from naïve T cells selected with higher affinity for non-self antigen have a role in suppressing responses against foreign antigens[211].

Four modes of action have been proposed for how Treg exert their suppression (Figure 1.9): suppression by secretion of inhibitory cytokines such as IL-10, TGF-β and IL-35; suppression by cytolysis through granzyme and perforin dependent mechanisms; suppression by metabolic disruption such as through consumption of local IL-2; and suppression by modulation of dendritic-cell (DC) maturation or function that then disrupts subsequent CD4 T cell activation[221]. No clear consensus of the single most important mechanism has been determined[178].

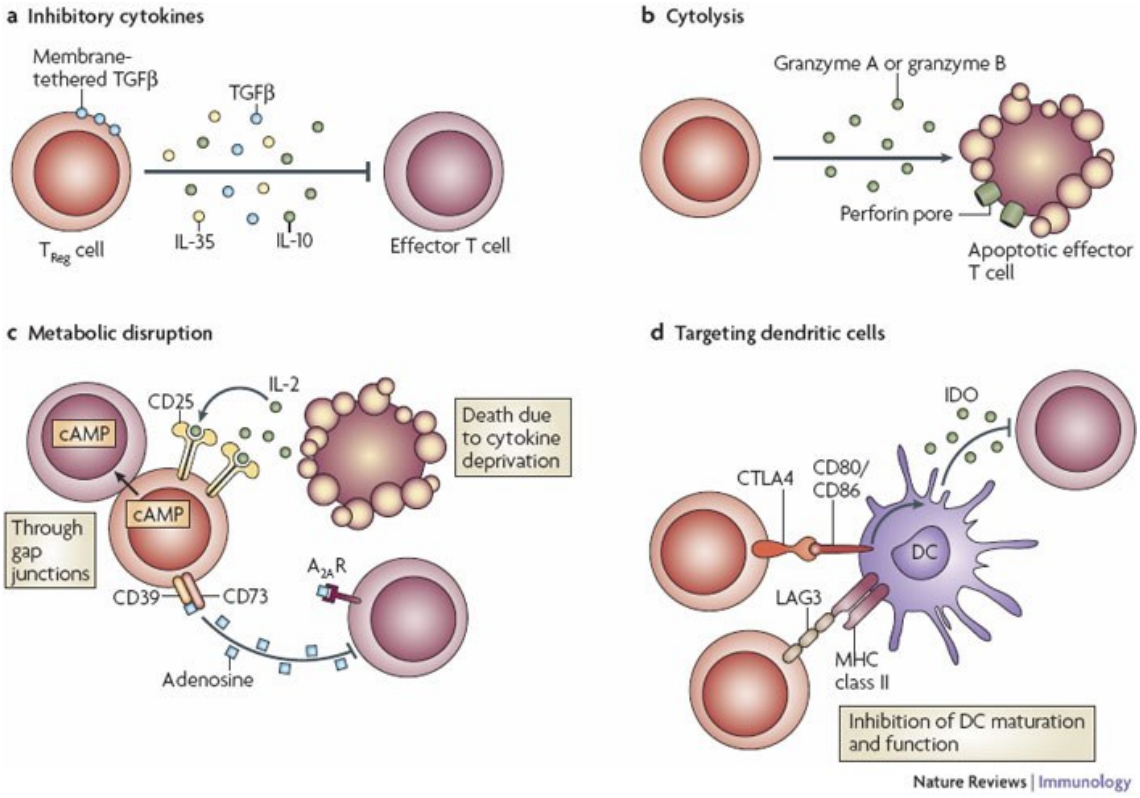


Figure 1.9 Suppressor mechanisms of regulatory T cells
Proposed mechanisms of Treg suppression. Reproduced from Vignali *et al.* Nature Immunology 2008[221]

There is now an extensive body of evidence that Treg have the ability to significantly prolong allograft survival following transplantation in animal models[209, 215, 222, 223]. This has involved development of techniques to isolate, induce and expand Treg *in vitro.* Furthermore, there is suggestion that particularly effective donor-specific tolerance can be established by administration of allospecific Treg, as compared to polyclonal Treg[165, 224].

The success in animal models has led to the beginnings of clinical translation of Treg therapy. Success with human Treg was first suggested by demonstration that *ex-vivo* expanded human Treg were effective at abrogating rejection in humanised mouse models[224, 225]. Several studies have now been performed in the setting of bone marrow transplantation, where Treg therapy has been shown to be safe with some efficacy in preventing development of GVHD[209, 226, 227].

In solid organ transplantation, very limited experience with Treg therapy has been reported so far. However, a multicentre phase I/IIa clinical trial funded by the European Union FP7 programme, the ONE study, is currently investigating the safety and production feasibility of regulatory cellular therapy, including several types of Treg, in living donor kidney transplant recipients[209, 215, 228]. The goal of developing cellular immunotherapy is to permit induction of long-lived donor-specific tolerance without the requirement for long-term chemical immunosuppression. The results of the ONE study are eagerly awaited.

1.9.2 Tolerogenic dendritic cells

Dendritic cells, as professional APC's, have an essential role in driving adaptive responses against foreign antigens. Nevertheless, their importance to the maintenance of central and peripheral tolerance has been increasingly appreciated[229, 230]. Tolerogenic DC's are immature, maturation resistant or alternatively activated DC's that express surface MHC molecules, have a low ratio of co-stimulatory to inhibitory signals, and an impaired ability to synthesise T helper 1 cell cytokines (such as IL-12)[229]. Tolerogenic DC's can be reproducibly generated *in vitro* through exposure to various anti-inflammatory and immunosuppressive agents, raising the potential for their use clinically in transplantation[229]. Tolerogenic DC's maintain peripheral tolerance using several mechanisms including: T cell deletion; inducing T cell anergy; inducing Treg; and by expressing immunoregulatory factors such as IL-10 and TGF-β[230].

Experimental administration of tolerogenic DC's in transplantation models has been undertaken with DC's of both donor and recipient origin, with prolonged allograft survival

demonstrated (reviewed in [229]). These cells differ in their ability to cognately interact with direct and indirect-pathway CD4 T cells. Recipient-derived tolerogenic DC's pulsed with donor antigen will only be able to interact with indirect-pathway T cells. By contrast, donor-derived tolerogenic DC's will only interact with direct-pathway T cells. It may be predicted therefore, that donor-derived tolerogenic DC's may not be effective at abrogating chronic rejection, however, this is not found to be the case[229].

Administration of *in vitro* generated donor-derived myeloid and plasmacytoid tolerogenic DC's 7 days prior to transplantation has been demonstrated to significantly prolong the survival of murine cardiac allografts, effects which can be enhanced by administering co-stimulatory blockade agents simultaneously[230-232]. Notably, in addition to significantly decreasing anti-donor direct-pathway responses, indirect-pathway responses are also impaired. After administration of donor-derived tolerogenic DC's, donor antigen is found to be presented by recipient DC's – suggesting that the tolerogenic DC's are acting as a source of donor antigen, that can be presented to indirect-pathway T cells, leading to their down-regulation, and contributing to the observed prolonged allograft survival[233, 234]. A recent study by Wang *et al.* has characterised this further, identifying that following administration of donor-derived tolerogenic DC's there was preferential deletion of indirect-pathway CD4 T cells which was dependent upon recipient DC's[235]. Wang *et al.* additionally identified that, surprisingly, the observed reduction in direct-pathway responsiveness was also dependent on the presence of recipient DC's highlighting that the interaction between the tolerogenic DCs and host cells is more complex than simply binding to direct-pathway CD4 t cells in the recipient.

Another strategy to target both direct and indirect-pathway CD4 T cells has been the use of semi-allogeneic DC's that co-express donor and recipient MHC molecules, typically by administering F1 (donor x recipient) DCs. These semi-allogeneic tolerogenic DC's appear particularly effective at regulating indirect-pathway responses, possibly by Treg induction, and have been shown to lead to indefinite survival of allografts[236, 237]. These cells could also interact with T cells in a semi-direct manner.

Although tolerogenic DC's offer some promise as a therapy, there remains the potential that these cells could revert to an immunogenic phenotype upon administration, with the inherent risk of sensitising the recipient and accelerating graft rejection.

1.9.3 Regulatory B cells

Regulatory B cells (Breg) are a more recently described population of immune cells with immunoregulatory function. Definitive characterisation of these cells has remained challenging because they are a rare subset of B cells and lack characteristic phenotypic or molecular markers analogous to those that define Treg such as *FOXP3*[238].

The suggestion that B cells may have an immunomodulatory role was first suggested upon observation that certain autoimmune models worsened upon B cell depletion[239]. The relevance to transplantation was similarly suggested when depletion of B cells at the time of kidney transplantation was surprisingly demonstrated to enhance acute T cell mediated rejection[240]. Two further studies suggesting a role for B cells in transplantation tolerance were published in 2010. Sagoo *et al.* observed that immunosuppression-free kidney transplant recipients are characterised by increased numbers of B cells, despite lacking donor-specific antibodies and demonstrated donor-specific T cell hypo-responsiveness *in vitro*, alluding to an immunoregulatory role for B cells in transplantation[241]. In the second, Newell *et al.* examined gene expression profiles and lymphocyte subsets of tolerant renal transplant recipients (off immunosuppression for greater than a year with stable renal function). Tolerant recipients had increased expression of multiple B cell differentiation markers and had elevated numbers of naïve and transitional B cells, again suggesting a central role for B cells in regulating alloimmunity[242]. More recent, functional, examination of B cells from similarly tolerant recipients has revealed a much higher percentage of IL-10 producing B cells, and quantity of IL-10 produced by B cells of tolerant recipients, and an impaired ability to differentiate into plasma cells[243].

It has been proposed that Breg may influence effector T cell responses either directly through antigen presentation and co-stimulation, or indirectly by cytokine production – particularly IL-10[243]. It is thought that interaction with Breg results in an increase in Treg development[244]. Although cognate interaction with Breg can only occur with indirect-pathway T cells, due to B cell presentation of antigens in the context of self-MHC, this pathway is particularly associated with chronic rejection and remains an important target of tolerogenic therapies.

Because of its apparent crucial role in mediating Breg suppression, IL-10 production is largely used as a marker for Breg identification. To date, a number of Breg subsets with overlapping phenotypes and functions have been identified in various mouse models[245]. As such, unlike for Treg and tolerogenic DC's, it has not yet been clarified how Breg can be reproducibly

generated *in vitro* for therapeutic use. A recent study from Rothsteins group has suggested that ligation of T cell immunoglobulin and mucin-domain-containing protein 1 (TIM-1) induces Breg expansion and regulatory activity[246]. Prolonged islet graft survival was observed in a murine model using a stimulatory anti-TIM-1 monoclonal antibody to induce Breg *in vivo* and following transfer of TIM-1^+ B cells[246].

The potential for Breg as an immunomodulatory therapy in transplantation has yet to be fully elucidated and currently there are no clinical studies with Breg as a cellular therapy, but this remains an active area of research[209].

1.9.4 Regulatory macrophages

Macrophages are cells of the innate immune system that respond to local inflammatory signals. Regulatory macrophages (Mreg) have been described as a population of macrophages that limit inflammatory responses and prevent prolonged activation of classical macrophages[247]. Mreg can be induced by stimulation with M-CSF and IFNγ and these cells have been shown to prolong survival of cardiac allografts in murine models, through inhibiting T cell proliferation through iNOS dependent mechanisms and through contact-dependent deletion of activated T cells[248]. Importantly, human Mregs have been successfully used as a cellular therapy in kidney transplantation. They proved to be safe and well tolerated and promising clinical outcomes were observed[249, 250]. Mreg are also being examined as part of the ONE study.

1.10 Limitations of murine models

Rodent models have been essential to the study of transplantation immunology, and have provided the opportunity to gain significant understanding of the process of allograft rejection and to discover novel therapeutic interventions. Several transplant models exist, the most frequently utilised being skin transplantation, heterotopic cardiac transplantation and kidney transplantation[251, 252]. In this thesis the heterotopic cardiac transplant model has been used. This has been the most widely utilised vascularised transplant model since its development in 1973 by Corry *et al.*[253]. There are several advantages of this model. It represents an immediately vascularised allograft, and allograft function is easily assessed by palpation of the recipient abdomen, with a defined endpoint for rejection – cessation of palpable beat. Unlike other vascularised transplant models, the recipient does not succumb to allograft rejection permitting examination of immunological responses even after the allograft has been rejected. Furthermore, the model has proven to be reproducible. However, there are shortcomings. The allograft does not contribute to cardiac output and is not truly physiological since blood only flows through the right side of the heart. The absence of functional contraction against an afterload results in progressive atrophy of the myocardium. This abnormal blood flow has been described as resulting in epicardial and endocardial inflammation due to chronic ischaemia. There are also anatomical differences between murine and human hearts. For example, coronary arteries are predominantly epicardial in humans, but are intramural in mice[251, 252].

Mice are the mainstay of *in vivo* immunological experimentation and have been instrumental to our understanding of the human immune system in health and disease. However, it must be borne in mind that there are significant differences between mice and humans in immune system development, activation and response to challenge[254]. Some examples with relevance to this thesis include: i) mice and humans differ in T cell co-stimulatory molecule expression - for example, 100% of murine T cells express CD28 whilst this is much lower in humans. This different pattern of co-stimulatory molecule expression is of relevance when considering extrapolation of experiments utilising co-stimulatory blockade agents. ii) Activated human CD4 T cells express MHC class II and may have a role in antigen processing and presentation which is not the case in mice. This may introduce some differences in characteristics of murine and human indirect-pathway CD4 T cell activity. iii) There is considerable evidence that human endothelial cells constitutively express MHC class II and can activate memory CD4 T cells. This is not the case in mice. This may result in significant differences in alloresponses against

vascularised allografts between humans and mice – with implications for both direct and indirect-pathway CD4 T cell responses since an on-going source of intact MHC class II is present in humans, but not in mice[254].

It should also be remembered that in human clinical transplantation recipients typically receive, at least initially, triple agent immunosuppression with corticosteroid, a calciuneurin inhibitor and an anti-proliferative agent such as azathioprine or mycophenolate mofetil. As is the case in most murine transplant immunology research, such immunosuppression has not been administered in the models reported in this thesis.

It is important to be aware of these potential limitations when considering extrapolating data from studies such as these in mice, to humans.

1.11 Aims and Objectives

The CD4 T cell alloimmune response is characterised by its unique ability to recognise alloantigen via two distinct pathways, the direct and indirect, which are thought to differ markedly in their respective duration and the mechanisms by which each contributes to allograft rejection. These differences however, remain largely unproven. In this thesis I aim to definitively examine the duration of the direct and indirect-pathways of allorecognition and understand the factors responsible for driving and limiting their longevity using a murine model of heterotopic cardiac transplantation.

Cellular immunotherapies are becoming an attractive treatment option in transplantation for attaining donor specific tolerance. Of these, regulatory T cell therapy has been studied in greatest detail, and clinical translation is not too distant. The second aim of this work is to examine if possessing a more detailed understanding of the alloimmune pathways will be useful to guide development of regulatory T cell therapy.

2 Methods

2.1 Animal work

2.1.1 Mouse strains and Animal Husbandry

C57BL/6 (H-2^b), BALB/c (H-2^d) and CB6F1 (C57BL/6 X BALB/c F1 – H-2^{bd}) mice were purchased from Charles River Laboratories (Margate, United Kingdom). Bm12 mice - B6(C)-H2-Ab1bm12/KhEgJ (H-2^{bm12})[255], T cell receptor-deficient C57BL/6 mice - B6.129P2-*Tcrb*^{tm1Mom}*Tcrd*^{tm1Mom}/J (H-2^b, TCRKO/*Tcrbd*$^{-/-}$)[256] and mice expressing the diphtheria toxin receptor gene (DTR) under the CD11c promoter on a C57BL/6 - B6.FVB-Tg (H-2^b – B6.DTR)[257] and BALB/c - C.FVB-Tg (H-2^d – BALB/c.DTR)[257] background were purchased from Jackson Laboratories (Bar Harbor, ME). Subsequently, BALB/c.DTR mice were crossed with C57BL/6 to obtain CB6F1 (H-2^{bd}) mice expressing the DTR gene. Similarly, *Tcrbd*$^{-/-}$ mice were backcrossed onto a bm12 background to obtain the bm12.TCRKO strain.

C57BL/6 *Rag2*$^{-/-}$ (H-2^b, RAG2KO) mice, lacking B and T cells were kindly gifted by Prof. T. Rabbitts (Laboratory of Molecular Biology, Cambridge, U.K.). Rag2IL2rg[258] (*Rag2*$^{-/-}$ mice with an additional IL-2 receptor γ chain knockout were kindly gifted by Dr Francesco Colucci (University of Cambridge, UK). These additionally lack NK cells.

C57BL/6 mice that lack expression of I-A^b, but express the I-Eα gene (ABOIE) and express I-E on the surface of their APCs were gifted by Prof C. Benoist (Joslin Diabetes Center, Boston, MA)[259]. C57BL/6-Tg(K^d)RPb mice (B6.Kd)[162] that additionally express the H-2K^d gene were gifted by Dr R.P. Bucy (University of Alabama, Birmingham, AL).

Bm12.Kd.IE mice were created in-house by first crossing bm12 x B6.Kd selecting from the offspring for those expressing I-A^{bm12} and H-2K^d but not I-A^b on PCR (bm12.Kd). These were then crossed with ABOIE animals and off-spring selected for expression of: I-A^{bm12}, I-E and H-2K^d. Bm12.IE mice were the F_1 offspring of bm12 x ABOIE.

Bm12.Kd.IE mice expressing the DTR gene under the CD11c promoter were created in-house by first crossing B6.FVB-Tg mice with ABOIE mice. The F_1 generation mice expressing DTR were back-crossed to ABOIE, with I-E^+ DTR^+ offspring selected (lacking I-A^b). These were then crossed with bm12.Kd mice and expression of DTR confirmed by PCR.

TCR transgenic animals (Table 2.1): *Rag2*$^{-/-}$ TEa mice (H-2^b, TEa)), specific for I-A^b restricted I-$E^d_{52\text{-}68}$ peptide were gifted by Prof A. Rudensky (University of Washington, Seattle, WA)[260]; *Rag1*$^{-/-}$ TCR75 mice (H-2b, TCR75), specific for I-A^b restricted H-2$K^d_{54\text{-}68}$ peptide were gifted by

Prof P. Bucy (University of Alabama, Birmingham, AL)[125]; *Rag2*$^{-/-}$ ABM mice (H-2^{b}, ABM), I-A^{bm12} restricted were gifted by Dr T. Crompton (Imperial College, London, UK)[261]; and *Rag1*$^{-/-}$ Marilyn mice (H-2^{b}, Mar) specific for I-A^{b} restricted *dby*$_{608\text{-}622}$ peptide were gifted by Dr Di Scott (Imperial College, London, UK)[123] .

Transgenic CD4 T-cell	**TCR variable segments**	**Epitope**
ABM[261]	Vα2, Vβ8	Recognition of intact I-A^{bm12} presenting undefined peptide
TCR75[162]	Vα1.1, Vβ8.3	Recognition of peptide 54-68 (QEGPEYWEEQTQRAK) derived from class I H-2K^{d} in the context of class II MHC I-A^{b}
TEa[260]	Vα2, Vβ6	Recognition of peptide 52-68 (ASFEAQGALANIAVDKA) derived from the class II I-E^{d}α chain in the context of class II I-A^{b}
Mar[123]	Vα1.1, Vβ6	Recognition of peptide 608-622 (NAGFNSNRANSSRSS) from H-Y minor histocompatibility antigen ***dby*** in the context of class II MHC I-A^{b}

Table 2-1 Summary of T cell receptor transgenic strains

A summary of animals used in this work is presented in Table 2.2. Mice weighed between 18-22g at the time of their use for *in vitro* experiments and transplants.

Genotyping and phenotyping of the mouse colonies was kindly performed by Mrs Margaret Negus, Mrs Jackie Higgins and Mrs Sylvia Rehakova. All animals were maintained in specific-pathogen free facilities and fed standard rodent diets at Central Biomedical Services (CBS), University of Cambridge. Animals were cared for and all experimental procedures performed were approved by the United Kingdom Home Office under the Animal (Scientific Procedures) Act 1986.

Strain	Description	MHC I	MHC II	CD8 T cells	CD4 T cells	B cells
C57BL/6	H-2^b wild-type	K^b D^b L^{null}	I-A^b I-E^{null}	+	+	+
BALB/c	H-2^d wild-type	K^d D^d L^d	I-A^d I-E^d	+	+	+
CB6F1	H-2^b x H-2^d F_1 cross	K^{bd} D^{bd} L^d	I-A^{bd} I-E^d	+	+	+
T*crbd*$^{-/-}$	TCR deficient C57BL/6	K^b D^b L^{null}	I-A^b I-E^{null}	-	-	+
RAG2$^{-/-}$	B and T cell deficient C57BL/6	K^b D^b L^{null}	I-A^b I-E^{null}	-	-	-
Rag2IL2rg	B, T and NK cell deficient C57BL/6	K^b D^b L^{null}	I-A^b I-E^{null}	-	-	-
B6.C-2^{bm12}/ KhEg bm12)	C57BL/6 mutant expressing I-A^{bm12}	K^b D^b L^{null}	I-A^{bm12} I-E^{null}	+	+	+
B6.Kd	C57BL/6 with K^d transgene	K^{bd} D^b L^{null}	I-A^b I-E^{null}	+	+	+
Bm12.Kd	Bm12 with K^d transgene	K^{bd} D^b L^{null}	I-A^{bm12} I-E^{null}	+	+	+
ABOIE	C57BL/6 with I-E^d transgene and I-A^b knockout	K^b D^b L^{null}	I-A^{null} I-E^d	+	+	+
Bm12.IE	Bm12 x ABOIE F_1	K^b D^b L^{null}	I-A^{bm12} I-E^d	+	+	+
Bm12.Kd.IE	Bm12.Kd x ABOIE F_1	K^{bd} D^b L^{null}	I-A^{bm12} I-E^d	+	+	+
Bm12.Kd.IE. DTR	Bm12.Kd.IE expressing DTR under CD11c promoter	K^{bd} D^b L^{null}	I-A^{bm12} I-E^d	+	+	+
B6.DTR	C57BL/6 expressing DTR under CD11c promoter	K^b D^b L^{null}	I-A^b I-E^{null}	+	+	+
BALB/c.DTR	BALB/c expressing DTR under CD11c promoter	K^d D^d L^d	I-A^d I-E^d	+	+	+
Mar	*Rag2*$^{-/-}$ TCR transgenic – recognising I-A^b restricted H-Y	K^b D^b L^{null}	I-A^b I-E^{null}	-	+	-
TEa	*Rag2*$^{-/-}$ TCR transgenic – recognising I-A^b restricted I-E^d	K^b D^b L^{null}	I-A^b I-E^{null}	-	+	-
TCR75	*Rag1*$^{-/-}$ TCR transgenic – recognising I-A^b restricted K^d	K^b D^b L^{null}	I-A^b I-E^{null}	-	+	-
ABM	*Rag2*$^{-/-}$ TCR transgenic – recognising I-A^{bm12} directly	K^b D^b L^{null}	I-A^b I-E^{null}	-	+	-

Table 2-2 Details of the murine strains used in this work
'+' indicates presence and '-' absence of cell type

2.1.2 Murine heterotopic cardiac transplantation

The technique used is based on that described originally by Corry and Russell in 1973 with some modifications[262, 263].

In this model the donor ascending aorta was anastomosed end-to-side to the recipient abdominal aorta and the donor pulmonary artery to the recipient inferior vena cava (IVC). The transplanted hearts function as aorto-caval shunts and do not contribute to the cardiac output of the recipient. Blood enters the donor ascending aorta retrogradely from the recipient abdominal aorta and is diverted into the coronary arteries by the closed, competent aortic valve. After the myocardium is perfused, venous blood drains into the right atrium through the coronary sinus and is pumped back through the donor pulmonary artery into the recipients IVC by the right ventricle (Figure 2.1).

The procedure was carried out using an operating microscope (Carl Zeiss OPM11-FC or OPM16-CH, Germany) at magnifications of between 6x to 40x. Operating time was approximately 60 minutes and an operative mortality of less than 5%.

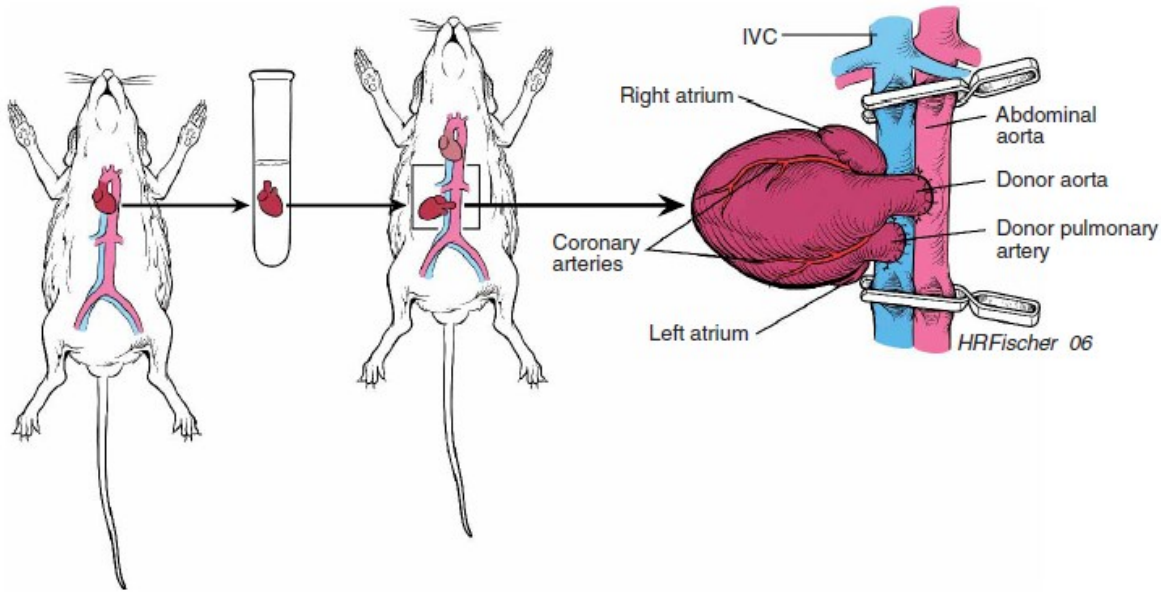


Figure 2.1 Murine heterotopic cardiac transplant model.
(from Hasegawa et al[263])

2.1.3 Donor operation

The donor was anaesthetised using a mixture of isoflurane and oxygen at 2 l/min administered via a nose cone. The animal was strapped to the operating board in the supine position using adhesive tape.

The abdominal cavity was opened via a large midline incision and the abdominal viscera deflected to the left exposing the IVC. 100 IU of unfractionated Heparin (Leo Pharma A/S, Ballerup, Denmark) was injected into the IVC using a 1 ml syringe attached to a 25 gauge needle and the puncture gently closed with a cotton bud. After about 5 seconds the IVC and abdominal aorta were transected, exsanguinating the animal.

The thoracic cavity was opened by dividing the diaphragm, cutting through the ribs along both sides of the thoracic spine up to the thoracic inlet and then across anteriorly to remove the entire anterolateral chest wall. The azygous vein, the superior vena cava (SVC) and IVC were ligated with silk ties and divided.

The ascending aorta was divided proximal to the brachiocephalic artery. The main pulmonary artery was also divided just distal to its bifurcation. Fat and connective tissue between the aorta and pulmonary artery was carefully teased away at this stage. The pulmonary veins were ligated en-mass with a circular tie around the base of the heart. The donor heart was then detached from the posterior mediastinal structures by blunt dissection and placed in ice cold saline.

2.1.4 Recipient operation

The recipient mouse was weighed before being anaesthetised using a mixture of isoflurane and oxygen at 2 l/min. A subcutaneous injection of 0.1 ml Temgesic diluted in 0.5 ml 0.9% saline was given at the nape of the neck. The animal was taped to an operating table at 37^{o}C in the supine position.

A midline abdominal incision was made from the pubic symphysis to the xiphisternum and a self-retaining retractor was inserted to expose the abdominal cavity. The urinary bladder was emptied by gentle pressure using sterile cotton buds.

Using cotton buds the intestines were gently deflected superiorly onto a piece of moistened sterile gauze on the anterior chest wall. They were covered with another strip of moist gauze. A small strip of connective tissue between the descending/left colon and the small bowel was

divided during this manoeuvre. The bladder and the internal genitalia were retracted downwards and covered with moist sterile gauze. Moisture was maintained throughout the procedure by periodic irrigation with warm saline.

Using two cotton buds the abdominal aorta and IVC were dissected free from the retroperitoneal connective tissue overlying the psoas muscles on both sides. The lumbar veins running in the posterior midline from the IVC between the renal vessels and the bifurcation were exposed and ligated using 7-0 vicryl™ to obtain adequate length for the application of the vascular clamps later.

The connective tissue overlying the great vessels was removed by a combination of blunt and sharp dissection. In the male, the gonadal vessels were dissected laterally at this point.

Non crushing clamps were applied across the great vessels just distal to the renal vessels and proximal to the bifurcation of the great vessels. The IVC was emptied via a venotomy using a 30G needle which was then used to make the arteriotomy, taking care not to damage the posterior wall. The arteriotomy was extended using micro-scissors to match the diameter of the donor aorta. The lumen was flushed with saline to remove any thrombi.

The donor heart was placed into the recipients abdomen with the remnant of the ascending aorta lying to the left, the base cranially and the left atrium caudally. Two stay sutures were placed anchoring the cranial and caudal ends of the donor ascending and recipient descending aorta. The lower one was used to construct a continuous end to side anastomosis in a counter-clockwise direction. This was done using a 10-0 nylon Bear™ surgical suture on a round bodied 4mm (3/8) needle (Bear Medic Corp., Tokyo, Japan). The heart was turned over to the right side when suturing the second wall, and constantly kept cool under a swab soaked with ice cold saline.

The venotomy was now extended to lie parallel but at a more caudal level in relation to the arteriotomy. The donor pulmonary artery was anastomosed to the recipient IVC in a similar manner using the same 10-0 sutures. The sutures were kept lax to prevent purse-stringing and constriction of the anastomosis.

Cut pieces of the moistened haemostatic agent Surgicel™ were placed around both anastomoses. The distal clamp was removed first to check the competency of the PA – IVC anastomosis. Once satisfactory, the proximal clamp was gently removed a few seconds later while applying pressure on the descending aorta with a cotton bud. This was gradually

withdrawn allowing distal blood flow and perfusion of the heart, any transient bleeding being controlled by gentle pressure with cotton buds.

Satisfactory perfusion was indicated by a bright red donor heart displaying strong and rhythmic contractions. Warm saline was infused into the peritoneal cavity and the viscera were replaced. The abdominal muscle and skin were closed in two layers of continuous 5-0 vivryl^{TM} sutures.

The cold ischaemia time (i.e. the duration from placement of the heart in ice cold saline to the transfer to the abdominal cavity) was approximately 20 minutes.

2.1.5 Recovery

On completion of the procedure, the recipient was placed in an incubator at 28^{o}C on soft dry bedding material. Recovery took about half-an-hour and the mice were checked for hind limb paralysis. If this was present they were killed immediately. Food and water were provided, and animals remained in the incubator overnight.

Grafts were assessed by abdominal palpation and rejection defined as the complete cessation of a palpable contraction.

2.1.6 Skin transplantation

Sterile techniques were maintained throughout. Full thickness tail skin was obtained from sacrificed donor animals, cut to 1 cm^2 and place in cold (4°C) saline. Recipient animal anaesthesia was induced and maintained with inhalational isoflurane, subcutaneous analgesia (Temgesic) was given and the animals placed prone on a heated (37°C) operating board. Forceps were used to lift the skin of the upper dorsum to allow a 1cm^2 area of skin to be removed in a single cut and the donor skin was laid over the defect. The donor skin was sutured to the recipients back with eight simple 5/0 sutures. The animal was placed in a 28°C incubator until recovery was complete. Rejection was defined as loss of skin graft at least two days post transplantation; loss of skin graft within 48 hours of grafting was defined as a technical failure.

2.1.7 Delivery of cells and antibodies

2.1.7.1 Intravenous (i.v.) injection

Where required, populations of lymphocytes or monoclonal antibodies were suspended in 100-200 µl Hartmann's solution (Animalcare Ltd, York, UK) and injected intravenously (i.v.) via the tail vein. Prior to injection, animals were placed in an incubator (37°C) for 10 minutes until veins were prominent.

2.1.7.2 Intraperitoneal (i.p.) injection

For some experiments, monoclonal antibodies or diphtheria toxin were suspended in 300 µl Hartmann's solution and injected intraperitoneally (i.p.).

2.1.8 Generation of bone-marrow chimeras

2.1.8.1 C57BL/6 x B6.DTR chimeras

To restrict expression of the DTR gene to haematopoietic lineage, C57BL/6 x B6.DTR bone-marrow chimeric mice were generated. C57BL/6 mice were lethally irradiated (13 Gy in two divided doses 3 hours apart) using a Caesium-137 source and reconstituted i.v. with $2x10^7$ B6.DTR bone marrow cells intravenously. Chimerism and DTR expression were confirmed by flow cytometric analysis and PCR of peripheral blood lymphocytes respectively (Figure 2.2a,b) at least 4 weeks after reconstitution.

2.1.8.2 Bm12.IE x Bm12.Kd.IE chimeras

To restrict expression of MHC class I H-$2K^d$ to haematopoietic lineage, bm12.IE x bm12.Kd.IE bone-marrow chimeric mice were generated. BM12.IE mice were lethally irradiated (13 Gy in two divided doses 3 hours apart) using a Caesium-137 source and reconstituted i.v. with $2x10^7$ bm12.Kd.IE bone marrow cells intravenously. Chimerism and haematopoietic H-$2K^d$ expression were confirmed by flow cytometric analysis of peripheral blood lymphocytes (Figure 2.2c,d) at least 4 weeks after reconstitution.

All chimeric animals were closely monitored and were sacrificed if severe signs of irradiation illness manifested.

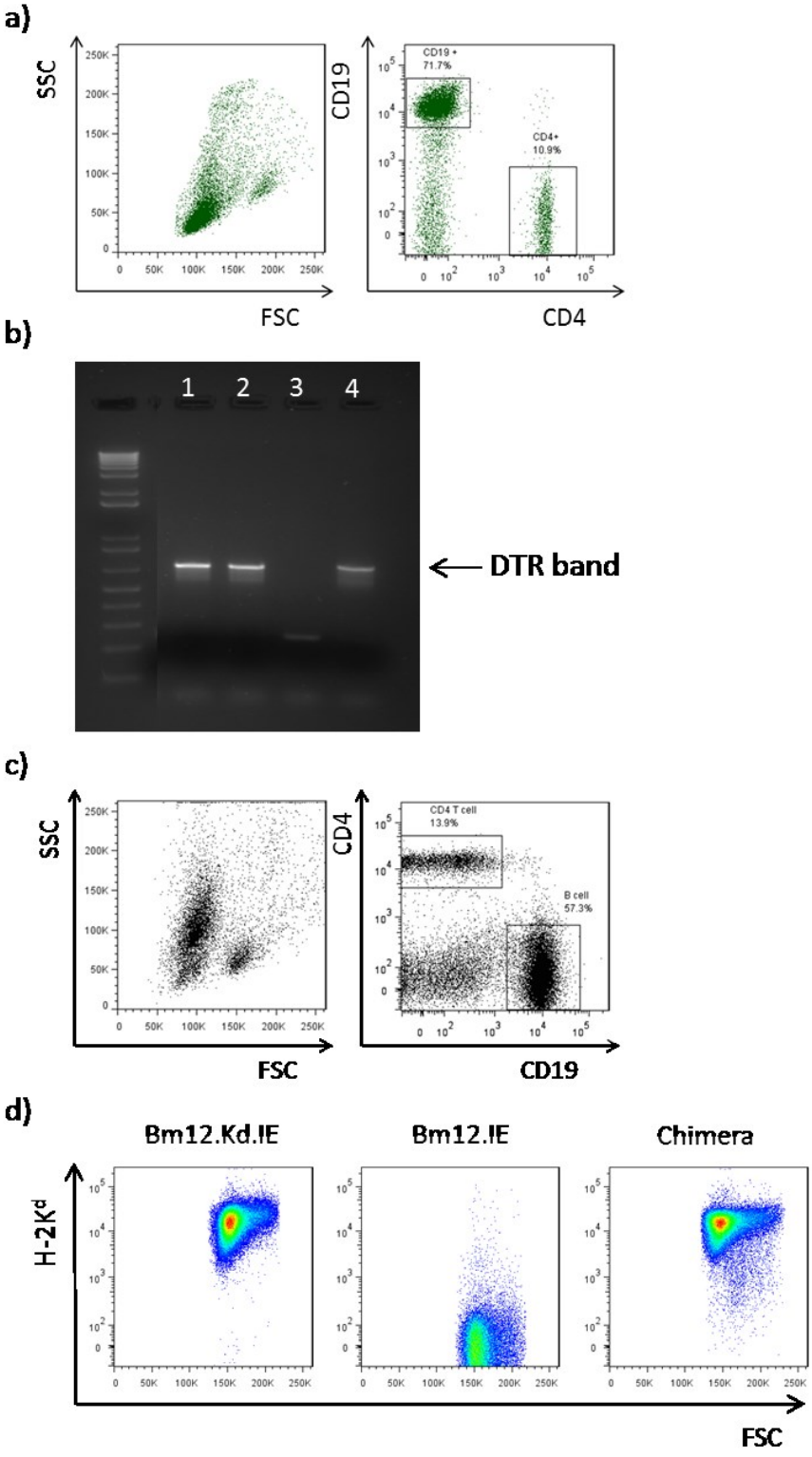


Figure 2.2 Confirmation of bone marrow chimeric status prior to use in experiments

(a) C57BL/6 x B6.DTR chimeric status was confirmed by demonstrating reconstitution of peripheral blood with lymphocyte populations 6 weeks following bone marrow transfer by flow cytometry. (b) Expression of the diphtheria toxin receptor was confirmed by PCR (performed by S Rehakova). (c) bm12.IE x bm12.Kd.IE chimeric status was confirmed by demonstrating peripheral blood reconstitution as above. (d) Acquisition of MHC class I H-2K^d expression (expressed by bm12.Kd.IE bone marrow donors but not expressed by bm12.IE bone marrow recipients) was confirmed by flow cytometry of peripheral blood lymphocytes.

2.1.9 Leukocyte depletion and co-stimulation blockade

2.1.9.1 Treatment protocol for depletion of dendritic cells (DC)

Donor DC depletion

Donor mice expressing the DTR gene under the CD11c promoter were injected i.p. with 10ng/mg diphtheria toxin (Quadratech Diagnostics Ltd, Epsom, UK) daily for seven days prior to the procurement of their hearts. The extent of depletion of DCs was measured by flow cytometry using the donor spleen on the day of retrieval.

Recipient DC depletion

C57BL/6 x B6.DTR bone marrow chimeras (Section 2.1.8.1) were used as recipients when depleting recipient DCs with diphtheria toxin due to the systemic effects experienced in non-chimeric diphtheria toxin sensitive mice[257, 264]. Mice were injected i.p. with 10g/mg diphtheria toxin (Quadratech Diagnostics Ltd, Epsom, UK) either i) daily for the seven days prior to transplantation and subsequently twice a week thereafter, or ii) daily for seven days, four weeks after transplantation and subsequently twice a week thereafter. The extent of DC depletion was measured by flow cytometry using splenocytes upon animal sacrifice.

2.1.9.2 Treatment protocol for depletion of Natural Killer (NK) cells

Donor and/or recipient mice were injected i.p. with 0.5 mg anti-NK1.1-depleting monoclonal antibody (mouse 1gG2a, clone PK13 – hybridoma) (ATCC-LGC Standards Parnership, Middlesex, UK) in 300 µl Hartmann's solution on days -2, 0 and +2 in relation to cardiac transplantation and weekly thereafter. Depletion of NK cells was confirmed by flow cytometry using splenocytes upon animal sacrifice for harvest of experimental tissues.

2.1.9.3 Treatment protocol for depletion of CD4 T cells

Donor mice were injected i.p. with two doses of depleting anti-CD4 mAb (1.0mg each dose; 2mg total) (YTS 191.1; European Collection of Animal Cell Cultures, Salisbury, UK) five and one day prior to procurement of their hearts. Depletion of CD4 T cells was confirmed by flow cytometry using peripheral blood lymphocytes (PBLs) the day before heart retrieval. In addition, depletion of CD4 T cells in the spleen and other tissues was confirmed by flow cytometry on the day of retrieval.

2.1.9.4 Treatment protocol for depletion of B cells

Mice were injected i.p. with a single dose of 250µg depleting anti-murine CD20 mAb (18B12 (pEAG2948/TOC262), IgG1 gifted by Cherie Butts at Biogen Idec Boston MA, US) seven days prior to requiring B cell depletion[265]. Depletion of B cells was confirmed by flow cytometry using peripheral blood lymphocytes and spleen of treated animals on the day of sacrifice.

2.1.9.5 Treatment protocol for depletion of macrophages

Mice were injected i.p. with 1mg of liposomal clodrinate (purchased from Prof van Rooijen: www.clodrinateliposomes.org[266-269]) seven and three days prior to transplantation. Efficacy of depletion was confirmed by flow cytometry using peripheral blood lymphocytes and spleens of treated animals on the day of sacrifice.

2.1.9.6 Treatment protocol for co-stimulation blockade

Mice were injected i.p. with 500µg anti-CD154 mAb (clone MR-1; BE0017-1; Bio X Cell West Lebanon, NH, US) on days -2, 0, 2 and 4 in relation to transplantation.

2.1.10 Collection of blood

Animals were pre-warmed at 37°C for 10 min to dilate tail veins. Approximately 20-30µl of blood was collected from the superficial tail vein of animals. When required, up to 1mL of blood could be obtained from animals at the time of sacrifice by direct cardiac puncture under terminal anaesthetic. In the case that peripheral blood lymphocytes were required, the tubes were pre-coated with heparin.

To collect serum from non-heparinised blood, samples were stored overnight at 4^{o}C to encourage clot formation. After one cycle of micro-centrifugation (14,000 revolutions per minute for 7 minutes), serum was aspirated and samples were aliquoted. All serum samples were heated at 56°C for 30 minutes in order to inactivate complement and stored at -20°C until analysed.

2.2 Cell Preparations and culture

All cell preparation and culture procedures were performed in a Microflow SE laminar flow hood (BIOQUELL, Andover, Hampshire, UK) with Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium (GibcoTM, Invitrogen, Paisley, UK), containing heat inactivated 10% foetal calf serum (FCS) (Biowest Ltd, Ringmer, East Sussex, UK), 2mM L-glutamine, 100 IU/ml penicillin, 100µg/ml streptomycin and 2-ME (2-mercapto-ethanol) unless stated otherwise. Centrifugation was performed in a Howe 6K10 Centrifuge (Sigma Laboratory Centrifuges GmbH, Osterode am Harz, Germany) at 200g for 7 minutes at 4^{o}C, unless otherwise stated. Cells were counted with a Neubauer chamber (Hawksley, Lancing, Sussex, UK).

2.2.1 Cell preparations

2.2.1.1 Single cell preparation from spleen

Spleens were harvested from sacrificed mice, mashed and teased through a 40µm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) with the rubber end of a 1 mL syringe plunger. The cells were washed with medium, centrifuged and re-suspended as a single cell suspension in an appropriate volume of medium.

2.2.1.2 Single cell preparation from bone marrow

Tibias and femurs were dissected from sacrificed animals. The marrow was flushed out with HBBS, and teased through a 40µm nylon cell strainer, as above.

2.2.1.3 Peripheral blood lymphocytes (PBLs)

Heparinised blood was diluted five-fold with 0.17M ammonium chloride for 5 minutes at room temperature (RT) to lyse erythrocytes and then centrifuged at 3000 rpm for 5 minutes. Supernatant was subsequently aspirated by a fine pipette, and the cell pellet re-suspended with medium.

2.2.1.4 Cell isolation from heart tissue

Hearts were cut in half and rinsed with HEPES buffered Dulbecco modified Eagle medium (DMEM) (Gibco®, Life technologies Ltd.) to remove all blood and blood clots. The tissue was dissected into 1-2mm pieces, transferred to a 5ml bijou and incubated in a 37°C water bath for 30 minutes with sufficient Collagenase Digestion mix (1mg/ml Collagenase A [Roche, USA], 1mg/ml DNAse1 [Roche, USA] and 2% FCS [Sigma] in DMEM) to cover the tissue. The resulting suspension was teased through a 40µm nylon strainer and incubated for a further 15 minutes. To stop the action of collagenase, the suspension was re-suspended in 50ml ice cold working medium containing 2-mercaptoethanol (2-ME) (10% FCS, 100IU/ml penicillin-streptomycin [Sigma-Aldrich Inc.], 2mM L-Glutamine [Sigma-Aldrich Inc.] and 0.05mM 2ME [Sigma-Aldrich Inc.] in DMEM) and centrifuged. Cells were washed with 50ml DMEM, and digested further with 3ml Trypsin/EDTA (Sigma-Aldrich Inc.) in a 37°C water bath for 10 minutes. The reaction was stopped with 50ml ice cold working medium, and the cells were passed through a 40µm nylon cell strainer to remove any remaining undigested tissue.

2.2.1.5 Cell subset enrichment using magnetic cell sorting

Single cell suspensions were re-suspended in MACS buffer (PBS + 0.5% BSA + 2mM EDTA +0.09% azide) and incubated at 4^{o}C for 15 minutes with antibody coated magnetic microbeads (Miltenyi Biotec, Bergisch, Germany) at 10 µl per 10^7 cells as per the manufacturers instruction.

Cells were washed, re-suspended in MACS buffer and the appropriate cell subset enriched by positive and negative selection using an AutoMACS^{TM} (Mitenyi Biotec, Bergisch, Germany) separator. The cells were counted and re-suspended in culture medium.

2.2.1.6 Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling of splenocytes

Single cell suspensions of splenocytes were re-suspended in PBS + 5% FCS at a concentration of 1 x10^7 cells per ml. A 10µl aliquot of CFSE [10mM] (Invitrogen, Paisley, UK) was diluted in an equal volume of PBS + 5% FCS. 2µl of this was added per 1 ml cell suspension and rapidly

mixed before incubation for 5 minutes at room temperature in the dark, with occasional agitation. At the end of this period the cells were washed thrice in 20ml of PBS + 5% FCS and reconstituted in an appropriate volume of 0.9% normal saline for adoptive transfer following counting[270].

2.2.2 Cell culture

2.2.2.1 Murine bone marrow dendritic cell (BMDC) culture

The method used was based on previously described protocols[271, 272]. Bone marrow was flushed with RPMI medium from the femurs and tibias, passed through a 40 µm nylon cell strainer and centrifuged at 300g for 7 minutes. The cells were re-suspended at a concentration of 1.5 x 10^6 cells/ml in full culture medium supplemented with murine granulocyte-macrophage colony stimulating factor (GM-CSF) (Peprotech Ltd, UK) at 10 ng/ml and recombinant murine IL-4 (Peprotech Ltd, UK) at 10 ng/ml. The suspension was distributed into flat bottom 6 well cell culture plates (Corning Incorporated, NY, USA) at 3 ml/well. The plates were incubated in a 5% CO_2-air mix at 37^{o}C in a SANYO MCO-17AI CO_2 incubator (SANYO Electric Corp, Japan).

On day 2, 3 ml of fresh GM-CSF and IL-4 supplemented culture medium was added to each well. On the 4th day, non-adherent cells were washed off and 3 ml of fresh GM-CSF and IL-4 supplemented culture medium was added. On the 6th day a further 3 ml of the culture medium - without IL-4 - was added to each well. The DCs were removed from the plates with a cell scraper on day 8, re-suspended in medium and counted.

2.2.2.2 Murine endothelial cell culture

Ten to fourteen day-old neonatal hearts were obtained and cells isolated as described in Section 2.2.1.4. Cells were re-suspended in MACS buffer and incubated with biotinylated monoclonal antibodies against endothelial cell markers: CD31, CD105 and Isolectin B4. After incubation, endothelial cells were enriched using anti-biotin magnetic Microbeads using an autoMACS separator as described above. The resultant cells were cultured in tissue culture flasks (Nunc^{TM}, Thermo Scientific Ltd.), pre-coated with 1% Gelatin (Sigma-Aldrich Inc.) in PBS,

and cultured in growth medium (Hepes buffered DMEM with 10% FCS, 100IU/ml penicillin-streptomycin, 2mM L-Glutamine) overnight. Non-adherent cells were washed away, and Endothelial Cell Growth Factor (E9640, Sigma-Aldrich Inc. St Louis, MO) added to the culture medium. Cells were passaged by incubation with Trypsin-EDTA Solution at 37°C, until cells were visibly detached under low power magnification.

2.2.2.3 Murine regulatory CD4 T cell culture

Ninety-six well, U-bottomed, cell culture plates (Corning Incorporated, NY, USA) were coated with anti-CD3 monoclonal antibody (Clone 145-2C11, BD Pharmingen, CA, USA) at 1µg/ml in bicarbonate buffer, incubating in a humidified chamber for 2 hours in a 5% CO_2-air mix at 37^{o}C in a SANYO MCO-17AI CO_2 incubator (SANYO Electric Corp, Japan). After incubation, the plates were washed 4 times with culture medium.

CD4 T cells were positively selected with anti-CD4 MicroBeads using an autoMACS separator as described in above. 1.5x10^{5} CD4 T cells were distributed into each well of the 96 well plate at a final volume of 200µl of full medium supplemented with IL-2 at a concentration of 2ng/ml (Life technologies, CA, USA), and TGF-β at a concentration of 5ng/ml (eBioscience, CA, USA). The plates were incubated in a humidified chamber for 5 days in a 5% CO_2-air mix at 37^{o}C as above. After five days, regulatory T cell phenotype was confirmed by flow cytometry, staining for expression of CD25 and *FOXP3* as detailed below.

2.3 Antibody quantification

2.3.1 Determining circulating anti MHC class I antibody—H-2K^d by ELISA

Serum samples were collected from experimental animals weekly and analysed for the presence of anti-H-2K^d IgG alloantibody by enzyme linked immunoabsorbent assay (ELISA). Ninety-six well ELISA plates (Immulon 4HBX; Thermo, Milford, MA) were coated with recombinant conformational H-2K^d (generated in house) at 5µg/ml in Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) and incubated overnight at 4^{o}C. Non-specific binding sites were blocked with 1% Marvel dried skimmed milk powder (Premier International Foods, UK) in PBS (hereafter referred to as 'Marvel') at 200 µl/well for 2 hours at 37°C. After washing (x6 with 0.05% tween 20 [Sigma, Poole, UK] in PBS throughout), samples were diluted 1:9 in Marvel and 50 µl/well added. Serial tripling dilutions were carried out down the remaining 8 rows of the plate, and the plate incubated at 37°C for 1 hour. After washing, 50 µl/well biotinylated Rabbit F(ab')2 anti-mouse IgG (STAR11B, AbD Serotec, Oxford, UK) at 1 µg/ml in Marvel was added and incubated at 37°C for 1 hour. After washing, 50 µl/well ExtrAvidin peroxidase conjugate (Sigma, Poole, UK) at 1 µg/ml in Marvel was added and incubated at room temperature (RT) for 1 hour. Plates were washed and 3,3', 5,5'-tetramethylbenzidene (TMB) microwell peroxidise substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was added. Colour was developed for 5 minutes and the reaction stopped by the addition of 0.2 M H_2SO_4. Absorbance at 450nm was measured on a FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK). For each sample, an absorbance versus dilution curve was plotted, and the area under the curve was calculated[273]. The AUC was then calculated as a percentage of the AUC of a standard of serial diluted pooled hyperimmune sera (sera pooled from C57BL/6 recipients of BALB/c cardiac allografts collected at week 5 post-transplant. Pooled serum from naïve C57BL/6 mice was used as a negative control). AUC percentages were normalised between plates by using an identical positive control on each plate.

2.3.2 Determining circulating anti MHC class II antibody— I-E by flow cytometry

Serum samples were collected from experimental animals weekly and analysed for an I-E alloantibody responses, using flow cytometry. Samples were diluted 1:9 in FACS buffer (1

µg/ml BSA [Sigma, Poole, UK] + 10 µl/ml 10% Azide [Sigma, Poole, UK] in PBS), 50 µl/well added to a 96-well U-bottomed plate (BD, Franklin Lakes, NJ, USA) and serial tripling dilutions carried out down the remaining 8 rows of the plate. BMDCs were cultured from ABOIE animals (as in Section 2.2.2.1). Upon harvest, BMDCs were blocked with anti-mouse CD16/CD32 (clone 2.4G2; BD Pharmingen, San Diego, CA, USA) at 5 µl/ml in FACS buffer for 30 minutes. The cells were washed and counted. BMDCs were then re-suspended at 1 x 10^7 cell/ml in FACS buffer and 50 µl transferred to each well of the plate, and incubated at 4°C for 30 minutes. The plate was washed twice by addition of 150 µl/well FACS buffer, centrifuged at 1200 rpm for 4 minutes, the supernatant discarded and cells re-suspended. Bound alloantibody was detected with 50 µl FITC-conjugated anti-mouse IgG mAb (STAR 70; Serotec, Oxfordshire, UK) at 10 µmg/ml in FACS buffer, and incubated for a further 30 minutes at 4°C. The plate was washed as above and the cells re-suspended. Cells contained within each well were then processed by flow cytometry, gating on the DC population and presenting as a histogram of FITC fluorescence. The geometric mean-channel fluorescence was obtained, and plotted against dilution, and the AUC was obtained and results analysed as above, against the same hyperimmune positive control. This approach, rather than ELISA, was adopted due to the unavailability of conformational MHC class II I-E during these studies.

2.3.3 Determining circulating antinuclear autoantibody by HEp-2 indirect immunofluorescence

The presence of antinuclear autoantibodies (ANA) was determined by HEp-2 indirect immunofluorescence (The Binding Site, Birmingham, UK). HEp-2 binding has been used traditionally for detecting autoimmune responses in human patients, but mouse autoantibody also binds to the target nuclear antigens, and this technique has been validated for this purpose[274].

Serum samples were diluted 1 in 10 in PBS and incubated for 30 minutes on slides coated with HEp-2 cells. Bound autoantibody was then detected with FITC-conjugated goat anti-mouse IgG (STAR70; AbD Serotec, Oxford, UK). Monospecific positive and negative pre-diluted controls were used according to the manufacturers instruction. Pooled sera from naïve C57BL/6 animals were used as an additional negative control. Serial two-fold dilutions (1 in 10 to 1 in 2560) of sera from TCRKO mice injected i.v. with 5x10^6 purified bm12 CD4 T cells, which

develop augmented antinuclear antibody responses[274], were used to generate a standard curve (Figure 2.3).

For each sample, four random photomicrographs were taken and the intensity of staining determined by morphometric analysis using MetaMorph software (Molecular Devices, Downingtown, PA, USA). The fluorescence intensity was then determined relative to a standard curve of serially diluted pooled hyperimmune serum that was assigned an arbitrary value of 1000 fluorescence units for the 1 in 10 dilution, 500 units for the 1 in 20 dilution and so on[274].

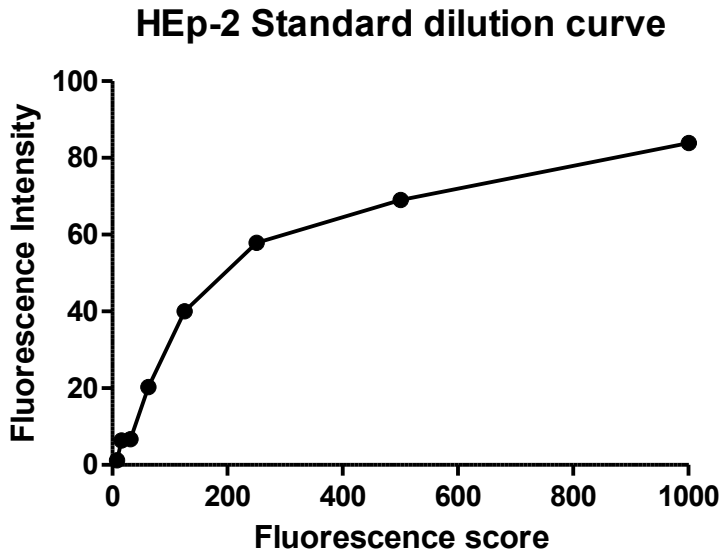


Figure 2.3 A representative example of a HEp-2 standard dilution curve

2.4 B cell H-2K^d ELISPOT

96-well MultiScreen® Filter Plates (Merck Millipore) were coated with recombinant conformational H-2K^d (generated in house) at 5µg/ml in Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) at 4°C overnight. Excess binding sites on the plates were blocked with 0.5% BSA in PBS (Blocking buffer) at 37°C for 1 hour, followed by five washes with sterile PBS, and one wash with ELISPOT culture medium (10% FCS, 1% penicillin-streptomycin + 2mM L-Glutamine and 0.05mM 2-mercaptoethanol [2ME; Sigma-Aldrich Inc] in RPMI culture medium [Gibco®, Life Technologies Ltd.]).

Single cell suspensions from spleen and bone marrow were re-suspended in culture medium as described above and added to the plate in triplicates of 1 x 10^6, 1 x 10^5 and 1 x 10^4 cells per well. Precautions were taken to ensure sterility during this stage. The plates were incubated undisturbed for 20 hours at 37°C in 5% CO_2 in a humidified chamber.

Plates were washed ten times with 0.05% Tween in PBS. Bound antibodies were detected with biotinylated Rabbit F(ab')2 anti-mouse IgG (STAR11B, AbD Serotec, Bio-Rad Laboratories Inc.) followed by ExtrAvidin Peroxidase conjugate (Sigma-Aldrich Inc.) diluted at 1:1000 concentration in Blocking buffer. Each incubation was performed at 37°C for 2 hours and the plates washed six times after each step.

For development of spots, 5mg of AEC (Sigma-Aldrich Inc.) dissolved in 2.5ml dimethylformamide (Sigma-Aldrich Inc.) was added to 10 ml 0.05 M Sodium acetate buffer (pH5.0). The solution was filtered through a 0.45µm filter (Sartorius Stedim Biotech) and mixed with Hydrogen peroxide solution (Sigma-Aldrich Inc.) (0.3%). Plates were incubated with the developing solution for 15-45 min in the dark at room temperature, until spots were visible in wells carrying the positive control with the naked eye. The plate was washed three times with distilled water and left to dry in the dark before being read on an AIDTM Elispot Reader version 3.5 (Autoimmun Dignostika, Strasberg, Germany). Data was expressed as mean number of responders per 10^6 cells.

2.5 CD8 ELISPOT

96-well MultiScreen® Filter Plates (Merck Millipore) were coated with anti-IFNγ monoclonal antibody (Clone RA-6A2, BD Bioscience) at 10µg/ml in Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) at 4°C overnight. Excess binding sites on the plates were blocked with 0.5% BSA in PBS (Blocking buffer) at 37°C for 1 hour, followed by five washes with sterile PBS, and one wash with ELISPOT culture medium (10% FCS, 1% penicillin-streptomycin + 2mM L-Glutamine and 0.05mM 2-mercaptoethanol [2ME; Sigma-Aldrich Inc] in RPMI culture medium [Gibco®, Life Technologies Ltd.]).

CD8 T cells from the spleens of recipient mice were purified from single cell suspensions of splenocytes using anti-CD8a (Ly-2) coated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the manufacturer's instructions. 2 $x10^5$ of these purified (responder) CD8 T cells were added to each well in triplicate. 8 $x10^5$ donor strain whole splenocytes (irradiated at 2000 rads) were also added to each well as stimulators. The plates were incubated undisturbed for 20 hours at 37°C in 5% CO_2 in a humidified chamber.

Plates were washed ten times with 0.05% Tween in PBS. Bound IFNγ was detected with a biotinylated anti-IFNγ monoclonal antibody (clone XMG1.2, BD Bioscience. [The recommended matched pair with the capture antibody]) at 40µg/ml, followed by ExtrAvidin Peroxidase conjugate (Sigma-Aldrich Inc.) diluted at 1:1000 concentration in Blocking buffer. Each incubation was performed at 37°C for 2 hours and the plates washed six times after each step.

For development of spots, 5mg of AEC (Sigma-Aldrich Inc.) dissolved in 2.5ml dimethylformamide (Sigma-Aldrich Inc.) was added to 10 ml 0.05 M Sodium acetate buffer (pH5.0). The solution was filtered through a 0.45µm filter (Sartorius Stedim Biotech) and mixed with Hydrogen peroxide solution (Sigma-Aldrich Inc.) (0.3%). Plates were incubated with the developing solution for 15-45 min in the dark at room temperature, till spots were visible in wells carrying the positive control with the naked eye. The plate was washed three times with distilled water and left to dry in the dark before being read on an AIDTM Elispot Reader version 3.5 (Autoimmun Dignostika, Strasberg, Germany). Data was expressed as mean number of spot counts for the triplicate wells, per 10^6 responder CD8 T cells.

2.6 Flow cytometry

All antibodies used in flow cytometry are detailed in Table 2.3. Prior to analysis, cells were transferred to flow cytometry tubes (BD Falcon, Franklin Lakes, NJ, USA). Samples were analysed on a FACS Canto™ II using FACS Diva software (BD Biosciences, San Jose, CA).

2.6.1 Surface antigen staining

Flow cytometry was carried out using a minimum of 2-5 x 10^5 target cells per well in 96-well U-bottomed plates (BD Falcon, Franklin Lakes, NJ, USA). Single cell suspensions in FACS buffer (100 µL/well of PBS with 0.1% BSA, 0.1% sodium azide) were added to the plates. The cells were blocked with anti-CD16/CD32 (FcRγ II/III, clone 2.4G2, BD Pharmingen, San Diego, CA) at a 1:200 dilution for 30 minutes to prevent non-specific antibody binding via cellular Fc receptors. All incubations with primary and secondary monoclonal antibodies were performed on ice and in the dark for 30 minutes, with two washes in FACS buffer between each step. Cell membrane of nonviable cells can trap fluorescent reagents, and therefore, 7-amino-actinomycin D (7-AAD) was routinely added to label dead cells dead cells which were excluded from the analysis. Control cells stained with each fluorescent antibody separately were used as single stain compensation controls to calculate any spectral overlap and calibrate the compensation gating for each fluorochrome.

2.6.2 Intracellular antigen staining

Single cell suspensions in FACS buffer were obtained and Fc receptors blocked with anti-CD16/CD32 as above at a 1:200 dilution for 30 minutes in 96 well U-bottomed plates on ice in the dark. Cells were then washed twice in PBS. 100µL fixable viability dye at 1/1000 concentration in PBS, was added to each well to stain dead cells and was incubated for 30 minutes (Fixable viability dye eFluor® 780, eBioscience, SanDiego, CA). Cells were washed twice in FACS buffer. Primary (and if necessary secondary) monoclonal antibodies against surface antigens were added and incubated for 30 minutes. Cells were washed twice in FACS buffer. Cells were fixed and permeabilised using the BD Cytofix/cytoperm™ fixation/permeabilisation kit (BD biosciences, San Jose, CA) as per the manufacturers instructions. 100 ml of fixation/permeabilisation solution was added to thoroughly

resuspended cells and incubated for 20 minutes. Cells were then washed in BD Perm/Wash™ solution from the kit. Monoclonal antibodies against intracellular antigens diluted in BD Perm/Wash™ solution were added and cells incubated for 30 minutes. Cells were washed twice more with BD Perm/Wash™ solution, and finally re-suspended in FACS buffer and analysed.

2.6.3 Trucount™ cell quantification

Single cell suspensions of splenocytes were re-suspended in a known volume of culture medium. An aliquot of defined volume was sampled for surface antigen staining as described above. Upon completion of staining, samples were transferred to Trucount™ tubes (BD Biosciences, San Jose, CA) containing a known number of fluorescent beads, and all events recorded. Gates were positioned around the bead and cell populations of interest, and cell number in the original sample calculated:

$$\frac{\text{Number of cells in defined gate recorded}}{\text{Number of Trucount™ beads recorded}} \times \frac{\text{Number of Trucount™ beads in tube}}{\text{Microlitres sampled from original cell suspension}} = \text{Number of cells per microlitre in original cell suspension}$$

2.6.4 Quantification of CFSE labelled CD4 T cell proliferation

Proliferation of CFSE labelled CD4 T cells (Section 2.2.1.6) was quantified using FlowJo™ (Tree Star, Oregon) flow cytometry analysis software. Upon cell division, the relative fluorescence intensity of CFSE in the daughter population is approximately half that of the parent. Therefore, upon division, a series of peaks is generated on a FITC channel histogram (Figure 2.4). FlowJo™ uses a non-linear curve-fitting algorithm to quantify proliferation and report a series of statistics. First, it models a Gaussian distribution at the highest fluorescence intensity peak (the parent population). By halving the fluorescence intensity, the daughter population distributions can be modelled, and this model is iterated until no further improvement in the model can be achieved to fit the data. Manual adjustments in defining the position of the parent peak can be made, and the results were taken for the position with the best quality of

fit to the data, as defined by the lowest Root Mean Square value. The statistic used to compare proliferation in this work is the 'percent divided' which represents the percentage of cells from the original sample that have undergone division[270, 275]. In all experiments where CFSE labelled transgenic CD4 T cells were transferred to experimental animals, cells were also transferred to a naïve C57BL/6 mouse as a negative control to confirm that any proliferation was consequent upon exposure to alloantigen due to the challenge presented to the experimental mice.

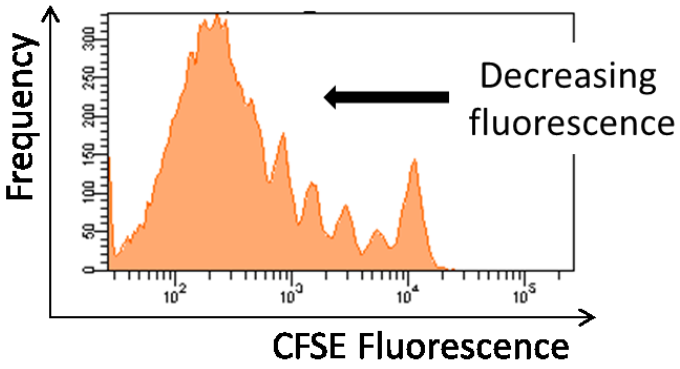


Figure 2.4 Representative histogram of T cell proliferation
Live CD4 T cells of interest gated and histogram against FITC fluorescence plotted to show CFSE dilution. Each peak to the left of the parent population on the far right represents daughter population peaks. Extent of proliferation is then quantified on FlowJo™ software.

2.6.5 Staining with MHC class II tetramer to identify antigen specific CD4 T cells

MHC class II I-A^b tetramers presenting peptide from the MHC class I H-$2K^d$ [peptide 54-68 (QEGPEYWEEQTQRAK)], MHC class II I-E^d [52-68 (ASFEAQGALANIAVDKA)] and control human CLIP (Class II-associated invariant chain peptide) [peptide 87-101 (PVSKMRMATPLLMQA)] conjugated to PE, were kindly gifted by the NIH Tetramer facility, Atlanta, GA. Whole spleen single cell suspensions in FACS buffer were obtained and Fc receptors blocked with anti-CD16/CD32 (FcRγ II/III, clone 2.4G2, BD Pharmingen, San Diego, CA) at a 1:200 dilution for 30 minutes in Falcon tubes. PE-conjugated tetramer was added at a concentration of 1:250 and cells incubated for 1 hour at 37°C. Subsequently the cells were washed and re-suspended in MACS buffer (PBS + 0.5% BSA + 2mM EDTA +0.09% azide). To enrich the tetramer bound cells, the single cell suspension was incubated with anti-PE magnetic MicroBeads (Miltenyi Biotec, Bergisch, Germany) as above. The PE labeled, and thus

tetramer bound cells, were extracted by positive selection using the program POSSEL on an AutoMACS™ (Miltenyi Biotech) separator. The enriched fraction was then washed and re-suspended in FACS buffer. The cells were then stained with flurochrome-labeled monoclonal antibodies specific for cell surface antigens CD19, CD4, CD11b, CD11c and CD8, and 7-AAD for dead cell exclusion, and incubated for 30 minutes at 4°C as in Section 2.6.1. Cells were washed twice more in FACS buffer and analysed.

2.6.6 Regulatory CD4 T cell suppression assay

To examine the ability of regulatory T cells (Treg) to exhibit regulatory behaviour, suppression assays were performed as described by Collison *et al.*[276].

Naïve C57BL/6 CD4 T cells were positively selected with anti-CD4 MicroBeads using an autoMACS separator as described above, and labelled with CFSE (Section 2.2.1.6). $2.5x10^4$ CFSE labelled CD4 T cells were distributed into each well of a 96 well plate at a final volume of 200µl of full medium supplemented with 1mM sodium pyruvate (Life technologies, CA, US), 5mM HEPES (Life technologies, CA, US) and 100mM non-essential amino acids (Life technologies, CA, US). CD3/CD28 CD4 T cell activating Dynabeads® were added to each well at a 1:1 ratio with the naïve CD4 T cells to induce proliferation (Figure 2.5) as per the manufacturers instruction (Life technologies, CA, US). Into column 12 of the 96 well plate $1.25x10^4$ cultured Treg were added to each well (representing a 1 Treg to 2 naïve CD4 T cell ratio). A 2-fold serial dilution was performed along the plate (Figure 2.6). Positive (Naïve CD4 T cell + Dynabead® only) and negative (Naïve CD4 T cell only) control columns were used. Plates were incubated in a humidified chamber for 3 days in a 5% CO_2-air mix at 37°C in a SANYO MCO-17AI CO_2 incubator (SANYO Electric Corp, Japan). Following incubation, cells were processed by flow cytometry and extent of naïve CD4 T cell proliferation quantified as described in Section 2.6.4.

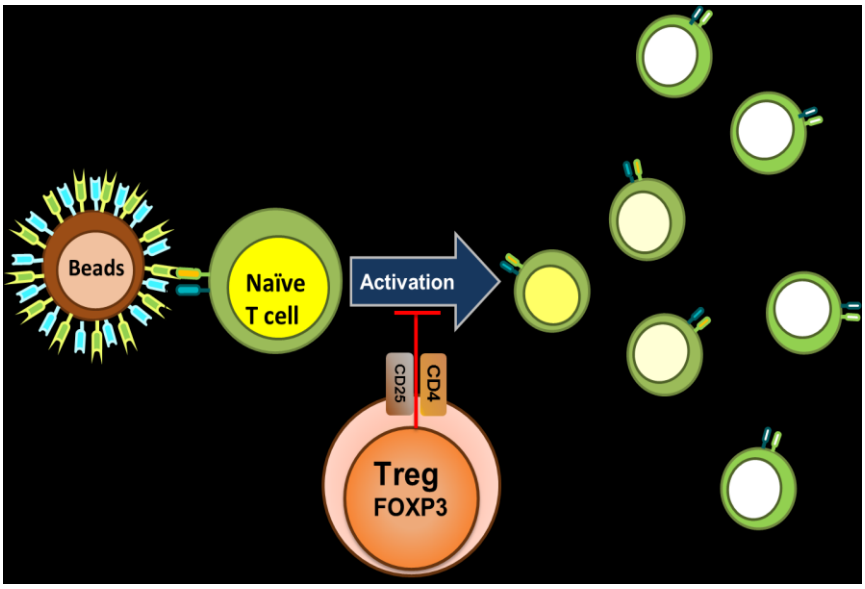


Figure 2.5 Regulatory CD4 T cell suppression assay
CD3/CD28 Dynabeads® (Life Technologies) activate naïve CD4 T cells and induce proliferation. The aim of this assay is to evaluate the efficacy of regulatory CD4 T cells in attenuating the induced polyclonal naïve CD4 T cell proliferation. This is assessed by quantification of proliferation on flow cytometry, since the naïve CD4 T cells are CFSE labelled.

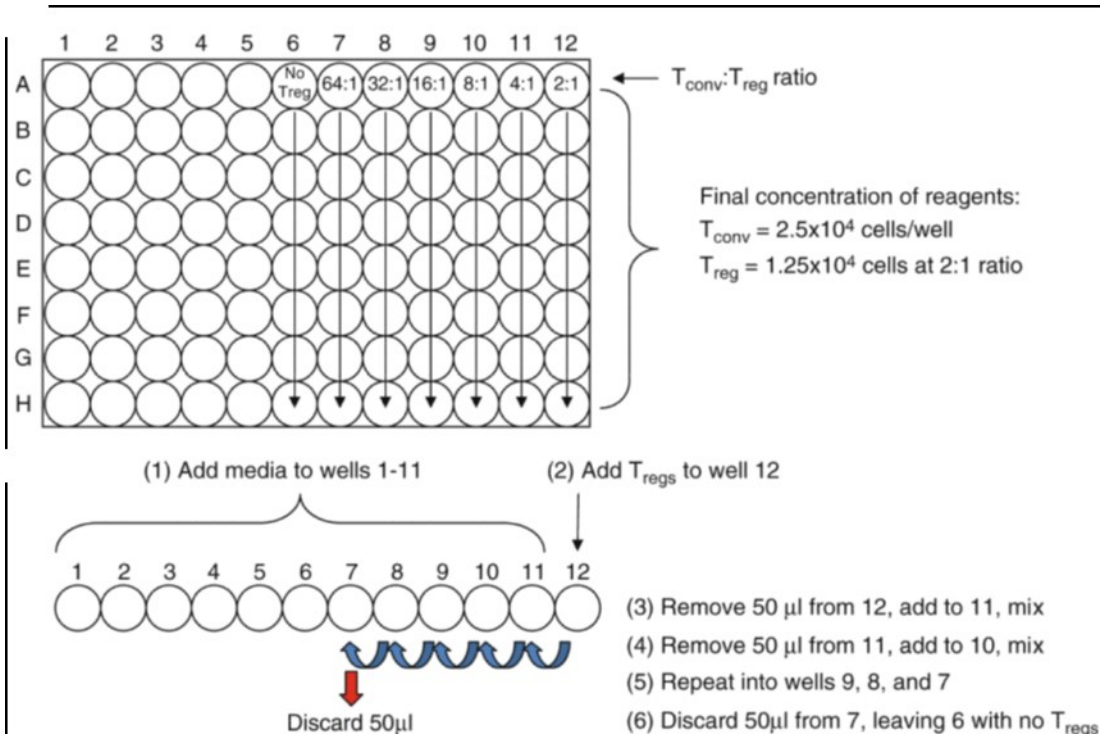


Figure 2.6 Regulatory T cell suppression assay plate diagram
96 well plate plan for suppression assay, reproduced from Collison *et al.*[276]. Tconv refer to CFSE labelled naïve C57BL/6 CD4 T cells in this work.

Antibody	Conjugate	Clone	Working dilution	Source	Catalogue number
7AAD	PerCP	-	1/500	BD Pharmingen	51-68981E
CD197 (CCR7)	PerCP	4B12	1/250	BD Pharmingen	560812
CD3	PE	145-2C11	1/100	BD Pharmingen	553083
CD3	FITC	145-2C11	1/100	BD Pharmingen	553061
CD4	APC	GK 1.5 L3T4	1/500	BD Pharmingen	553051
CD4	PE Cy7	RM4-5	1/2000	BD Pharmingen	552775
CD4	BIO	GK 1.5	1/500	BD Pharmingen	553728
CD4	FITC	GK1.5	1/100	BD Pharmingen	553729
CD4	APC Cy7	GK1.5	1/100	BD Pharmingen	560181
CD8	PE	53-6.7	1/100	BD Pharmingen	553032
CD8	PerCP	53-6.7	1/100	BD Pharmingen	551162
CD8	APC Cy7	53-6.7	1/200	BD Pharmingen	561092
CD11b	APC Cy7	MI/70	1/200	BD Pharmingen	557657
CD11c	FITC	HL3	1/50	BD Pharmingen	557400
CD11c	PE	HL3	1/50	BD Pharmingen	557401
CD16/CD32 Fc Block™	-	2.4G2	1/200	BD Pharmingen	553141
CD19	APC	ID3	1/250	BD Pharmingen	550992
CD19	FITC	1D3	1/100	BD Pharmingen	553785
CD19	PE	1D3	1/200	BD Pharmingen	553786
CD19	PE Cy7	1D3	1/200	BD Pharmingen	552854
CD25	PE	PC61	1/100	BD Pharmingen	553866
CD25	PercP	PC61	1/200	BD Pharmingen	551071
CD40	BIO	HM40-3	1/100	BD Pharmingen	553721
CD40	FITC	HM40-3	1/50	BD Pharmingen	553723
CD44	APC	IM7	1/200	BD Pharmingen	561862
CD44	FITC	IM7	1/50	BD Pharmingen	553133
CD45.1	APC Cy7	A20	1/200	BD Pharmingen	560579
CD45.2	APC Cy7	104	1/200	BD Pharmingen	560694
CD49b	BIO	DX-5	1/1000	BD Pharmingen	553856
CD62L	APC	MEL-14	1/100	BD Pharmingen	561919
CD69	APC	H1.2F3	1/100	BD Pharmingen	560689
CD69	FITC	H1.2F3	1/50	BD Pharmingen	553236
CD80	FITC	B7-1	1/100	BD Pharmingen	553768
CD86	BIO	GL-1	1/50	BD Pharmingen	553690
CD105	BIO	MJ7/18	1/200	BioLegend	120403
CXCR5	BIO	APC	1/200	BD Pharmingen	560615
Foxp3	PE	MF23	1/100	BD Pharmingen	560408
Isolectin B4	BIO	B-1205	1/25	Vector	W1208
Kd	PE	SF1-1	1/100	BD Pharmingen	553566
Ki 67	Alexa Fluor 647	B56	1/100	BD Pharmingen	558615
CD279 (PD1)	APC	J43	1/500	BD Pharmingen	562871
APC Streptavidin conjugate	Secondary	-	1/1000	Cantag Laboratories	SA1005
PECy7 Streptavidin conjugate	Secondary	-	1/1000	BD Pharmingen	557598

Thy 1.1	PE	OX-7	1/1000	BD Pharmingen	554898
Thy 1.1	Biotin	OX-7	1/20,000	BD Pharmingen	554896
Thy 1.1	PerCP	OX-7	1/2000	BD Pharmingen	557266
Thy 1.1	APC Cy7	OX-7	1/200	BD Pharmingen	561401
Vα2	Biotin	B20.1	1/1000	BD Pharmingen	553287
Vα2	FITC	B20.1	1/100	BD Pharmingen	553288
Vβ6	PE	RR4-7	1/200	BD Pharmingen	553194
Vβ6	Biotin	RR4-7	1/500	BD Pharmingen	553192
Vβ8	PE	OX-7	1/100	BD Pharmingen	553862
Vβ8.3	PE	1B3.3	1/100	BD Pharmingen	553664
Anti-mouse IgG	Biotin	-	1/100	Serotec	Star 70
Anti-mouse IgG	FITC	-	1/1000	Serotec	Star 11b
NK1.1	BIO	PK136	1/2000	BD Pharmingen	553163
I-E	PE	14-4-45	1/250	BD Pharmingen	553544
$I\text{-}A^b$	FITC	AF6-120.11	1/100	BD Pharmingen	553551
YA-e	Biotin	YAe	1/50	Produced in lab	-
YA-e	Biotin	YAe	1/100	LifeSpan Biociences	LS-C106473
Rat IgG2b Isotype control	PE	-	1/100	BD Pharmingen	556925
Rat IgG1 Isotype control	FITC	-	1/100	BD Pharmingen	554684
Rat IgG1 Isotype control	APC	-	1/100	BD Pharmingen	554686

Table 2-3 Monoclonal antibodies used for flow cytometry staining in this thesis

2.7 Histology: Staining

2.7.1 Paraffin sections and staining

Tissue was placed in 10% formalin solution in PBS and paraffin-mounted. The paraffin sections were then stained with either haematoxylin and eosin (H&E) or elastin van Gieson (EVG) using Weigert's method to delineate the internal elastic lamina (IEL), by the Department of Pathology, Papworth Hospital, Cambridge, UK.

2.7.2 Frozen sections

Tissue was embedded in OCT compound (VWR International, USA), flash frozen in liquid nitrogen, and stored at -80°C. Serial 7µm cryostat sections of the frozen tissue were placed onto Poly-l-lysine coated slides (Sigma-Aldrich Inc.) and fixed in chilled acetone for 10 minutes. Sections were air-dried for 30 minutes and stored at -80°C.

2.7.3 Immunohistochemistry staining

Immunohistochemistry was performed on cryostat sections using the avidin-biotin-peroxidase technique (Vector Laboratories Inc.) for detection of the complement by-product C4d. Sections were thawed and rehydrated in PBS, and tissue peroxidase activity was quenched by incubation with 0.3% H_2O_2 in PBS for 10 min. The sections were washed in excess PBS and incubated with Avidin D blocking solution (Vector Laboratories Inc.) for 15 minutes, re-washed, and then incubated with Biotin blocking solution (Vector Laboratories) for 15 minutes. Non-specific binding sites were blocked by incubating sections with 5% Rabbit serum (Sigma) in PBS (blocking solution) for 20 minutes. Sections were incubated with primary antibody - purified anti-C4d (1D62, ABCAM) diluted in the blocking solution at room temperature for 30 minutes. Bound primary antibody was detected by incubation with biotinylated secondary antibody (anti-rat IgG [ABCAM]) for 30 min followed by incubation with ABC [avidin and biotinylated horseradish peroxidase (HRP) complex] solution (Vector Laboratories Inc.) for 30 minutes. All incubations were carried out in a humidified chamber and followed by two 5 minute washes in excess PBS.

For development, sections were incubated with 0.3mg/ml DAB with 0.03% hydrogen peroxide for 30-45s. The sections were subsequently counterstained with Harris' haematoxylin (Sigma-Aldrich Inc.) for 1s. Sections were dehydrated with increasing concentrations of ethanol, cleared in xylene, and mounted in DPX (Sigma-Aldrich Inc.). This technique was performed by Sylvia Rehakova

2.7.4 Immunofluorescence staining

Sections were incubated with 5% BSA (Sigma-Aldrich Inc.) and 4% goat serum (Sigma-Aldrich Inc.); or 5% Rabbit serum in PBS (In the case if C4d staining) for 1 hour at room temperature to block non-specific binding sites. Sections were incubated with primary antibodies conjugated to a fluorochrome; or antibodies conjugated to biotin, followed by incubation with fluorochrome-conjugate secondary antibody (Table 2.4). Antibodies were diluted in blocking solution and each incubation was carried out in a humidified chamber in the dark for 1 hour, followed by two washes in excess PBS for 10 minutes. Sections were counterstained with 20% Harris' haematoxylin (Sigma-Aldrich Inc.) for 20 seconds, washed in excess distilled water and mounted in FluorSave™ reagent (Calbiochem®, Merck-Millipore).

Monoclonal antibodies used for immunofluorescence staining are detailed in Table 2.4.

Antibody	Conjugate	Clone	Working dilution	Source	Catalogue number
Streptavidin	Cy2	-	1:500	Jackson	016-220-684
Goat anti-rat IgG	Cy3	-	1:500	Jackson	112765-143
B220	Purified	RA3-6B2	1:200	BD Pharmingen	553084
PNA	FITC		1:100	Vector Labs	Y0626
GL-7	Biotin	Gl-7	1:100	E Bioscience	13-5902-85
IgG2 ακ isotype	Biotin	FC-1071	1:250	BD Pharmingen	553982
IgM κ1 isotype	Biotin	R4-22	1:250	BD Pharmingen	550342
CD31	Purified	MEC 13.3	1/250	BD Pharmingen	557355
I-E	FITC	14-4-45	1/50	BD Pharmingen	558846
$I\text{-}A^b$	FITC	AF6-120.11	1/50	BD Pharmingen	553551
C4d	Purified	16D2	1/100	ABCam	ab11863
C4d secondary (anti-rat IgG)	Biotin	-	1/100	ABCam	ab6673

Table 2-4 Antibodies used for immunofluorescence staining in this work

2.8 Histology: Imaging and analysis

2.8.1 Microscopic imaging

Sections were viewed using an IX81 microscope with a 20X0, 70 UplanApo lense (Olympus, Japan). Images were photographed using an ORCA-ER digital camera (Hamamatsu Photonics, Japan) and acquired with CellR 2.6 software (Olympus Imaging Solutions, Germany).

2.8.2 Quantification of chronic allograft vasculopathy (AV)

Chronic allograft vasculopathy was assessed by morphometric analysis of EVG stained vessels using CellR digital imaging software (Olympus, Tokyo, Japan). Percentage cross-sectional area luminal stenosis was calculated as demonstrated in Figure 2.7 using the equation:

$$\frac{\text{Area within internal elastic lamina} - \text{Area of lumen}}{\text{Area within internal elastic lamina}} \times 100\%$$

All elastin-positive vessels in each section were evaluated, with an average of 10 vessels/ heart examined. The severity of vasculopathy, parenchymal damage and cellular infiltration were also qualitatively assessed by an experienced cardiothoracic histopathologist (Dr. Martin Goddard, Consultant Cardiothoracic Histopathologist, Papworth Hospital, Cambridge, UK) who was blinded to the study groups.

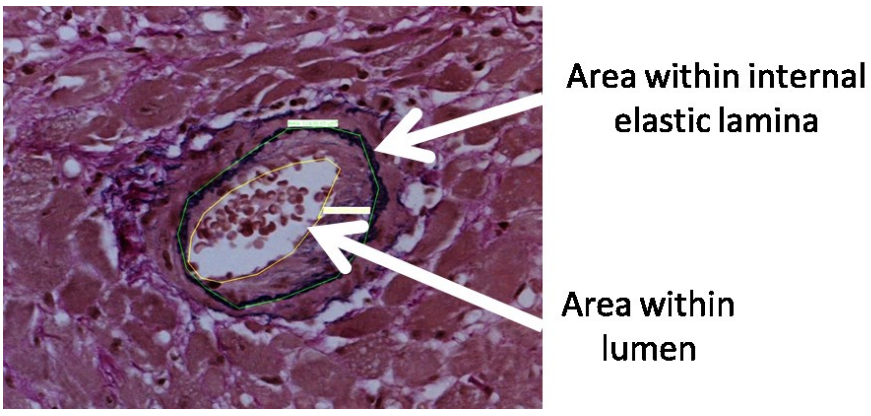


Figure 2.7 Calculating luminal stenosis
A representative light photomicrograph of an EVG stained paraffin section demonstrating the areas quantified for calculation of luminal stenosis (the area within internal elastic lamina and the luminal area).

2.8.3 Quantification of germinal centre activity

To assess the degree of germinal centre (GC) formation within the spleens of allograft recipients, 7µm sections were stained with B220 and GL7 as described in Section 2.7.4. The percentage of follicles with GCs was derived by dividing the total number of B220+ follicles within a random section by the number of GL7+ GCs:

$$\frac{\text{Number of GL7 positive GCs}}{\text{Number of B220 positive follicles}} \times 100\%$$

2.9 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism (v 5.03; GraphPad Software Inc, CA, US.). The Kaplan-Meier method was used to plot graft survival, with the Log rank (Mantel-Cox) test used to compare groups. For other variables, data was analysed using the unpaired *t*-test, or the Mann-Whitney test for non-parametric data. Comparison of antibody levels over the course of an experiment was performed using two-way analysis of variance. $p < 0.05$ was considered statistically significant.

3 Model to study direct and indirect allorecognition reveals a role for passenger lymphocytes in augmenting alloimmune responses following transplantation

3.1 Introduction

3.1.1 Transgenic CD4 T cells

The development of T cell receptor (TCR) transgenic mice has been a significant advance in immunology. Several CD4 TCR transgenic mice have been developed to permit detailed analysis of specific immunological pathways. These mice possess insertion of a transgene encoding a defined, antigen specific, rearranged TCR. The mice are then crossed onto a *Rag*$^{-/-}$ background to prevent endogenous TCR rearrangement, thus eliminating non-transgenic specificity CD4 T cell expression. This also prevents B cell and CD8 T cell development. Thus, the mice possess a monoclonal population of CD4 T cells of defined specificity. These mice, and their CD4 T cells, can be used to undertake detailed investigation of antigen specific responses, and have been widely used to study the alloimmune response following transplantation. Transgenic CD4 T cells can be adoptively transferred in a number large enough to permit detection and tracking by knowledge of their specific Vα and Vβ subunit expression, but small enough to behave in a physiological manner[277]. In this work, four TCR transgenic strains have been utilised: ABM, TCR75, TEa and Mar (Figure 3.1).

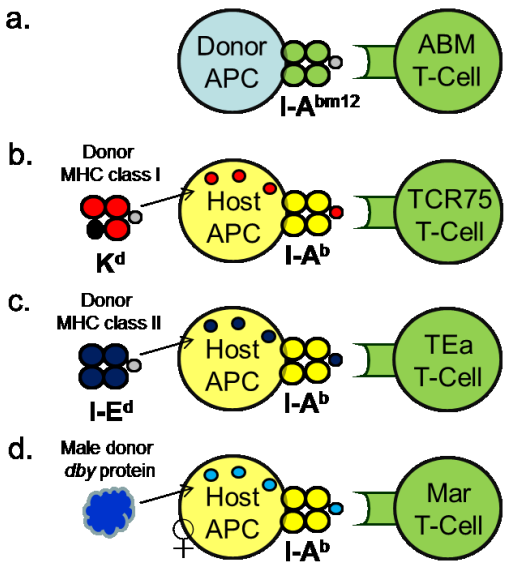


Figure 3.1 Antigen specificity of T cell receptor transgenic strains
a) ABM – direct-pathway, b) TCR75 – indirect-pathway: MHC class I H-2K^{d}, c) TEa – indirect-pathway: MHC class II I-E and d) Mar – indirect-pathway: minor histocompatibility H-Y antigen.

3.1.1.1 ABM

The ABM strain was generated by Palmer and colleagues from a TCR shown to be positively selected in C57BL/6 mice and negatively selected in bm12 mice. In the initial report describing their production, ABM mice were used to study TCR motifs which regulate positive selection[261]. This strain carries Vα2.1 and Vβ8.1 gene segments as transgenes, from the 3BBM74 CD4 T cell clone that recognises I-A^{bm12} by the direct-pathway, in a peptide degenerate fashion.

3.1.1.2 TCR75

The TCR75 transgenic strain was developed by Bucy and colleagues for detailed study of indirect-pathway CD4 T cell responses[162]. This strain carries the Vα1.1/Jα33 and Vβ8.3/Jβ1.4 transgenes from the IκB7/5 CD4 T cell clone that reacts to amino acids 54-68 of the H-$2K^d$ Class I MHC presented in the context of the Class II MHC I-A^b (K^d54-68/I-A^b epitope). An allelic polymorphism at the CD90 locus has also been introduced in this strain to distinguish its cells from those of other C57BL/6 strains by cell surface expression of the Thy1.1 antigen.

3.1.1.3 TEa

The TEa transgenic strain was developed by Rudensky and colleagues originally for the detailed study of positive and negative thymocyte selection[260]. This strain carries the Vα2.3Jα11 and Vβ6Dβ2Jβ2.6 transgenes from the p8.3 CD4 T cell clone that reacts to amino acids 52-68 of the I-E MHC class II α chain presented in the context of the Class II MHC I-A^b (I-Eα52-68/I-A^b epitope).

3.1.1.4 Mar

The Mar transgenic strain was developed by Di Santo and colleagues originally to investigate the role of γ chain-dependent cytokines in the homeostasis of antigen-specific CD4 T cells *in vivo*[123]. This strain carries the Vα1.1-Jα35 and Vβ6-Jβ2.3 transgenes from the Marylin CD4 T cell clone that reacts to amino acids 608-622 of the H-Y minor histocompatibility antigen *dby* presented in the context of the Class II MHC I-A^b (*dby* 608-622/I-A^b epitope).

3.1.2 Bm12 to C57BL/6 model of chronic rejection

Experimental work in this thesis is based upon the bm12 to C57BL/6 model of chronic rejection. The bm12 strain was identified as a naturally occurring spontaneous mutant variant of C57BL/6 which differs by only three amino acids in the third hypervariable region of the peptide binding groove of the MHC class II I-A^b, creating the so-called I-A^{bm12} class II molecule[278-280]. The amino acid changes consist of codon 67 (isoleucine to phenylalanine), codon 70 (arginine to glutamine) and codon 71 (threonine to lysine) (Figure 3.2). As a consequence of the Eα gene being deleted in the H-2^b haplotype, these two strains cannot produce functional MHC class II I-E molecules[255]. Thus, the bm12 strain possesses only a limited class II mismatch, but is identical at MHC class I and minor histocompatibility loci.

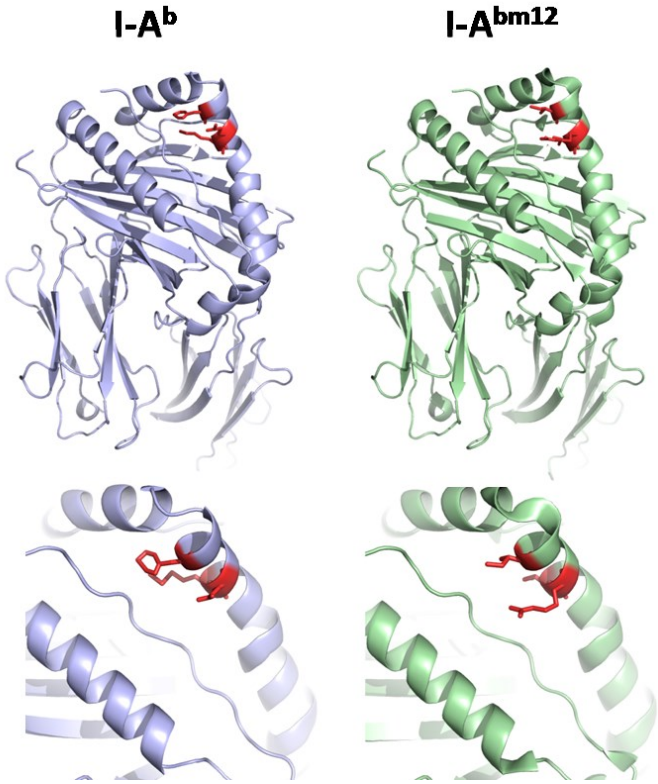


Figure 3.2 I-A^{bm12} MHC class II is mismatched from I-A^b at just three amino acid loci
Molecular ribbon diagram demonstrating the position and structural differences between the I-A^{bm12} and I-A^b MHC class II molecules consequent upon the three amino acid disparity (highlighted red). Courtesy of Dr Dermot Mallon, Department of Surgery, University of Cambridge

3.1.2.1 Rejection in the bm12 to C57BL/6 model

As a consequence of this limited antigenic mismatch, a much reduced proportion of recipient T cells are found to be reactive to bm12 allografts – a precursor frequency of ~2.5% compared to the 21% typical of complete MHC mismatches[50]. Despite this, bm12 skin grafts are acutely rejected by C57BL/6 recipients[255, 279, 281] and cardiac allografts are rejected chronically with development of allograft vasculopathy (AV) and consequent parenchymal injury[274, 281, 282].

Rejection in this model appears to be dependent upon recipient T cells. Early work in this model demonstrated that AV could be observed following transplantation into RAG2KO recipients following reconstitution with recipient T cells (CD4 and CD8), in the absence of B cells[283]. Furthermore, they demonstrated that this was dependent upon IFNγ, since no rejection was observed when IFNγ knock-out T cells were transferred, and AV is markedly attenuated when bm12 allografts were transplanted into IFNγ knock-out recipients[281]. In keeping with these findings, AV is significantly diminished in CD8 T cell knock-out recipients suggesting that CD8 T cells play a role in allograft rejection through their cytotoxic activity and IFNγ secretion[284]. It is worth considering that donor and recipient strains share MHC class I loci, and so CD8 T cells cannot recognise allograft cells by the direct-pathway. Instead, they presumably recognise disparate I-A^{bm12} peptides presented in the context of shared MHC class I molecules (indirect-pathway CD8 T cell recognition) presented by donor APCs.

Further examination of this model has highlighted that both direct and indirect-pathway CD4 T cells are important to allograft rejection, where CD4 T cells recognise intact I-A^{bm12} or self-restricted processed I-A^{bm12} allopeptide respectively. The direct-pathway appears capable of driving allograft rejection since AV is observed when bm12 cardiac allografts are transplanted into H2-M C57BL/6 mice which express normal levels of MHC class II but possess a defect in peptide loading[285]. In these recipients, CD4 T cells can only recognise I-A^{bm12} by the direct-pathway. However, the importance of indirect-pathway CD4 T cells is demonstrated by long-term survival and absence of AV in bm12 cardiac allografts when transplanted into ABM.$RAG^{-/-}$ mice – possessing a monoclonal population of CD4 T cells recognising I-A^{bm12} by the direct-pathway[102]. Further evidence was provided by demonstrating accelerated rejection in recipients previously primed with bm12 peptide[274]. It is not clear how indirect-pathway CD4 T cells themselves would mediate allograft injury since their epitope is not expressed by the allograft. It is more likely that indirect-pathway CD4 T cells drive cytotoxic CD8 T cell or humoral responses.

Although rejection has been seen to occur in the absence of B cells[283], humoral immunity also appears to contribute, since rejection is significantly delayed in C57BL/6.μ-MT recipients which lack B cells[274]. Furthermore, endothelial IgG deposition in bm12 cardiac allografts has been confirmed[102] and detailed histological examination of donor cardiac allografts reveals additional features characteristic of humoral alloimmunity such as vascular proliferation, fibrinoid necrosis and complement C4d deposition[274].

3.1.2.2 Antinuclear autoantibody development

Despite the above evidence of humoral alloimmunity work from our laboratory, and others, has demonstrated that no anti-bm12 alloantibody develops following challenge[274, 279]. Considering the limited nature of the MHC class II mismatch, this likely reflects the absence of a conformational epitope for B cell allorecognition. However, high levels of antinuclear autoantibody were discovered in recipients, as detected by HEp-2 indirect immunofluorescence[274]. Different patterns of HEp-2 staining were observed for different recipients, suggesting that a range of autoantibody specificities are generated and that this differs between recipients. This work also implied an effector role for autoantibody in allograft rejection, as suggested by reduction in severity of AV when autoantibody responses were prevented, and by accelerated rejection in recipients primed for humoral autoimmunity.

It is not immediately obvious why a bm12 cardiac allograft should drive the development of autoantibody. Eisenberg's group has contributed significantly to our understanding of this. They observed that adoptive transfer of bm12 splenocytes resulted in development of antinuclear autoantibodies and an SLE like autoimmune phenotype[286]. They extended this finding to demonstrate that this was a Graft versus Host (GvH) cognate 'direct-pathway' interaction between the donor CD4 T cells and recipient B cells[286, 287]. They generated mixed C57BL/6 and bm12 bone marrow chimeras and demonstrated that upon transfer of bm12 splenocytes only C57BL/6 B cells produced autoantibody, despite bm12 B cells being subject to potential bystander activation[287]. In C57BL/6 recipients they observed that the entire recipient B cell population becomes activated – as would be expected since all recipient B cells express allogeneic I-A^b, regardless of BCR specificity, and so should receive equal direct-pathway help, which should in theory lead to a polyclonal antibody response[288]. That only autoantibody is produced was postulated to be due to antigen availability at the time of B cell activation[289].

Work from our laboratory has extended these findings to the cardiac transplant model. In a similar manner, when T cell depleted bm12 donors (bm12.TCRKO strain or T cell depletion with anti-CD4) were transplanted into C57BL/6 recipients, no autoantibody developed and reduced AV observed[274]. These findings suggest that passenger CD4 T cells carried with the cardiac allograft activate recipient B cells in a GvH manner by cognate direct-pathway recognition of recipient I-A^b, driving the development of autoantibody. This process can occur because murine CD4 T cells do not express MHC class II and therefore are not recognised as allogeneic by the recipient in this strain combination.

One question arising from these observations is why does global GvH activation of recipient B cells only result in autoantibody generation? Eisenberg alluded to the concept that specificity of antibody generated in the context of GvH B cell activation is dependent upon antigen availability[289]. Recent work from our laboratory has sought to examine the hypothesis that plasma cell differentiation requires concurrent BCR ligation in addition to direct-pathway cognate donor CD4 T cell help. T cell deficient C57BL/6 mice (*Tcrbd*$^{-/-}$) were adoptively transferred with bm12 CD4 T cells and immunised with ovalbumin. Control mice received bm12 CD4 T cells only. Whilst both groups developed autoantibody, only those immunised with ovalbumin developed IgG anti-ovalbumin antibody (Ines Harper, Department of Surgery, manuscript in preparation). This has also been observed with alloantigen challenge. These results suggest that although global B cell activation is observed, differentiation into antibody secreting plasma cells required concurrent ligation of the BCR, conforming to the 2-signal hypothesis of B cell activation[290].

3.1.2.3 How does autoantibody mediate allograft rejection and is autoantibody important in clinical transplantation?

There has been increasing appreciation that autoantibodies may contribute to allograft injury and rejection in clinical transplantation[291-293]. Indeed the development of humoral autoimmunity has been reported for all solid organ allografts and there is increasing association with poorer allograft outcomes. For example, anti-vimentin autoantibody (a cytoskeletal protein found within the intima and media of coronary arteries) has been observed in some human cardiac transplant recipients and has been associated with accelerated AV development[294, 295]. Similarly, anti-endothelial cell autoantibody has been associated with higher rates of cellular rejection following renal transplantation[296].

What remains unclear though is how autoimmunity develops in human transplant recipients. The prevailing view is that following transplantation the inflammatory milieu, perhaps exacerbated by ischaemia-reperfusion injury, and ongoing tissue remodelling, leads to exposure of cryptic self-antigens on graft endothelium, normally not available for immune recognition[297, 298]. This may also be a consequence of damaging innate and adaptive alloresponses[299]. It has also been observed that intra-cellular proteins can be translocated to the cell-surface during apoptosis[300]. The exposure of cryptic autoantigens in the context of activated innate and adaptive alloresponses may be sufficient to trigger autoimmunity and activate auto-reactive CD4 T cells.

Why the damaging effects of autoimmunity, both in human and experimental transplant recipients, appear restricted to the donor allograft remains incompletely understood. Target autoantigens are predominantly intracellular proteins that will be expressed by both donor and recipient, but transplant recipients do not appear to experience detectable systemic autoimmunity.

It is proposed that exposed autoantigens on endothelium as a consequence of the processes described above may then permit endothelial autoantibody binding and subsequent initiation of downstream signalling, as has been reported for anti-HLA antibodies, activating the cells as a possible initiating lesion driving AV and thus mediate its progression directly[301]. It has also been observed that antibody molecules can be internalised by cell surface receptors, and this may permit an alternative route of access for autoantibody to autoantigens and possible activation of endothelial cells[302]. Alternatively, bound autoantibody may act indirectly and target endothelial cells for complement fixation and/or NK cell mediated antibody-dependent cellular cytotoxicity, acting as an opsonin for more efficient antigen processing and presentation to recipient alloreactive CD4 T cells, in turn enhancing humoral and cellular alloimmunity[301, 303].

3.1.2.4 Is there a role for donor CD4 T cells in human transplantation

It is now widely recognised that populations of memory and naïve CD4 T cells reside in non-lymphoid organs and tissues[304-306]. As a result it is likely that solid organ transplantation will deliver substantial numbers of donor lymphocytes to the recipient. Unlike the bm12 to C57BL/6 model, human transplant recipients are almost always significantly more mismatched

and so how long donor lymphocytes survive following transplantation is not clear. Although immunosuppression may prevent them from being rapidly cleared by adaptive responses, innate immune recognition by NK cells would be expected to lead to early clearance[307].

Nevertheless, it is clear from early human transplantation studies that circulating donor lymphocytes are often detectable in recipients, although in small numbers[308]. The impact of passenger donor lymphocytes is incompletely understood, although clinical manifestations have been described. In some patients donor CD4 T cells manifest as devastating acute GvH disease, which is an uncommon but fatal complication following solid organ transplantation[309]. Similarly, several cases of 'passenger lymphocyte syndrome' have been reported, whereby haemolysis in the recipient is triggered by donor B cell recognition of mismatched blood group antigens leading to antibody production[310-312]. It has been postulated that donor CD4 T cells provide help to these B cells since recipient CD4 T cells would be tolerant to these self-antigens.

Thus, the impact passenger lymphocytes have on the alloimmune response to a solid organ transplant, both in humans and mismatched experimental models, remains incompletely understood.

3.2 Aims

The aims of the research described in this chapter are as follows:

1. To develop and characterise a murine model of chronic rejection with which to further examine the direct and indirect-pathways of allorecognition using the described TCR transgenic CD4 T cells.

2. Evaluate the impact of passenger donor CD4 T cells on adaptive alloimmunity in a model based on the bm12 to C57BL/6 but which possesses additional MHC class I and II mismatches*.

* In addressing the second aim there was some overlap with the work of a colleague (I. Harper). Although some of the experiments have been performed in conjunction, all the data presented here is my own experimental work. A manuscript on both our findings is in preparation on which we are joint first authors.

3.3 Results

3.3.1 The direct-pathway *is short-lived, whilst longevity of the in*direct-pathway *is variable*

Longevity of direct and indirect allorecognition pathways was initially examined using four murine cardiac transplant models, each with a single major or minor histocompatibility antigen mismatch (Table 3.1). Heterotopic cardiac transplantation was utilised, since this is the standard vascularised allograft model used in transplant immunological research, having the advantage over other potential vascularised organs that allograft function can be easily assessed by abdominal palpation. Donor and recipient strains were selected to ensure that transferred monoclonal TCR transgenic CD4 T cell populations recognised their alloantigen either directly or indirectly.

Donor	Recipient	Donor antigen mismatch	Transgenic T cell recognising mismatch
bm12	C57BL/6	Class II I-A^{bm12}	ABM - directly
ABOIE	C57BL/6	Class II I-E^{d}	TEa - indirectly
B6.K^{d}	C57BL/6	Class I H-$2K^{d}$	TCR75 - indirectly
C57BL/6 Male	C57BL/6 Female	H-Y Minor antigen	Mar - indirectly

Table 3-1 Donor and recipient mouse strains

To examine longevity of the direct-pathway fluorescently labelled (CFSE) ABM CD4 T were adoptively transferred either on the day of transplant (early) or 28 days later (late). Since CFSE is equally distributed amongst daughter cells at division, each daughter cell possesses half the fluorescence of the parent and CFSE dilution can be used to quantify proliferation as described in Section 2.6.4. For this work, 4-6x10^{6} TCR transgenic splenocytes were adoptively transferred. CD4 T cells represent 20-25% of splenocytes in these transgenic strains (Figure 3.3). Whilst robust proliferation was observed at the early time-point, this was absent at the late time, suggesting priming of the direct-pathway is short-lived (Figure 3.4a).

In a similar manner, priming of the indirect-pathway against donor MHC class I, MHC class II and minor histocompatibility antigen was examined. Indirect-pathway allorecognition of MHC class I derived peptide was found to be long-lived, being equally strong at the early and late

time-points. Indirect-pathway priming against minor histocompatibility, male H-Y, antigen was also long-lived, but there was significant attenuation at the late time point. In contrast, whilst indirect allorecognition of peptides derived from donor MHC class II occurred early, proliferation was absent at the late time point, suggesting, as for direct allorecognition, indirect-pathway priming against MHC class II is short-lived (Figure 3.4b-d). This variability in the longevity of indirect allorecognition has not been previously described.

In this experiment transgenic CD4 T cells, except TCR75, were identified by staining for their TCR α or β subunits (ABM - Vβ8; TEa Vβ6; Mar Vα1.1). Since these subunits are expressed by endogenous CD4 T cells, by chance, a population of CFSE negative cells can be seen on the histograms. TCR75 CD4 T cells were more specifically identified by staining for a congenic marker: CD90.1/Thy 1.1, thus avoiding the endogenous populations.

Since allogenicity of the four donor allografts is potentially different, this may have contributed to the variable proliferation observed since extent of CD4 T cell proliferation will not solely depend upon antigen availability, but other factors such as cytokine milieu. To control for this potentially confounding factor, it was necessary to confirm the longevity of indirect-pathway allorecognition in recipients of a single donor allograft expressing all of the above mismatched alloantigens.

Furthermore, because B6.K^{d} and male C57BL/6 donors also express the recipient MHC class II, I-A^{b}, it would be possible for donor APCs to present alloantigen to the transgenic CD4 T cells via the direct-pathway, potentially complicating interpretation of the previous results.

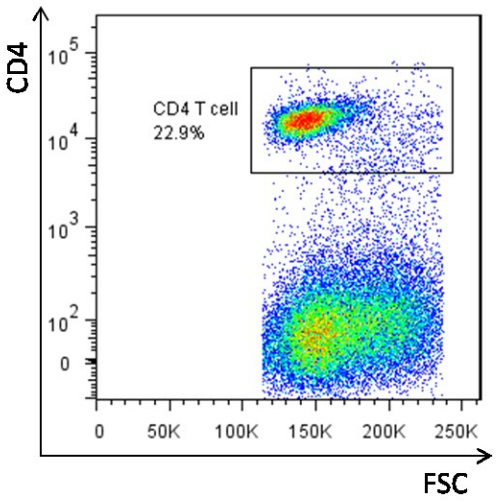


Figure 3.3 Proportion of CD4 T cells in TCR transgenic splenocytes
Representative flow cytometry plot of TEa splenocytes gated on live lymphocytes.

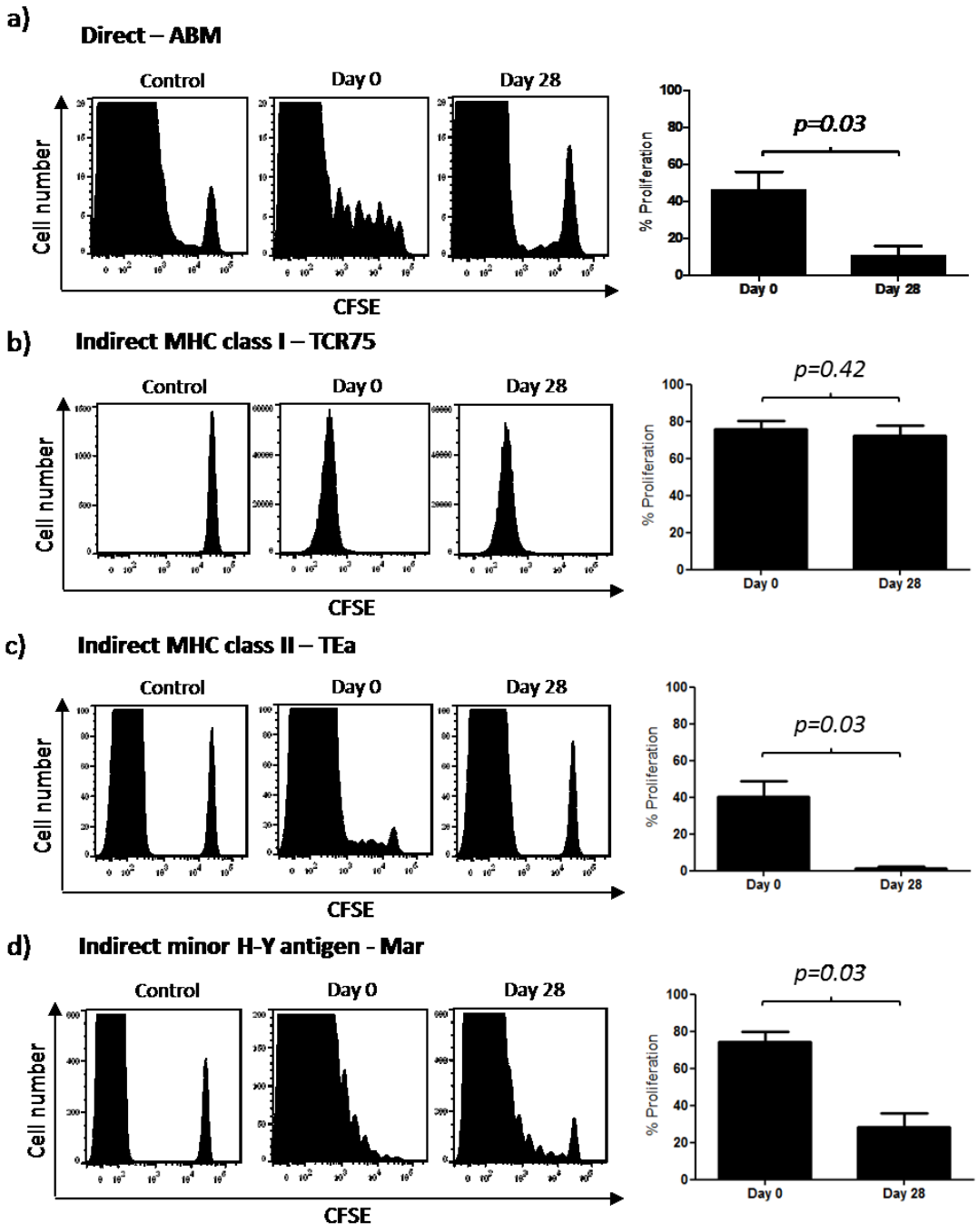


Figure 3.4 Longevity of direct and indirect-pathway allorecognition in individual models

Representative flow cytometry plots and bar chart summarising proliferation following adoptive transfer of 4-6x10^6 CFSE labelled a) ABM splenocytes into C57BL/6 recipients of bm12 hearts b) TCR75 splenocytes into C57BL/6 recipients of B6.K^d hearts; c) TEa splenocytes into C57BL/6 recipients of ABOIE hearts and d) Mar splenocytes into female C57BL/6 recipients of male C57BL/6 hearts, 0 or 28 days following transplantation or naïve C57BL/6 control mice (all groups n=3). Animals were sacrificed 6 days later and splenocytes analysed by flow cytometry. Proliferation of transgenic CD4 T cells quantified using FlowJo software (Tree Star, Oregon). *P* values using the Mann Whitney test.

3.3.2 Developing a new donor strain: bm12.Kd.IE

To overcome the shortfalls of the individual models presented above, a new donor strain was developed that allowed for simultaneous examination of the direct and three indirect-pathway targets. To achieve this, the donor strain was required to express the MHC class II: I-E and I-A^{bm12}, class I: H-$2K^d$ and must NOT express MHC class II I-A^b to prevent any direct-pathway presentation to the indirect-pathway TCR transgenic CD4 T cells. This was achieved by crossing a bm12.Kd strain (I-A^{bm12}, H-$2K^d$) with an ABOIE (I-E^d, I-A^b knock out) – both of which lack expression of I-A^b. The resulting F_1 offspring, referred to henceforth as bm12.Kd.IE, possess the required complement of MHC molecules (Figure 3.5a). Expression of I-A^{bm12} and absence of I-A^b was confirmed by PCR. Prior to experimental use, the expression of H-$2K^d$ and I-E was confirmed by flow cytometry on peripheral blood mononuclear leukocytes (Figure 3.5b,c). When a male bm12.Kd.IE is transplanted into a female C57BL/6, indirect allorecognition of class I, class II and minor H-Y antigen can be examined simultaneously.

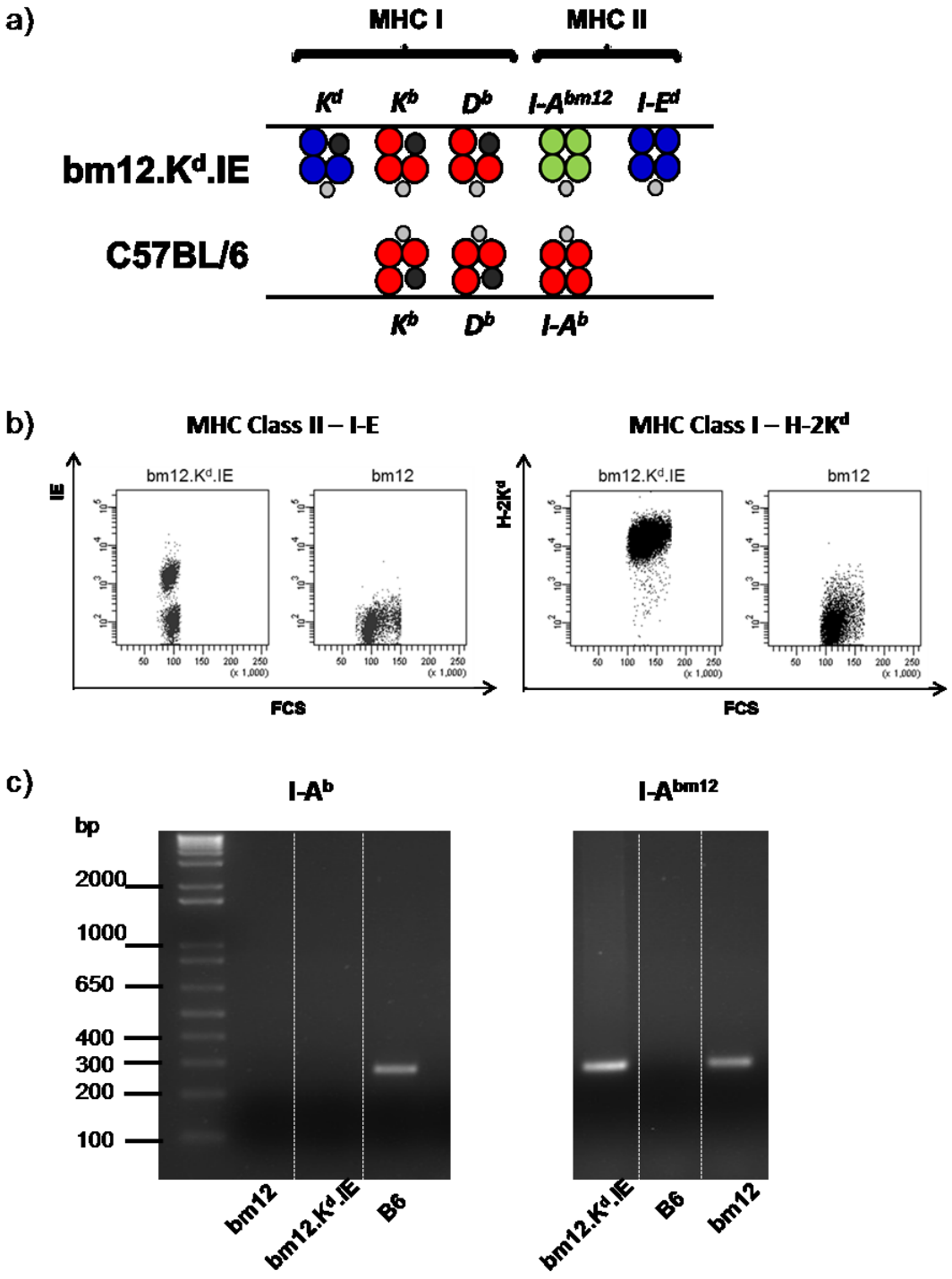


Figure 3.5 Characterisation of the bm12.Kd.IE donor strain

A series of intercrosses were performed between bm12, B6.Kd and ABOIE strains to derive the bm12.Kd.IE strain. (a) Comparison of MHC complement in BM12.Kd.IE and C57BL/6 strains. (b) Flow cytometric demonstration of expression of MHC class I H-2K^{d} (left) and class II I-E antigens (right) on live peripheral blood mononuclear leukocytes from bm12.Kd.IE strain; these are not expressed on parent bm12 strain. (c) PCR genotyping of I-A loci of bm12, B6 and bm12.Kd.IE strains using different primer pairs specific for β chain of the I-A alpha-helix region. The bm12.Kd.IE strain expresses the I-A^{bm12}, but not the I-A^{b}, antigen. (PCR performed by S Rehakova)

3.3.3 Characterisation of the bm12.Kd.IE to C57BL/6 model

3.3.3.1 Graft survival and histopathology

Bm12.Kd.IE hearts demonstrate long-term survival in C57BL/6 recipients with a median survival time of >100 days (n=12). Serial graft histology was reviewed by a consultant cardiothoracic histopathologist (Dr Martin Goddard, Papworth Hospital, Cambridge, UK). At one week following transplantation an aggressively proliferating dense cellular infiltrate was identified. At this early time, minimal myocyte damage was observed and there was no evidence of vascular injury. By three weeks, the infiltrate had subsided with only a few residual inflammatory cells observed, suggesting there is an early 'hit' that resolves. There was evidence of early injury, with areas of myocyte drop-out, and early vascular changes, with endothelialitis seen in some intra-myocardial vessels. Florid endothelialitis was identified in some major coronary vessels. By week seven the grafts showed some areas of replacement fibrosis indicating chronic parenchymal injury. There were also larger areas of myocyte loss. The majority of intra-myocardial vessels demonstrate the presence of concentric proliferative vascular lesions of endothelial and smooth muscle origin, suggesting a vasculopathic process, with associated areas of injury, likely a consequence of chronic ischaemic injury. This is in keeping with the macroscopic scarred appearance, although the majority continue to demonstrate rhythmical contraction. At day 100, although the majority continue to beat, there is a rhythm irregularity in many, and the hearts appear swollen and scarred. Microscopically, the replacement fibrosis, myocyte drop out and vasculopathy have significantly progressed, with some vessels occluded. These histological features are compatible with chronic antibody-mediated damage[149] (Figure 3.6).

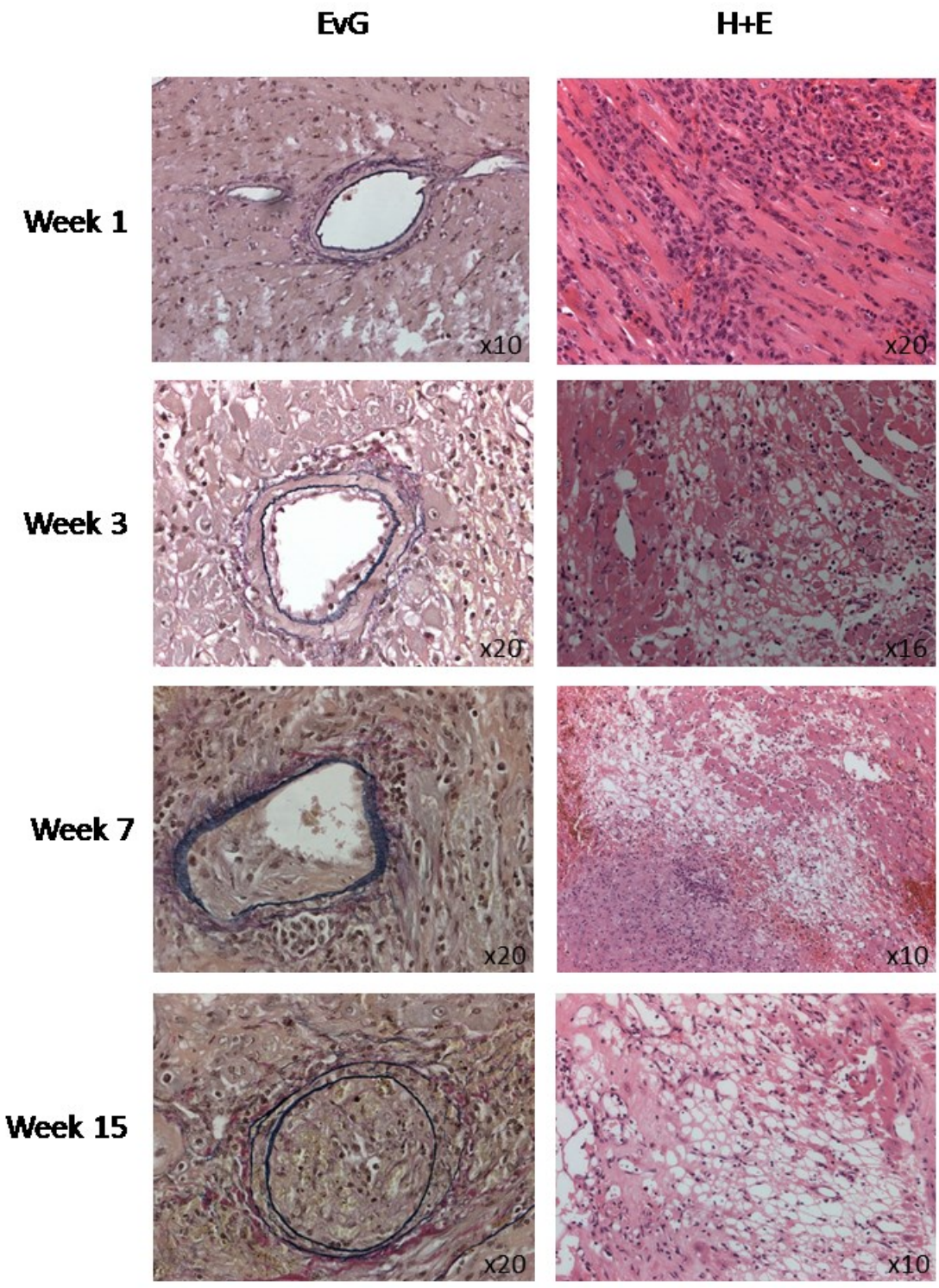


Figure 3.6 Histological evaluation of donor bm12.Kd.IE cardiac allografts following transplantation
Representative photomicrographs of paraffin sections of donor cardiac allografts at 1, 3, 7 and 15 weeks following transplantation. Left panels stained with Elastin van Gieson (EVG) demonstrating the progression of allograft vasculopathy. Right panels stained with haematoxylin and eosin (H+E) demonstrating the early inflammatory infiltrate (week 1) and subsequent progressive myocyte drop-out and replacement fibrosis. Magnification indicated on panels.

In comparison, syngeneic transplanted hearts continue to beat indefinitely. Microscopically, there is no evidence of an early inflammatory infiltrate, nor injury to either the myocardium or the intra-myocardial vasculature, supporting that the findings in the bm12.Kd.IE donor hearts to be attributed to the alloimmune response (Figure 3.7)

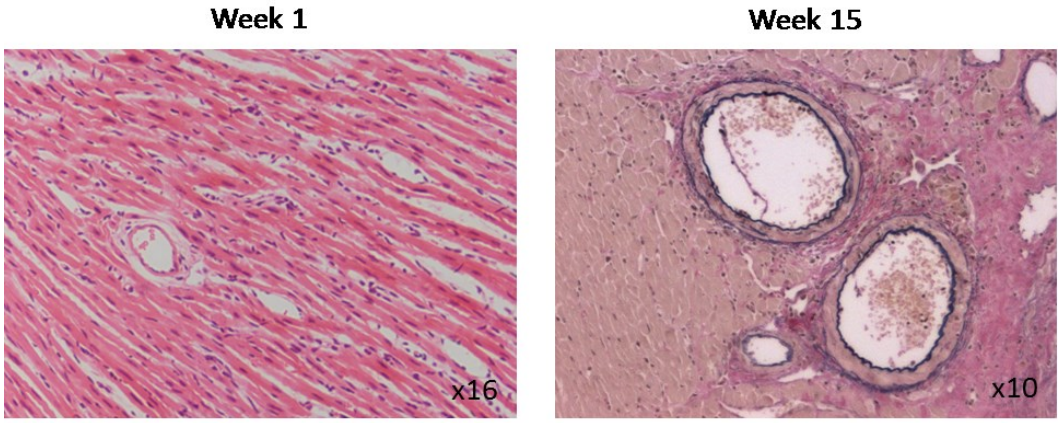


Figure 3.7 Histological evaluation of syngeneic donor cardiac allografts
Representative photomicrographs of paraffin sections of syngeneic donor cardiac allografts. No early inflammatory infiltrate is observed (left panel, haematoxylin and eosin) and there is no development of allograft vasculopathy by 100 days following transplantation (right panel, elastin van Gieson). Magnification indicated on panel.

The extent of AV present in day 100 donor bm12.Kd.IE and syngeneic hearts was quantified morphometrically, measuring the mean luminal stenosis of intra-myocardial vessels (Section 2.8.2). A substantial degree of AV was observed in allogeneic hearts.

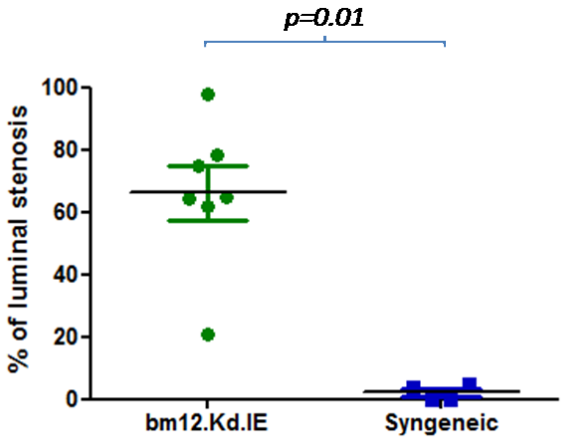


Figure 3.8 Allograft vasculopathy in donor bm12.Kd.IE cardiac allografts
Allograft vasculopathy quantified by calculating luminal stenosis from elastin van Gieson stained paraffin sections of bm12.Kd.IE and syngeneic donor allografts 100 days following transplantation. n=7 in each group. *P* values using the Mann Whitney test.

3.3.3.2 Humoral alloimmune effector responses

Having established that bm12.Kd.IE cardiac allografts provoke a chronic alloimmune response in C57BL/6 recipients, the effector arms of this response were next investigated. The disparate MHC possessed by the donor are: class I H2-K^d, and class II I-E and I-A^{bm12}. Work from our laboratory, and previous studies, have highlighted that due to the similarity to I-A^b, and the absence of a conformational epitope for B cell recognition, C57BL/6 mice do not form anti-I-A^{bm12} alloantibody[274, 279]. In contrast, long-lasting alloantibody responses were detected against both the MHC I H-2K^d and MHC II I-E (Figure 3.9).

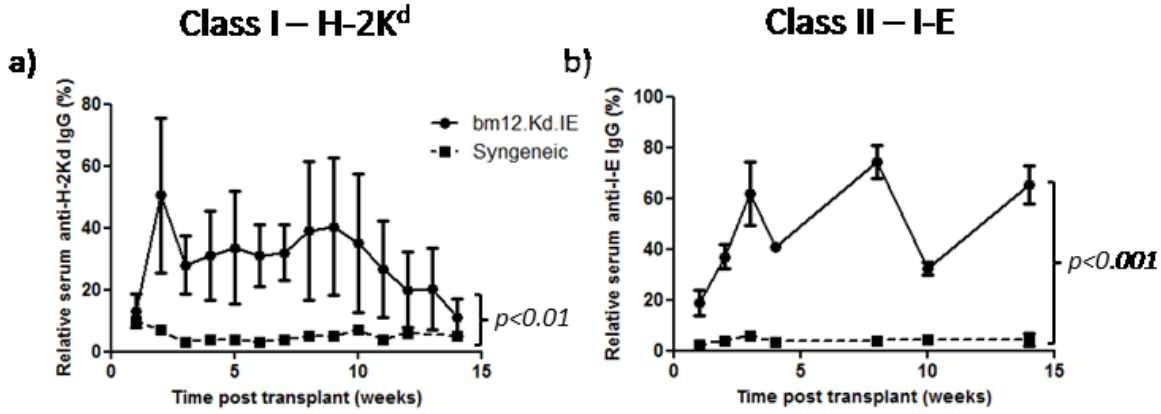


Figure 3.9 Alloantibody generation in recipients of bm12.Kd.IE cardiac allografts
Alloantibody levels were quantified in weekly serum samples from C57BL/6 recipients of bm12.Kd.IE and syngeneic cardiac allografts. Anti-H-2K^d alloantibody (a) quantified by ELISA and anti-I-E alloantibody (b) quantified by flow cytometric labelling of I-E expressing ABOIE strain BMDCs. N=7 in all groups. *P* values using two-way ANOVA

Our laboratory have previously demonstrated that in the bm12 to C57BL/6 model, donor CD4 T cells are responsible for driving the development of autoantibody, through 'graft-versus-host' mediated provision of 'direct-pathway' help to recipient B cells[274]. It was therefore anticipated that a bm12.Kd.IE donor heart may provoke a similar autoimmune humoral response. Indeed this was found to be the case, as significant long-lived autoantibody levels were detected in recipients of bm12.Kd.IE hearts by HEp-2 indirect immunofluorescence (Figure 3.10).

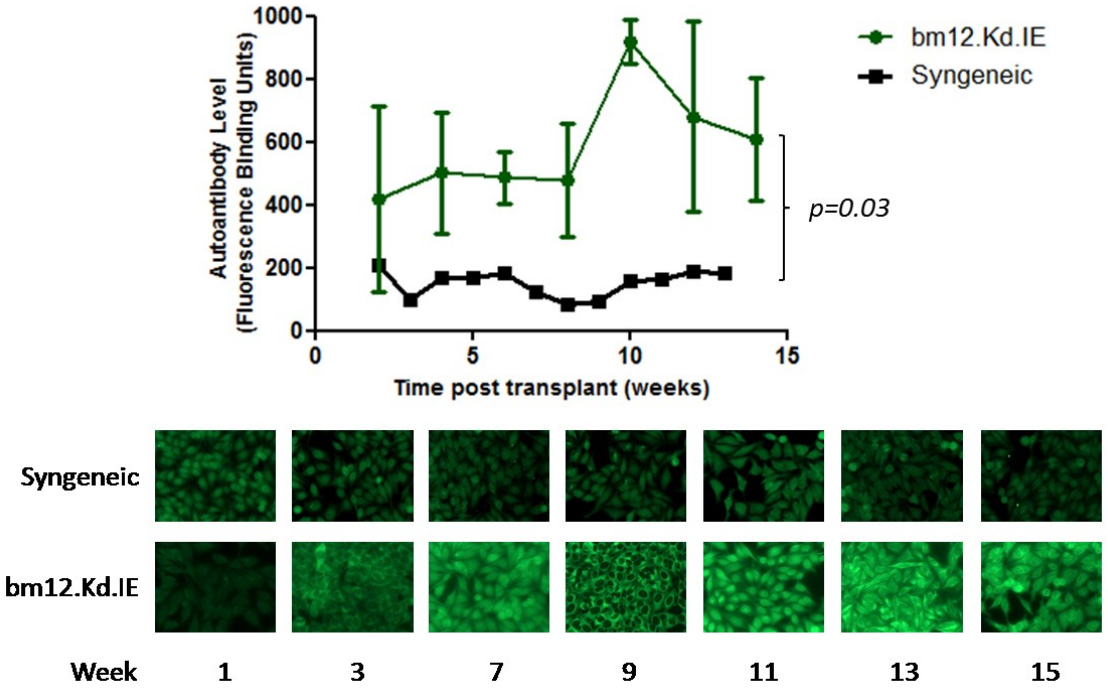


Figure 3.10 Autoantibody quantification using HEp-2 indirect immunofluorescence
Antinuclear autoantibody levels were quantified in weekly serum samples from C57BL/6 recipients of bm12.Kd.IE and syngeneic cardiac allografts. Autoantibody was quantified using HEp-2 indirect immunofluorescence. Representative immunofluorescence photomicrographs shown. N=7 in all groups. *P* values using two-way ANOVA

In keeping with the allo- and autoantibody responses detected, significant germinal centre (GC) activity was detected by immunofluorescence. Germinal centres were quantified by staining cryostat sections of recipient spleen 50 days following transplantation. The proportion of GCs was indicated by identifying the proportion of $B220^{+}$ B cell follicles that also stained positive for GC activity as indicated by GL-7 staining (Section 2.8.3; Figure 3.11).

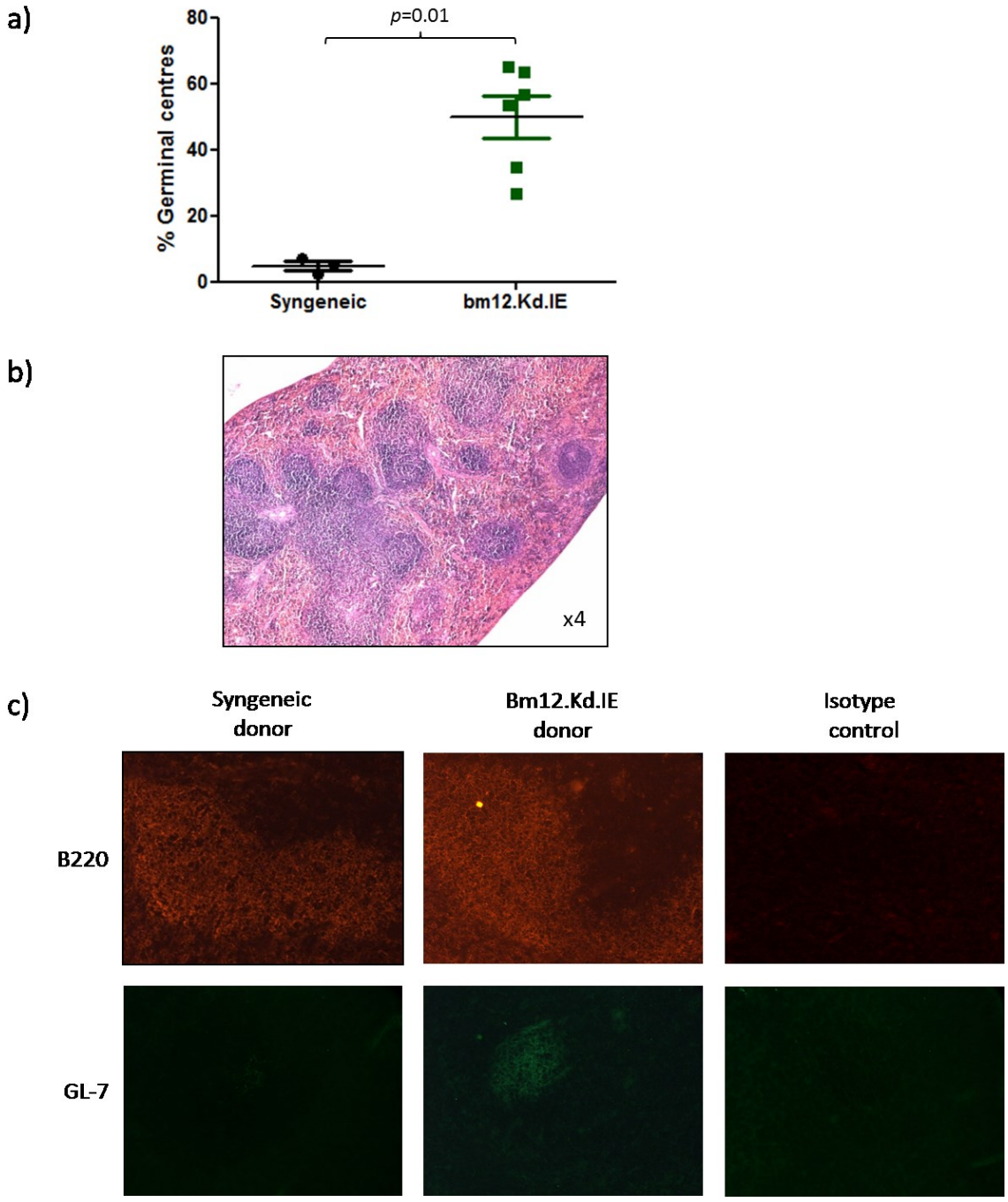


Figure 3.11 Germinal centre activity in cardiac allograft recipients

Analysis of germinal centres in recipients of bm12.Kd.IE and syngeneic cardiac allografts 50 days following transplantation. (a) Germinal centres quantified in recipients of bm12.Kd.IE and syngeneic allografts (n=6, *p* value using Mann-Whitney test); (b) Representative haematoxylin and eosin stained paraffin section of recipient spleen demonstrating follicles; (c) Representative immunofluorescence cryostat sections of recipient spleen stained with B220 (B cell), and GL-7 (germinal centre B cells) at 40X magnification for syngeneic donors (top panels) and bm12.Kd.IE donors (lower panels).

Further examination of the alloantibody response was undertaken by performing an ELISPOT to detect the presence of anti-H-2K^{d} antibody secreting cells, both in the spleen, but also in the bone marrow (although this did not reach statistical significance), where long-lived plasma cells reside. Detection of antibody secreting cells was identified in both locations, suggesting productive GC output with deposition of long-lived plasma cells in the recipient bone marrow.

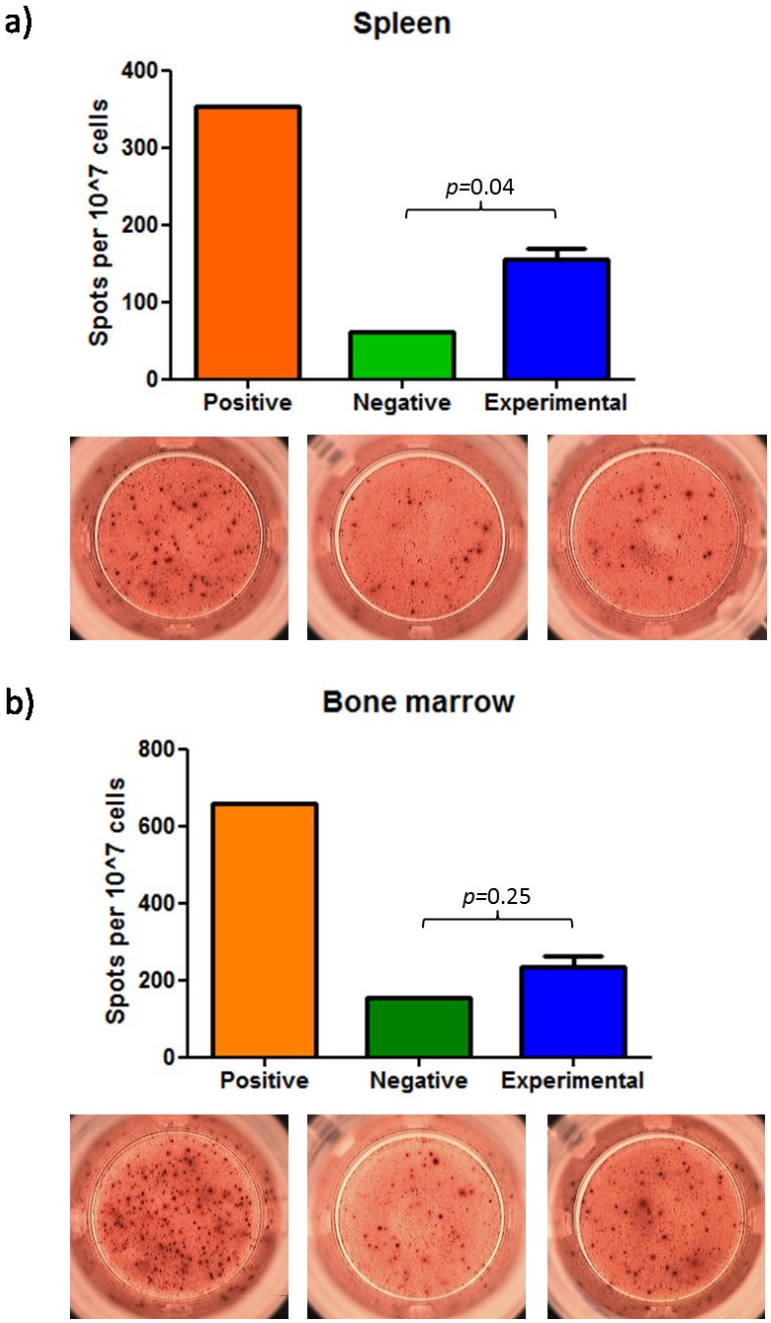


Figure 3.12 H-2K^{d} ELISPOT

Quantification of H-2K^{d} antibody secreting cells in spleen (a) and bone marrow (b) of C57BL/6 recipients of bm12.Kd.IE cardiac allografts 50 days following transplantation by ELISPOT (n=5). Representative images of well shown. *P* values using Mann-Whitney test. (Positive control C57BL/6 recipients of BALB/c cardiac allografts at 7 days following transplantation; negative control naïve C57BL/6)

In keeping with the detection of long-lived humoural responses, histological examination of the hearts, described above, is suggestive of chronic antibody mediated damage. To further confirm a role for antibody in mediating AV, grafts were stained for the complement by-product C4d, which is a well-established clinical marker of antibody-mediated rejection, and C4d staining is recommended by current International Society for Heart and Lung Transplantation guidelines for the diagnosis of antibody mediated rejection[149]. Endothelial C4d deposits could be readily identified, along with diffuse parenchymal staining for C4d, which has been described as a generic feature of ischaemic death, a consequence of the vasculopathic process in these hearts (Figure 3.13).

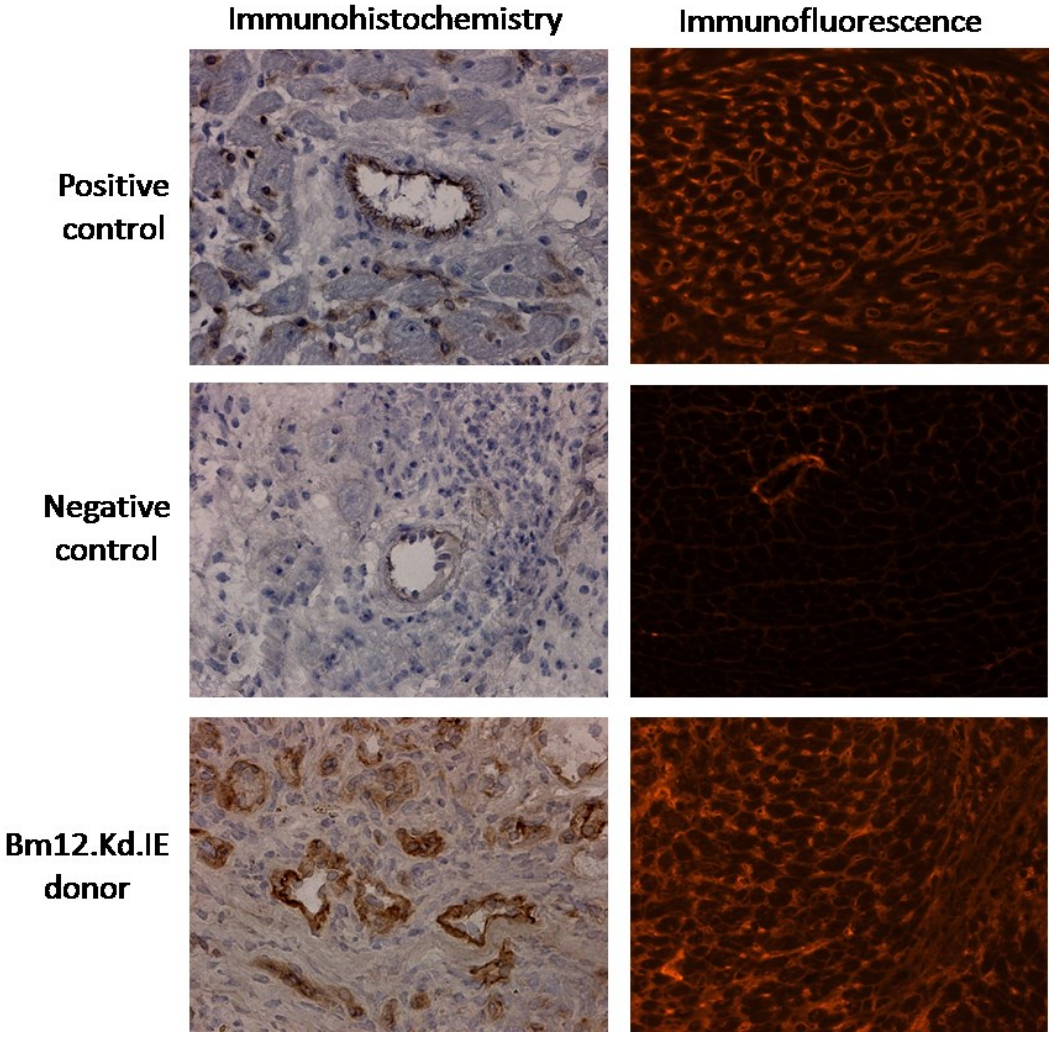


Figure 3.13 Immunofluorescence and immunohistochemical staining for C4d complement deposition
Representative photomicrographs of immunohistochemistry (left panels) and immunofluorescence (right panels) staining for complement by-product C4d of cryostat sections of donor bm12.Kd.IE cardiac allografts harvested 50 days following transplantation (n=7). As negative and positive controls, representative images from syngeneic heart grafts and BALB/c heart allografts that provoked strong humoral alloimmunity in T cell deplete TCRKO recipients reconstituted with TCR75 CD4 T cells respectively (sections courtesy of M Chhabra). All panels x40 magnification.

3.3.3.3 Cell-mediated alloimmune responses

To investigate the presence of a cytotoxic CD8 T cell-mediated arm to the alloimmune response in this model, a CD8 IFNγ ELISPOT was performed. Whilst a robust CD8 T cell alloresponse was detected 7 days following transplantation, by day 12 only minimal reactivity was seen. This may reflect the histological finding of an intense inflammatory infiltrate at 1 week, which almost completely resolves by week 3 following transplantation.

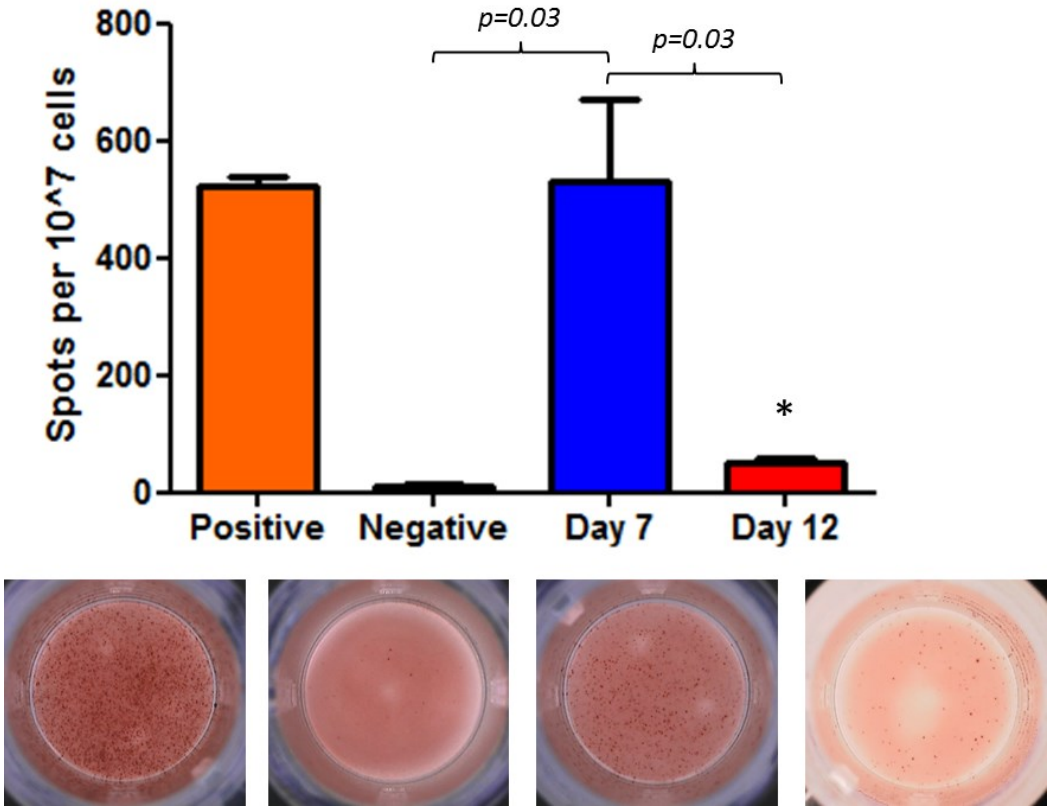


Figure 3.14 CD8 T cell IFNγ ELISPOT
Quantification of cytotoxic CD8 T cell alloimmunity in C57BL/6 recipients of bm12.Kd.IE cardiac allografts 7 and 12 days following transplantation using IFNγ ELISPOT assay (n=5). Irradiated bm12.Kd.IE splenocytes were used as stimulators. Responses compared to those observed in C57BL/6 recipients of a BALB/c cardiac allograft (positive) and naïve C57BL/6 (negative). Representative images of wells presented. *P* values using Mann-Whitney test

3.3.4 Innate immune reactivity

Having characterised the adaptive alloimmune response, I was interested to understand the contribution of the innate immune response to allograft rejection. To examine this, RAG2KO mice, which lack adaptive immunity due to deletion of the recombination-activating 2 gene (*Rag*), preventing maturation of T and B cells, were utilised as recipients. First, bm12.Kd.IE B cells were adoptively transferred i.v. into RAG2KO recipients. B cells, rather than CD4 T cells, were chosen due to their expression of the full allogeneic MHC repertoire. Murine CD4 T cells are thought not to express MHC class II[254], and would therefore not express the allogeneic I-E or I-A^{bm12}. Fully allogeneic BALB/c and syngeneic C57BL/6 B cells were transferred as positive and negative controls respectively. Mice were bled weekly for 7 weeks. Similar levels of circulating bm12.Kd.IE and C57BL/6, but not BALB/c, B cells were identified over this period. Similarly, bm12.Kd.IE B cells were present in RAG2KO recipient splenocytes following sacrifice on day 50 (Figure 3.15). These results suggest that bm12.Kd.IE cells evade innate immune surveillance, perhaps a consequence of the shared expression of MHC I K^b and D^b.

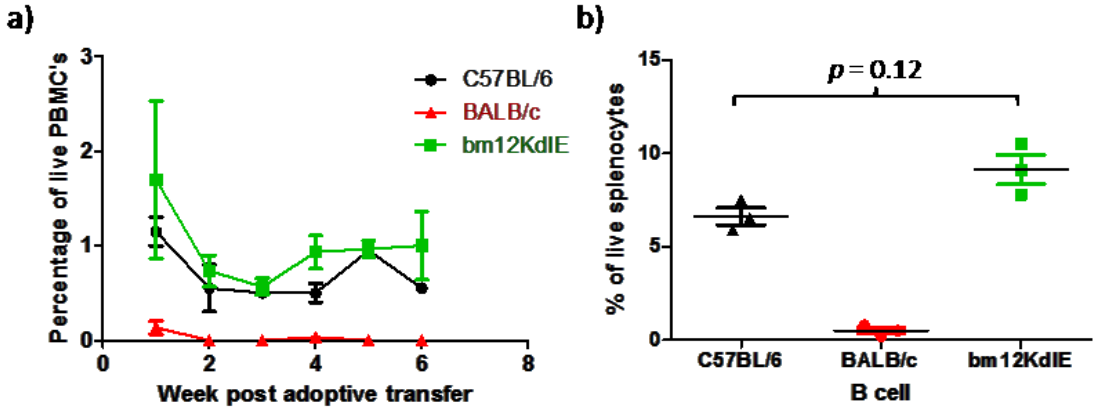


Figure 3.15 Bm12.Kd.IE cells evade innate immune recognition
Percentage of CD19 positive cells within the live lymphocyte gate on (a) weekly bleeds and (b) in splenocytes at 50 days, following adoptive transfer of 1x10^6 MACS purified B cells i.v. into RAG2KO recipients (n=3 in each group). Despite the small percentages of B cells attained, very distinct populations were observed. *P* values using Mann Whitney test. PBMC – peripheral blood mononuclear cells.

Bm12.Kd.IE hearts were then transplanted into RAG2KO recipients, with the expectation that in the absence of innate immune recognition, and without adaptive immunity, long term graft survival should be observed. All grafts continued to beat beyond day 100, and histological examination revealed no evidence of vascular or parenchymal injury (Figure 3.16a). In keeping with the results for B cell transfer, analysis of recipient peripheral blood 7 days following transplant into wild-type and RAG2KO recipients, revealed a circulating population of passenger donor CD4 T cells in RAG2KO but not in wild-type recipients (Figure 3.16b). These results further support the absence of innate immune reactivity to bm12.Kd.IE allografts.

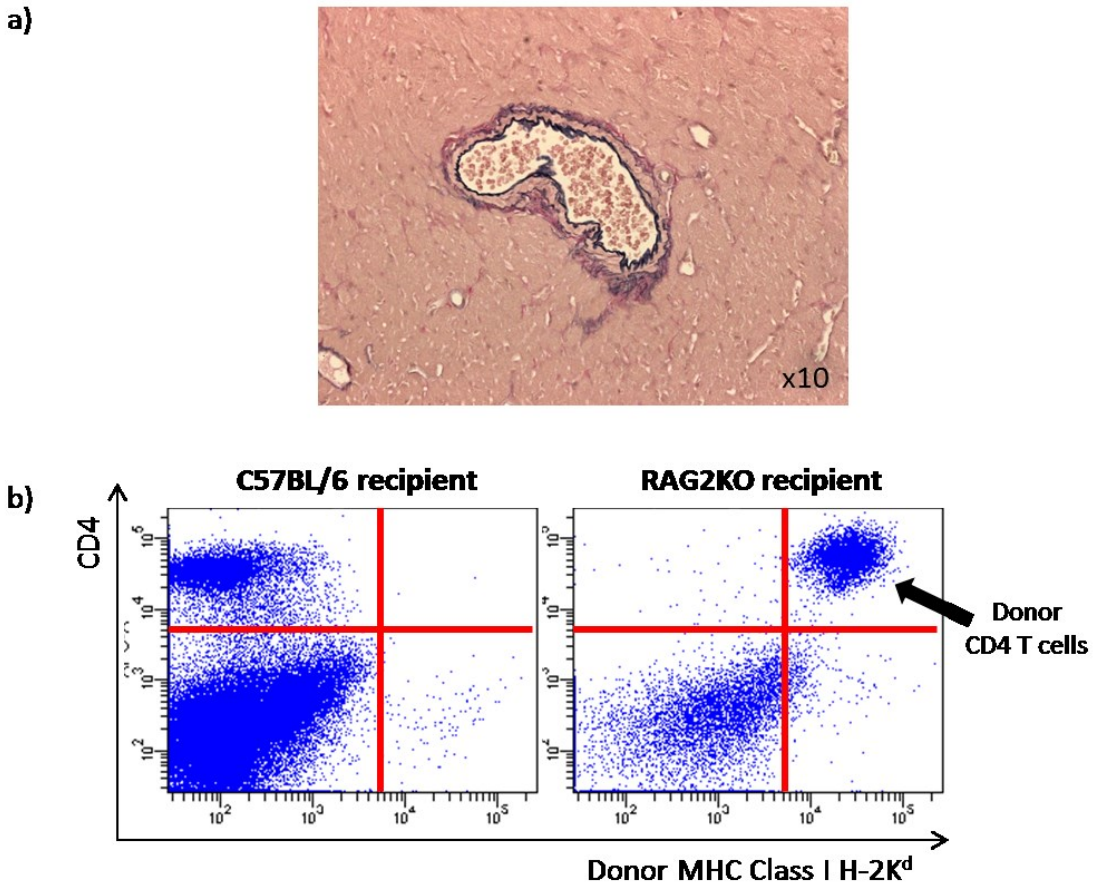


Figure 3.16 Bm12.Kd.IE cardiac allografts do not develop evidence of rejection in RAG2KO recipients
(a) Representative photomicrograph of paraffin sections of syngeneic donor cardiac allografts stained with elastin van Gieson. No evidence of allograft vasculopathy at 100 days following transplantation was observed - magnification indicated on panel. (b) Representative flow cytometry plots of CD4 T cells in recipient peripheral blood 7 days following transplantation of bm12.Kd.IE cardiac allografts into a wildtype (left) or RAG2KO (right) recipient (n=3). A population of donor CD4 T cells (as evidenced by H-2K^d expression) is only identified in RAG2KO recipients.

3.3.5 Donor CD4 T cells augment alloimmune responses

Work previously published from our laboratory has identified donor bm12 CD4 T cells to have a central role in driving the observed autoantibody responses in C57BL/6 recipients by providing direct-pathway GvH help to recipient B cells. Upon depletion of donor CD4 T cells, autoantibody responses do not develop and grafts survive long term with no evidence of myocardial or vascular injury[274]. I was interested to evaluate the role of donor CD4 T cells in this model, aware that unlike bm12 CD4 T cells which survive long-term, bm12.Kd.IE CD4 T cells are cleared by the recipient adaptive autoimmune response within the first week following transplantation (Figure 3.16b). Nevertheless, long-lived autoantibody responses develop in this model and the presumption was that this is similarly driven by donor CD4 T cells.

3.3.5.1 The role of donor CD4 T cells in the bm12.Kd.IE to C57BL/6 model

Donor CD4 T cells were depleted by administration of a depleting anti-CD4 antibody (YTS 191.1; European Collection of Animal Cell Cultures, UK). Two 0.5mg i.p. doses administered to donors, 5 and 1 day prior to transplant, resulted in a significant depletion of circulating, splenic and cardiac parenchymal CD4 T cells (Figure 3.17).

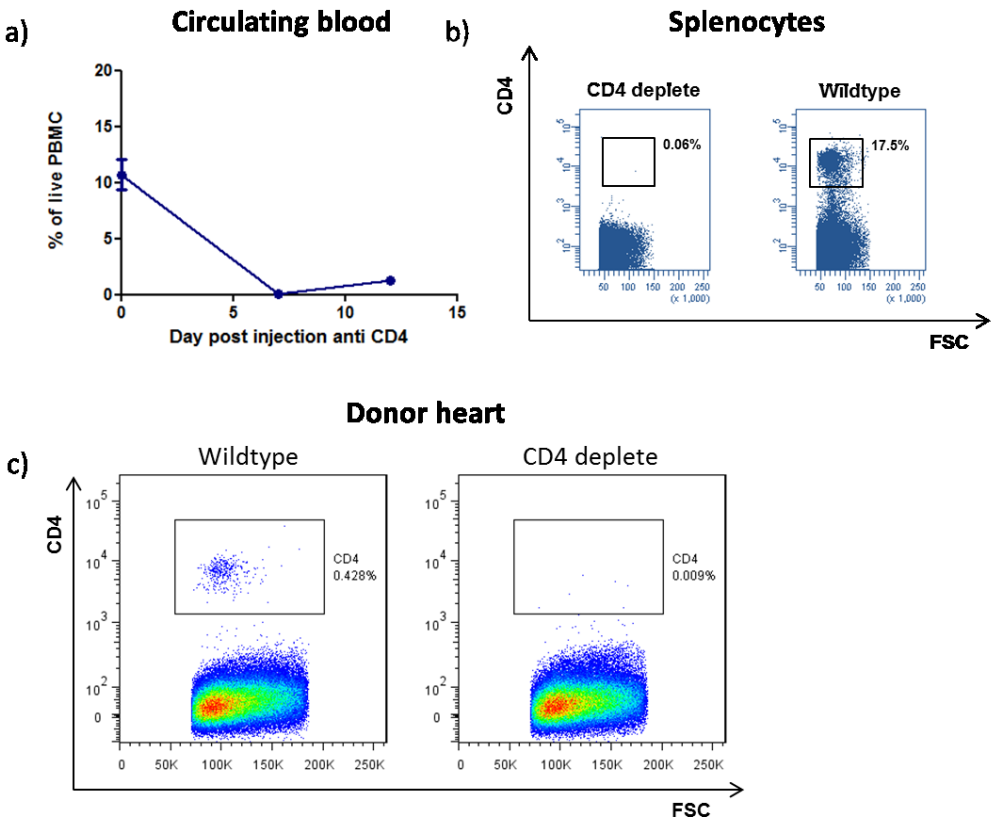
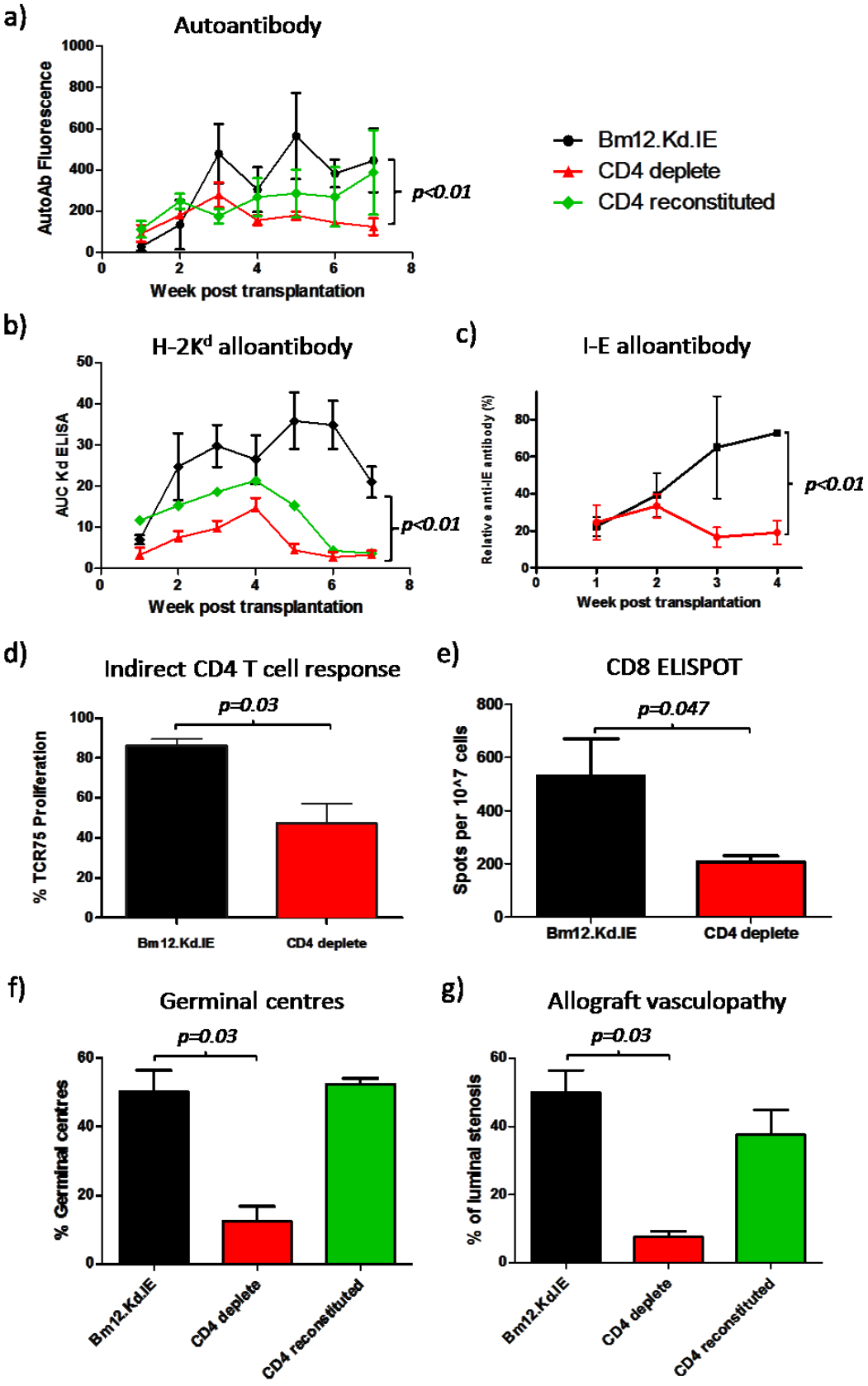


Figure 3.17 Effective depletion of donor CD4 T cells is achieved with depleting anti-CD4 antibody
Depleting anti-CD4 antibody (YTS 191.1) was administered i.p. to recipient mice (0.5mg day -5 and -1 prior to transplantation). Effective depletion was confirmed with flow cytometry (all plots gated on live lymphocytes). (a) No circulating CD4 T cells are present 6 days following administration. On the day of transplantation effective donor depletion of CD4 T cells was observed in both splenic tissue (b) and in cardiac homogenates (c) as compared to an untreated, wildtype donor.

Donor CD4 T cell depletion had a significant impact on the adaptive responses against bm12.Kd.IE cardiac allografts and rejection kinetics. Long-term graft survival was seen with all hearts beating strongly at 100 days (n=8). As was anticipated, from our knowledge of the bm12 to C57BL/6 model, no autoantibody developed in recipients of CD4 T cell deplete donors (Figure 3.18a).

Perhaps more surprisingly was the impact on the alloimmune response. A significant reduction in the anti-H-2K^d and anti-I-E alloantibody responses was observed following donor CD4 T cell depletion (Figure 3.18b,c). Furthermore, depletion of donor CD4 T cells resulted in reduced CD8 alloimmunity as quantified on IFNγ CD8 ELISPOT, and reduced indirect CD4 T cell responses, examined by evaluating the proliferation of TCR75 CD4 T cells transferred four weeks following transplantation to indicate ongoing immunogenic presentation of H-2K^d allopeptide (Figure 3.18d-f). In keeping with this reduction in the autoimmune and alloimmune response, there was a significant reduction in donor myocardial and vascular injury (Figure 3.18g). In support of this role of donor CD4 T cells augmenting the alloimmune responses, reconstituting recipients of CD4 deplete donors with donor CD4 T cells on the day of transplantation restored alloimmune effector responses and consequent chronic rejection (Figure 3.18). These results suggest that donor CD4 T cells, in addition to driving the autoantibody responses observed, also have a role in enhancing the adaptive alloimmune responses.

Figure 3.18 Donor CD4 T cell depletion significantly attenuates auto- and alloimmune responses
Following transplantation of CD4 deplete bm12.Kd.IE donor cardiac allografts into C57BL/6 recipients a significant reduction in auto and alloimmune responses is observed. Autoantibody levels were significantly reduced on weekly serum collections [(a), HEp-2 indirect immunofluorescence]. Additionally, significant reductions are seen in anti H-2K^d [(b), ELISA] and anti-I-E [(c), flow cytometric analysis of binding to I-E expressing BMDCs] alloantibody on weekly serum collection. (d) Indirect CD4 T cell alloresponses were assayed by adoptive transfer of 4-6x10^6 CFSE labelled TCR75 CD4 T cells (recognising H-2K^d allopeptide indirectly) on the day of transplantation and extent of proliferation quantified 5 days later by flow cytometry using FlowJo software for analysis (n=3). (e) Cytotoxic CD8 T cell alloimmunity was assayed with an IFNγ CD8 ELISPOT 7 days following transplantation. (f) Percentage of follicles with germinal centre activity were quantified by immunofluorescence staining (B220 and GL-7) of cryostat sections of recipient spleen at 50 days following transplantation. (g) Allograft vasculopathy was quantified by calculating luminal stenosis on elastin van Gieson stained paraffin sections. Adoptive transfer of 5x10^6 donor (bm12.Kd.IE) enriched CD4 T cells (MACS sorted using anti-CD4 microbeads) on the day of transplantation restored humoral responses and allograft vasculopathy (a,b,f,g). *P* values using two-way ANOVA (a-c) and Mann Whitney test (d-g). n=5 unless otherwise stated.


a)
Autoantibody
AutoAb Fluorescence
Week post transplantation
p<0.01
Bm12.Kd.IE
CD4 deplete
CD4 reconstituted
b)
H-2K^d alloantibody
AUC Kd ELISA
Week post transplantation
p<0.01
c)
I-E alloantibody
Relative anti-IE antibody (%)
Week post transplantation
p<0.01
d)
Indirect CD4 T cell response
% TCR75 Proliferation
p=0.03
Bm12.Kd.IE
CD4 deplete
e)
CD8 ELISPOT
Spots per 10^7 cells
p=0.047
Bm12.Kd.IE
CD4 deplete
f)
Germinal centres
% Germinal centres
p=0.03
Bm12.Kd.IE
CD4 deplete
CD4 reconstituted
g)
Allograft vasculopathy
% of luminal stenosis
p=0.03
Bm12.Kd.IE
CD4 deplete
CD4 reconstituted

3.3.5.2 Memory donor CD4 T cells

In the setting of specific pathogen free mice negligible immunological memory exists. However, in clinical transplantation donors will possess a wide repertoire of memory lymphocytes. Since memory lymphocytes respond more vigorously and have reduced co-stimulatory requirements, the possibility that memory donor CD4 T cells would augment the alloimmune response to a greater extent than naïve CD4 T cells was next explored.

Donor bm12.Kd.IE mice were transplanted with tail skin from C57BL/6 (future recipient strain) mice. Six weeks later, hearts were procured and transplanted into naïve C57BL/6 recipients (Figure 3.19a). Analysis of splenocytes of the donor mice at the time of transplantation demonstrated an increase in proportion of CD4 T cells with a $CD44^{+}$ $CD62L^{-}$ memory phenotype (Figure 3.19b).

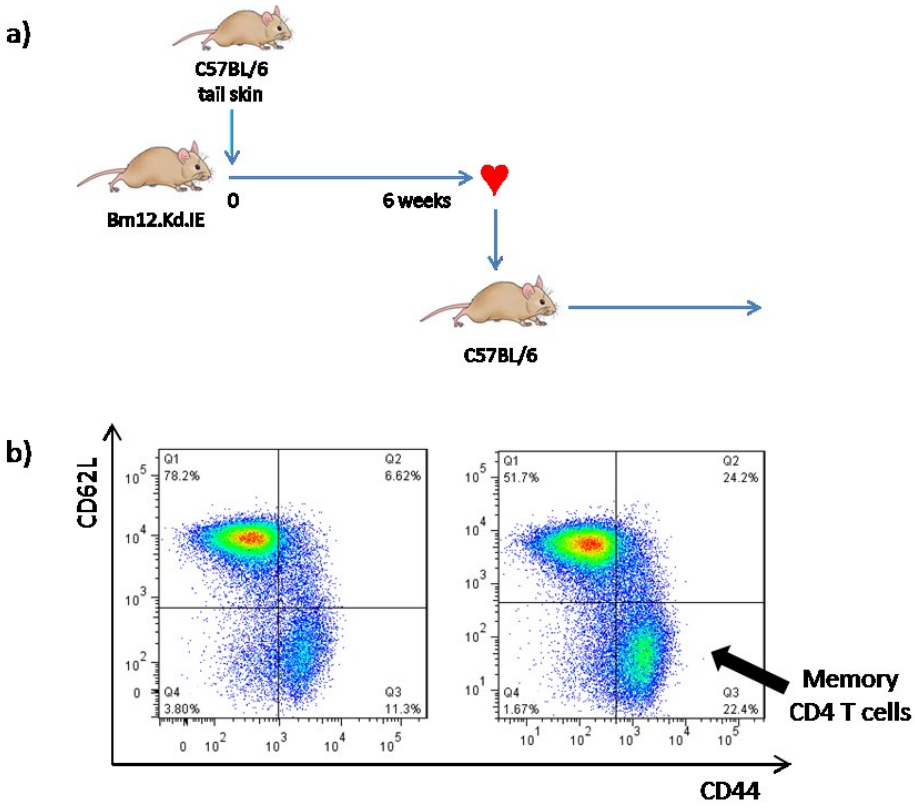


Figure 3.19 Priming of donors to generate recipient strain donor CD4 T cell memory
(a) Donor bm12.Kd.IE mice were transplanted with C57BL/6 tail skin 6 weeks prior to their use as cardiac donors for transplantation into C57BL/6 recipients. (b) At 6 weeks following skin transplantation there was an increase in the proportion of antigen experienced $CD44^{+}$ $CD62L^{-}$ CD4 T cells reflecting this antigenic challenge

In recipients of primed donor cardiac allografts there was a notable acceleration in rejection kinetics, with all hearts rejected by 60 days with a median survival of just 30 days (n=4) (Figure 3.20a). The autoantibody levels quantified in these recipients were significantly augmented in keeping with a primed GvH interaction between donor CD4 T cells and recipient B cells. There was also augmentation of anti-H-2K^{d} alloantibody, and a corresponding increase in germinal centre activity. The degree of AV though was similar to that observed with un-primed donors and this may reflect augmented cytotoxic CD8 alloimmunity contributing to the accelerated rejection observed – since the allografts are rejected within this time (Figure 3.20b-e).

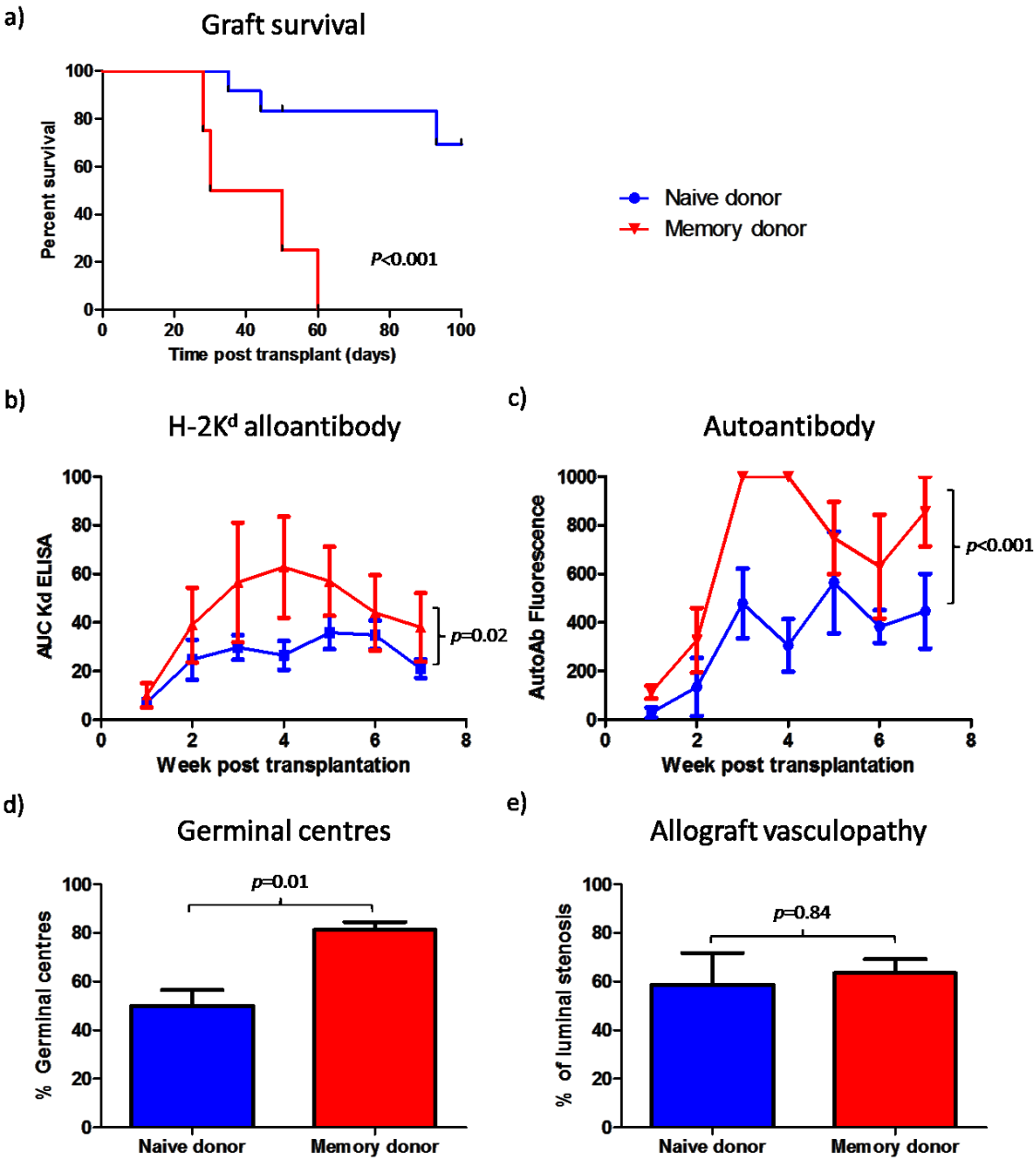


Figure 3.20 Primed donors drive augmented alloimmune responses and accelerate allograft rejection
Bm12.Kd.IE donor hearts, primed with a recipient strain 6 weeks prior, were transplanted into C57BL/6 recipients (n=5). (a) Allograft survival was significantly poorer for primed donor allografts, as assessed by thrice weekly abdominal palpation. There were augmented humoral responses with significantly greater anti-H-2K^d alloantibody [(b), ELISA] and autoantibody [(c), HEp-2 indirect immunofluorescence] levels detected in weekly serum samples. A greater proportion of splenic follicles demonstrated germinal centre activity (d) as assessed by immunofluorescence staining of cryostat sections with B220 and GL-7. Allograft vasculopathy, as assessed on elastin van Gieson stained paraffin sections, was not significantly different (e). *P* values using Log Rank (Mantel Cox) test (a), two-way ANOVA (b,c) and Mann Whitney test (d,e).

3.3.5.3 Donor CD4 T cells become activated, but their survival in recipients is short-lived

Unlike in the bm12 to C57BL/6 model, bm12.Kd.IE donor haematopoietic cells are rapidly cleared by recipient alloimmune effector responses as confirmed by identifying no donor CD4 T cells in recipients at seven days following transplantation (Figure 3.16). This excludes the possibility that donor CD4 T cells are surviving long-term and mediating direct effects against the graft, or providing long-term help to recipient effector alloimmune responses. Rather, it suggests that donor CD4 T cells act to enhance the alloimmune response early and that this enhancement persists long-term despite rapid clearance of the initiating cells.

To confirm that donor CD4 T cells do become activated and undergo proliferation in recipients, CFSE labelled donor bm12.Kd.IE CD4 T cells were adoptively transferred into recipients on the day of transplantation. As above, by seven days following transfer, these were not identifiable as they had presumably been cleared. However, at three days following transfer, there was significant CFSE dilution in a subset of the transferred cells, presumably the alloreactive component of the polyclonal donor CD4 T cell population. This confirms that a proportion of donor CD4 T cells become activated and divide vigorously early after transplantation (Figure 3.21).

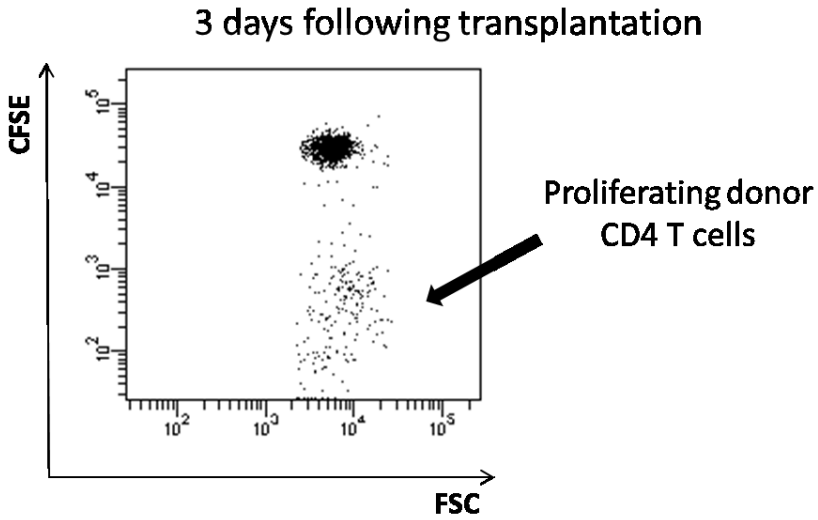


Figure 3.21 Alloreactive donor CD4 T cells undergo significant proliferation following transplantation
$5x10^6$ enriched bm12.Kd.IE CD4 T cells (MACS separated with anti-CD4 microbeads) were CFSE labelled and adoptively transferred into C57BL/6 recipients of CD4 T cell deplete bm12.Kd.IE cardiac allografts on the day of transplantation. Three days later, mice were sacrificed and splenocytes analysed by flow cytometry. Donor CD4 T cells were identified by gating on CD4 T cells that stained positive for H-$2K^d$. Vigorous proliferation of the alloreactive subset of donor CD4 T cells is observed with significant CFSE dilution.

3.3.6 Recipient T and B cells are essential mediators of the accelerated rejection triggered by early graft-versus-host recognition

Since donor CD4 T cells are short-lived, but their impact long-lasting, it appears likely that the augmented alloimmune responses and accelerated rejection observed is dependent upon alloreactive recipient T and B cell populations. To explore this, bm12.Kd.IE cardiac allografts were transplanted into T or B cell deplete recipients and the alloimmune responses examined.

3.3.6.1 Recipient CD4 T cells are essential for allograft rejection

To examine the role of recipient T cells, bm12.Kd.IE hearts were transplanted into TCRKO recipients: C57BL/6 with targeted disruptions of the TCR β and δ genes (*Tcrbd*$^{-/-}$)[256]. These mice possess a normal B cell repertoire, but are deficient in CD4 and CD8 T cells.

Work from our laboratory using these mice as recipients of bm12 cardiac allografts has revealed that strong autoantibody responses develop, driven by donor CD4 T cells. Despite high levels of autoantibody, no GCs develop suggesting the antibody to be of extra-follicular origin. Furthermore, analysis of donor cardiac allografts reveals the absence of AV suggesting that extra-follicular autoantibody responses are unable to drive the development and progression of AV (Saeed Quereshi, manuscript in preparation).

Bm12.Kd.IE hearts were transplanted into TCRKO recipients to investigate the role of recipient CD4 T cells in the alloimmune response. Similar to previous results with bm12 donor hearts, high levels of autoantibody were detected, driven by GvH recognition of recipient B cells by donor CD4 T cells. There was only a weak early anti-H-2K^{d} alloantibody response observed. Despite the high levels of autoantibody, no GCs were detected, and as with bm12 donors, no C4d deposition nor AV or myocardial injury were evident (Figure 3.22). These data suggest that recipient T cells are essential for allograft rejection and whilst donor CD4 T cells play a critical role in augmenting the alloresponses, they are insufficient alone to drive allograft injury. Recipient CD4 T cells appear essential to drive the sophisticated GC responses resulting in development of class-switched, high affinity long-lived antibody responses, responsible for driving the predominantly humoral chronic rejection in this model. They may also provide help for CD8 cytotoxic alloresponses although these appear restricted to the first week following transplantation in this model. The CD4 T cells themselves may, of course, additionally contribute directly to allograft damage.

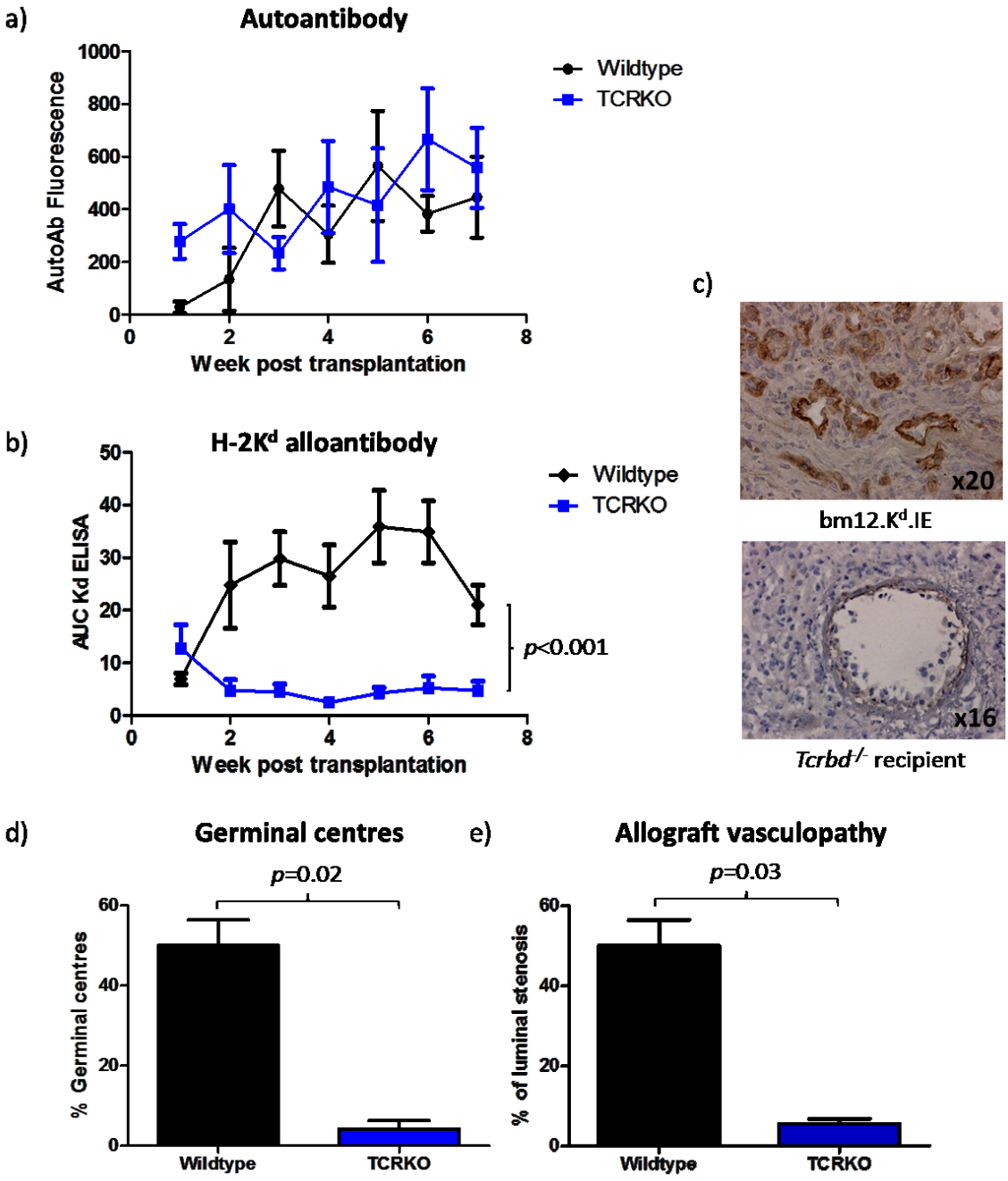


Figure 3.22 Absence of recipient CD4 T cells prevents development of damaging humoral alloimmunity
Bm12.Kd.IE cardiac allografts were transplanted into T cell deficient *Tcrbd*$^{-/-}$ recipients and alloimmunity evaluated over 50 days (n=4). Equivalent autoantibody responses were observed (a), despite absence of recipient CD4 T cells (HEp-2 indirect immunofluorescence) and a weak short-lived anti-H-2K^{d} alloantibody response was seen (b). Despite high levels of autoantibody, unlike in wildtype recipients, no C4d deposition was observed on immunohistochemical staining of cryostat sections of day 50 donor cardiac allografts (c), and no germinal centre response was present (immunofluorescence staining with B220 and GL-7 on cryostat sections of day 50 recipient spleen) (d). Furthermore, no allograft injury was observed with minimal allograft vasculopathy observed on elastin van Gieson stained paraffin sections of day 50 donor hearts. *P* values using two-way ANOVA (a,b) and Mann Whitney test (d,e).

3.3.6.2 Absence of recipient B cells

To further examine the dominant role humoral immunity appears to play in this model, recipients were depleted of B cells using a depleting murine anti-CD20 antibody (clone 18B12 Biogen Idec, MA, US). A single 250 µg i.p. dose of this monoclonal antibody results in effective depletion of circulating and splenic B cells for greater than 14 days (Figure 3.23).

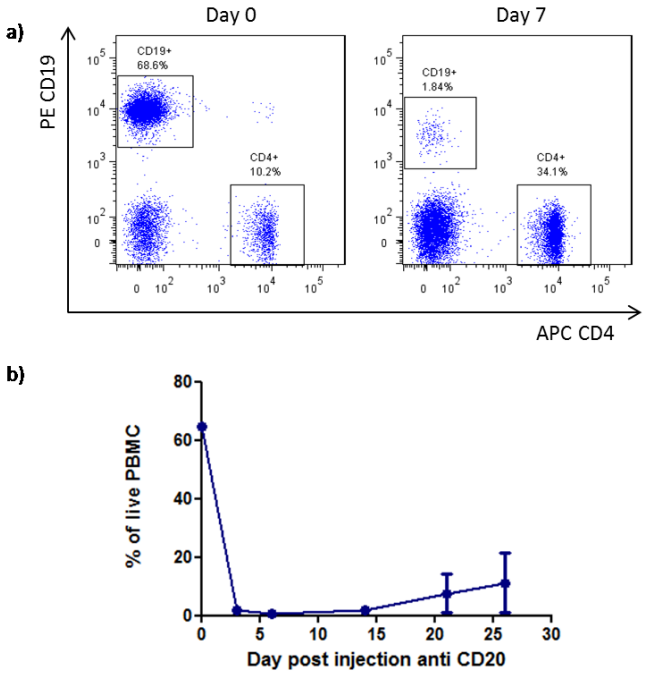


Figure 3.23 Effective B cell depletion is achieved with murine anti-CD20 antibody administration
(a) Representative flow cytometry plots of peripheral blood obtained prior to (left) and 7 days following (right) i.p. administration of 250µg murine anti-CD20 antibody (Biogen Idec) (n=3). Effective depletion of B cells is achieved as soon as 3 days following injection and reconstitution begins after 14 days (b).

Recipients were treated 7 days prior to transplant, the day of transplant and every 14 days thereafter. As to be expected, there was a significant reduction in anti-K^d alloantibody and autoantibody. There was a corresponding reduction in the degree of vasculopathy present in intra-myocardial vessels when examined day 50 post-transplant (Figure 3.24a,b).

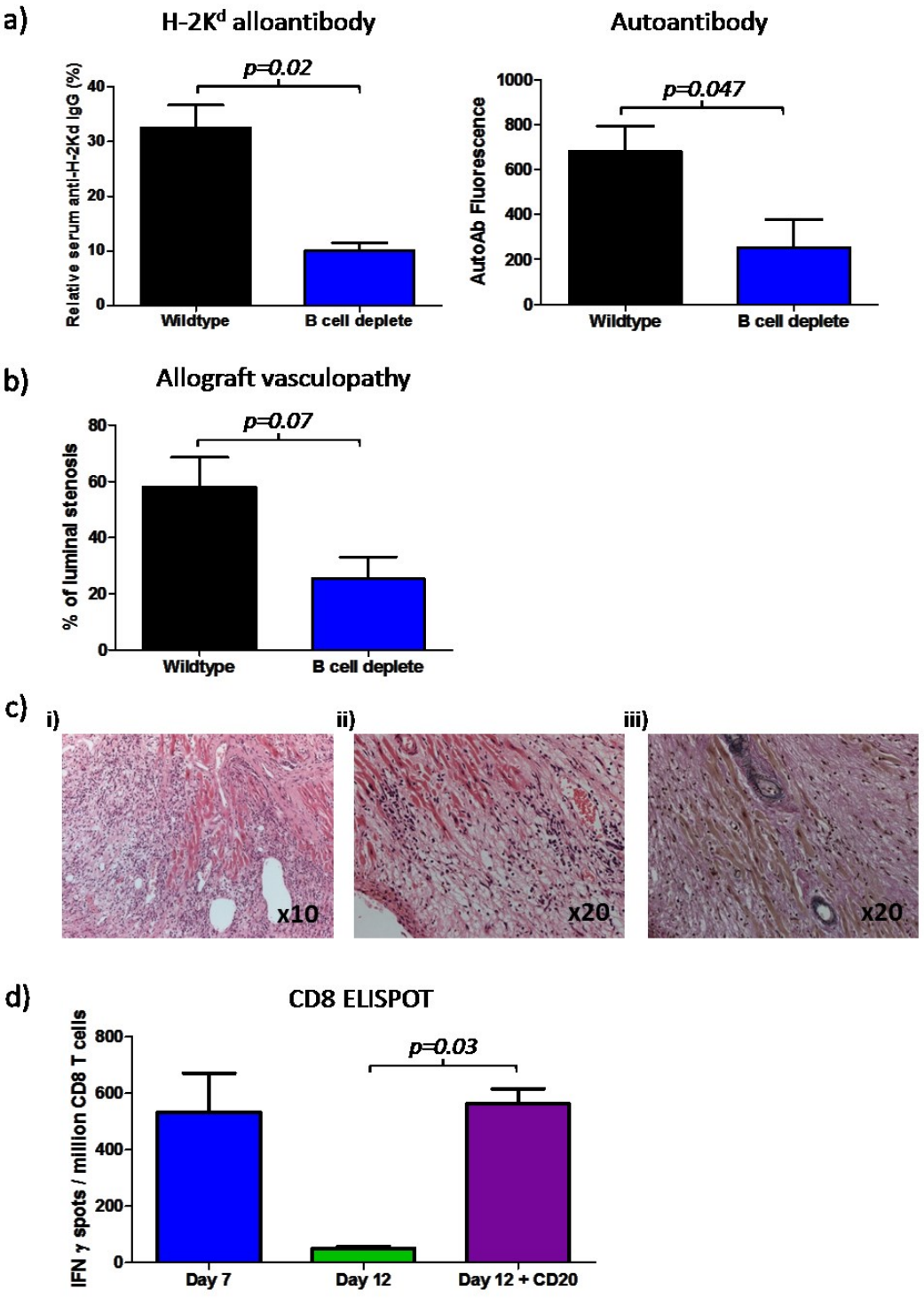


Figure 3.24 Humoral alloimmunity is significantly attenuated following recipient B cell depletion
(a) Following recipient B cell depletion a significant reduction in anti H-2K^d alloantibody (ELISA, left) and anti-nuclear autoantibody (HEp-2 indirect immunofluorescence, right) at 50 days following transplantation of bm12.Kd.IE cardiac allografts into C57BL/6 recipients is observed (n=4). Histological examination of haematoxylin and eosin (i,ii) and elastin van Gieson (iii) stained sections of donor cardiac allografts 50 days following transplantation reveal minimal vasculopathy (b), but significant lymphocytic infiltrate (c). (d) CD8 IFNγ ELISPOT whilst negative in wildtype recipients 12 days following transplantation, is strongly positive in B cell deplete recipients at this time-point (n=4). *P* values using Mann Whitney test.

Interestingly though, the donor myocardium was not preserved. There was a significant lymphocytic infiltrate and more extensive myocyte damage and drop-out and replacement fibrosis than seen in untreated recipients (Figure 3.24c). This suggests that disrupting humoral immunity reveals a vigorous cell-mediated alloimmune response not observed in the presence of B cells. In keeping with this histological appearance, significantly increased CD8 alloreactivity was detected on ELISPOT at day 12 following transplantation when compared to B cell replete recipients, where no CD8 alloreactivity was seen at this time point (Figure 3.24d). This could simply reflect extended survival of donor haematopoietic cells, such as DCs, in the absence of adaptive humoral alloimmunity that could prime CD8 T cells by the direct-pathway.

3.3.7 Donor CD4 T cells require recipient B cells for augmentation of CD4 indirect responses

In the bm12 to C57BL/6 model, the GvH interaction between donor CD4 T cell and recipient B cells is central to the development of autoantibody and allograft injury. However, recipient CD4 T cells are clearly required for the ongoing provision of help, and my experiments suggest the recipient CD4 T cells provide help for driving GC development and CD8 T cell alloimmunity. Depletion of recipient B cells in this model results in abrogation of auto- and alloimmune humoral responses. However, what is less clear is if recipient B cells are required for the augmentation of other arms of the adaptive immune response, driven by the recipient CD4 T cells. It is possible, for example, that donor CD4 T cells could interact with other professional APCs priming them for enhanced antigen presentation and activation of indirect-pathway CD4 T cells.

To examine this, indirect-pathway CD4 T cell proliferation was examined in recipients four weeks following transplantation, by quantifying CFSE labelled TCR75 CD4 T cell proliferation. Proliferation was significantly reduced in B cell deplete recipients and notably this was reduced to a similar extent as was observed in recipients of CD4 deplete donors suggesting that the additional proliferation caused by the donor CD4 T cell fraction is mediated via host B cells. Furthermore, transplanting CD4 deplete donors into B cell deplete recipients resulted in no reduction of indirect-pathway CD4 T cell proliferation.

Transfer of CFSE labelled donor bm12.Kd.IE CD4 T cells into B cell deplete recipients, as in Figure 3.21, revealed an equivalent degree of proliferation of the alloreactive component, suggesting that the role of recipient B cells is not simply as a source of recipient MHC class II for GvH activation of the donor CD4 T cells, since they appear to achieve similar activation

even in the absence of recipient B cells – but the interaction with non-B cell APCs is insufficient to drive augmented alloresponses (Figure 3.25).

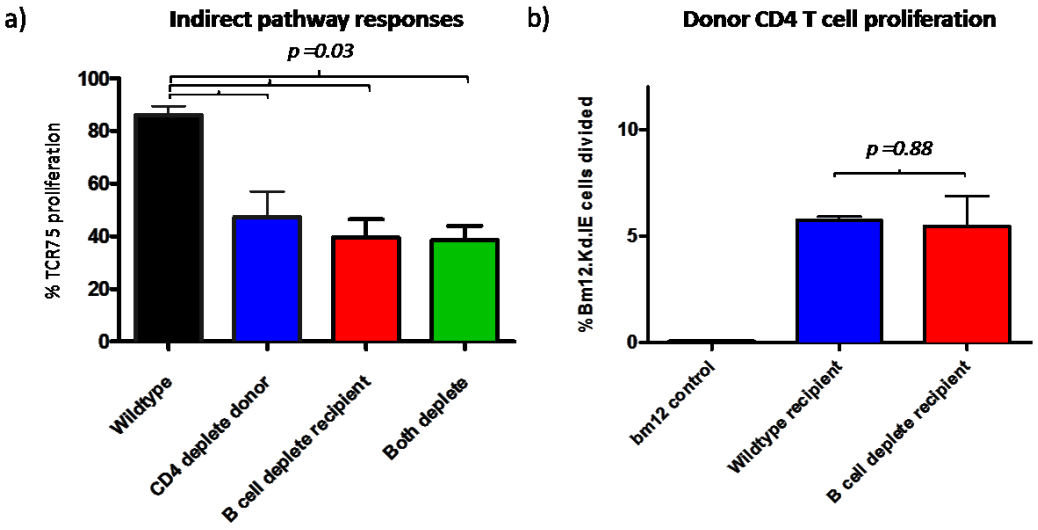


Figure 3.25 B cell depletion prevents donor CD4 T cell augmentation of recipient CD4 T cell activity
(a) Wildtype or donor CD4 T cell deplete Bm12.Kd.IE cardiac allografts were transplanted into wildtype or B cell deplete recipients (250µg i.p.murine anti-CD20 antibody day -7) (n=4). Recipient indirect-pathway CD4 T cell responses were examined by transfer of $5x10^6$ CFSE labelled TCR75 splenocytes 4 weeks following transplantation. Proliferation was quantified by flow cytometry after 6 days. Whereas donor CD4 T cell depletion significantly reduced proliferation in wildtype recipients, this was not observed in B cell deplete recipients, where proliferation was reduced even in the presence of donor CD4 T cells. (b) $5x10^6$ enriched bm12.Kd.IE CD4 T cells (MACS separated with anti-CD4 microbeads) were CFSE labelled and adoptively transferred into wildtype or B cell deplete C57BL/6 recipients of CD4 T cell deplete bm12.Kd.IE cardiac allografts on the day of transplantation (n=4). Three days later, mice were sacrificed and splenocytes analysed by flow cytometry. Donor CD4 T cells were identified by gating on CD4 T cells that stained positive for $H\text{-}2K^d$. Vigorous proliferation of the alloreactive subset of donor CD4 T cells is observed and a similar degree of proliferation was observed regardless of whether recipient B cells were present. *P* values using Mann Whitney test.

Together, these results position the interaction between donor CD4 T cells and recipient B cells central to the augmentation of recipient CD4 T cell responses that are in turn responsible for augmentation of adaptive humoral and cellular immune responses that effect allograft rejection.

3.3.8 No evidence of systemic autoimmunity / graft-versus-host disease

It is notable that despite significant levels of autoantibody and the significant contribution GvH recognition of recipient by donor CD4 T cells makes, the recipient mice show no overt signs of systemic autoimmunity or GvH disease. To further confirm this, recipient heart, kidneys and liver were analysed histologically. Analysis of these slides was performed by a consultant histopathologist (Dr Sue Davies, Addenbrookes Hospital, Cambridge, UK). No evidence of parenchymal injury nor evidence of GVHD were found in any of these tissues, suggesting the donor cardiac allograft is the sole target of the autoantibody response.

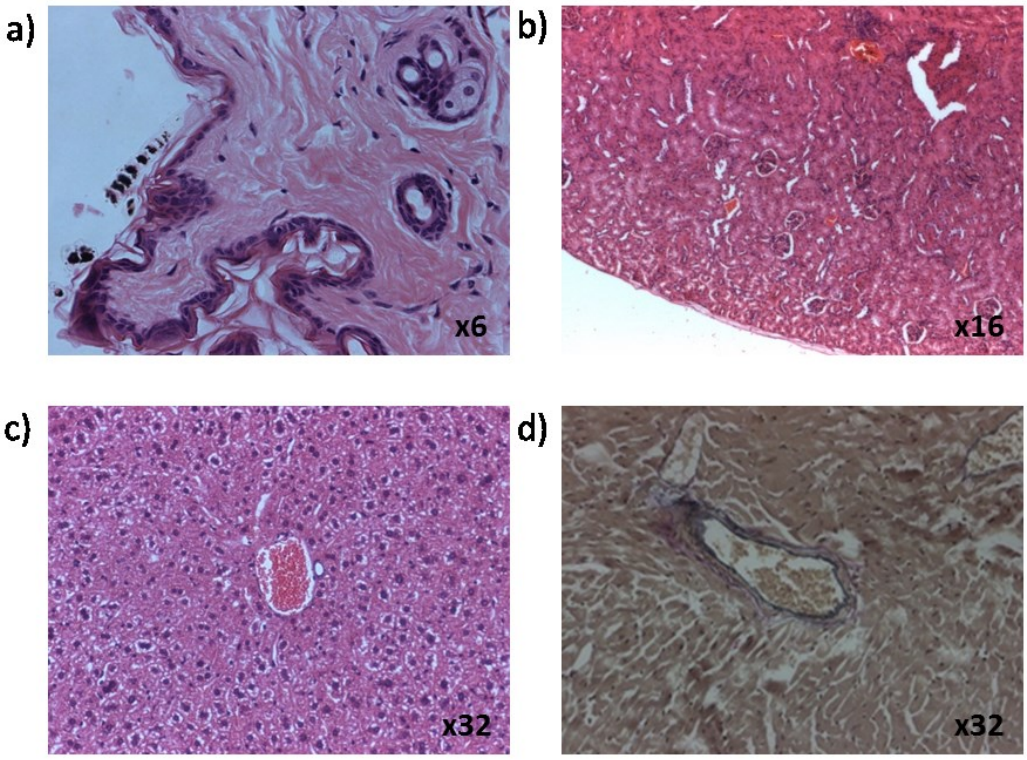


Figure 3.26 No evidence of systemic graft versus host disease is apparent in recipients
Representative photomicrographs of skin (a), kidney (b), liver (c) and native heart (d) retrieved from C57BL/6 recipients of bm12.Kd.IE cardiac allografts 100 days after transplantation and either haematoxylin and eosin staining (a-c) or elastin van Gieson's staining (d) of paraffin sections assessed for presence of inflammation. All tissues sampled were unremarkable and free from inflammation. Magnification detailed in panels.

3.3.9 Wider relevance of these findings to clinical transplantation

The wider relevance of these observations to clinical transplantation is not immediately clear, because in this murine model the minimally mismatched nature of I-A^{bm12} to I-A^{b} is unusual. Nevertheless, there is increasing appreciation of the role of autoimmunity in transplantation[291-293], and donor CD4 T chimerism has been described, and implicated in GvH disease[309], and rarer clinical scenarios, such as passenger lymphocyte syndrome[310-312].

In the current model, whilst it is observed that donor cells evade innate immune surveillance, they are eventually cleared by recipient adaptive alloimmune responses. It is therefore likely that with more mismatched models, NK cells would recognise donor cells as non-self due to the absence of self MHC class I expression. Thus, in the bm12.Kd.IE to C57BL/6 model, lack of NK cell allorecognition may permit donor CD4 T cell survival long enough for them to interact with recipient B cells in a GvH manner and thus trigger augmented alloimmunity. One would therefore expect that this effect would not be observed in more mismatched models.

To test this hypothesis, a fully mismatched BALB/c to C57BL/6 strain combination was studied. As demonstrated in Section 3.3.4, BALB/c cells are rapidly cleared by RAG2KO recipients due to innate immune recognition and clearance. However, if NK cells are depleted from RAG2KO recipients, with anti NK 1.1 monoclonal antibody (clone PK136) prior to cell transfer, long term survival of BALB/c CD4 T cells was observed, highlighting the importance of NK cell allorecognition (Figure 3.27a).

In keeping with their rapid clearance by NK cells, no autoantibody was observed following adoptive transfer of BALB/c CD4 T cells into C57BL/6 recipients (Figure 3.27). However, when BALB/c CD4 T cells were transferred into C57BL/6 recipients that had been treated to deplete NK cells, strong autoantibody levels were observed suggesting that donor CD4 T cell GvH recognition of recipient B cells driving autoantibody is not simply a peculiarity of the bm12 to C57BL/6 model (Figure 3.27b). Rather, these results suggest that the GvH response will be relevant in any MHC class II mismatched donor-recipient combination in which NK cell allorecognition does not occur, and donor CD4 T cells survive for long enough to make cognate interaction with intact allogeneic MHC class II on recipient B cells.

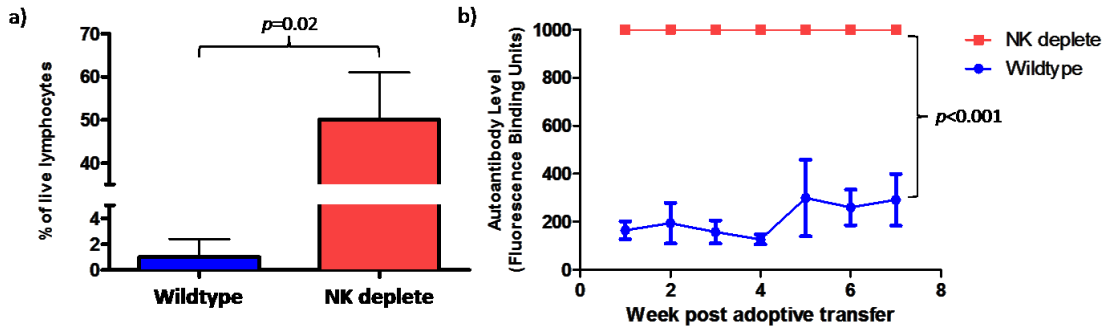


Figure 3.27 NK allorecognition limits donor CD4 T cell GvH activity and development of autoantibody
(a) $5x10^6$ enriched BALB/c CD4 T cells (MACS separated with anti-CD4 microbeads) were adoptively transferred i.v. into untreated or NK cell deplete RAG2KO recipients (administration of anti-NK1.1 antibody, clone PK136, 0.5mg i.p. day -2, 0 and +2). Flow cytometric analysis of peripheral blood at two weeks revealed survival of CD4 T cells only in NK deplete recipients. (b) Similarly, whilst i.v. administration of BALB/c CD4 T cells into wildtype C57BL/6 recipients resulted in minimal autoantibody development, a strong autoimmune response was observed when cells were transferred into NK deplete C57BL/6 recipients (HEp-2 indirect immunofluorescence).

3.3.9.1 Augmentation of alloimmunity occurs in the absence of NK cell allorecognition

The observation that autoantibody is detected after BALB/c CD4 T cell transfer into NK deplete C57BL/6 recipients suggests that donor CD4 T cell GvH interaction with recipient B cells is normally prevented in MHC-mismatched transplant models by host NK cell alloreactivity. The possibility that, in the absence of NK cell alloreactivity, augmentation of recipient alloimmune effector responses would occur with any donor-recipient mismatch was next evaluated.

BALB/c cardiac allografts are acutely rejected within 7-10 days following transplantation and I thought it likely was too rapid to be potentially influenced by NK cell depletion. Therefore, the model was manipulated to become a model of chronic alloantibody-mediated rejection.

Here, BALB/c cardiac allografts were transplanted into C57BL/6 T cell deplete TCRKO recipients which were reconstituted at transplantation with TCR75 CD4 T cells (H-$2K^d$ antigen-specific indirect-pathway CD4 T cells), but at limiting numbers (10^3 per mouse), such that rejection occurs slowly and is mediated by anti-H-$2k^d$ germinal centre alloantibody responses, with help provided by differentiation of the transferred TCR75 T cells to follicular helper T cells (Figure 3.28a; model developed and characterised by M Chhabra, manuscript in preparation).

Such transplants were performed into untreated and NK deplete TCRKO recipients. In NK deplete recipients, as anticipated from the previous finding, development of autoantibody was

observed. Furthermore, the anti-H-2K^d alloantibody response was significantly augmented. Perhaps most striking though was the impact on allograft rejection. Whilst a median survival time of >50 days is achieved in untreated TCRKO recipients, depletion of NK cells resulted in acute rejection of the allografts, with all rejected in under 9 days. To confirm that this was due to donor CD4 T cell GvH interactions, a further group of NK deplete recipients received CD4 deplete BALB/c cardiac allografts. When compared to NK cell-depleted recipients of unmodified BALB/c heart allografts, recipients of CD4 T cell-depleted BALB/c heart allografts developed auto- and alloantibody responses that were comparable to those observed in NK cell-replete recipients of unmodified heart grafts (Figure 3.28). Kinetics of graft rejection were similarly delayed (Figure 3.28).

These results suggest that through preventing donor CD4 T cell GvH interactions in the recipient, NK cell allorecognition and clearance of passenger donor leukocytes may paradoxically prolong the survival of allografts. This clearly may have implications for donor and recipient combinations in which NK cell allorecognition does not occur.

Putting together the previous observations with the bm12 to C57BL/6 model and the current findings, a model for how donor CD4 T cells may influence the alloimmune effector responses is presented in Figure 3.29.

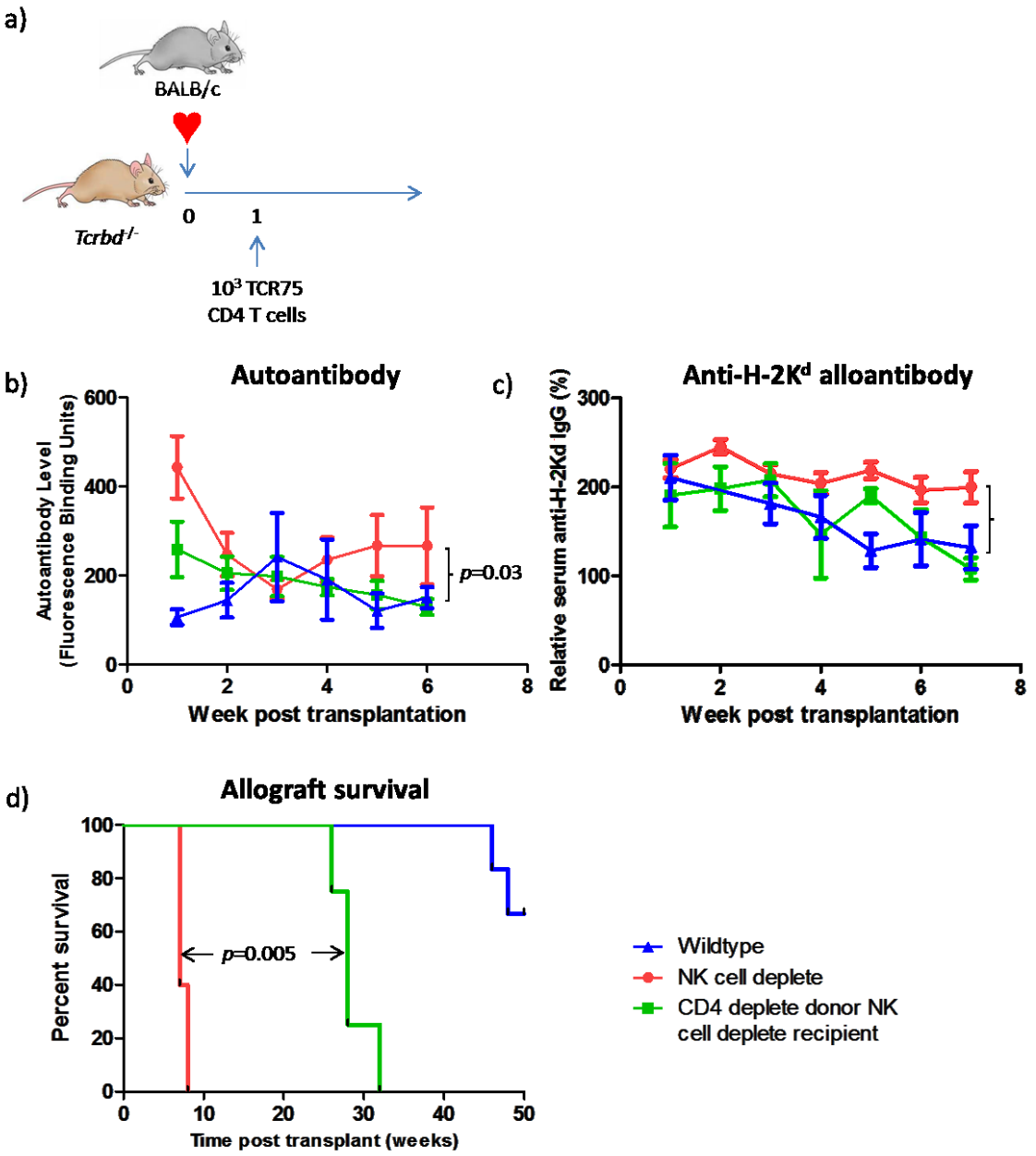


Figure 3.28 Host NK cell alloreactivity is critical for preventing passenger donor lymphocytes from augmenting host adaptive alloimmunity

(a) A model of chronic alloantibody-mediated vasculopathy was developed in which 10^3 TCR75 CD4 T cells are transferred into T cell deplete TCRKO mice at transplant with a BALB/c cardiac allograft. Some recipients were treated to deplete NK cells (administration of anti-NK1.1 antibody, clone PK136, 0.5mg i.p. day -2, 0 and alternate days thereafter) and some donors were depleted of CD4 T cells prior to transplantation (administration of anti-CD4 antibody, clone YTS, 1mg day -5 and -1). Weekly serum samples were obtained and autoantibody [(b), HEp-2 indirect immunofluorescence] and anti-H-$2K^d$ alloantibody [(c), ELISA] was quantified. Autoantibody and a significant augmentation in the alloantibody response was observed following recipient NK cell depletion. A corresponding acceleration in allograft rejection was observed in NK cell deplete recipients (d). Depletion of donor CD4 T cells abrogated the augmented antibody responses and significantly delayed rejection. All groups n=5. *P* values using two-way ANOVA (b,c) and log-rank analysis (d).

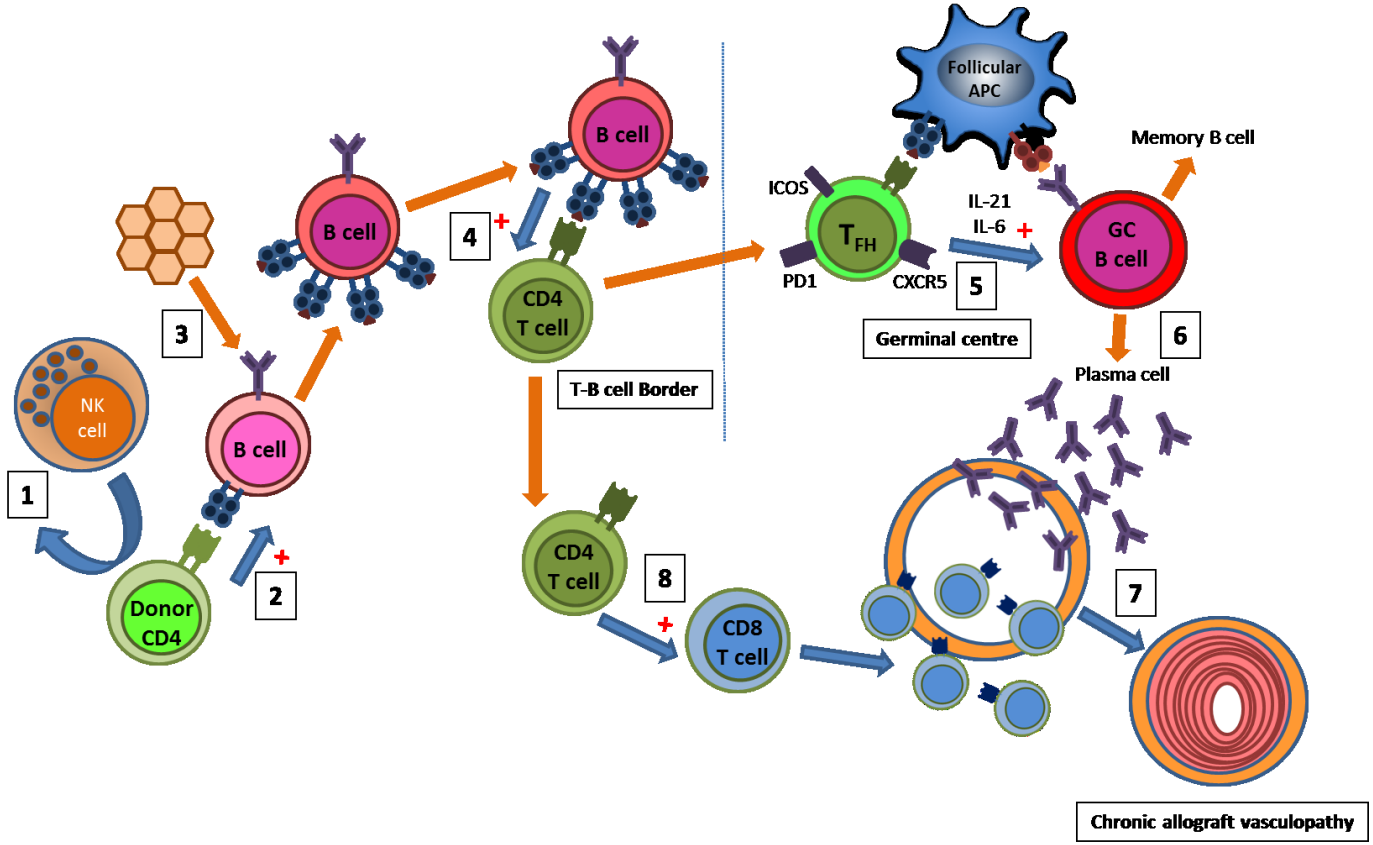


Figure 3.29 Proposed model for augmentation of host adaptive alloimmunity by passenger lymphocytes.

NK cell allorecognition normally results in rapid destruction of passenger donor lymphocytes within solid organ allografts (1). If NK cell allorecognition is avoided, 'direct-pathway' graft-versus-host recognition by donor CD4 T cells can activate all recipient B cells (2), but class-switched antibody secretion is dependent upon simultaneous ligation of their B cell receptor with target antigen (3). Activated B cells drive enhanced activation of host CD4 T cells with indirect-pathway allospecificity (4), which provide reciprocal help for development of germinal centre alloantibody responses (5), presumably reflecting unique T follicular helper cell function of host CD4 T cells in providing cognate, allopeptide-specific help (5). This results in long-term augmentation of humoral alloimmunity (6), with more rapid progression of allograft vasculopathy and early allograft failure (7). Enhanced activation of indirect-pathway CD4 T cells may also contribute to allograft rejection through the provision of help for generating heightened host CD8 T cell cytotoxic alloresponses (8).

3.4 Discussion

Although the presence of passenger donor lymphocytes in the circulation of recipients of solid organ transplants was first demonstrated over two decades ago, the extent to which they impact on recipient alloimmunity has remained unclear[308, 313]. The realisation that significant populations of memory T lymphocytes are resident within non-lymphoid organs and tissues, including all solid organs transplanted in humans, has made understanding their role more pertinent[304-306, 314]. In human clinical transplantation, donor CD4 T cells have already been implicated in development of invariably fatal GVHD[309] and passenger leukocyte syndrome[311, 312]

In this chapter the bm12.Kd.IE to C57BL/6 model of heterotopic cardiac transplantation has been fully examined as a model of chronic rejection. The presence of mismatched MHC class I H-2K^{d} and MHC class II I-E and I-A^{bm12} lead to chronic rejection characterised by development of GC antinuclear autoantibody, anti-H-2K^{d} and anti-I-E alloantibody responses and short-lived CD8 cytotoxicity which resulted in the development of AV. Further characterisation revealed a significant role for passenger donor CD4 T cells in augmenting all arms of the alloimmune response through a GvH interaction with recipient B cells, with markedly reduced humoral and cellular alloresponses and AV evident in recipients of CD4 depleted donors. The presence of memory CD4 T cells augmented the alloimmune response, and significantly accelerated rejection kinetics. Examination of the role of donor CD4 T cells revealed that the alloreactive fraction of the polyclonal donor CD4 T cell population undergo several rounds of proliferation within the first few days of transplantation, but also that these cells are cleared within 7 days. Donor CD4 T cells therefore augment long-lived adaptive alloresponses within this early post-transplant period, but are themselves cleared by the adaptive responses they augment, with a requirement for recipient T and B cells to drive the ongoing alloresponse. Bm12.Kd.IE donor cells evade innate immune recognition, and this was found to be critical for the passenger donor CD4 GvH interaction with recipient B cells. Depletion of recipient NK cells was found to reveal this role for passenger donor CD4 T cells in the BALB/c to C57BL/6 model, highlighting a novel role for NK cells in suppressing alloimmunity in NK cell receptor mismatched donor/recipient pairs.

This work extends previous observations from the laboratory that attributed a role for passenger donor CD4 T cells in the development of autoantibody in the bm12 to C57BL/6 model[274]. The results highlight that the cognate GvH interaction between donor CD4 T cells and recipient B cells additionally drives augmented alloimmune responses. This unusual form

of global B cell help arises due to the peptide-degenerate nature of direct-pathway recognition[48, 63]. Thus, all recipient B cells receive equivalent help, irrespective of their specificity, and show upregulated MHC class II expression (Saeed Qureshi, unpublished data). Differentiation into an antibody secreting plasma cell is determined by the availability of target antigen, as described in the introduction. However, it is clear that this interaction does not replicate conventional CD4 T cell help as in the absence of recipient CD4 T cells the auto and alloantibody responses were not sustained, GCs did not develop and allograft rejection did not occur.

Recipient CD4 T cells therefore play a critical role in directing the augmented alloimmune responses. It would be predicted that donor CD4 T cell GvH interaction will drive increased allopeptide presentation by allospecific B cells (as evidenced by upregulation of MHC class II), resulting in increased indirect-pathway activation of recipient CD4 T cells. This was evidenced by increased TCR75 proliferation in CD4 T cell replete donors. Indirect-pathway CD4 T cells are known to be required for development of alloantibody responses[100], and it is conceivable that the activated CD4 T cells will provide reciprocal help to those B cells with which they have interact, thus explaining the central role for recipient CD4 T cells in the process[315]. An additional role likely played by recipient CD4 T cells, is as follicular helper T cells (Tfh), which are pivotal for the development of GC responses providing antigen-specific cognate help to B cells driving the GC reaction[316]. Work underway in our laboratory has begun to dissect out the respective roles of recipient and donor CD4 T cells in initiating these GC reactions. Utilising a series of models including incorporating signaling lymphocyte activation molecule-associated protein (SAP) knock-out mice (in which T cells are unable to initiate GC reactions)[317], we have demonstrated that donor CD4 T cells are not required to drive the GC responses observed (Saeed Qureshi, manuscript in preparation). Why donor CD4 T cells are unable to adopt a Tfh role is unclear. However, there is some evidence suggesting that the affinity of peptide-MHC – TCR interaction influences CD4 T cell differentiation into a Tfh program and subsequent function[318]. Direct-pathway interactions are peptide degenerate and may therefore not be of high enough affinity to initiate Tfh differentiation.

It was notable that in the absence of recipient T cells, although alloantibody and autoantibody responses developed, there was no evidence of AV. Other work in our laboratory has demonstrated that the development of AV correlates with the development of GC responses (Saeed Qureshi, manuscript in preparation). GCs are responsible for the development of long-lived, high-affinity antibody responses[316], and it is plausible that high affinity antibody is more able to initiate the effector pathways upon binding to donor endothelium responsible for the

development of AV[27]. Alternatively, GC responses are also required to drive epitope diversification and it may be that diversification of the allo and autoantibody repertoires is required to develop the pathological humoral response[132, 319]. In support, we have demonstrated that anti-vimentin autoantibody only develops when GC responses occur (Saeed Qureshi, manuscript in preparation), and anti-vimentin autoantibody has previously been associated with development of AV in human cardiac transplant recipients[297].

Recipient B cells are central to the augmented alloimmune responses observed, both enhancing indirect-pathway CD4 T cell alloresponses and as humoral effectors. As expected, depletion of recipient B cells resulted in significantly reduced development of AV. Interestingly though, histological evaluation of the allografts revealed significant lymphocytic infiltration and parenchymal injury, and CD8 cytotoxic alloresponses were augmented. There are several possible explanations for this observation. In addition to production of antibody, B cells have other essential roles in adaptive immunity. B cells have an important role as antigen presenting cells for initiating adaptive immunity as they are able to efficiently detect and internalise their target antigen[320]. It is recognised that through this cognate interaction with naïve CD4 T cells, B cells are able to influence and direct the nature of helper CD4 T cell differentiation favouring Th2 commitment[315]. In the absence of B cells, perhaps Th1 cell mediated responses are favoured. A similar observation was made in human kidney transplantation where depletion of recipient B cells with rituximab (anti-CD20 mAb) resulted in increased rates of acute cellular rejection resulting in early suspension of a clinical trial[240]. Alternatively, rituximab administration may also deplete regulatory B cells that may play an important regulatory role in the alloimmune response[321]. Another possibility is that in the absence of humoral alloimmunity, donor APCs are able to survive longer term, and perhaps this permits ongoing direct-pathway CD8 T cell activation accounting for the augmented CD8 cytotoxicity observed.

The most interesting observation in this chapter is that the development of autoantibody through the GvH interaction between donor CD4 T cells and recipient B cells is not simply a quirk of the minimal nature of the MHC class II mismatch between I-A^{bm12} and I-A^{b} antigens. Rather, autoantibody development and augmentation of adaptive alloimmunity is limited by NK cell allorecognition and rapid clearance of passenger donor leukocytes. This perhaps helps to explain the inconsistent autoantibody development observed in human transplant recipients[291-293]. The results in this chapter suggest a novel mechanism by which NK cells inhibit allograft rejection. As described in Chapter 1, the role of NK cells in alloimmunity remains to be completely understood. There is both evidence that NK cells promote allograft rejection, for

example through the killing of opsonised cells, and evidence supporting a role in allograft tolerance[197]. The results in this chapter support that NK cells play an important role in limiting destructive cellular and humoral alloimmunity, but through a novel mechanism – the rapid deletion of passenger donor CD4 T cells. The tolerogenic mechanisms employed by NK cells previously described include rapid deletion of passenger DCs, therefore reducing adaptive immune priming, and deletion of activated recipient CD4 T cells[202, 205, 207]. It could be argued that clearance of donor CD4 T cells is a more important tolerogenic role for NK cells considering the impact that they can have on adaptive alloimmunity. For example, irrespective of NK cell allorecognition, allogeneic DCs would be expected to be cleared promptly by adaptive immune allorecognition, limiting their effect. In this regard it is notable that the model used to demonstrate NK deletion of donor DCs to influence CD4 T cell alloimmunity utilised $CD8^{-/-}$ mice preventing their clearance by CD8 T cell cytotoxicity[208]. In contrast, adaptive immune clearance is too slow to prevent the donor CD4 T cell GvH mediated augmentation of adaptive alloimmunity and there is a critical window within the first few days after transplantation during which evasion of NK cell mediated killing is essential.

These results are of interest when considering the recent focus of some research which aims to improve allograft outcomes by achieving mixed haematopoietic chimeric states in recipients to achieve donor-specific tolerance[322]. The persistence of donor lymphocytes in recipients would imply that augmentation of recipient alloimmunity is not occurring. This may be due to the impact of myeloablative therapy the recipient is often subjected to prior to attainment of mixed chimerism[322]. In support of this, a study in murine skin transplant recipients demonstrated that chimerism was associated with stronger alloimmunity and more rapid allograft rejection in immunologically mature recipients, but tolerance in immature recipients[323]. It is also interesting to note that autoantibody, using HEp-2 immunofluorescence, has been observed in human kidney and bone-marrow transplant recipients in which mixed haematopoietic chimerism was achieved by non-myeloablative conditioning, supporting that donor CD4 T cell GvH interaction with recipient B cells may be an important factor to consider in these approaches.

The prediction from this work would be that those with an NK KIR match (as is reported to occur in approximately 50% of kidney transplant pairs[198]) may have inferior outcomes because the avoidance of immediate host NK cell detection would enable donor lymphocytes to potentiate host alloimmunity. These findings may then, have important clinical relevance, which could be elucidated by undertaking a clinical study. HLA and NK KIR typing of donors and recipients could be performed, and clinical outcomes following transplantation compared

between patients who do and do not possess an NK KIR mismatch. A retrospective clinical study which has partially addressed this, observed that a KIR mismatch was in fact associated with poorer clinical outcomes following kidney transplantation[198]. However, this analysis was restricted to minimally mismatched donor/recipient pairs with the aim of investigating the role of NK allorecognition in isolation of adaptive alloimmunity. The findings in this chapter though, would predict KIR matching to compromise outcomes only when donor and recipient are additionally mismatched at MHC class II loci (for direct-pathway GvH interaction). Furthermore, understanding of NK cell receptors and what constitutes a 'match' remains incompletely understood[324].

A further intriguing consideration is mother to child living organ donation. Should a mother become sensitised during pregnancy, it is conceivable that they would possess tissue-resident memory CD4 T cells targeted against the mismatched HLA of their child. The results in this chapter would predict that if there was a KIR match between the mother and child, following transplantation passenger memory lymphocytes may impact deleteriously on clinical outcome. This is a hypothesis that could be tested by examining sensitisation status, KIR type and clinical outcomes of relevant living donor-recipient pairs.

4 CD4 Direct-pathway allorecognition is short-lived and limited by both innate and adaptive immunity

4.1 Introduction

4.1.1 Direct CD4 allorecognition

Direct-pathway allorecognition is unique to transplantation. Here, recipient CD4 T cells recognise intact allogeneic MHC class II on passenger donor APCs[48]. There is an unusually high precursor frequency for direct-pathway allorecognition and the strength of mixed lymphocyte reactions *in vitro* have led many to conclude that direct-pathway allorecognition is responsible for acute rejection[49-51]. However, the precise contribution that direct-pathway allorecognition makes to allograft rejection remains uncertain. In animal models where allorecognition is restricted to the indirect-pathway, acute rejection can still be observed[120, 121]. Furthermore, clearance of passenger donor APCs soon after transplantation by innate immunity is thought to limit direct-pathway allorecognition to the period immediately following transplantation[48]. The development of TCR transgenic technology has enabled the development of mouse strains that permit investigators to track individual T cells of known antigenic specificity, thus providing additional insight into the in vivo fate and function of individual antigen-reactive T cells. Two TCR transgenic strains have been developed to investigate direct-pathway allorecognition: 4C and ABM

4.1.2 TCR transgenic models to study direct allorecognition – 4C and ABM

The 4C TCR transgenic CD4 T cell is on a *Rag*$^{-/-}$ C57BL/6 background and recognises intact I-A^{d}, an MHC class II molecule expressed by BALB/c[81]. *In vitro* these CD4 T cells could be activated by BALB/c DCs, B cells, macrophages and activated endothelial cells. Following transplantation of BALB/c skin or cardiac allografts into *Rag*$^{-/-}$ recipients significant proliferation of adoptively transferred 4C cells was observed. Skin allografts rejected acutely, whilst cardiac allografts developed AV. Priming of the 4Cs prior to challenge with a cardiac allograft resulted in acute rejection, suggesting that direct-pathway CD4 T cells alone have the potential to mediate allograft injury and rejection[81].

The ABM TCR transgenic CD4 T cell is similarly on a *Rag*$^{-/-}$ C57BL/6 background and recognises intact I-A^{bm12}, the MHC class II expressed by bm12 mice[261]. Early work with this model demonstrated that the presence of ABM CD4 T cells could accelerate rejection of bm12 skin[325].

This was extended to demonstrate that bm12 cardiac allografts could be acutely rejected by *Rag*$^{-/-}$.ABM recipients, but only if the recipients were primed with bm12 skin prior to the cardiac allograft. Surprisingly, without priming, cardiac allografts survived long-term and did not develop significant injury[326]. These results again supported the assertion that direct-pathway CD4 T cells may have the potential to mediate allograft rejection in certain circumstances.

The studies described above using TCR transgenic direct-pathway CD4 T cells have focused on examining their ability to reject allografts in isolation. However, this does not replicate the clinical setting where there will be interplay of multiple adaptive immune pathways. Furthermore, the work has focused on examining proliferation in the early period following transplantation and has not examined the duration of these responses.

The relevance of this to clinical transplantation is seen when considering the use of cellular immunotherapies, for example Treg. Research is currently underway to examine the relative efficacy of different Treg products – including direct and indirect-pathway antigen specific Treg, such as the One study[215]. The current focus is administration to recipients of living donor organs where the product can be prepared for administration on the day of transplantation. Looking forward, it is likely that Treg therapy will be extended to cadaveric organ recipients, administered later following transplantation. Having an insight into the relative duration of direct and indirect-pathway allorecognition may guide rational development of antigen-specific Treg therapies at these later times following transplantation.

4.2 Aims

The purpose of the research described in this chapter is to extend the findings reported with direct-pathway TCR transgenic CD4 T cells and to examine direct-pathway allorecognition in wildtype recipients of cardiac allografts in which the adaptive immune response is not restricted to direct-pathway allorecognition.

The aims are:

1) To determine the duration of direct-pathway CD4 T cell alloresponses.

2) To examine the factors responsible for limiting direct-pathway allorecognition.

4.3 Results

4.3.1 Challenges encountered using ABM transgenic CD4 T cells

Direct-pathway CD4 T cell allorecognition was suggested to be short-lived in section 3.3.1, using ABM TCR transgenic CD4 T cells in C57BL/6 recipients of bm12 cardiac allografts. To further investigate direct-pathway allorecognition, proliferation of ABM CD4 T cells was evaluated in C57BL/6 recipients of bm12.Kd.IE cardiac allografts. It was notable that a much larger number of splenocytes were required for adoptive transfer experiments in order to detect a population 6 days later, as compared to the 3 other transgenic populations used (TCR75, TEa and Mar), raising question as to the survival of ABM CD4 T cells in C57BL/6 mice. To investigate this, ABM CD4 T cells were adoptively transferred into naïve C57BL/6 mice with a congenic marker (CD45.1) to permit more definitive identification of transferred cells. Only a small population were identified 6 days after transfer (Figure 4.1a). Of more concern, a proportion of the ABM cells had undergone division, suggesting an element of cross-reactivity with I-A^b, and this was in keeping with the greater than expected proliferation of ABM in naïve C57BL/6 detected in Figure 4.1b, approaching 15% in comparison with <2-3% for the other TCR transgenic CD4 T cells (Figure 3.4). Despite these limitations, the time period following transplantation during which adoptively transferred ABM CD4 T cells underwent proliferation was short-lived, with proliferation being back to baseline at week 5 following transplantation (Figure 4.1b).

Proliferation of ABM CD4 T cells was also evaluated in RAG2KO recipients of bm12.Kd.IE hearts. Since bm12.Kd.IE cells are not cleared by the innate immune response (Section 3.3.4), it was anticipated that donor APCs would continue to drive direct-pathway allorecognition long term, limited by their natural senescence. The hypothesis therefore, was that direct-pathway allorecognition would be longer-lived in these recipients. The experimental data supported the hypothesis; in the absence of adaptive immune clearance, significant proliferation was observed at the late time-point (Figure 4.1c). As above, some proliferation was seen upon transfer into the naïve control mouse, which was not observed for the other transgenic CD4 T cell clones, raising concern over continued use of ABM CD4 T cells in this work.

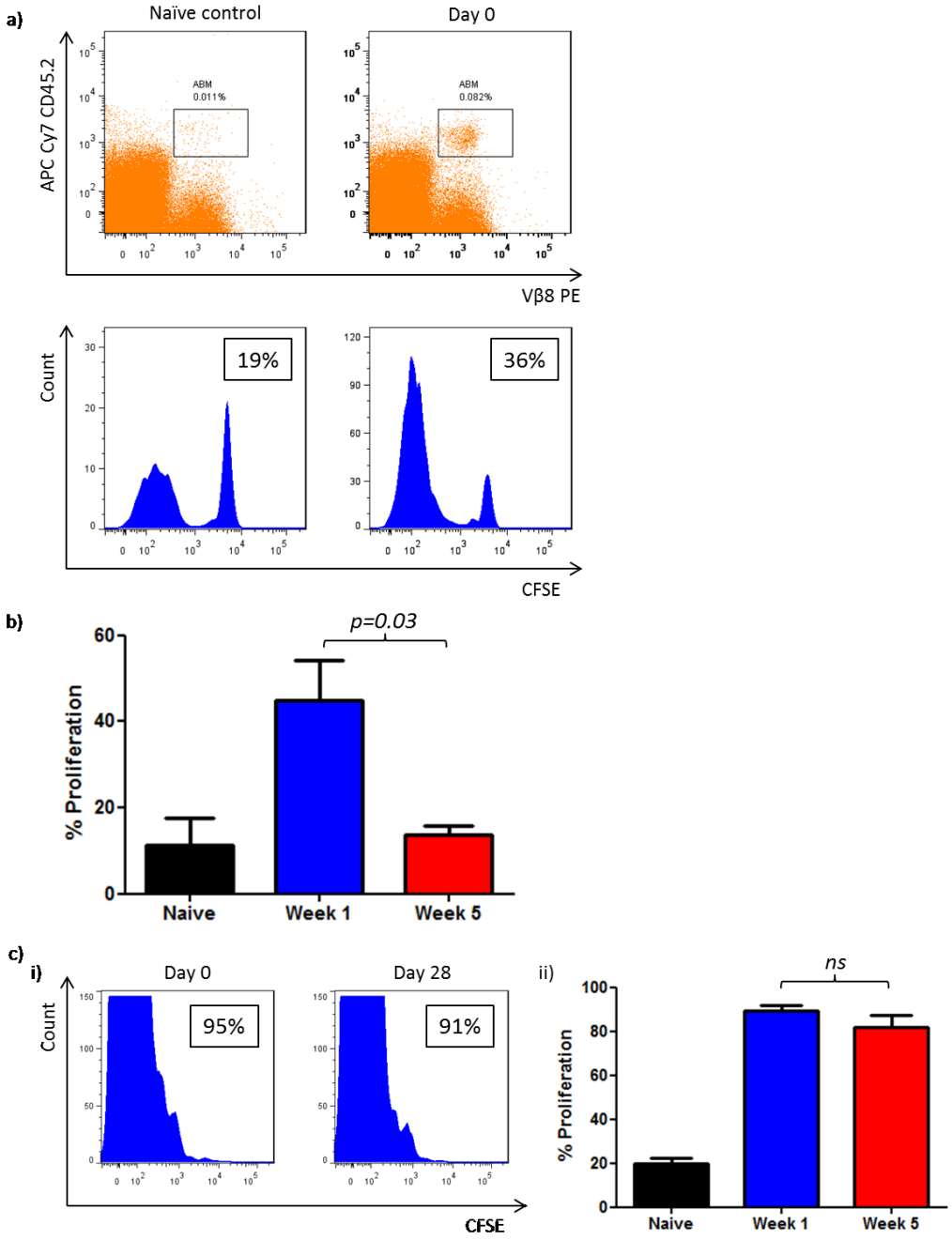


Figure 4.1 ABM proliferation is short-lived in wildtype but long-lived in RAG2KO recipients

(a) 6-8x10^6 CFSE labelled ABM splenocytes were adoptively transferred i.v. into naïve control (left panels) or bm12.Kd.IE cardiac allograft recipient congenically marked CD45.1 C57BL/6 mice on the day of transplantation (day 0, right panels). Six days following transfer, spleens were harvested and analysed by flow cytometry. ABM CD4 T cells were gated on the basis of CD45.2 and Vβ8 expression (upper panels) and presented as histograms against CFSE (lower panels) Proliferation was quantified by FlowJo software (percentage proliferation as inset of panels). Of concern, a significant degree of proliferation was observed in naïve recipients. (b) Proliferation of CFSE labelled ABM CD4 T cells transferred at week 1 or week 5 was quantified as above, following bm12.Kd.IE cardiac transplantation into wildtype C57BL/6 (n=3). (c) A significant degree of proliferation (inset percentages) was observed when ABM splenocytes were adoptively transferred, as above, but into RAG2KO recipients following transplantation at both early (day 0) and late (day 28) time-points (n=3). In RAG2KO recipients ABM CD4 T cells were identified by Vβ8 expression since RAG2KO lack T cells. *P* values using Mann Whitney test.

4.3.2 Using TCR75 transgenic CD4 T cells for investigating direct allorecognition

In view of the difficulties encountered with the ABM CD4 T cells, an alternative model of direct-pathway allorecognition was developed using TCR75 transgenic CD4 T cells. These cells recognise a peptide from MHC class I H-2K^{d} presented in the context of class II I-A^{b}, and have been used to evaluate indirect-pathway allorecognition[162]. TCR75 CD4 T cells can be restricted to direct-pathway allorecognition if the donor expresses both the MHC class I H-2K^{d} and the class II I-A^{b}, and if neither are expressed by the graft recipient (Figure 4.2). This scenario could be achieved by grafting a heart from a B6.Kd or a C57BL/6 x BALB/c F1 (CB6F1) donor into a bm12 recipient.

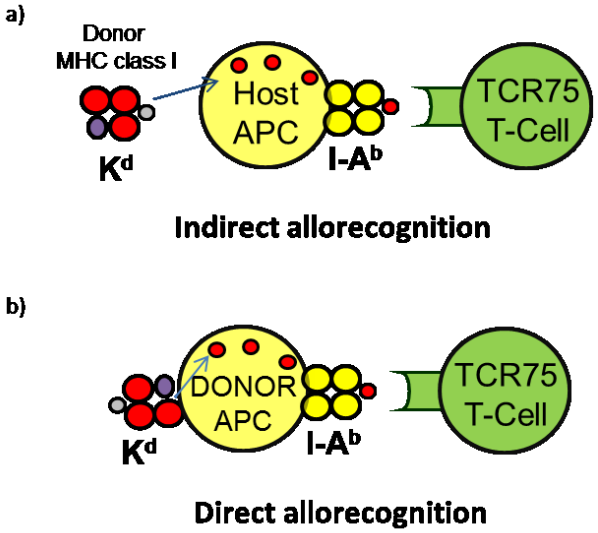


Figure 4.2 Using TCR75 CD4 T cells to examine direct-pathway allorecognition
(a) TCR75 CD4 T cells recognise I-A^{b} restricted H-2K^{d} allopeptide, and have been widely used to examine indirect-pathway CD4 T cell allorecognition in C57BL/6 recipients. (b) However, if a donor strain co-expresses I-A^{b} and H-2K^{d} then donor APCs will present the TCR75 TCR epitope. Should the recipient strain lack I-A^{b} then the TCR75 can only become activated and induced to divide by recognition of intact donor class II and therefore represent direct-pathway allorecognition (albeit restricted to presentation of a single defined peptide, whereas direct-pathway recognition is usually considered peptide degenerate.)

B6.Kd mice express the additional MHC class I H-2K^{d} as a transgene. Cardiac allografts are rejected chronically in C57BL/6 recipients[153], with development of anti-H-2K^{d} alloantibody[100]. It would be anticipated that rejection kinetics will be similar if B6.Kd cardiac allografts are transplanted into bm12 recipients, identical to C57BL/6 other than the mutated class II I-A^{bm12}, and therefore suitable for studying late direct-pathway allorecognition with TCR75 CD4 T cells.

Although I-A^{bm12} differs from I-A^{b} by only 3 amino acids, TCR75 CD4 T cells do not proliferate in response to H-2K^{d} allopeptide presented by I-A$^{bm12\ 100}$. To evaluate the ability of TCR75 to recognise antigen by the direct-pathway, B6.Kd cardiac allografts were transplanted into bm12 recipients, and TCR75 splenocytes adoptively transferred either on day 0 or day 28. As anticipated, proliferation was observed early confirming the strategy to employ TCR75 for examining direct-pathway allorecognition to be feasible. Proliferation was notably weaker than when TCR75 are utilised to examine the indirect-pathway (Section 3.3.1), likely reflecting lower antigen availability since the epitope will only be presented by passenger donor APCs. Furthermore, no proliferation was detected at the late point following transplantation in keeping with the earlier results. Consistent with the prevailing view that direct-pathway CD4 T cell allorecognition reflects recognition of intact donor MHC class II on professional APCs, subjecting B6.Kd donors to lethal irradiation prior to transplantation (to ablate the haematopoietic compartment) resulted in abrogation of direct recognition (Figure 3.3).

As with bm12.Kd.IE donors, B6.Kd donor cells are sufficiently similar to 'self' to preclude innate immune recognition and clearance of donor cells[125]. Whilst it is commonly held that direct-pathway allorecognition, although strong, is short-lived due to rapid clearance of donor DCs by recipient innate immunity – in particular NK cells[76], the absence of a late direct-pathway response observed with ABM CD4 T cells with bm12.Kd.IE donors and with TCR75 CD4 T cells here indicate that adaptive immunity may play a role in clearing donor cells, limiting the duration of direct-pathway allorecognition.

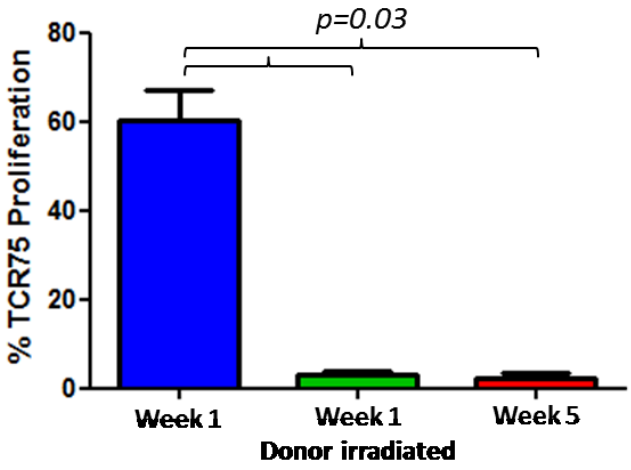


Figure 4.3 The direct-pathway is short lived and can be limited by adaptive immune responses
B6.Kd cardiac allografts were transplanted into bm12 recipients and 4-6x10^{6} CFSE labelled TCR75 splenocytes adoptively transferred i.v. at week 1 or 5 (n=4). TCR75 proliferation was quantified by flow cytometry 6 days later. Some donors were subjected to lethal irradiation with 13 Gray as a split dose and transplanted after 7 days. *P* values using Mann Whitney test.

4.3.3 Evaluating the role of innate immunity in limiting direct-pathway allorecognition

4.3.3.1 Validating a model of TCR75 direct -pathway allorecognition in which there is NK mediated recognition

To evaluate the role of NK cells in limiting direct-pathway allorecognition an alternative model was sought in which a greater mismatch results in NK mediated immune recognition of donor cells. CB6F1 animals (the F_1 offspring of a cross between BALB/c and C57BL/6 mice) express both I-A^b and H-2K^d and if transplanted into bm12 recipients would be predicted to drive TCR75 direct-pathway allorecognition in similar fashion to that described in the previous section. The CB6F1 donor is a haplotype mismatch, with both shared and mismatched MHC molecules with the bm12 recipient. It was first important to confirm that NK recognition and killing of CB6F1 cells by donor mice occurs. This was achieved by adoptive transfer of purified CB6F1 CD4 T cells i.v. into H-2^b RAG2KO recipients (lacking adaptive immunity) and Rag2IL2rg (RAG2KO mice with an additional IL-2 common γ chain knock-out, which renders the animals additionally deplete of NK cells)[258]. These experiments confirmed that CB6F1 CD4 T cells are subject to NK cell mediated clearance. Whilst CD4 T cells did not survive in RAG2KO recipients, survival was equivalent to syngeneic cells in Rag2IL2rg mice (Figure 4.4).

Having confirmed that CB6F1 cells are subject to NK mediated clearance, the ability of TCR75 CD4 T cells to recognise their epitope by the direct-pathway in this model was next evaluated. Adoptive transfer of CFSE labelled TCR75 splenocytes into bm12 recipients of CB6F1 cardiac allografts on the day of transplantation revealed that direct-pathway allorecognition does occur, although TCR75 proliferation was weaker than in the above B6.Kd to bm12 model, possibly due to the reduced proportion of direct 'alloantigen' present on donor cells as a consequence of the cells expressing a greater number of different class I and II molecules. TCR75 proliferation was restricted to the first week following transplantation reaffirming the observation that direct-pathway allorecognition is short-lived (Figure 4.4).

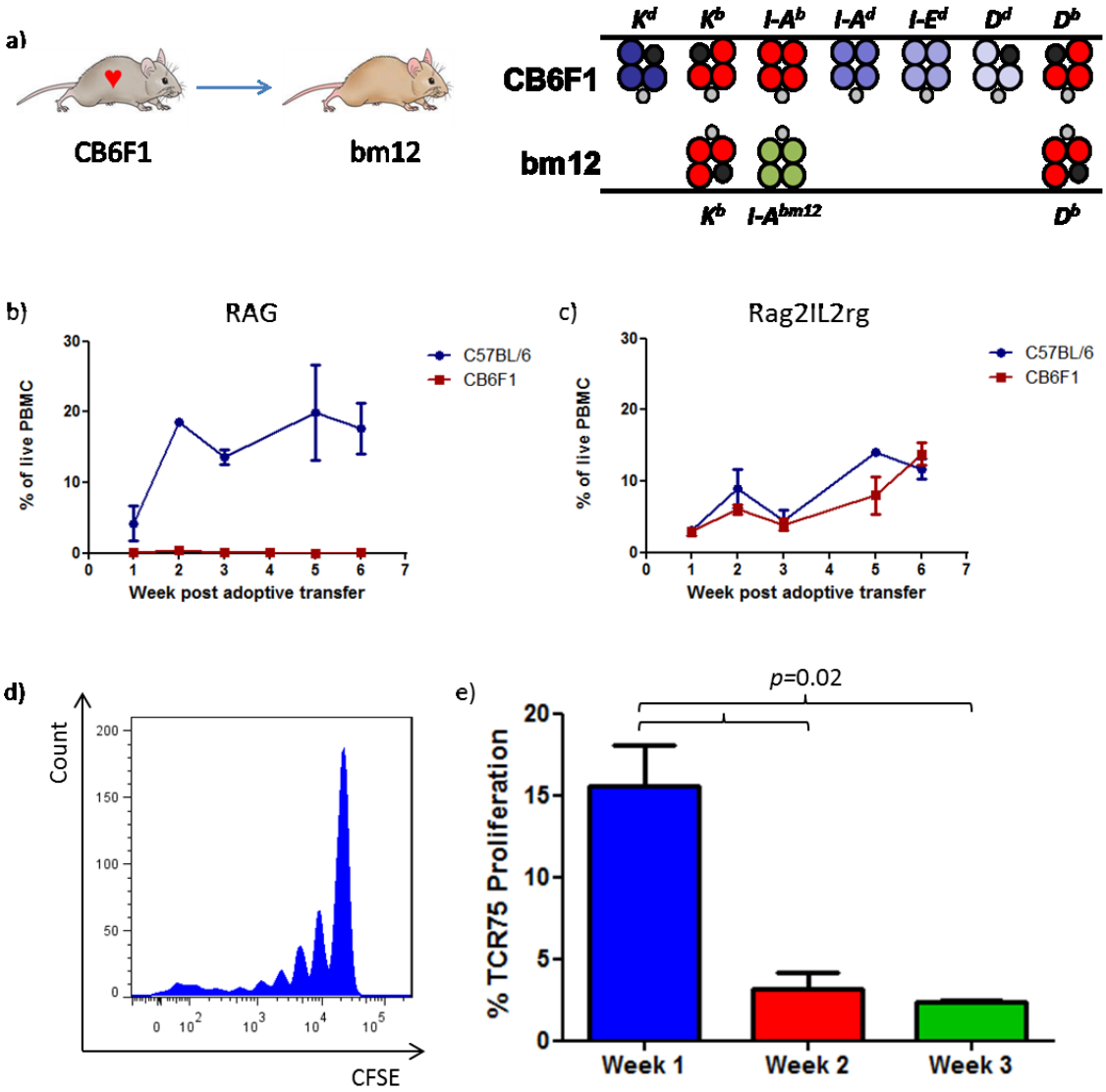


Figure 4.4 Evaluating direct-pathway allorecognition in the CB6F1 to bm12 model

(a) CB6F1 donor cardiac allografts were transplanted into bm12 recipients, representing a haplotype mismatch, but with some common MHC class I molecules. $5x10^6$ enriched CB6F1 or syngeneic C57BL/6 CD4 T cells (MACS separated with anti-CD4 microbeads) were adoptively transferred i.v. into RAG2KO [(b) lacking adaptive immunity] or RagIL2rg [(c) additionally lacking NK cells] recipients (n=3). Weekly blood samples were processed by flow cytometry to quantify the surviving CD4 T cell population. CB6F1 cardiac allografts were then transplanted into bm12 recipients and $4\text{-}6x10^6$ CFSE labelled TCR75 splenocytes were adoptively transferred on the day of transplant. Spleens were harvested 6 days later TCR75 proliferation was observed on flow cytometry confirming TCR75 direct-pathway allorecognition occurs (d). Subsequently this was repeated with transfer of TCR75 at week 1, 2 and 3 (n=5) and extent of proliferation quantified with FlowJo software. *P* values using Mann Whitney test.

4.3.3.2 The role of donor APCs in priming direct allorecognition

The observation in bm12 recipients of B6.Kd donor cardiac allografts, that direct-pathway allorecognition is abrogated by donor irradiation (Figure 4.3) alludes to direct-pathway allorecognition being principally dependent upon donor APCs as has been previously suggested[76]. To further investigate that donor APCs are the predominant donor cell type presenting MHC class II for recipient direct-pathway allorecognition, transplants were performed with donors that had been treated to deplete APC populations. For these experiments, CB6F1 mice expressing the diphtheria toxin receptor (DTR) gene under the CD11c promoter (CB6F1.DTR) were utilised, allowing for donor DC depletion with administration of Diphtheria toxin (DTx), in addition to depletion of other leukocyte subsets with depleting antibody administration[257, 327, 328]. It should be noted that although widely used, the CD11c.DTR model is not without significant limitations. It has been reported that expression of the transgene is 'promiscuous' and administration of DTX results in depletion of cells other than DCs – including some macrophage subtypes, CD8 T cells and even some GC B cells[329-331]. Therefore caution must be exercised in concluding that all differences observed following DTX administration are attributed to the absence of DCs. Furthermore, not all DC subsets express CD11c.

First, the passenger leukocyte populations carried with donor cardiac allografts were examined to estimate the number of cells transferred. Donor hearts were harvested as if for use in transplantation and homogenates were analysed by flow cytometry. The most prevalent leukocytes were B cells which were present in 1000 fold greater number than CD4 T cells, $CD11c^+$ (predominantly DCs), or $CD11b^+$ (predominantly macrophages) cells (Figure 4.5). One technical difference between murine heterotopic cardiac transplantation and clinical transplantation is that donor organs used in the clinic are thoroughly flushed with organ preservation fluid following procurement. This is not typically performed in murine experimental models. To confirm that the identified leukocyte populations did not simply reflect blood remaining within the coronary vasculature, donor hearts were flushed immediately after procurement with 0.9% saline introduced retrogradely through the ascending aorta, and homogenates then similarly analysed by flow cytometry. Only a modest reduction in cell numbers was observed in the flushed hearts, suggesting that retained donor leukocytes in the coronary circulation are not artificially exaggerating the passenger leukocyte load administered to recipients (Figure 4.5).

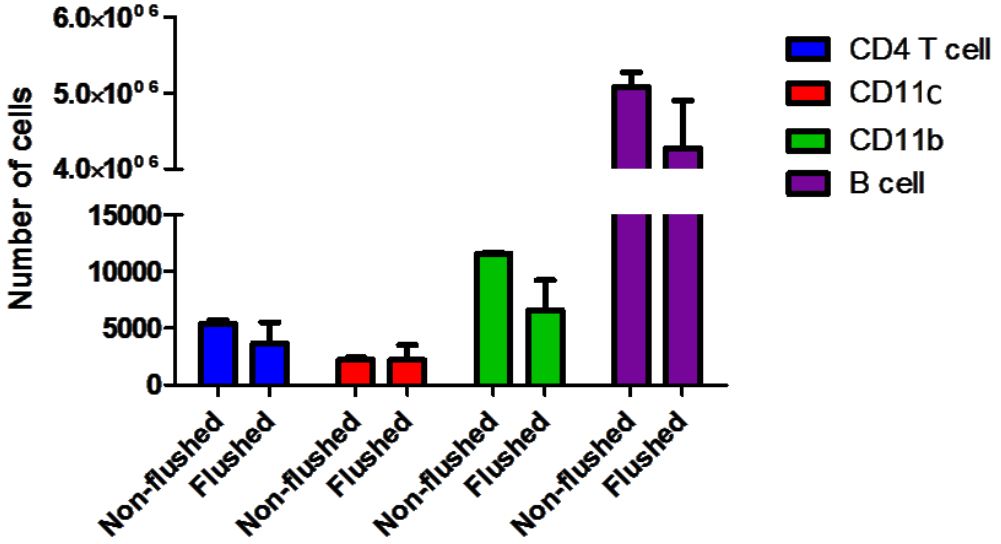


Figure 4.5 Passenger leukocyte populations in cardiac allografts
Donor CB6F1 cardiac allografts were procured as for transplantation. In half of the cases (n=3), 1ml of 0.9% saline was flushed retrograde through the ascending aorta. Hearts were homogenised and incubated with a collagenase digestion mix. The samples were then analysed by flow cytometry using Trucount™ tubes (BD Bioscience), staining for live CD4, CD19, CD11b and CD11c, and the number of cells quantified.

CB6F1.DTR donors were either lethally irradiated 7 days prior to transplantation, or treated to deplete DCs (DTx administration 10ng/g daily from 7 days prior to transplantation) and/or B cells (depleting murine anti-CD20 antibody 250µg 7 days prior to transplantation), prior to transplantation into bm12 recipients. It was hypothesised that direct-pathway responses would be reduced or abrogated with these treatments due to clearance of the donor APC populations, as the major source of donor MHC class II.

Analysis of peripheral blood by flow cytometry on the day of transplantation confirmed haematopoietic depletion following irradiation, and depletion of DCs and B cells following administration of diphtheria toxin or anti CD20 antibody (Figure 4.6). An attempt to deplete macrophages was also made utilising liposomal clodronate as has been described in the literature[266-268]. Despite following the manufacturer's instructions and several attempts, a significant population of $F4/80^+$ $CD11b^+$ cells remained in both the circulation and spleen of treated animals and it was decided not to pursue this further (Figure 4.6).

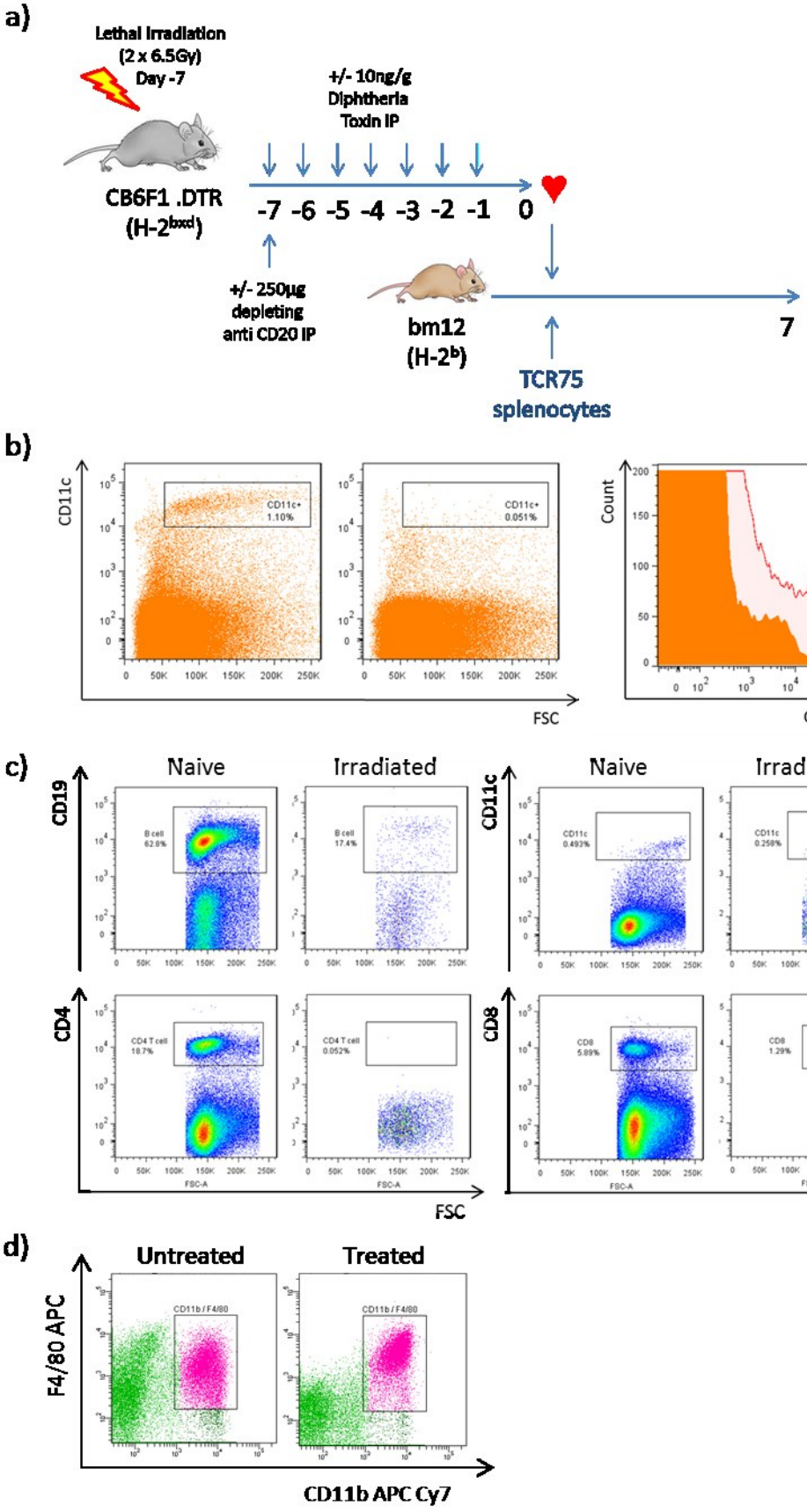

a)
Lethal Irradiation
(2 x 6.5Gy)
Day -7
+/- 10ng/g
Diphtheria
Toxin IP
CB6F1 .DTR
(H-2bxd)
-7 -6 -5 -4 -3 -2 -1 0
+/- 250µg
depleting
anti CD20 IP
bm12
(H-2b)
7
TCR75
splenocytes
b)
CD11c
CD11c+
1.10%
CD11c+
0.051%
FSC
Count
CD11c
c)
Naive
Irradiated
Naive
Irradiated
CD19
B cell
62.8%
B cell
17.4%
CD11c
CD11c
0.493%
CD11c
0.258%
CD4
CD4 T cell
18.7%
CD4 T cell
0.052%
CD8
CD8
5.89%
CD8
1.29%
FSC-A
FSC
FSC
d)
Untreated
Treated
F4/80 APC
CD11b / F4/80
CD11b / F4/80
CD11b APC Cy7

Figure 4.6 Donor antigen presenting cell depletion

(a) Untreated, or antigen presenting cell (APC) subset depleted donor CB6F1.DTR cardiac allografts were transplanted into bm12 recipients. 4-6X10^6 CFSE labelled TCR75 splenocytes were adoptively transferred and proliferation quantified after 6 days by flow cytometry. Representative flow cytometry data from splenocytes of treated and untreated donors on the day of transplantation (n=4): (b) following administration of 10ng/g diphtheria toxin i.p. daily for 7 days prior to transplantation there is a significant reduction in CD11c DCs as seen on dot plot, and overlay histogram data; (c) following lethal irradiation (13 Gray split dose) 7 days prior to transplantation there is a substantial paucity of leukocytes present and a significant reduction in CD4 T cells, CD8 T cells, B cells and CD11c DCs; (d) following administration of 1mg liposomal clodronate i.p. 7 and 2 days prior to transplantation an insignificant reduction in macrophage population (double gated on F4/80 and CD11b) was observed despite several attempts.

Lethal Irradiation of donors resulted in complete abrogation of direct-pathway TCR75 proliferation, suggesting haematopoietic cells to be the major source of intact MHC class II antigen for direct-pathway allorecognition. This result was extended by demonstrating that depletion of both B cells and DCs resulted in a significant reduction of direct allorecognition, with depletion of both populations simultaneously appearing to achieve the greatest reduction. That this did not fall to the level achieved with irradiation likely reflects the incomplete nature of clearance of cells with diphtheria toxin and anti-CD20, and contribution from additional professional APCs for example, CD11c negative macrophages. Whilst it is widely held that donor DCs are the major driver of direct-pathway allorecognition, these results suggest that B cells, in addition to DCs, are important sources of alloantigen for CD4 T cell direct-pathway allorecognition.

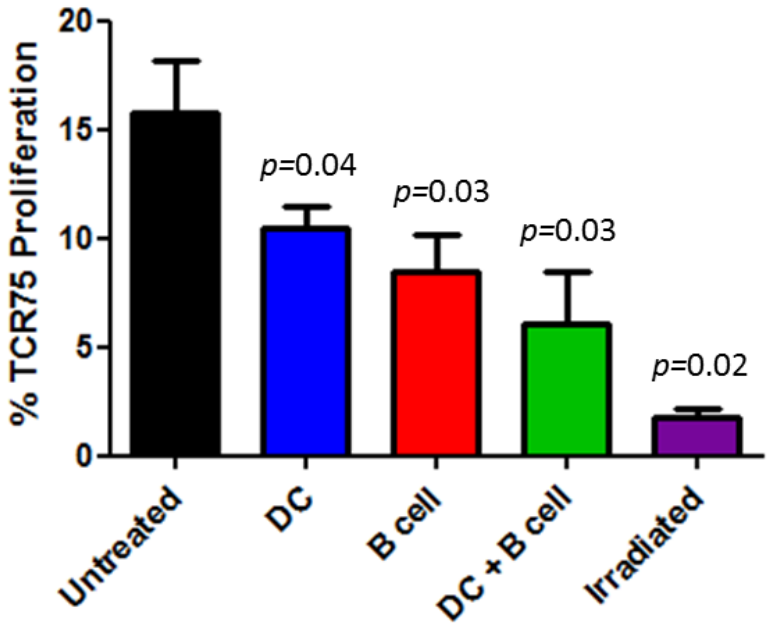


Figure 4.7 Donor APC depletion significantly attenuates direct-pathway allorecognition
Untreated, lethally irradiated (13 Gray split dose) or APC subset depleted (DC with administration of DTx and B cells by administration of depleting murine anti-CD20 antibody) donor CB6F1.DTR cardiac allografts were transplanted into bm12 recipients. 4-6X10^6 CFSE labelled TCR75 splenocytes were adoptively transferred and proliferation quantified after 6 days by flow cytometry. *P* values in comparison to proliferation observed with untreated donors, using Mann Whitney test. All groups n=4.

4.3.4 The role of NK cells in limiting direct-pathway allorecognition

Having demonstrated that NK cells recognise CB6F1 cells as foreign and clear them rapidly (Figure 4.4b,c), the role of NK cells in limiting the duration of direct-pathway allorecognition was next examined. NK cells can be depleted by i.p. administration of anti-NK 1.1 antibody (clone PK136) with 0.5mg administered days -2, 0 and +2 following transplantation (Figure 4.8a). To further investigate the role of NK cells in deleting passenger donor leukocytes, CD4 T cells were administered to RAG2KO mice that were, or not, treated with anti-NK 1.1 antibody (Figure 4.8b). CB6F1 CD4 T cells survived and expanded significantly in the NK depleted RAG2KO mice; the mice, moreover, demonstrated signs consistent with severe GVHD. This is therefore consistent with the findings in NK depleted RagIL2rg mice (Figure 4.8c) and verifies anti-NK 1.1 antibody to be efficacious.

Having demonstrated the ability of NK cells to clear donor cells, and anti-NK 1.1 antibody to be effective at prolonging their survival, bm12 recipients of CB6F1 hearts were NK depleted and TCR75 cells adoptively transferred at various points following transplantation.

When TCR75 cells were transferred on the day of transplant there was a small, and not statistically significant, increase in TCR75 proliferation in the NK depleted recipients. NK depletion was unable to prolong direct-pathway allorecognition, as no proliferation was observed when TCR75 cells were transferred on day 3, as was observed in the untreated recipients (Figure 4.8c). Although it is widely held that innate immune responses are predominantly responsible for limiting direct-pathway allorecognition, these results suggest that adaptive immune responses rapidly ensue and also contribute to donor APC clearance. Thus, in keeping with the results presented for the B6.Kd to bm12 model, even in the absence of innate immune recognition of donor cells, direct-pathway allorecognition was restricted to the immediate period following transplantation.

To confirm a role for adaptive immunity in limiting direct-pathway allorecognition, T cell deplete recipients were used – bm12.TCRKO (bm12 mice were crossed with *Tcrbd*$^{-/-}$ mice and backcrossed to bm12). When transferred into bm12.TCRKO recipients of CB6F1 cardiac allografts, TCR75 cells underwent a significantly greater degree of proliferation than in wildtype recipients, suggesting a role for adaptive immune responses in clearing donor APCs. When NK cells were depleted from bm12.TCRKO recipients, to ablate both adaptive and innate immune responses, a substantially increased proliferation of TCR75 cells was observed and the response was prolonged, persisting into the second week, highlighting that both arms of the

immune response play a role in limiting the longevity of direct-pathway allorecognition (Figure 4.8c).

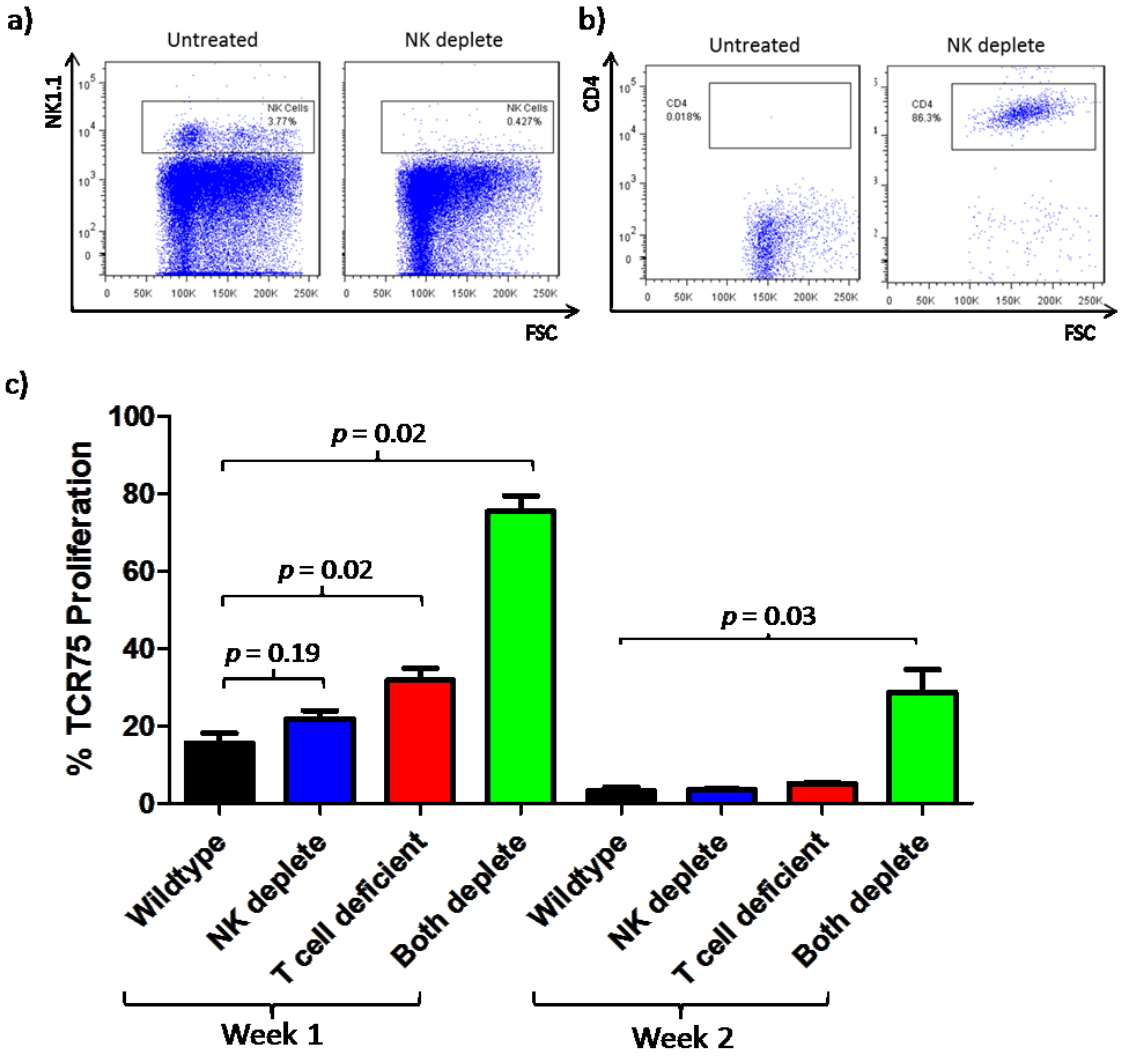


Figure 4.8 Adaptive immune responses in addition to NK killing limit direct-pathway allorecognition
(a) Anti-NK 1.1 antibody (clone PK136, 0.5mg i.p.) was administered to wildtype C57BL/6 recipients (n=3). Analysis of splenocytes by flow cytometry after 3 days revealed a substantial reduction in NK cell number compared with untreated mice. (b) $5x10^6$ CB6F1 CD4 T cells (MACS separated using anti-CD4 microbeads) were adoptively transferred i.v. into RAG2KO recipients that were untreated, or treated with anti-NK 1.1 antibody (0.5mg i.p. day -2, 0, 2 in relation to injection). Flow cytometry performed after 7 days revealed a substantial CD4 T cell population in NK depleted recipients. (c) CB6F1 cardiac allografts were transplanted into bm12 or bm12.TCRKO (T cell deplete) recipients that had, or not, been treated with anti-NK 1.1 to deplete NK cells (0.5mg days -2, 0 and +2). All groups n=4. $4\text{-}6X10^6$ CFSE labelled TCR75 splenocytes were adoptively transferred on the day of transplantation (week 1) or after 7 days (week 2) and proliferation quantified 6 days later by flow cytometry. Whilst absence of NK cells or T cells resulted in modest increases in proliferation, absence of both resulted in significant enhancement and prolongation of proliferation. *P* values using Mann Whitney test.

4.4 Discussion

Traditionally, direct-pathway presentation of alloantigen by donor-derived DCs has been a primary focus of transplant immunology and has been regarded as the driving mechanism underlying T cell-mediated allograft rejection. However, in this chapter, adoptive transfer of ABM and TCR75 TCR transgenic CD4 T cells has enabled confirmation that direct-pathway alloresponses are very short-lived, limited by the rapid clearance of donor professional APCs. These results suggest that there is no parenchymal source of MHC class II sufficient to drive direct-pathway CD4 T cell activation, at least beyond the first week following transplantation. The results have also demonstrated that there is a role for both innate and adaptive immunity in clearance of donor APCs. Only in the absence of both arms of the immune response were direct-pathway responses significantly augmented and prolonged in duration. These results suggest that despite the high precursor frequency and strong mixed lymphocyte reactions observed *in vitro*[50], the direct-pathway may only have a limited role in the alloimmune response.

This position is in agreement with the results of Brennan *et al.* who similarly examined direct-pathway allorecognition in the context of other adaptive immune responses[142]. They simultaneously transferred direct-pathway and indirect-pathway specific CD4, and direct-pathway specific CD8 TCR transgenic T cells following cardiac transplantation. There was significantly greater proliferation of indirect-pathway than direct-pathway CD4 T cells, suggesting that there is preferential priming of indirect-pathway alloresponses. Examination of endogenous responses with ELISPOT verified these findings and excluded that intrinsic differences between the TCR transgenic cells were responsible for the findings. The authors also compared the ability of direct and indirect-pathway CD4 T cells to reject healed skin grafts, 100 days following transplantation. Only indirect-pathway CD4 T cells were able to mediate rejection of the skin at this late time-point. However, if the direct-pathway CD4 T cells were primed, rejection of the skin was observed. This is consistent with results in this chapter, where direct-pathway CD4 T cell priming only occurs early. Brennan *et al.* have extended this by confirming that the lack of priming late following transplantation has implications for their ability to mediate allograft injury. They conclude by questioning the role of direct-pathway allorecognition in allograft rejection, suggesting that their findings reconcile previous observations that elimination of direct-pathway responses does not significantly alter experimental allograft rejection kinetics.

The absence of late priming of direct-pathway CD4 T cells is due to rapid clearance of passenger donor APCs, since irradiation of donors prior to transplantation abrogated direct-pathway priming. Whilst murine endothelial cells have been demonstrated to express MHC class II early following transplantation – both in the literature[86] and Chapter 5, and activated endothelial cells have been demonstrated to prime direct-pathway CD4 T cells *in vitro*[326], this is clearly insufficient to prime direct-pathway CD4 T cells beyond the first week following transplantation *in vivo*. Even if expression persists beyond this time, lack of appropriate co-stimulation may prevent productive cognate interactions from taking place[82].

Early reports highlighted the important role of NK cells in clearing donor APCs suggesting this occurs over the first days to weeks following transplantation[207, 332]. NK cells are thought to mediate this clearance through recognising 'missing-self' (absence of self MHC class I)[179, 180]. This was more definitively examined using two-photon microscopic studies, where live clearance of allogeneic DCs by NK cells was visualised[76, 333]. This process was much more rapid than initially appreciated – with ~80% of DCs cleared by just 12 hours following transfer or skin transplantation[76]. T cell priming by DCs occurs in three successive stages in a process that takes in excess of 24 hours: initial transient serial encounters during the activation phase, a second phase of stable contacts culminating in cytokine production, and a third phase of high motility with transient interactions and rapid proliferation[334]. In keeping with this, rapid NK cell clearance of allogeneic DCs has been confirmed to limit the ability of allogeneic DCs to efficiently stimulate cognate CD4 T cells[335].

This is in keeping with the observation in this chapter that proliferation of TCR75 CD4 T cells (in bm12 recipients of CB6F1 cardiac allografts) was only observed when transferred on the day of transplantation – transfer just 2 days later was too late to yield TCR75 proliferation.

In this chapter it is clearly demonstrated that adaptive immune responses also play a role in clearing donor APCs. In this regard it is interesting to note another study examining the role of NK cells in priming alloreactive CD4 T cell responses. Coudert *et al*. demonstrated that depleting NK cells from recipients, although resulting in enhanced early direct-pathway CD4 T cell priming, did not impact upon allograft rejection[336]. Although it was not examined, it is conceivable that this may reflect that although augmented initially, direct-pathway priming remained short-lived due to adaptive immune clearance of passenger donor APCs, thus limiting the duration of direct-pathway allorecognition which therefore failed to impact upon allograft rejection kinetics.

One argument in favour of direct-pathway CD4 T cells having an important role in the alloimmune response is the observation that direct-pathway CD8 T cells are one of the principal mediators of allograft injury and rejection[337]. CD8 T cell activation is thought to require formation of a three-cell cluster with a 'licensed' APC and a CD4 T cell[92, 93]. The APC must present antigen to both the CD4 and CD8 T cell in order to elicit CD4 T cell help. Therefore, activation of direct-pathway CD8 T cells requires an interaction with a donor APC, and requires direct-pathway CD4 T cells to licence the donor APC. Indeed, murine studies have confirmed that restricting CD4 T cell help to the direct-pathway generates strong cytotoxic CD8 T cell alloresponses that effect rapid allograft rejection[87]. However, it is clearly not that simple, since direct-pathway CD8 T cell cytotoxicity has been demonstrated in the absence of direct-pathway CD4 T cells, suggesting that indirect-pathway CD4 T cells can also provide the necessary help[96]. This has prompted the proposal that direct-pathway cytotoxic CD8 T cell alloimmunity may be initiated by the 'semi-direct' pathway, whereby intact donor MHC class I alloantigen is recognised after its acquisition onto the surface of recipient DCs. This phenomenon provides a possible mechanism by which the same recipient APC as re-presents intact MHC class I alloantigen could simultaneously present processed allopeptide for recognition by indirect-pathway CD4 T cells[47]. Thus, the semi-direct-pathway may enable disconnect between direct-pathway CD4 and CD8 T cells, explaining why direct-pathway CD8 cytotoxicity appears an important long-lived effector arm of alloimmunity, whilst direct-pathway CD4 T cell activity appears restricted to the period immediately following transplantation.

It is notable that human endothelial cells are thought to constitutively express MHC class II, and so caution must be exercised in extrapolation of these findings to human clinical transplantation[338]. However, two clinical studies have attempted to examine direct-pathway allorecognition in long-term recipients of solid organ transplants. Baker *et al.* have studied kidney transplant recipients[77, 148]. They performed *in vitro* assays to examine direct and indirect-pathway CD4 T cell responsiveness. Significant widespread donor-specific direct-pathway hypo-responsiveness was observed both in recipients with stable renal function and those with evidence of chronic allograft nephropathy. In contrast, high frequencies of CD4 T cells were identified responding to the indirect-pathway, with significantly higher responsiveness in those with evidence of chronic allograft nephropathy implicating indirect-pathway responses in chronic rejection. Similarly, Hornick *et al.* studied cardiac transplant recipients[78, 339]. They too demonstrated direct-pathway donor-specific hypo-responsiveness and concluded that direct-pathway allorecognition appears unlikely to be responsible for the

progression of chronic rejection, implicating indirect-pathway allorecognition as the predominant immunological driving force.

Therefore, despite potential differences in donor MHC class II expression, the findings in this chapter mirror observations in human clinical transplantation, and put into question the role of direct-pathway CD4 T cells in the on-going alloimmune response following transplantation.

5 Duration of indirect-pathway alloresponses vary

5.1 Introduction

5.1.1 Indirect-pathway allorecognition and allograft rejection

The indirect-pathway of allorecognition is now recognised to be central to allograft rejection[48]. This stems from early experimental data supporting a role for indirect-pathway allorecognition – such as rejection of leukocyte depleted donors[70, 71], and rejection of MHC class II knock-out donors[120, 121], both of which precluded direct-pathway CD4 T cell allorecognition.

Whether indirect-pathway CD4 T-cells can themselves directly mediate rejection of vascularised allografts is controversial, because although one paper has described independent effector function[340], there is no cellular target within the allograft to which indirect-pathway CD4 T-cells that have developed cytolytic potential could bind. In theory, replacement of graft endothelium by ingress from recipient progenitor cells could result in expression of a self-restricted allopeptide target for indirect-pathway CD4 T-cell allorecognition, but the limited extent to which this occurs is likely to limit its relevance[341]. Damage may alternatively occur through a delayed-type hypersensitivity response from interaction of indirect-pathway CD4 T-cells with recipient APCs within the parenchyma of the graft[342] but again, it is not clear whether this is sufficient to cause allograft rejection.

Indirect-pathway CD4 T-cells are more likely to influence rejection by providing help for generating CD8 T-cell and humoral alloimmunity. As described in Chapter 1, the provision of help to CD8 T cell for responses against conventional protein antigen involves CD4 T cell recognition of MHC class II restricted target antigen, presented by the same APC as presents MHC class I restricted antigen to the CD8 T cell[343-345]. One would therefore expect indirect-pathway CD4 T cells to readily provide help for indirect-pathway CD8 T cells responses against self MHC class-I restricted allopeptide presented by host APCs. Nevertheless, as discussed above, it is unclear whether indirect-pathway CD8 T cell responses can mediate allograft injury, due to absence of expression of target epitope on the allograft. Instead, indirect-pathway CD4 T cells have been demonstrated to be able to provide help to direct-pathway CD8 T cells, which are capable of directly interacting with the allograft[96]. Evidence is accumulating that the 'semi-direct'-pathway may reconcile these observations[48]. In contrast, indirect-pathway CD4 T cells appear pivotal for the development of humoral alloimmunity. Murine models incorporating either MHC class II deficient recipients or mice harbouring monoclonal populations of

alloreactive CD4 T-cells have now demonstrated conclusively that alloreactive B-cells can only receive help from indirect-pathway CD4 T-cells [100, 346].

The contribution of antibody to allograft rejection was, for many decades, thought negligible. Previous work from our laboratory examining allograft rejection in an MHC class I-mismatched rat cardiac allograft model was amongst the first to suggest otherwise[135, 347-349], because acute rejection was found to be dependent upon CD4, but not CD8, T-cells, and could be restored in T-cell-deficient 'nude' rats by passive transfer of immune serum. Subsequently, the introduction of staining for complement split products[350], has led to a re-evaluation of the contribution of alloantibody to clinical transplantation[351]; acute antibody-mediated rejection is now a well-recognised cause of early kidney graft loss[352], and may be encountered more frequently as HLA antibody incompatible kidney transplants are more widely performed using induction strategies that incorporate B-cell depletion[240, 353-355]. Furthermore, increasing numbers of human clinical studies have correlated the presence of donor-specific alloantibody development with failure of kidney[356-359] and cardiac allografts[360-362]. Thus probably by far the most important role for indirect-pathway CD4 T-cells in acute graft rejection is as helpers for promoting humoral alloimmunity.

5.1.2 Duration of allorecognition pathways

Although the two main allorecognition pathways were first recognised over three decades ago, their respective contribution to allograft rejection remains unclear. Because donor DCs are thought to be eliminated early after transplantation, only indirect-pathway responses are thought to occur at late time points after transplantation and it is frequently stated that the direct-pathway is responsible for acute rejection, and the indirect for chronic rejection. Evidence to support this statement is, however, limited to a small number of clinical studies showing that in patients suffering from chronic rejection, self-restricted allopeptide responses are augmented whereas direct-pathway T-cell responses are unchanged [363-366].

Much effort is currently being expended on developing cellular immunotherapies for the clinic in transplantation, such as Treg therapy. It is likely that possessing a greater understanding of allorecognition may facilitate the development of these cellular therapies, such as in decisions surrounding the optimal Treg product administered. For example, if it becomes clear that

direct-pathway alloreactivity is restricted to early following transplantation, then this would impact on Treg product development for administration late after transplantation.

5.2 Aims

The purpose of the research described in this chapter is to examine the indirect-pathway of allorecognition using the bm12.Kd.IE to C57BL/6 model of chronic rejection, exploring the preliminary finding that duration of indirect allorecognition is variable dependent upon target alloantigen.

The aims are:

1. Examine the duration of indirect-pathway alloresponses against different groups of alloantigen

2. Examine the factors responsible for limiting the duration of indirect-pathway alloresponses

3. Examine the significance of indirect-pathway duration for the development of late humoral alloresponses

5.3 Results

5.3.1 Duration of indirect allorecognition is variable

Earlier results using individual models demonstrated that indirect-pathway allorecognition of donor MHC I, MHC II and minor H-Y antigen have variable duration (Section 3.3.1). The bm12.Kd.IE model was used to examine these three responses simultaneously. Since the donor class II I-A^{bm12} is very similar to I-A^{b} of the recipient, it was first necessary to confirm that the transgenic CD4 T cells (I-A^{b} restricted) did not recognise their target allopeptide presented by I-A^{bm12}. If this were the case, donor APCs could present antigen and induce division by the direct-pathway. Male BALB/c hearts (possessing both class I H2-K^{d} and class II I-E^{d}) were grafted into female bm12 recipients and TCR75, TEa and Mar splenocytes adoptively transferred on the day of transplant. No proliferation was observed confirming allorecognition by these transgenic cells is restricted to I-A^{b} (Figure 5.1).

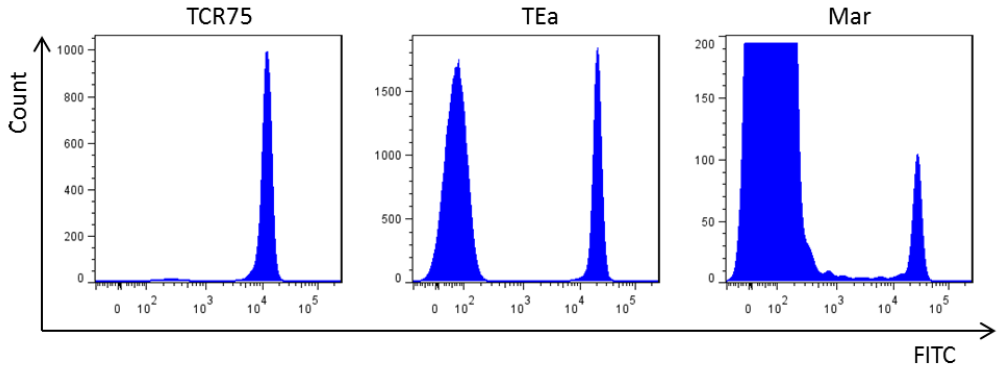


Figure 5.1 I-A^{b} restricted TCR transgenic CD4 T cells do not recognise antigen presented by I-A^{bm12}
Male BALB/c cardiac allografts were transplanted into female bm12 recipients (n=2). 4-6x10^{6} CFSE labelled TCR75, TEa and Mar splenocytes were adoptively transferred and proliferation examined after 6 days by flow cytometry. No proliferation was observed, confirming the transgenic CD4 T cells are unable to recognise their allopeptide presented by the I-A^{bm12} MHC class II molecule.

To control for differences between the individual models presented previously (Section 3.3.1), the three indirect-pathway TCR transgenic CD4 T cell populations were transferred simultaneously. Mar and TEa cells are identified using antibodies against their specific Vα and Vβ subunits, whilst TCR75 cells express a congenic marker: CD90.1/Thy 1.1 (Table 2.1). Notably, TEa and Mar possess an identical Vβ subunit (Vβ6) so it was important to confirm the ability to distinguish between the two populations on flow cytometry. This was achieved using an antibody directed against the Vα2 subunit of the TEa TCR (Figure 5.2). It is noteworthy that double gating on Vα and Vβ subunits substantially reduced the number of non-transgenic, endogenous CD4 T cells present within the gate, resulting in cleaner proliferation histograms, similar to that achieved with TCR75 (Figure 5.1 left panel). This approach was adopted for TEa detection throughout the remainder of this thesis.

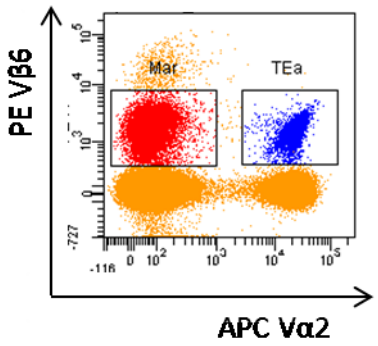


Figure 5.2 Ability to distinguish TEa and Mar TCR transgenic cells, despite shared Vβ subunit
A Bm12.Kd.IE cardiac allograft was transplanted into a C57BL/6 recipient and 4-6x10^6 CFSE labelled TEa and Mar splenocytes adoptively transferred on the day of transplantation. After 6 days the spleen was harvested and processed by flow cytometry. Despite sharing Vβ6 subunit, TEa and Mar CD4 T cells could be distinguished by double staining TEa with Vα2.

Examination of early (Week 1) and late (Week 5) proliferation following transplantation confirmed the previous findings. At the early time point robust indirect-pathway responses were detected against donor MHC class I, MHC class II and minor H-Y alloantigen. At the late time point the responses against MHC class I and minor H-Y antigen were preserved, albeit the anti-H-Y response was attenuated. In contrast, an indirect-pathway response against MHC class II was undetectable (Figure 5.3).

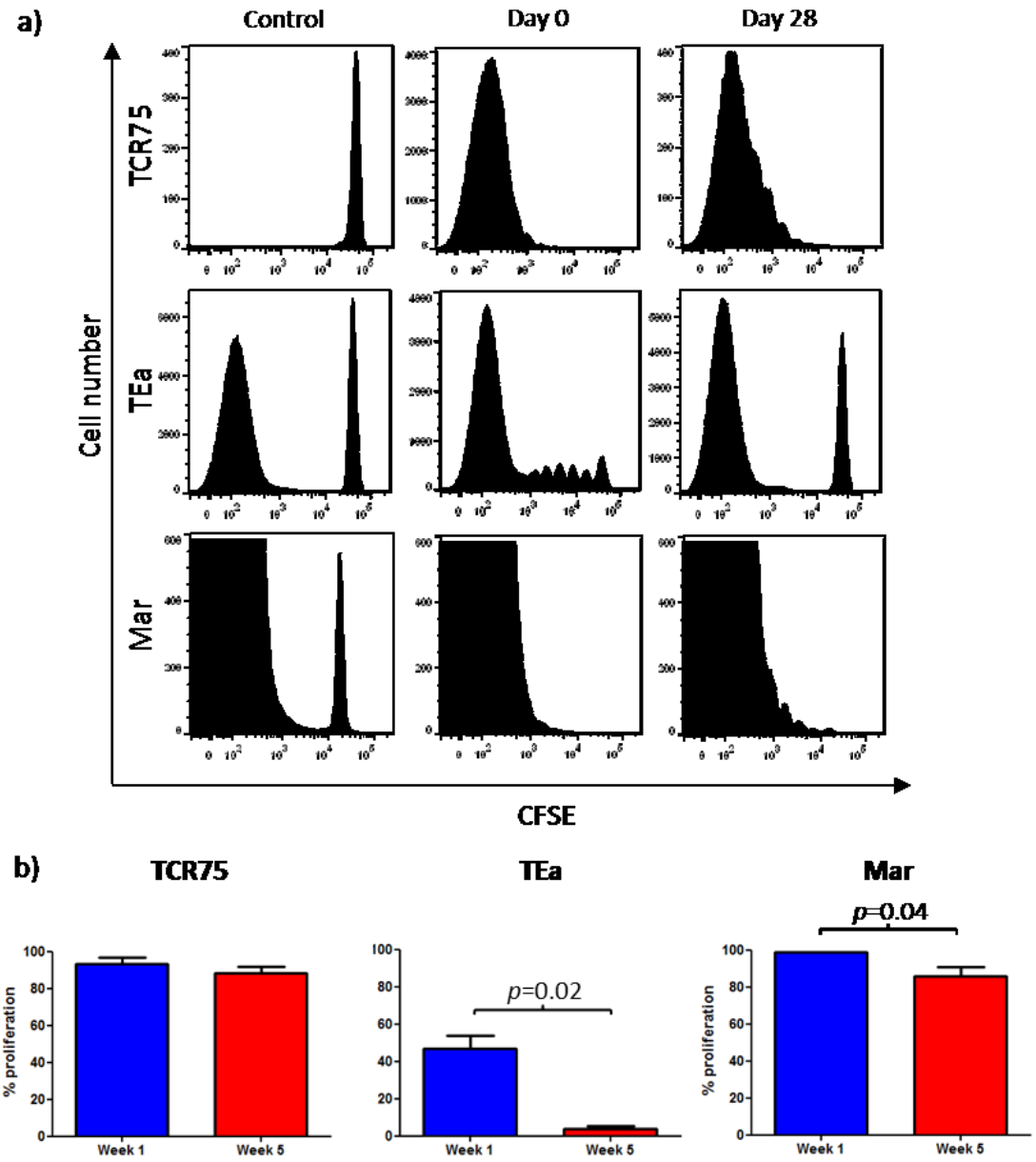


Figure 5.3 Duration of indirect-pathway allorecognition is variable depending on the alloantigen
Representative flow cytometry plots (a) and bar chart summarising proliferation (b) following adoptive transfer of 4-6x10^6 CFSE labelled TCR75, TEa and Mar splenocytes i.v. into C57BL/6 recipients of bm12.Kd.IE cardiac allografts, 0 (Week 1) or 28 days (Week 5) following transplantation or naïve C57BL/6 control mice (all groups n=5). Animals were sacrificed 6 days later and splenocytes analysed by flow cytometry. Proliferation of transgenic CD4 T cells quantified using FlowJo software (Tree Star, Oregon). *P* values using Mann Whitney test.

5.3.2 *Indirect-pathway responses against MHC Class II are limited by antigen availability*

Examining MHC II indirect-pathway allorecognition more closely revealed that even at week 2 following transplant no proliferation of TEa CD4 T cells was identified, suggesting the response decays within the first week following transplantation (Figure 5.4). It was hypothesised that the short-lived nature of MHC class II indirect-pathway allorecognition reflects rapid clearance of donor MHC class II expressing cells following transplantation.

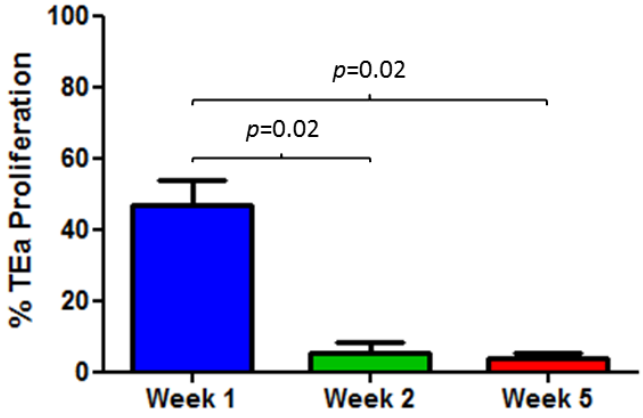


Figure 5.4 MHC class II indirect allorecognition is restricted to the first week following transplantation
Bm12.Kd.IE cardiac allografts were transplanted into C57BL/6 recipients and 4-6x10^6 CFSE labelled TEa splenocytes adoptively transferred i.v. at week 1, 2 and 5 (n=5). Six days following transfer recipient splenocytes were analysed by flow cytometry. Proliferation of transgenic CD4 T cells was quantified using FlowJo software (Tree Star, Oregon). *P* values using Mann Whitney test. Proliferation was only observed when transferred at week 1 suggesting MHC class II indirect allorecognition to be short-lived

5.3.2.1 Primed transgenic TEa cells do not proliferate late after transplantation

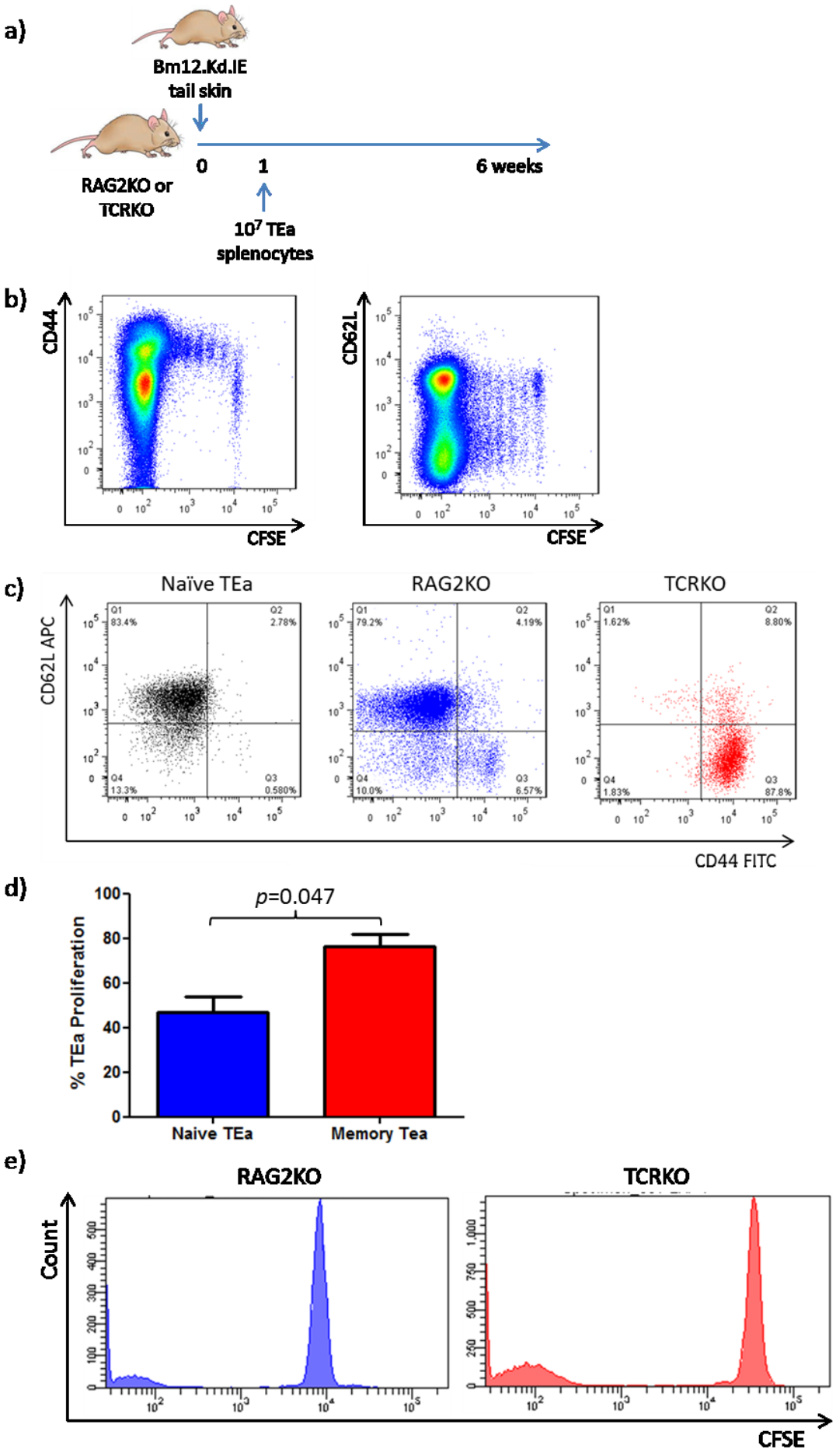
In experiments thus far only naïve transgenic CD4 T cells have been transferred. There is the possibility that beyond the first week following transplantation a source of donor class II persists and initiated indirect-pathway alloresponses are on-going. However, in the absence of adequate co-stimulation, naïve TEa CD4 T cells are insufficiently activated to undergo division. To examine this, antigen experienced, memory, TEa CD4 T cells were generated.

Bm12.Kd.IE skin transplants were performed on to RAG2KO recipients. TEa splenocytes (1x10^7) were adoptively transferred the day following transplantation. Six weeks following skin

transplantation, CD4 T cells were stained by flow cytometry for surface expression of CD44 and CD62L (Figure 5.5a). Antigen experienced, memory, CD4 T cells are reported to up-regulate CD44 and down-regulate CD62L expression[81]. In keeping with this, when naïve TEa CD4 T cells are induced to undergo proliferation in transplant recipients, an up-regulation of CD44 and down-regulation of CD62L is observed (Figure 5.5b).

However, CD4 T cells isolated from the skin-grafted RAG2KO recipients did not exhibit a memory phenotype and instead, appeared phenotypically similar to naïve TEa CD4 T cells (Figure 5.5c). Given the report that optimal CD4 T cell memory generation requires B cells[367], the experiment was repeated using T cell deplete TCRKO recipients, which retain a functional B cell compartment[256]. Six weeks following skin transplantation, isolated CD4 T cells were found to have up-regulated CD44 and down-regulated CD62L, supporting a requirement for B cells in the development of CD4 T cell memory (Figure 5.5c). In support of their memory phenotype, these cells proliferated more vigorously upon transfer into C57BL/6 recipients of bm12.Kd.IE hearts when transferred on the day of transplantation (Figure 5.5d). However, when transferred four weeks following transplantation, no proliferation was detected (Figure 5.5e). These results suggest that beyond the first week following transplantation there is an absence of target processed I-E allopeptide epitope, rather than expression in a non-stimulatory fashion. However, it must be noted that constitutive human endothelial MHC class II expression may limit extrapolation of these findings to clinical transplantation.

Figure 5.5 Antigen experienced TEa CD4 T cells do not proliferate late following transplantation
To overcome the limitation of transferring naïve TEa CD4 T cells late after transplantation, antigen experienced, memory, TEa were generated. Bm12.Kd.IE tail skin was transplanted onto RAG2KO or TCRKO recipients and $1x10^7$ TEa splenocytes adoptively transferred i.v. on the day of transplantation (a). Antigen experienced CD4 T cells upregulate CD44 and downregulate CD62L[81]. This is demonstrated by staining TEa splenocytes for flow cytometry analysis 6 days following adoptive transfer into C57BL/6 recipients of bm12.Kd.IE cardiac allografts on the day of transplantation, as described above. The parent population is $CD44^{low}$ $CD62L^{hi}$, and with proliferation, daughter population expression of CD44 increases and CD62L falls (b). Six weeks after skin transplantation, recipient RAG2KO and TCRKO spleens were processed by flow cytometry (c). An antigen experienced, memory, population of CD4 T cells was observed in TCRKO but not RAG2KO recipients. To confirm a memory phenotype $4\text{-}6x10^6$ antigen experienced TEa CD4 T cells (MACS separated with anti-CD4 microbeads) from TCRKO skin transplant recipients were CFSE labelled and adoptively transferred into C57BL/6 recipients of bm12.Kd.IE cardiac allografts on the day of transplantation (n=3). Augmented proliferation was observed when compared to naïve TEa on flow cytometry 6 days later (d). When similarly purified and CFSE labelled CD4 T cells (from both RAG2KO and TCRKO skin transplant recipients) were adoptively transferred into C57BL/6 recipients of bm12.Kd.IE cardiac allografts 4 weeks following transplantation, no proliferation was observed (representative flow cytometry histogram plots from n=4). *P* values using Mann Whitney test.


a)
Bm12.Kd.IE
tail skin
RAG2KO or
TCRKO
0
1
6 weeks
10^7 TEa
splenocytes
b)
CD44
CFSE
CD62L
CFSE
c)
Naïve TEa
RAG2KO
TCRKO
CD62L APC
Q1 83.4%
Q2 2.78%
Q4 13.3%
Q3 0.580%
Q1 79.2%
Q2 4.19%
Q4 10.0%
Q3 6.57%
Q1 1.62%
Q2 8.80%
Q4 1.83%
Q3 87.8%
CD44 FITC
d)
p=0.047
% TEa Proliferation
100
80
60
40
20
0
Naive TEa
Memory Tea
e)
RAG2KO
TCRKO
Count
CFSE

5.3.2.2 Y-Ae antibody supports no ongoing MHC class II allopeptide presentation

The Y-Ae antibody is a clonotypic monoclonal antibody specific for donor derived I-E α-chain peptide 52-68, presented in the context of recipient MHC II I-A^b (Figure 5.6a)[368]. This epitope is identical to the specificity of the TEa TCR. The Y-Ae antibody permits examination of indirect-pathway donor MHC II antigen presentation in this model.

The BALB/c x C57BL/6 F1 (CB6F1) strain provides a positive control for Y-Ae staining, since there is co-expression of MHC II I-E and I-A^b on all class II expressing cells. A naïve C57BL/6 and a C57BL/6 recipient of a bm12.Kd cardiac allograft (identical to bm12.Kd.IE, but lacking the MHC II I-E) were used as negative controls, to exclude that Y-Ae is cross-reactive with any non-IE donor antigen. A degree of increased staining was observed with $CD11b^+$ macrophages in the transplanted negative control, perhaps reflecting the previously reported degree of cross-reactivity of the Y-Ae antibody[368].

Recipients of bm12.Kd.IE cardiac allografts revealed strongly positive Y-Ae staining of $CD11c^+$ dendritic cells and $CD11b^+$ macrophages at 4 days following transplantation, confirming antigen presentation to be occurring at this early time (Figure 5.6b,c). However, two weeks following transplantation, the majority of APCs stained negative for Y-Ae, further supporting that there is only limited, if any, MHC class II allopeptide presentation occurring, commensurate with the lack of TEa proliferation observed at this time.

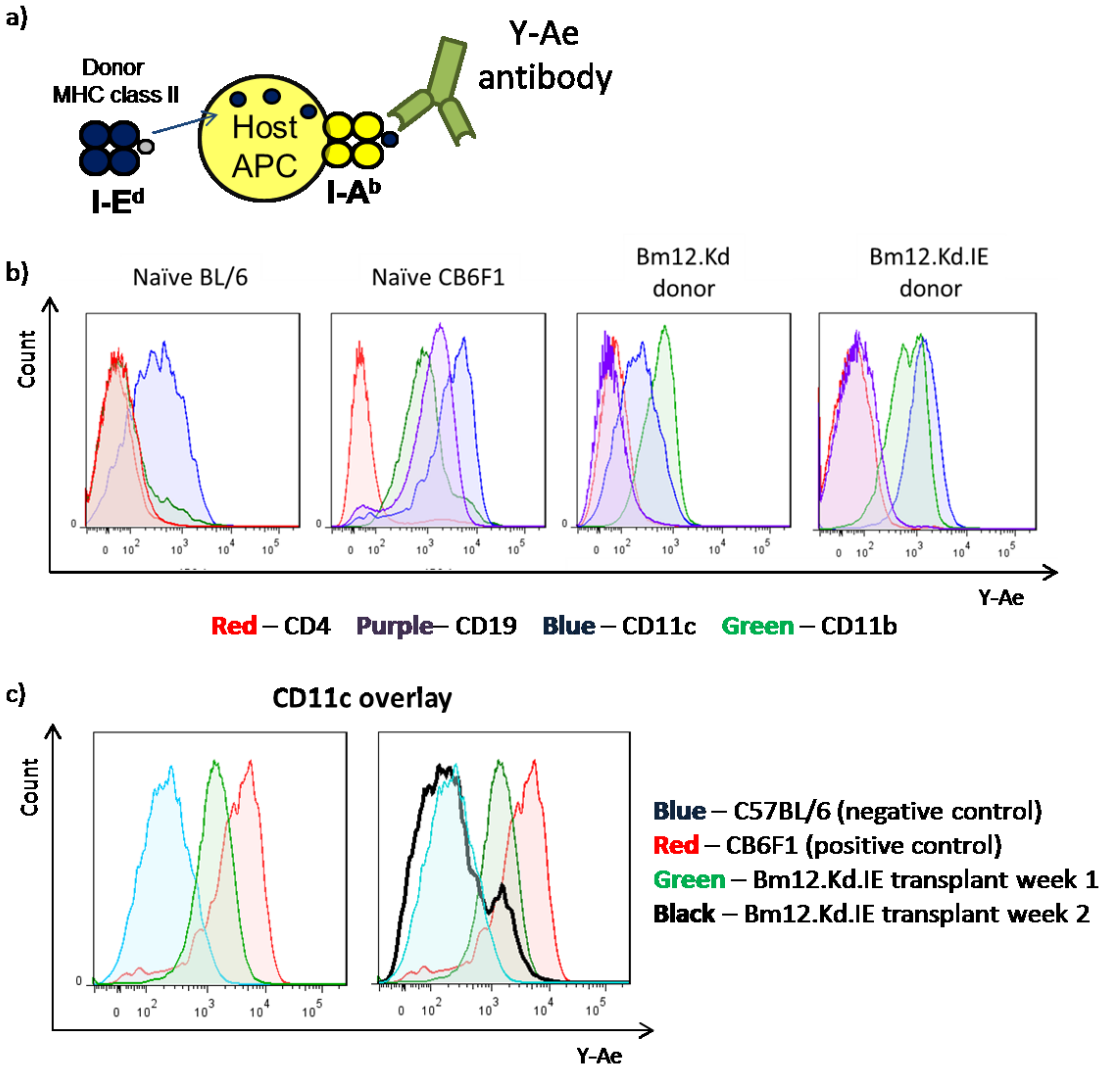


Figure 5.6 Y-Ae clonotypic antibody permits assessment of allopeptide presentation

(a) The epitope of the Y-Ae antibody is created by presentation of MHC class II I-E allopeptide presented by MHC class II I-A^b, which in the bm12.Kd.IE to C57BL/6 model represents indirect-pathway presentation of donor mismatched MHC class II. (b) Splenocytes from naïve C57BL/6 (BL/6) and CB6F1, and from C57BL/6 recipients of bm12.Kd and bm12.Kd.IE cardiac allografts, in the first week following transplantation, were processed by flow cytometry and staining with Y-Ae of leukocyte subsets presented as histogram overlays (representative of n=3). (c) Left panel displays an overlay of CD11c positive leukocyte Y-Ae staining from naïve C57BL/6 (negative control), CB6F1 (positive control) and experimental C57BL/6 recipients of bm12.Kd.IE cardiac allografts 4 days following transplantation, demonstrating positive staining of the experimental group. Right panel additionally overlays C57BL/6 recipients of bm12.Kd.IE cardiac allografts 2 weeks following transplantation demonstrating a marked reduction in Y-Ae staining by this time.

5.3.2.3 Donor APCs are the major source of MHC II alloantigen

MHC class II is predominantly expressed by professional APCs (DC's, macrophages and B cells), although endothelial expression has also been described[86, 369]. To examine the hypothesis that donor APCs are the major source of antigen for indirect-pathway priming, donors were depleted of professional APCs and the resultant proliferation of transgenic CD4 T cells quantified – in a similar manner to the previous chapter for direct-pathway allorecognition.

To study the role of APCs, donor mice were either subjected to lethal irradiation to deplete their haematopoietic compartment, or were treated to deplete APC populations. $CD11c^+$ DCs were depleted by administering diphtheria toxin to mice expressing the diphtheria toxin receptor under the CD11c promoter, and B cells by administering depleting murine anti-CD20 antibody. An attempt was made to deplete macrophages using liposomal clodronate, but this did not prove successful and was not pursued (Section 4.3.3.2).

These experiments required development of a bm12.Kd.IE-CD11c.DTR donor strain in which $CD11c^+$ DCs would be susceptible to diphtheria toxin and could be depleted (Figure 5.7).

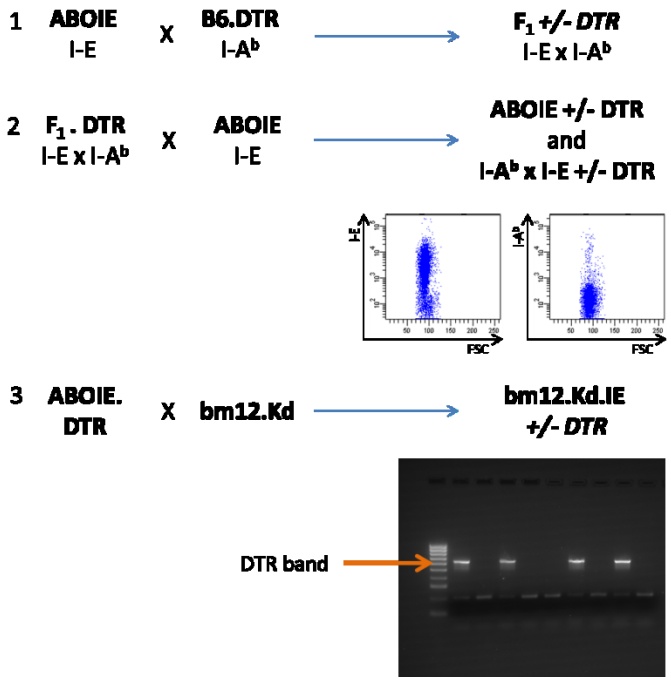


Figure 5.7 Developing bm12.Kd.IE.DTR strain
(1) First, ABOIE were crossed with B6.DTR. (2) The F_1 offspring expressing the DTR gene were backcrossed with ABOIE, selecting for offspring expressing: DTR gene and the MHC class II I-E but not I-A^b (by flow cytometry of peripheral blood – left panel confirming I-E expression, right panel showing absence of I-A^b). (3) These 'ABOIE.DTR' mice were then crossed with bm12.Kd to generate bm12.Kd.IE.DTR donors. Expression of the DTR gene was confirmed by PCR (performed by S Rehakova).

In this series of experiments, both TCR75 and TEa CD4 T cells were transferred in order to compare the impact of donor treatment on the donor MHC class I and II indirect-pathway responses. Lethally irradiating donors abrogated TEa proliferation whilst TCR75 proliferation was unchanged. This suggests that passenger donor professional APCs are the major source of MHC class II, but not class I, for indirect-pathway presentation. This finding was confirmed by depletion of donor APC subsets. DC or B cell depletion reduced TEa proliferation, although DC depletion had a greater impact. Depleting *both* DCs and B cells resulted in a similar degree of proliferation as was observed with lethal irradiation. In contrast, depletion of donor APCs had a negligible impact upon TCR75 proliferation (Figure 5.8a,b).

Commensurate with absent TEa proliferation, depletion of donor DCs and B cells prevented binding of the clonotypic Y-Ae antibody to recipient DCs confirming absence of I-E allopeptide presentation in the recipients (Figure 5.8c).

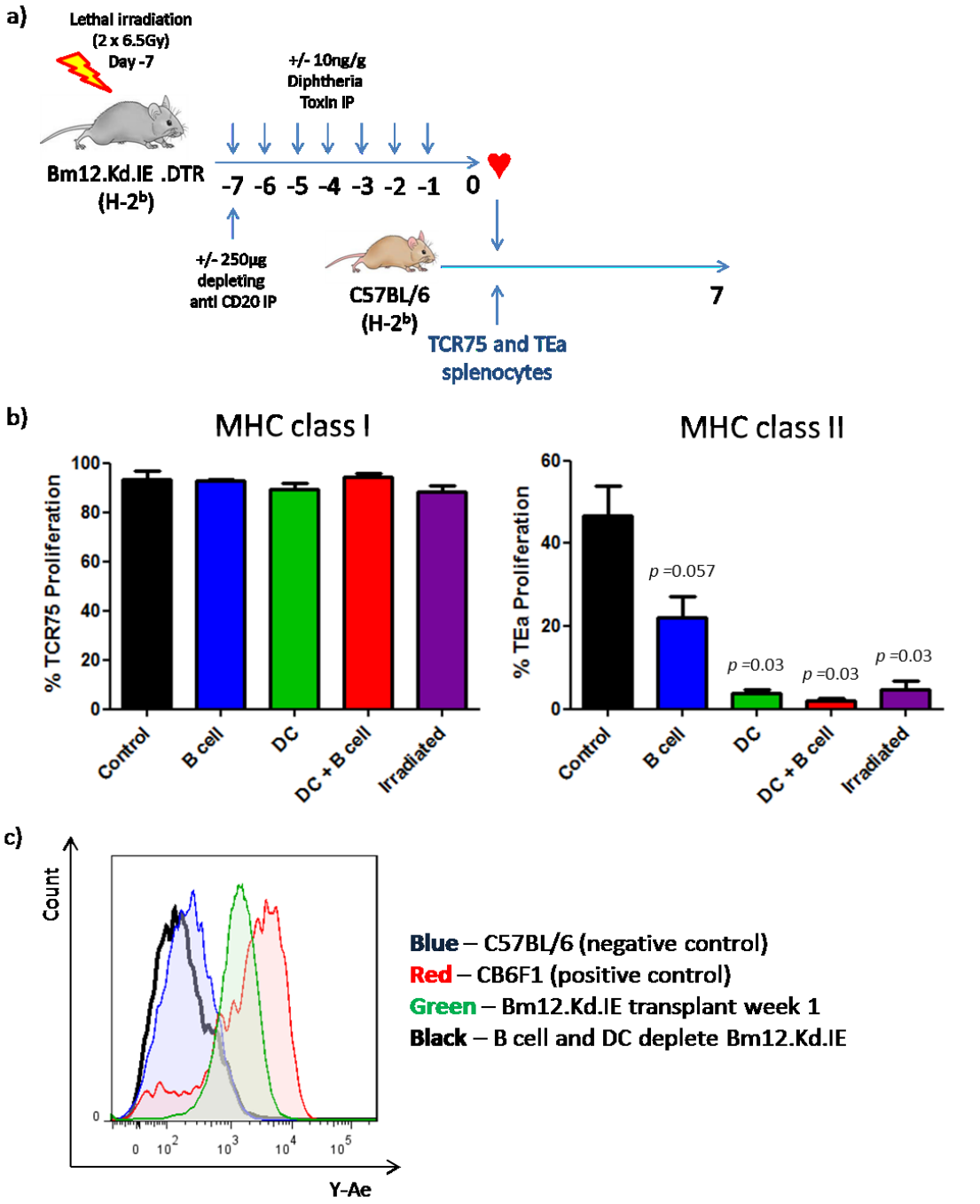


Figure 5.8 Depletion of donor APCs significantly abrogates MHC class II indirect allorecognition

(a) Bm12.Kd.IE.DTR cardiac allografts were transplanted into C57BL/6 mice following either lethal irradiation (13 Gray split dose) or treatment to deplete donor APC subsets (administration of 10ng/g diphtheria toxin daily for 7 days prior to transplantation to deplete DCs and/or administration of 250µg murine anti-CD20 antibody to deplete B cells). 4-6x10^6 CFSE labelled TCR75 and TEa splenocytes were adoptively transferred i.v. on the day of transplantation and recipient spleens were harvested after 6 days for analysis of transgenic CD4 T cell proliferation by flow cytometry. All groups n=4 (b) TCR75 and TEa proliferation quantified by FlowJo, revealing that donor APC depletion and irradiation significantly attenuate TEa proliferation, but TCR75 proliferation is unchanged. (c) Further confirmation that donor APCs are the major source of MHC class II allopeptide is provided by Y-Ae staining of recipient CD11c DCs. Following transplantation with DC and B cell deplete donors, no Y-Ae staining is detected in the first week, unlike with untreated donors (green line). *P* values using Mann Whitney test.

5.3.2.4 Innate immunity is not responsible for limiting class II indirect allorecognition

Having confirmed passenger donor APCs to be the major source of donor MHC class II for indirect-pathway allorecognition, further experiments were designed to clarify what limits their survival. It is widely held that donor APCs are rapidly cleared by recipient innate immune responses and that this accounts for the short lived nature of the direct-pathway[76]. To examine the roles of the innate and adaptive responses in limiting the MHC II indirect-pathway response in this model, RAG2KO mice lacking adaptive immunity were utilised[370].

Cell transfer experiments described previously (Section 3.3.4) demonstrated that bm12.Kd.IE donor cells survive long-term, equivalent to syngeneic cells, in RAG2KO recipients, identifying that innate immunity is not responsible for donor APC clearance in this model. From this, it was hypothesised that class II indirect allorecognition may be long-lived in RAG2KO recipients.

Bm12.Kd.IE hearts were therefore transplanted into RAG2KO recipients and TEa proliferation quantified at early and late time-points. Unlike in C57BL/6 recipients, class II indirect-pathway allorecognition occurred at the late time-point (Figure 5.9a). The degree of proliferation observed at the late time point though was attenuated, and likely reflects natural senescence of donor DCs, since they have an expected half-life of at most a few weeks depending on subtype[371, 372]. In contrast, B cells appear to establish a long-term splenic population in these RAG2KO mice, and may be the major source of class II at this late time point (Figure 3.15). In keeping with TEa proliferation at the late time-point in RAG2KO recipients, Y-Ae staining was demonstrated on recipient DCs confirming donor MHC II antigen processing and presentation to be occurring at this time (Figure 5.9b).

To extend these findings an attempt was made to abrogate late MHC II indirect allorecognition in RAG2KO recipients by depleting donor B cells or DCs late after transplantation. Bm12.Kd.IE-CD11c.DTR mice were used as donors, permitting sensitivity of donor DCs to diphtheria toxin; B cells were depleted using murine anti-CD20 antibody. No significant impact on late TEa proliferation was observed following this treatment (Figure 5.9c). Although surprising, in the case of DC depletion this may reflect incomplete depletion[264], or that donor DCs have senesced by this point. Alternatively, it may reflect the difficulty in completely depleting donor B cells once they have established as the dominant splenic population[373].

Together, these results highlight a critical role for the adaptive immune response in limiting the survival of donor APCs, and therefore MHC class II indirect-pathway allorecognition.

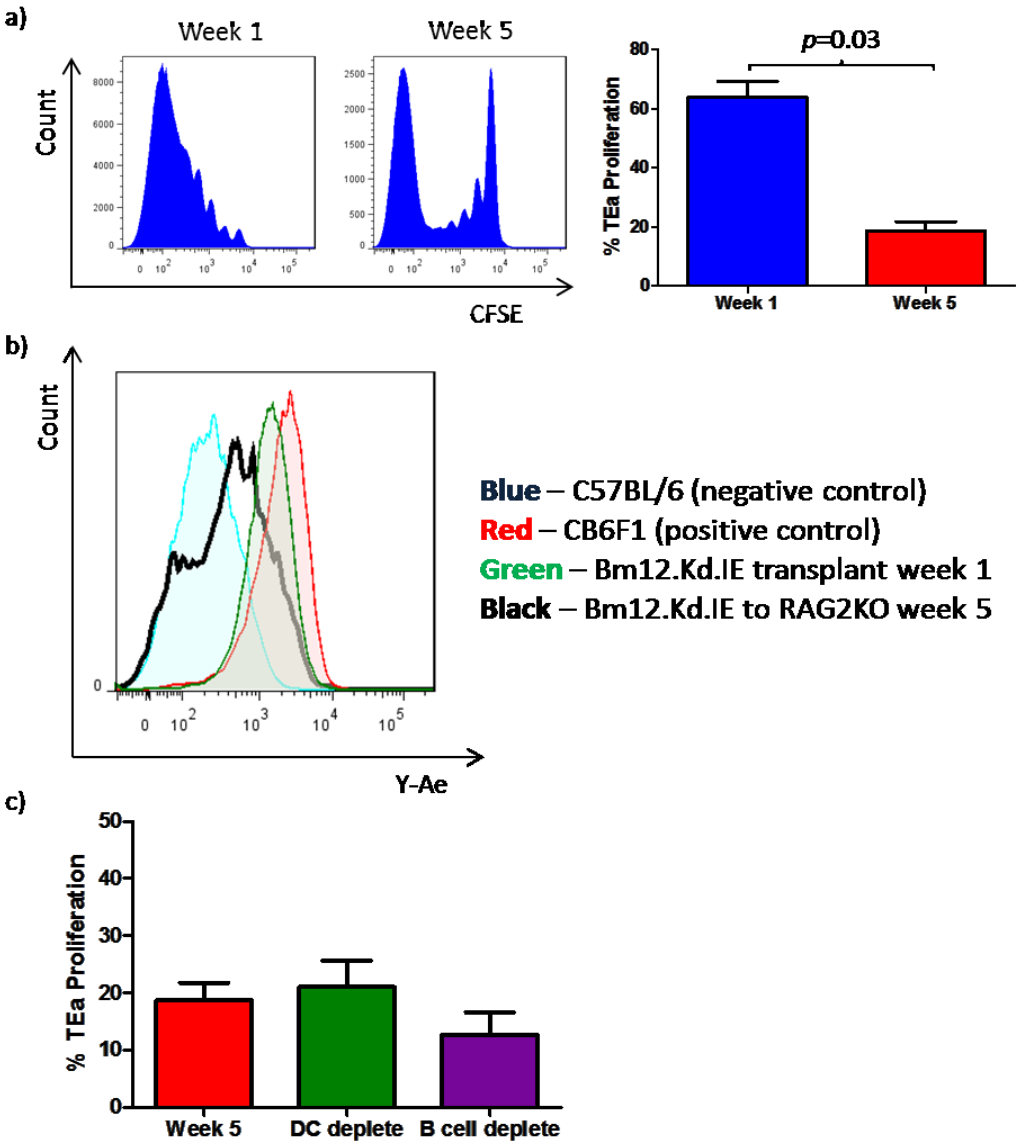


Figure 5.9 Indirect-pathway recognition of donor MHC class II is long-lived in RAG2KO recipients
(a) Bm12.Kd.IE cardiac allografts were transplanted into RAG2KO recipients (lacking adaptive immunity) and 4-6x10^6 CFSE labelled TEa splenocytes were adoptively transferred i.v. on the day of transplantation (week 1) or week 5. Six days following transfer recipient spleens were harvested and processed by flow cytometry to quantify TEa proliferation. Left panel shows representative CFSE histograms and right panel comparing week 1 and week 5 proliferation (n=5 in each group) where it is notable that proliferation occurs at the late time-point, although it is significantly attenuated. (b) In contrast to wildtype recipients, where no Y-Ae staining of recipient DCs is observed beyond the first week following transplantation, positive staining was observed in RAG2KO recipients 5 weeks following transplantation (black line). (c) In an attempt to demonstrate longevity of passenger APCs in RAG2KO recipients as the reason for longevity of MHC class II allorecognition in this model, donor APCs were depleted in recipients 4 weeks following transplantation, prior to CFSE labelled TEa splenocytes transfer at week 5. Bm12.Kd.IE.DTR donors were utilised and donor DCs depleted by administration of 10ng/g diphtheria toxin daily for 7 days prior to TEa transfer and alternate days thereafter. B cells were depleted by administration of 250µg murine anti-CD20 7 days prior to TEa transfer. (RAG2KO mice do not possess B cells, therefore targeting only passenger donor B cells). Recipient spleens were analysed as above by flow cytometry to quantify TEa proliferation (all groups n=4). *P* values using Mann Whitney test.

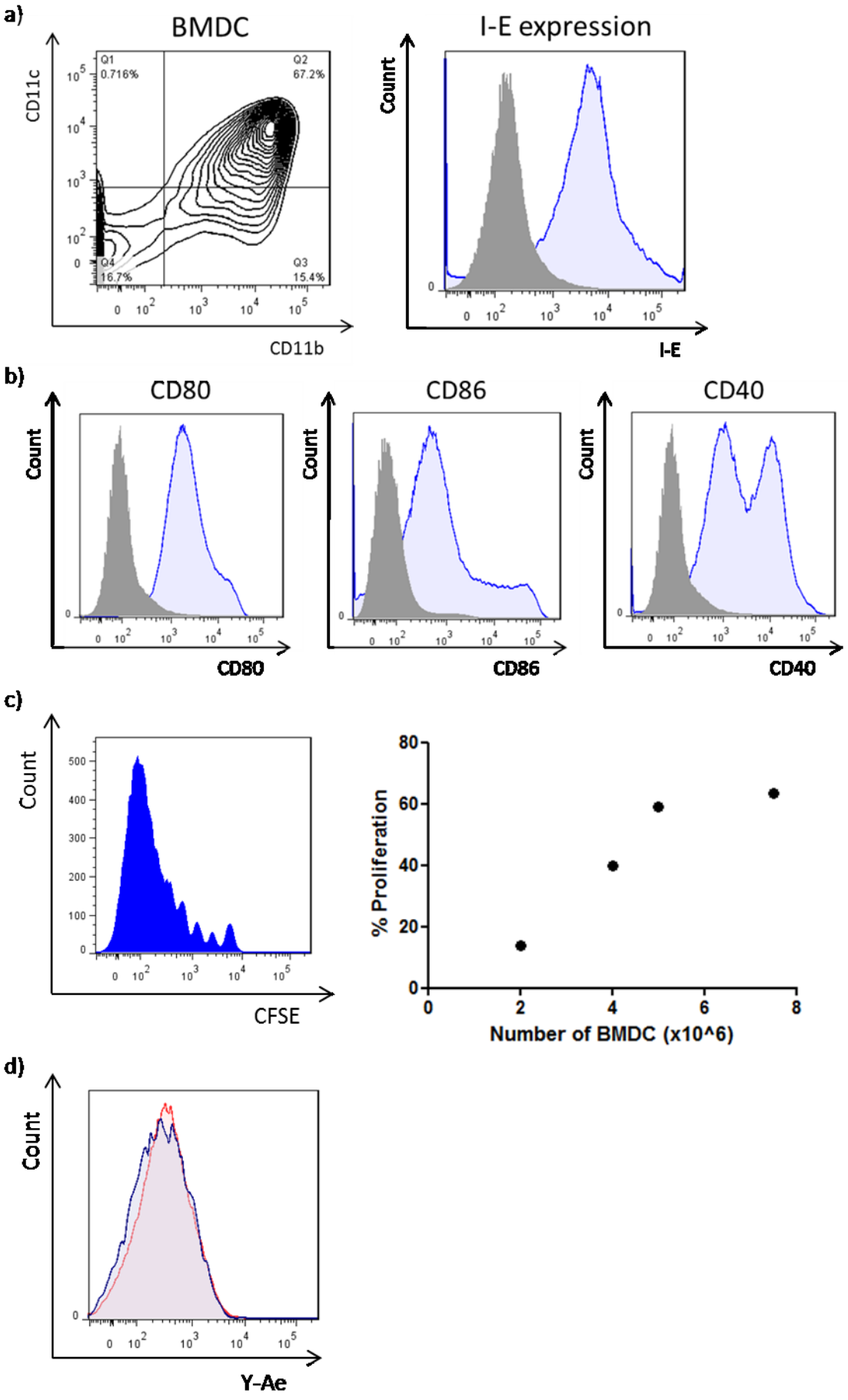
5.3.2.5 Late class II indirect allorecognition can be reconstituted with antigen transfer

To further confirm that lack of ongoing late MHC class II indirect-pathway allorecognition is a consequence of early antigen clearance, the possibility of reconstituting the response was next considered. Using an established protocol, bone marrow-derived DCs (BMDC's) were cultured from donor bm12.Kd.IE bone marrow as a source of donor MHC class II antigen[271, 272]. Following an eight day period of culture, BMDCs were harvested and expression of the MHC class II I-E was confirmed by flow cytometry. These cells also expressed the activation and co-stimulatory markers CD80, CD86 and CD40 (Figure 5.10a,b). The ability of donor BMDCs to act as a source of MHC class II that could be processed and presented by the indirect-pathway was confirmed by adoptive transfer into naïve C57BL/6 mice, where TEa proliferation was observed, in a dose-dependent manner (Figure 5.10c). The BMDCs demonstrated no Y-Ae reactivity themselves confirming that any TEa proliferation is a consequence of allopeptide processing and presentation by recipient APCs (Figure 5.10d).

If BMDCs were adoptively transferred 28 days prior to injection of TEa splenocytes into a RAG2KO animal, no proliferation was observed suggesting that *in vitro* cultured BMDCs do not demonstrate long term survival *in vivo*, likely due to natural senescence.

Figure 5.10 Bm12.Kd.IE bone marrow derived DCs (BMDCs) can act as a source of MHC class II for indirect-pathway processing and presentation

Bm12.Kd.IE bone marrow was cultured *in vitro* under conditions encouraging DC differentiation (IL-4 and GM-CSF)[271, 272]. (a) Upon harvest on day 8, BMDC phenotype was confirmed on flow cytometry with the majority of cells expressing CD11b and CD11c surface markers (left panel). Furthermore, strong expression of MHC class II I-E was observed (right panel, blue line; grey shaded histogram represents isotype control). (b) The BMDCs also demonstrated surface expression of co-stimulatory molecules: CD80, CD86 and CD40. (c) Increasing numbers of BMDCs were adoptively transferred i.v. into naïve C57BL/6 mice, with 4-6x10^6 CFSE labelled TEa transferred i.v. the following day and proliferation quantified 6 days later. TEa proliferation was observed (left panel) in a dose dependent manner (right panel) following BMDC transfer confirming BMDCs can act as a source of antigen for recipient processing and presentation by the indirect-pathway (Proliferation was not observed in control animals not transferred BMDCs). All groups n=2. (d) BMDCs themselves did not bind the clonotypic Y-Ae antibody confirming they themselves do not possess the epitope for TEa recognition by the direct-pathway.


a)
BMDC
CD11c
CD11b
Q1
0.716%
Q2
67.2%
Q4
16.7%
Q3
15.4%
I-E expression
Counrt
I-E
b)
CD80
CD86
CD40
Count
CD80
CD86
CD40
c)
Count
CFSE
% Proliferation
Number of BMDC (x10^6)
d)
Count
Y-Ae

Having confirmed that BMDCs are sufficient to drive an indirect-pathway class II alloresponse, next they were adoptively transferred into animals that had previously been transplanted to assess the possibility of reconstituting the late class II indirect-pathway response, thus supporting that the absence of a late class II alloresponse reflects a lack of donor antigen.

Donor BMDCs were adoptively transferred into RAG2KO and C57BL/6 recipients of bm12.Kd.IE cardiac allografts 28 days following transplantation (Figure 5.11a). In RAG2KO recipients, proliferation of TEa CD4 T cells was restored to a level similar to that observed at the early time point, confirming that the late MHC class II indirect-pathway response had been reconstituted. Proliferation was greater in the grafted animals than the naïve positive controls receiving an identical number of cells, presumably due to the fact that the transferred BMDCs were supplementary to the natural donor APCs surviving in the RAG2KO animal, responsible for the indirect-pathway allorecognition usually observed at this late time point.

Although late TEa proliferation was observed in C57BL/6 recipients following BMDC transfer, it was significantly attenuated compared with the extent of proliferation observed in non-transplanted, naïve mice receiving the same number of BMDCs (Figure 5.11b,c). The difference in proliferation observed in naïve C57BL/6 and RAG2KO recipients reflects the lower number of BMDCs transferred in the RAG2KO experiment due to BMDC culture yield. When an identical number of BMDCs were transferred to naïve C57BL/6 and RAG2KO mice, similar proliferation was observed (Figure 5.11d).

Why there was reduced proliferation in a transplanted, as compared to naïve, C57BL/6 mouse receiving the same number of BMDCs was not clear (Figure 5.11b). One possibility is that there was competition from endogenous, antigen experienced CD4 T cells which were primed to respond more vigorously and thus out-competed the naïve transferred TEa cells. To investigate this, the experiment was repeated but 7 days prior to BMDC transfer, recipient CD4 T cells were depleted by administration of a depleting anti-CD4 antibody. There was no significant increase in TEa proliferation observed despite removing the potential competition, suggesting this not to be the reason (Figure 5.11e). Perhaps, then, this previously encountered donor antigen is rapidly cleared in a quiescent manner not resulting in activation of naïve CD4 T cells by sensitised CD8 T cells or circulating alloantibody. In this regard, it is recognised that in the absence of inflammatory stimuli DCs can present antigen in a non-stimulatory manner[374].

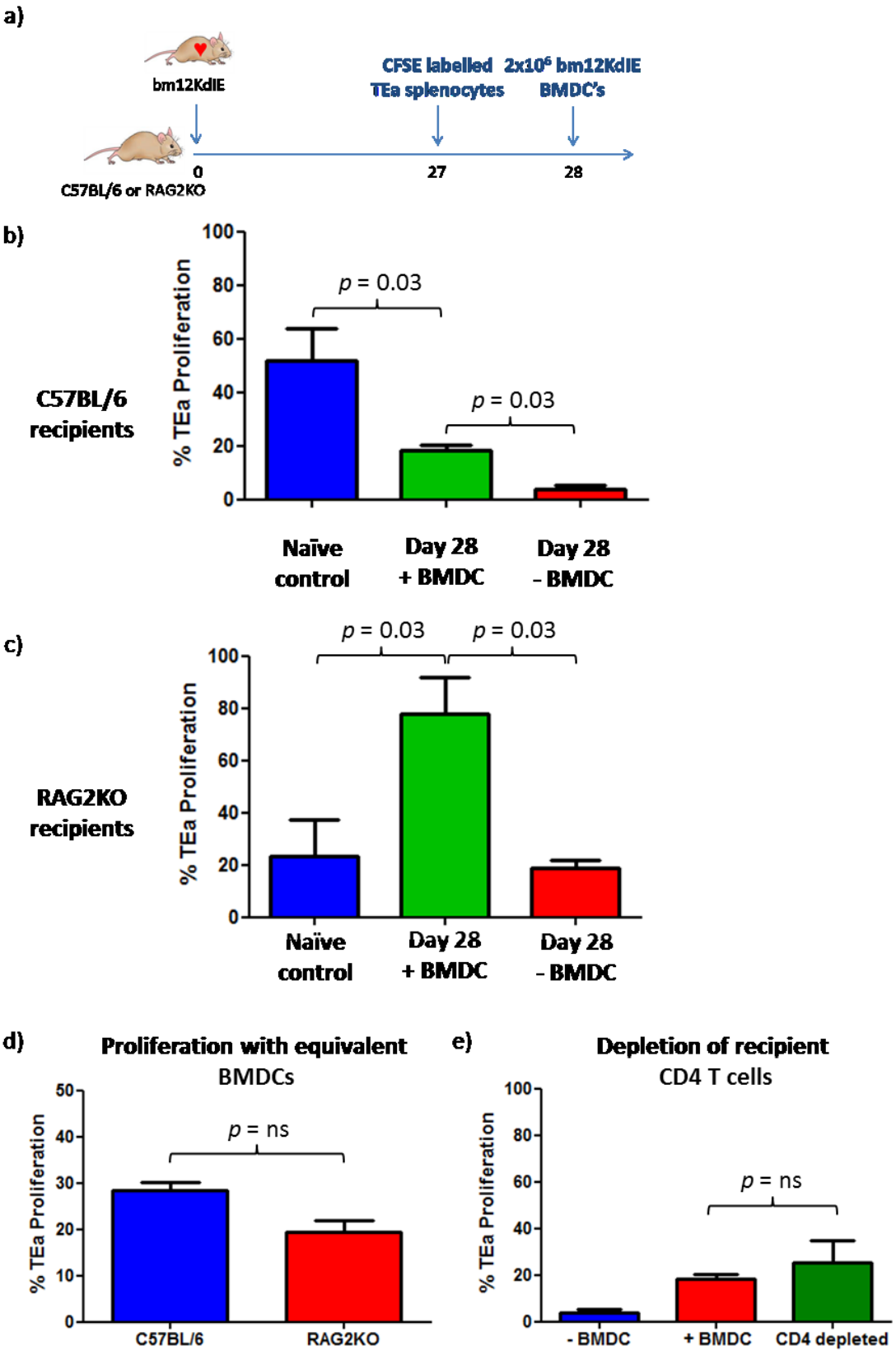

a)
bm12KdIE
CFSE labelled TEa splenocytes
2x10⁶ bm12KdIE BMDC's
C57BL/6 or RAG2KO
0
27
28
b)
C57BL/6 recipients
% TEa Proliferation
p = 0.03
p = 0.03
Naïve control
Day 28 + BMDC
Day 28 - BMDC
c)
RAG2KO recipients
% TEa Proliferation
p = 0.03
p = 0.03
Naïve control
Day 28 + BMDC
Day 28 - BMDC
d)
Proliferation with equivalent BMDCs
% TEa Proliferation
p = ns
C57BL/6
RAG2KO
e)
Depletion of recipient CD4 T cells
% TEa Proliferation
p = ns
- BMDC
+ BMDC
CD4 depleted

Figure 5.11 BMDC transfer into recipients following transplantation reconstituted MHC class II indirect-pathway allorecognition

(a) Bm12.Kd.IE cardiac allografts were transplanted into wildtype or RAG2KO C57BL/6 recipients. 4-6x10^6 CFSE labelled TEa splenocytes were adoptively transferred i.v. 27 days later. The following day 2x10^6 BMDCs were transferred i.v. and TEa proliferation quantified 6 days later. (b) In naïve wildtype C57BL/6 mice, proliferation was again observed following BMDC transfer. Whilst minimal proliferation is seen at this late time point usually, transfer of BMDCs reconstituted TEa proliferation – although not to the same extent as observed in non-transplanted, naïve, C57BL/6 mice. (c) In naïve RAG2KO mice, BMDC transfer similarly resulted in TEa proliferation. In RAG2KO recipients a degree of proliferation is usually observed at the late time point due to donor cell survival. Transfer of BMDCs augmented the extent of proliferation observed in these recipients. (d) To compare the extent of proliferation driven by BMDCs in naïve wildtype and RAG2KO C57BL/6 mice, 2x10^6 BMDCs were simultaneously transferred the day after CFSE labelled TEa. An equivalent degree of proliferation was observed. (e) To examine if endogenous CD4 T cell proliferation competes with TEa at the late time point following BMDC transfer (following the observation that less proliferation occurs in transplanted than naïve BMDC recipients), the experiment was repeated but this time, recipients were treated with 1mg depleting anti-CD4 7 and 5 days prior to TEa transfer. No substantial increase in TEa proliferation was observed. All groups n=4; *p* values using Mann Whitney test.

5.3.3 Persistent indirect-pathway CD4 T cell activation is driven by graft parenchymal alloantigen expression

The data presented suggests that the short-lived nature of MHC class II indirect-pathway allorecognition is a consequence of alloantigen clearance. In contrast, donor APC clearance had little impact on allorecognition of H-2K^{d} allopeptide (Figure 5.8b). To confirm that late anti-MHC class I indirect-pathway responses are due to graft parenchymal expression of alloantigen (and not due to an intrinsic difference between TCR75 and TEa transgenic CD4 T cells such as affinity for epitope), the following series of experiments were performed.

5.3.3.1 Ongoing proliferation is driven by recipient APC presentation of allopeptide

Recipient B cells and DCs were depleted in order to confirm that late proliferation of TCR75 CD4 T cells reflects ongoing antigen presentation by host professional APCs. For this experiment C57BL/6 x BL6.DTR chimeric mice were used as recipients to avoid the systemic complications of prolonged administration of DTX when administered to B6.DTR mice[257]. Recipients were treated with either depleting anti-CD20 mAb or diphtheria toxin from three weeks following transplantation, and week 5 proliferation of TCR75 assessed. Depletion of either cell type resulted in a significant abrogation of TCR75 proliferation, confirming the importance of recipient professional APCs to ongoing indirect-pathway alloresponses (Figure 5.12). Administration of diphtheria toxin to a non-DTR gene expressing C57BL/6 had no impact on TCR75 proliferation, excluding the possibility that diphtheria toxin interferes with T cell proliferation directly (data not shown). The inability to completely abrogate indirect-pathway presentation with both B cell and DC depletion likely reflect both the incomplete nature of depletion and the contribution of additional professional APCs not depleted, for example macrophages.

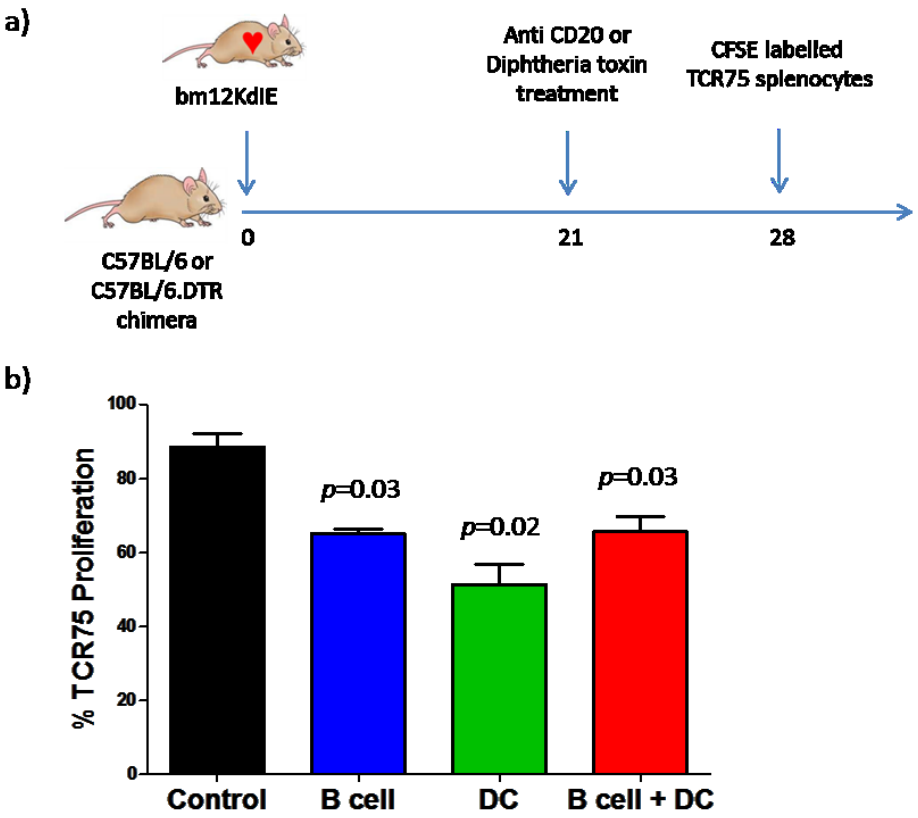


Figure 5.12 Ongoing professional APC presentation is required for late indirect-pathway alloresponses
(a) Bm12.Kd.IE cardiac allografts were transplanted into C57BL/6.DTR bone marrow-chimeric mice. Twenty-one days following transplantation, recipient APCs were depleted: DCs by administration of 10ng/g diphtheria toxin on alternate days and/or B cells by administration of 250µg depleting anti-CD20 antibody, both i.p.. 4-6x10^6 CFSE labelled TCR75 splenocytes were adoptively transferred on day 28 and proliferation quantified 6 days later. (b) Depletion of either APC subset resulted in attenuation of TCR75 proliferation confirming a role in indirect-pathway presentation. All groups n=5. *P* values using Mann Whitney test.

5.3.3.2 *Restricting H-2K^d alloantigen expression to haematopoietic lineage limits the longevity of class I indirect-pathway responses*

To confirm that ongoing indirect-pathway allorecognition of donor MHC class I reflects parenchymal expression, bone-marrow chimeric mice were developed in which H-2K^d expression was restricted to the haematopoietic lineage. The aim of this experiment was to provide the opportunity to use TCR75 transgenic CD4 T cells in a setting in which it would be predicted that proliferation would be short-lived from the findings with TEa CD4 T cells. This would then act as a control for any differences between the two transgenic CD4 T cells, and enable a direct comparison between indirect-pathway responses against MHC class I and MHC class II in this model using the same transgenic CD4 T cell population.

Bm12.IE mice, identical to bm12.Kd.IE other than lacking the expression of the MHC class I H-2K^d, were lethally irradiated and reconstituted with bm12.Kd.IE bone marrow. Chimerism was confirmed with expression of H-2K^d identified on lymphocytes 6 weeks following generation (Section 2.1.8). It was hypothesised that MHC class I indirect-pathway allopeptide presentation, and therefore TCR75 proliferation, would be restricted to the early phase following transplantation of these chimeric donors, because transferred haematopoietic cells will be rapidly cleared by the recipient. As expected, there was no TCR75 proliferation following transplantation of bm12.IE donors since they lack H-2K^d expression. Following transplantation of the chimeric mice, TCR75 CD4 T cells underwent robust proliferation at the early time-point, but did not proliferate when transferred late, unlike when transferred following transplantation of bm12.Kd.IE donors (Figure 5.13). This further confirms that ongoing indirect-pathway alloresponses are targeted against antigens expressed by graft parenchyma.

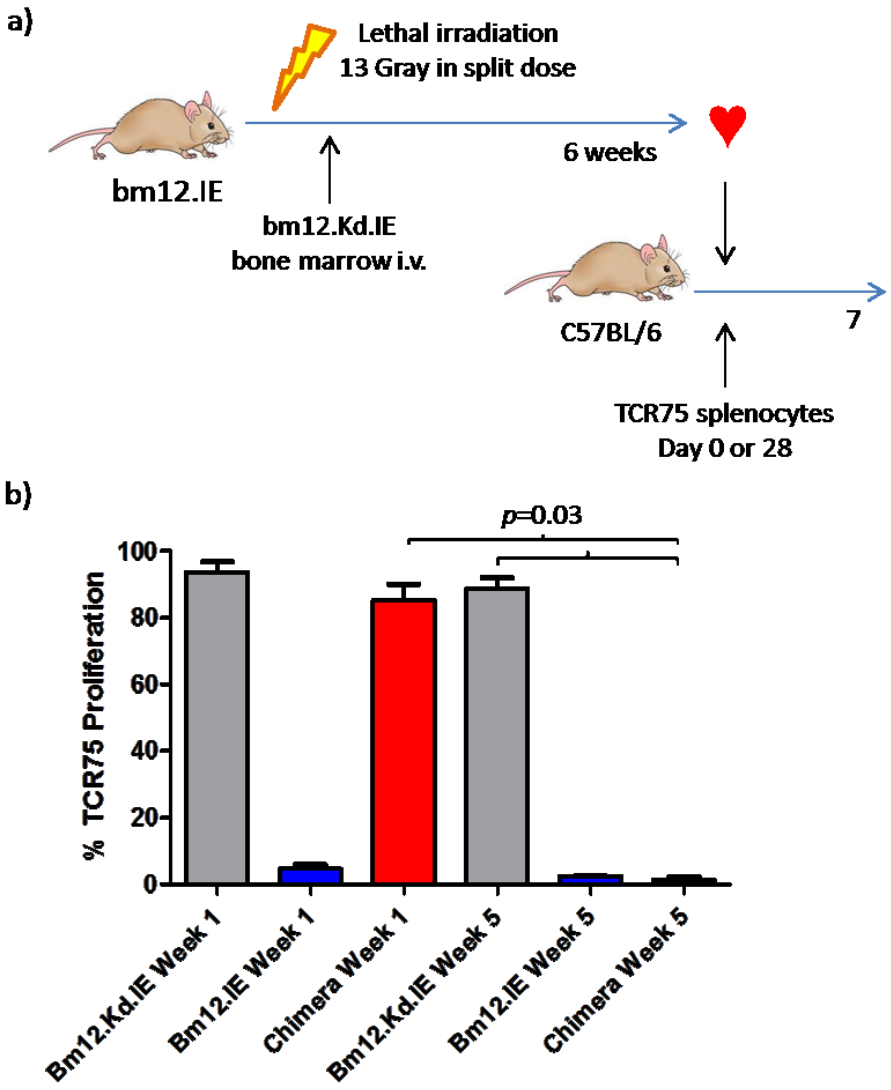


Figure 5.13 MHC class I indirect-pathway alloresponses are short-lived when antigen expression is restricted to haematopoietic lineages

(a) Bm12.IE mice were lethally irradiated (13 Gray split dose) and adoptively transferred with $2x10^7$ bm12.Kd.IE bone marrow cells. Reconstitution was confirmed by flow cytometry on peripheral bleeds prior to experimental usage. After six weeks, these chimeras were used as donors for cardiac transplantation into C57BL/6 recipients and $4\text{-}6x10^6$ CFSE labelled TCR75 splenocytes adoptively transferred on the day of transplantation (week 1) or after 28 days (week 5). (b) No proliferation was observed when bm12.IE mice were used as donors prior to bone-marrow chimerism. Robust TCR75 proliferation was observed early following transplantation with chimeric donors, but this was short-lived. All groups n=5. *P* values using Mann Whitney test.

5.3.3.3 Acute rejection of allografts restricts duration of indirect allorecognition

The archetypal and most widely studied model of acute allograft rejection is the BALB/c to C57BL/6 model. Here, cardiac allografts are acutely rejected with a median survival time of 7-10 days[375] and presumably do not continue to shed alloantigen.

Since BALB/c mice express the MHC class I H-2K^d (in addition to the MHC II I-E), following transplantation, unlike in the bm12.Kd.IE to C57BL/6 model, MHC class I alloantigen would not be continually shed in the recipient and it was hypothesised that this would restrict the duration of the H-2K^d-specific indirect-pathway response. To examine this, TEa and TCR75 CD4 T cells were transferred at early and late time points following transplantation. As anticipated, whilst there was proliferation of both early, no proliferation was observed at the late time-point, after the graft had been rejected (Figure 5.14).

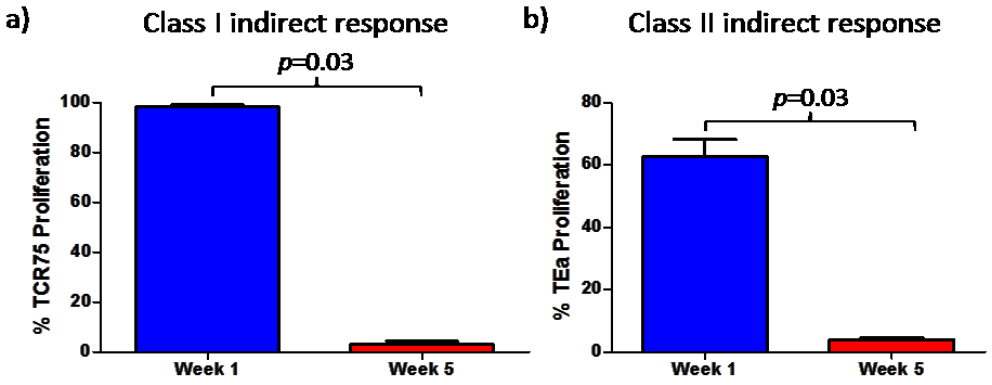


Figure 5.14 MHC class I and II indirect-pathway responses are short-lived in an acute-rejection model
BALB/c cardiac allografts were transplanted into C57BL/6 recipients. 4-6x10^6 CFSE labelled TCR75 and TEa splenocytes were adoptively transferred i.v. at either week 1 or week 5 (n=4). Both MHC class I (a) and MHC class II (b) indirect-pathway allorecognition were short-lived, with no late proliferation observed. *P* values using Mann Whitney test.

These last two experiments suggest that ongoing indirect-pathway responses are driven by graft parenchymal antigen expression. They also strengthen the findings above in relation to class II indirect-pathway responses, excluding that differences in duration of TEa and TCR75 proliferation are simply due to inherent differences between the transgenic cells, for example in affinity of the TCR for the respective allopeptide epitopes.

5.3.4 Endothelial MHC II expression

There has been some debate in the literature regarding murine endothelial expression of MHC class II. Whilst it is accepted that human endothelial cells express MHC class II it is not generally thought to be expressed by resting murine endothelium[338]. However, activation-induced up-regulation, as typically occurs within the endothelium of rejecting allografts, has been described in murine cardiac allografts[86, 369, 376-378].

An attempt was thus made to demonstrate the ability of donor murine endothelium to express MHC class II in the bm12.Kd.IE to C57BL/6 model. First, endothelial cells were cultured *in vitro*. Both bm12.Kd.IE and C57BL/6 strain endothelial cells were generated and expressed appropriate endothelial cell markers. MHC class II expression was evident, with bm12.Kd.IE expressing the class II: I-E but not I-A^b as expected (Figure 5.15a).

Confirmation of *in vivo* MHC class II expression by endothelium was obtained by immunofluorescence staining of donor bm12.Kd.IE cardiac allografts explanted at one week following transplantation. Expression of donor MHC class II was evident on the endothelium at this early time-point (Figure 5.15b). By week 5 however, no such expression was identified, perhaps reflecting the reduced pro-inflammatory milieu.

That clearance of haematopoietic cells from donor allografts abrogates class II indirect-pathway responses is therefore potentially surprising since an endothelial source appears to exist, at least transiently; presumably endothelial I-E alloantigen is expressed at too low a level, and for too short a period, to drive a detectable indirect-pathway CD4 T cell response with TEa CD4 T cells.

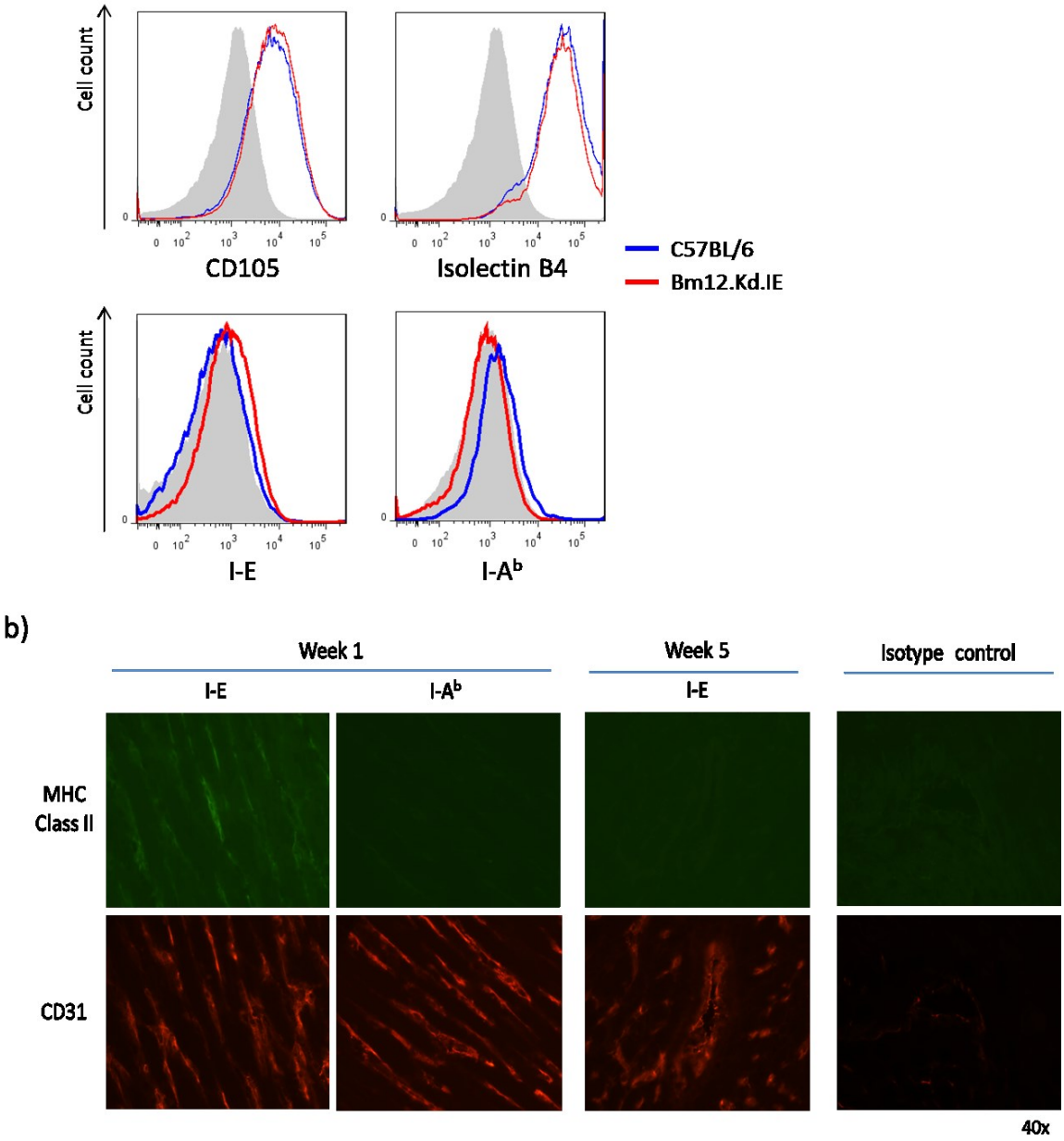


Figure 5.15 Donor endothelial MHC class II expression

(a) Bm12.Kd.IE (red) and C57BL/6 (blue) endothelial cells were cultured *in vitro*. Flow cytometric analysis of these cells revealed surface expression of endothelial cell markers: CD105 (top left panel) and isolectin B4 (top right panel) confirming them to be phenotypically endothelial cells. Surface expression of MHC class II was observed, compared to isotype control staining (grey). Bm12.Kd.IE endothelial cells expressed I-E and not I-A^b and the reciprocal was true of C57BL/6 endothelial cells (bottom panels). (b) Immunofluorescence staining (with CD31, I-E and I-A^b) of cryostat sections of donor bm12.Kd.IE cardiac allografts harvested at week 1 and 5 following transplantation was performed. Endothelial expression of donor MHC class II was observed at week 1, but not at week 5. Representative photomicrographs at 40x magnification presented.

5.3.5 Development of late MHC class II humoral alloresponses

Development of *de novo* donor specific MHC II class alloantibody has been detected in human transplant recipients[379-381]. Because the late development of MHC class II alloantibody would not be expected in the absence of a simultaneous MHC class II allospecific indirect-pathway CD4 T cell response, these clinical findings question the relevance of the above findings in the murine model.

However, recent work from our laboratory has introduced the concept of 'unlinked-help' in transplantation, whereby an antigen-specific B cell can receive help to produce alloantibody by a T cell of different allospecificity[382]. It was demonstrated that CD4 T cells specific for another alloantigen expressed by a graft could provide this unlinked-help.

Upon ligation of its BCR, a B cell will internalise antigen[320]. Since alloantigen is membrane bound, it is proposed that an alloantigen specific B cell will internalise its antigen and membrane from target donor cells, which may contain additional cell surface donor antigens. These will also be processed and presented by the B cell which could therefore, potentially be able to receive help from an allospecific CD4 T cell specific for a different antigen, from the graft, to that recognised by the B cell itself[382].

To examine the possibility that this process could account for late MHC class II alloantibody responses in the bm12.Kd.IE to C57BL/6 model, bm12.Kd.IE hearts were transplanted into TCRKO mice, providing the ability to limit antigen specificity of CD4 T cell help by adoptive transfer of TCR transgenic CD4 T cells.

Earlier experiments revealed that such transplants lead to the development of autoantibody and weak, short-lived, alloantibody responses in TCRKO recipients with no evidence of allograft rejection (Figure 3.22). Despite the short-lived nature of the alloantibody response, donor haematopoietic cells were cleared from recipients rapidly, and no TEa CD4 T cell proliferation was observed upon transfer four weeks after transplantation (Figure 5.16a,b).

To examine the development of late alloantibody, TCR transgenic CD4 T cells were adoptively transferred 4 weeks following transplantation of bm12.Kd.IE cardiac allografts into C57BL/6 recipients, and weekly serum samples collected. As predicted from their lack of proliferation, transfer of MHC class II specific indirect-pathway TEa CD4 T cells resulted in neither anti-IE nor anti-H-2K^{d} alloantibody (Figure 5.16c,d).

In contrast, transfer of MHC class I specific TCR75 CD4 T cells resulted in development of robust anti-H-2K^d *and* anti-IE alloantibody responses and the hearts were rapidly rejected (Figure 5.16c,d). This suggests, as previously described by our laboratory, that MHC class I specific CD4 T cells can provide help to MHC class II specific B cells and drive an anti-IE alloantibody response.

To confirm that TEa CD4 T cells do possess the ability to drive alloantibody responses, TEa CD4 T cells were transferred on the day of transplantation. In this experiment, TEa CD4 T cells drove a robust anti-IE alloantibody response. Furthermore, a short-lived low level anti-K^d alloantibody response was observed, confirming the reciprocal ability of these transgenic CD4 T cells to provide help for B cells of a different allospecificity (Figure 5.16e,f).

These findings potentially reconcile the observation that late class II alloantibody responses can develop *de novo* in human transplant recipients at a time when class II allospecific indirect-pathway CD4 T cell responses appear not to remain active.

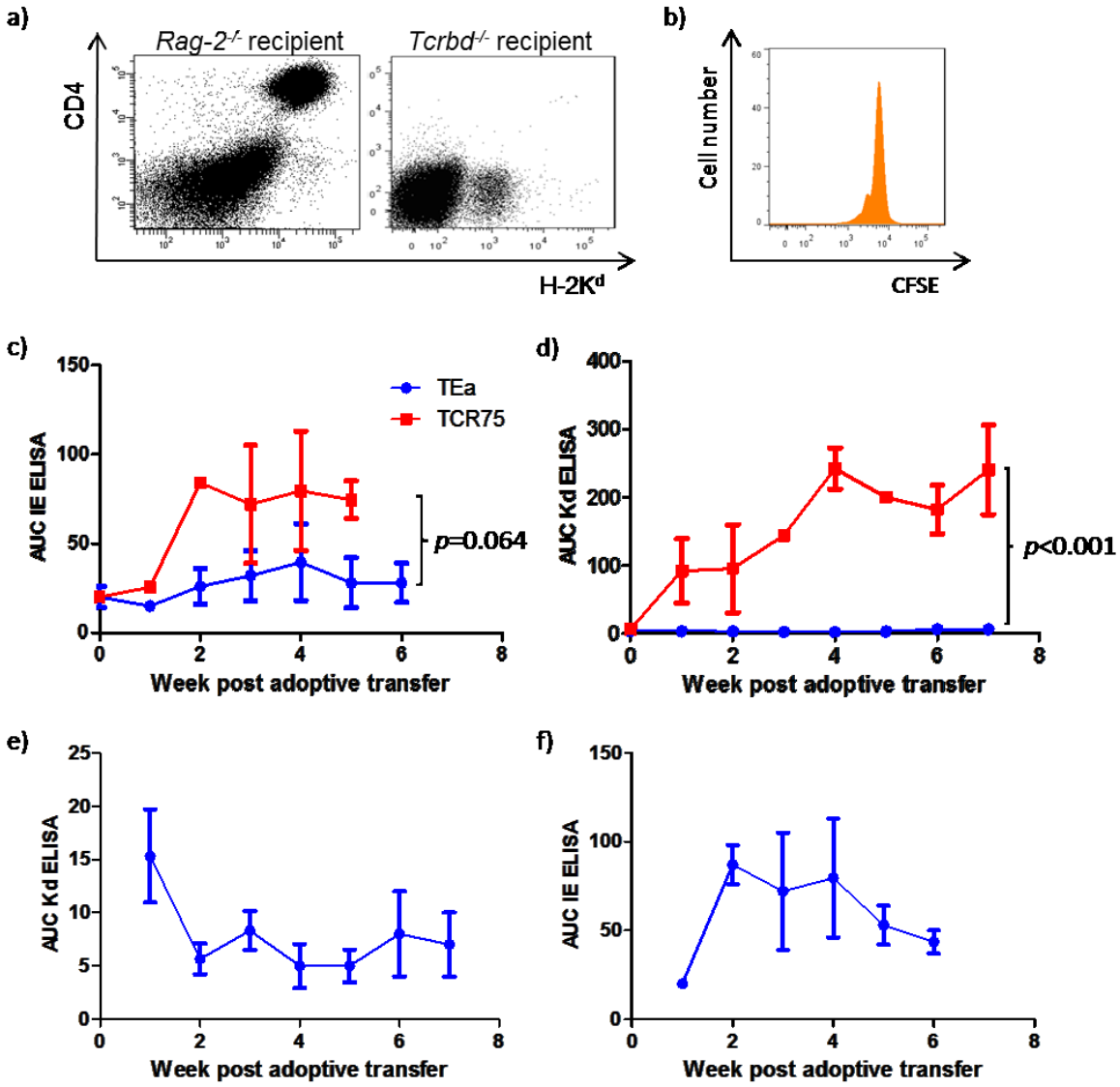


Figure 5.16 Linked presentation permits development of late MHC class II alloantibody responses

(a) Peripheral bleeds of RAG2KO and TCRKO recipients of bm12.Kd.IE cardiac allografts 4 weeks following transplantation reveals that donor CD4 T cells do not survive long term in TCRKO recipients, unlike in RAG2KO recipients (absence of $CD4^+$ $H\text{-}2K^{d+}$ population). (b) In keeping with absence of donor haematopoietic cell survival in TCRKO recipients, no TEa proliferation was observed when 4-6x10^6 splenocytes were adoptively transferred i.v. 4 weeks following transplantation (n=2). In subsequent experiments 1x10^6 purified TCR75 (red lines) or TEa (blue lines) CD4 T cells (MACS separated using anti-CD4 microbeads) were adoptively transferred into recipients either 4 weeks following transplantation (c,d) or on the day of transplantation (e,f). All groups n=4. I-E alloantibody was quantified by flow cytometric labelling of I-E expressing BMDCs and $H\text{-}2K^d$ alloantibody by ELISA, on weekly serum samples. When transferred late, TEa CD4 T cells did not drive alloantibody generation, whilst TCR75 CD4 T cell transfer resulted in both I-E and $H\text{-}2K^d$ alloantibody production, suggesting that the TCR75 CD4 T cells provide unlinked-help to I-E specific B cells (c,d). TEa CD4 T cells are capable of driving alloantibody responses, because when transferred early a robust I-E alloantibody response was observed (f), and they also provided unlinked-help to $H\text{-}2K^d$ specific B cells to drive a short lived $H\text{-}2K^d$ alloantibody response (e). *P* values using two-way ANOVA.

5.4 Discussion

In this chapter, the indirect-pathway of allorecognition has been explored. The use of the bm12.Kd.IE to C57BL/6 model allowed simultaneous comparison of TCR transgenic proliferation controlling for potential differences between individual models. Results have confirmed that the indirect-pathway, rather than being a single entity, is in fact a heterogeneous collection of responses against different donor alloantigens that can have different duration. Specifically, indirect-pathway responses against donor MHC class II are restricted to the immediate post-transplant period, with even memory responses not being primed at later time-points. Beyond the first week following transplantation there was no presentation of MHC class II allopeptide, confirmed with the Y-Ae clonotypic antibody. The short-lived nature of this response reflects the fact that donor professional APCs are the major source of MHC class II alloantigen. Although allograft endothelial cells can express MHC class II this was found to be short-lived, likely reflecting dampening of the inflammatory milieu with time following transplantation. Furthermore, the contribution of this antigen to MHC class II indirect-pathway responses is likely limited considering the abrogation of TEa proliferation when leukocyte depleted lethally irradiated donors were used. Use of chimeric mice with expression of MHC class I restricted to haematopoietic lineages supported that ongoing indirect-pathway alloresponses are targeted against alloantigens expressed by graft parenchymal cells, and it is plausible that these are the alloimmune pathways responsible for driving chronic rejection.

One key concern in extrapolating these results to human transplantation surrounds endothelial expression of MHC class II. Whilst it is accepted that human endothelial cells can upregulate expression of MHC class II in the context of inflammatory stimulation with IFNγ[383], murine endothelial expression has remained controversial[338, 376]. However, there are now several studies which report murine endothelial expression of MHC class II upon inflammatory stimulation – both *in vitro* and in rejecting allografts *in vivo*[86, 376-378]. These findings were replicated in this chapter, although expression appeared short-lived. Despite the fact that endothelial cells may be able to express MHC class II, if this is short-lived the relevance to the alloimmune response may be limited. Several studies have examined the ability of human donor endothelium to prime allogeneic CD4 T cells and the evidence suggests that they are unable to do so – even memory CD4 T cells with their reduced costimulatory requirements[338, 384-386]. In these experiments, although direct-pathway recognition and activation specifically was being examined, the results led the authors to question the contribution endothelial MHC

class II expression can make to the alloimmune response. Similar findings have been observed in murine studies where endothelial cells were found unable to prime allogeneic CD4 T cells *in vitro* despite MHC class II expression being evident[376]. In this study the authors also developed chimeric mice in which MHC class II expression was restricted to haematopoietic lineages and observed no impact upon allograft rejection. Together, these observations question the importance of endothelial MHC class II expression to the alloimmune response in both humans and mice. However, endothelial MHC class II expression was found to be transient in this thesis, which differs from the constitutive expression observed in humans. Therefore, further studies to clarify the role of MHC class II in the human alloimmune response would be warranted prior to extrapolating these findings to clinical transplantation.

Another important consideration when interpreting these results is epitope diversification[387-389]. This is the process whereby the focus of the T cell response to an antigenic challenge may spread from the initial dominant determinant to other, initially cryptic, epitopes on either the same protein (intramolecular) or a different protein (intermolecular). Analysis of indirect - pathway allorecognition in this chapter has been performed utilising monoclonal TCR transgenic CD4 T cell proliferation. There is the possibility that epitope diversification may occur within recipients resulting in reduced presentation of earlier dominant determinant epitopes with time. In support, a study examining indirect-pathway responses in human transplant recipients revealed evidence of epitope diversification, although this did not occur until relatively late (>6 months) following transplantation[132]. Interestingly, the identification of epitope diversification in these recipients correlated with poorer outcomes. It is unlikely that this could account for the absence of TEa proliferation just one week following transplantation since epitope diversification appears to be a manifestation of chronic immune responses. Furthermore, even in the human study described above, CD4 T cells reactive against the original dominant pathway remained prevalent despite diversification[132].

To date there have been only limited attempts to examine indirect-pathway responses in human transplant recipients, which may reflect the limited experimental techniques available for such a study. Examination of alloimmune pathway responses in human transplant recipients has tended to demonstrate that indirect-pathway CD4 T cell responses are long-lived and remain active in recipients many years following transplantation[77, 78, 148, 339]. Furthermore, there is some correlation between these responses and development of allograft vasculopathy and chronic rejection. These studies have tended to examine the global indirect-pathway response by pulsing stimulator APCs with donor cell homogenates, rather than considering indirect-pathway responses against individual alloantigens. One study though,

looked specifically at indirect-pathway responses against donor HLA-DR MHC class II molecules using synthetic peptides to prime stimulator APCs[132]. This study demonstrated recipient CD4 T cell reactivity against donor MHC class II late following transplantation. However, only a single snapshot was assessed rather than the evolution of these indirect-pathway CD4 T cells over time. Additionally it is important to consider that the use of *in vitro* limiting dilution analysis does not necessarily confirm that these CD4 T cells are activated and continue to proliferate *in vivo* and may represent the memory population of CD4 T cells that develop following an antigenic challenge. There are additional concerns regarding the techniques adopted in these studies[390]. When using cell fragments as a source of donor allopeptide it is, for example, possible that membrane containing intact MHC molecules could be trogocytosed and integrated within the APC membrane. This could potentially result in direct-pathway CD4 T cells, present at high precursor frequency, being erroneously measured as an indirect-pathway response. Similarly, there are significant concerns about studies using synthetic peptides as a source of donor allopeptide to prime APCs. For example, reactivity is often observed using synthetic peptides from self-MHC molecules. It is proposed this may relate to the creation of neo-epitopes – either due to the absence of post-translational modifications, or the creation of peptides that would not occur *in vivo* because of the absence of natural splicing sites in the full protein[390].

A recent publication has described the use of synthetic HLA monomers as a tool to monitor indirect allorecognition which the authors claim avoid the pitfalls described above[391]. The authors demonstrated that HLA monomers were able to detect indirect-pathway CD4 T cells with high sensitivity and specificity and may permit more reliable examination of indirect-pathway CD4 T cell responses in human transplant recipients – although the current high cost may be prohibitive[390].

Another question that arises from the observations in this chapter relates to the development of *de novo* anti-MHC Class II antibody that is sometimes observed in human recipients late following transplantation[379-381]. One of the central tenets of immunology is the essential requirement for an antigen-specific B cell to present internalised, processed antigen in a self-MHC restricted manner to permit a cognate interaction with antigen-specific CD4 T cells for effective delivery of help[97]. From this, it would be expected that in order to receive help, a donor MHC class II specific B cell would require an indirect-pathway, donor MHC class II specific CD4 T cell. However, the results in this chapter suggest that the MHC class II indirect-pathway CD4 T cell response is quiescent late following transplantation, and raises the possibility that these results are not relevant to human transplantation. One possibility is that

the graft does continue to express MHC class II alloantigen but at very small levels that cannot be detected by the techniques employed in this work, but is nonetheless present in sufficient quantities to drive MHC class II alloantibody production. Against this, transfer of TEa CD4 T cells that would recognise this epitope do not drive an anti-MHC class II alloantibody response. An alternative explanation stems from work recently published from our laboratory which demonstrated that CD4 T cells specific for one alloantigen could provide help to B cells whose allospecificity is for a different alloantigen expressed by cells within the graft – a form of 'unlinked' help[382]. In this work monoclonal Mar cells (recognising H-Y antigen *dby* by the indirect-pathway) were found capable of providing help for the development of anti-MHC class I alloantibody. This help required a cognate interaction between Mar CD4 T cells and MHC class I specific B cells, and required that both the *dby* antigen and the MHC class I were co-expressed by the same donor cell – appearing to contravene the maxim of 'linked recognition'. To examine this same phenomenon and offer a means of reconciling the observation in human transplantation regarding late development of anti-MHC class II alloantibody, an experiment was designed to examine the possibility of 'unlinked-help' in the bm12.Kd.IE to C57BL/6 model. The results confirmed that monoclonal TCR75 CD4 T cells (recognising H-2K^{d} indirectly) could provide help at the late time-point for the development of anti-I-E alloantibody (and interestingly this could not be provided by TEa CD4 T cells which recognise I-E indirectly). These results demonstrated that 'unlinked-help' could provide a mechanism for development of *de* novo late MHC class II alloantibody.

Although on the surface, these results appear similar to the published results described, there is an important difference, in that there is apparently no longer a source of donor MHC class II. In the study described above, both the *dby* and MHC class I were simultaneously expressed by donor cells, and such simultaneous expression of the target and helper alloantigen was essential. In considering these findings, it was postulated that BCR-mediated internalisation of antigen was central. It is recognised that B cells can internalise their target antigen in a BCR-mediated fashion, permitting them to present antigen cognately to antigen-specific CD4 T cells in order to receive help[320, 392]. It is conceivable that upon internalisation of target antigen, B cells will simultaneously internalise surrounding fragments of donor cells permitting a means of capturing additional antigen. Against this, one study visualising B cell synapse formation real-time observed BCR mediated concentration of antigen in the synapse suggesting that non-selective acquisition would be unlikely[393]. However, another study examining capture of cognate antigen from follicular dendritic cells (FDC), using a PE-HEL conjugate and anti-HEL transgenic B cells, identified that B cells also acquired FDC surface markers as they acquired

their target antigen, suggesting that acquisition of adjacent membrane proteins during B cell antigen uptake may be possible[394].

Whilst this mechanism appears to offer an explanation for the observations in the published work, with simultaneous expression of *dby* and MHC class I by the donor cells, the situation in this chapter is different since there is no longer a source of donor MHC class II. One possible explanation for this is that the MHC class II-specific B cell has internalised both I-E and neighbouring H-2K^d from donor cells around the time of transplantation and continues to present H-2K^d peptide long-term enabling the receipt of help when the TCR75 CD4 T cells are transferred – in effect the B cell is primed by engagement of target antigen, but antibody production is delayed until CD4 T cell help is provided. This raises several questions though. Is the alloantigen internalised and stored intact by I-E-specific B cells, or broken down but allopeptide presented long-term, or could another cell type act as the antigen store? Also, how long can a B cell retain in a dormant state having acquired antigen: for example, would anti-MHC class II alloantibody still develop if the T helper cell population was adoptively transferred into the T cell deficient recipient several months after the transplant?

Long term sequestration of antigen is not without precedent. FDCs for example, are known to act as 'antigen libraries' for B cell sampling, and long term antigen storage has been reported[395, 396]. It is possible then that I-E molecules are retained on the surface of FDCs which present intact alloantigen to B cells, but remains inaccessible to CD4 T cells. It is possible that I-E specific B cells continue to acquire antigen from this source even late after transplantation.

Alternatively, Bergtold *et al* have demonstrated that DCs possess two different antigen processing pathways for immune complexes[397]. Typically, immune complexes are internalised by activating Fc receptors and are targeted for lysosomal degradation[398]. However, they observed that immune complexes internalised by inhibitory receptor FcγRIIB were internalised into a recycling vesicular system and excluded from the degradative compartment[399]. This antigen could then be recycled intact to the DC cell surface for B cell priming some time after encounter and internalisation. This raises the possibility that DCs may act as a source of antigen, or perhaps even that B cells possess a similar antigen processing pathway permitting long-term storage of antigen, that is degraded and presented upon appropriate stimulation at a later time. Long-term storage of antigen could be explored by leukocyte subset depletion several weeks following transplantation and transfer of CD4 T cells following reconstitution.

A related question though is: presumably I-E allopeptide would be presented as much as the H-2K^d, so why are TEa CD4 T cells unable to provide sufficient help? Perhaps this relates to

activation status. Late following transplantation, results in this chapter have demonstrated no TEa proliferation occurs, whilst TCR75 proliferate vigorously. B cell activation and production of alloantibody likely requires antigen experienced CD4 T cell help, potentially due to down-regulation of B cell surface expression of costimulatory molecules preventing these B cells from being in a position to activate naïve CD4 T cells. This could be confirmed by transferring antigen experienced memory TEa which may be able to provide help to B cells without a requirement for costimulation. Alternatively this could be tested by transplanting bm12.IE x bm12.Kd.IE chimeric mice in which H-2K^{d} expression is restricted to haematopoietic lineages. It would be predicted that in this setting, late after transplantation, neither TEa nor TCR75 would be activated and able to provide help to I-E specific B cells.

It is clear that there are many unanswered questions around this observation that will require further exploration. What though is the potential clinical significance? A situation could exist whereby a patient receives a transplant containing an alloantigen to which they have been previously sensitised through pregnancy, blood transfusion or previous transplant. Memory CD4 T cell responses against this alloantigen could drive augmented alloantibody responses against all other donor alloantigens. Alternatively, T cell recognition could be of non-transplant antigens for example against viruses such as CMV. Viral-specific memory CD4 T cells could, through 'unlinked-help', drive alloantibody responses whilst responding against CMV infection of the allograft. Interestingly, strong associations have been highlighted between CMV infection and graft failure[400], with accelerated AV development observed possibly suggesting augmented humoral alloimmunity[401].

6 Phenotypic examination of indirect-pathway CD4 T cells

6.1 Introduction

In the previous chapter, study of the indirect-pathway of allorecognition has been performed by adoptive transfer of exogenous monoclonal populations of TCR transgenic CD4 T cells. This approach has been used widely for study of non-transplant antigen[277], but my approach differs in that the TCR-transgenic population was transferred at various time-points after the transplant. This raises the question how the division of the transferred T cell population relates to events occurring within the endogenous indirect-pathway CD4 T cell population. I therefore sought to optimise an experimental approach that would enable identification and tracking of endogenous antigen-specific CD4 T cells.

6.1.1 Detecting antigen-specific CD4 T cells

Detecting antigen-specific CD4 T cells has traditionally been challenging. Due to the diversity of the T cell repertoire, the frequency of T cells specific for a single peptide-MHC ligand is very low within the naïve repertoire – estimated to be between 0.2-60 cells/10^6 naïve T cells[402]. The second challenge to their identification has been characterisation of the T cell epitope for a particular antigen since the TCR recognises only peptide fragments of protein antigens, unlike B cells which recognise conformational epitopes.

As a result, efforts to identify antigen-specific T cells have involved challenging cells with antigen *in vivo* or *ex vivo* and examining for a response. Several strategies have been described[402, 403]:

Examining proliferation – T cell proliferation in response to antigen can be detected by incorporation of radioactive (^{3}H)-thymidine or bromodeoxyuridine (BrdU) into DNA. Alternatively proliferating T cells can be identified by isolating T cells that have undergone CFSE dilution. Limitations of this approach include the inability to exclude the contribution of bystander T cell proliferation and identification of only the proliferating antigen-specific T cells.

Examining cytokine expression or secretion – Flow cytometric intracellular staining for cytokine expression and analysis of cytokine secretion from live cells are both techniques that have been adopted to examine T cell effector function in response to an antigenic challenge. Again the problem of bystander activation exists and it is difficult to quantify the antigen-specific population using this approach.

Examining activation markers – Detecting antigen-specific T cells on the basis of up-regulation of activation markers following antigen challenge. This permits identification of responding T cells regardless of their functional specialisation (and whether they proliferate or secrete cytokines). A number of activation markers have been used for this including CD69, CD25, CD71, CD137, CD154. However, bystander activation can be a problem as well as marker expression not being limited to activated T cells.

All of these techniques have the advantage that the antigenic peptide epitope does not need to be characterised. However, the culture conditions required for many of these techniques themselves may influence T cell phenotype and function, precluding robust phenotypic assessment following identification of the responding antigen-specific T cells. Furthermore, it has been difficult to accurately quantify the antigen-specific population using these approaches[402].

6.1.2 Development and use of peptide-MHC tetramers

To overcome the challenges detailed above, efforts turned to utilising TCR epitope, in a similar manner to the use of fluorochrome-conjugated protein antigen to detect antigen-specific B cells. Initially, peptide-MHC monomers were utilised but success was limited because of insufficient affinity for TCR to detect antigen-specific T cells[404]. However, in 1996 Altman *et al.* demonstrated that tetramerisation of biotinylated peptide-MHC monomers with avidin resulted in sufficient avidity to reliably detect antigen-specific T cell populations[405]. These tetramers are fluorochrome labelled permitting detection by flow cytometry. Much of the early work with MHC tetramers used MHC class I molecules to detect antigen-specific CD8 T cells. The development and success with MHC class II tetramers has lagged. It is thought that structural differences between MHC class I and II molecules and lower avidity of binding between the CD4 TCR and peptide-MHC complex are responsible for this[402, 403]. Over recent years there has been an expansion in MHC class II tetramer availability and they have been widely used to study antigen-specific CD4 T cell responses against bacterial and viral infection[406, 407]. There are limitations of this technique though. One challenge is the requirement to identify the immunodominant peptide for a particular protein antigen in order to load the MHC molecules. Analysing the TCR specificities of T cells responding to *in vitro* antigen challenge and using computer algorithm predictions, have both been employed to overcome this[403]. However, epitope spreading is known to occur during chronic immune

responses and restricting analysis to an initial immunodominant epitope may not therefore, be representative of the entire T cell response against protein antigen[132].

One further challenge has been the low precursor frequency of naïve T cells and the small proportion of responding T cells that are detected with tetramer. To overcome this, enrichment steps have been developed to allow for rapid processing of samples whilst increasing the sensitivity of antigen-specific T cell detection. Enrichment steps have involved magnetic separation of cell populations based upon tetramer binding (using anti-fluorochrome magnetic microbeads). The positive fraction additionally contains significant numbers of non-target cells. Gating out non-T cells and dead cells, permits sensitive and specific identification of antigen-specific T cells[408-411].

Peptide-MHC tetramers have revolutionised the ability to detect and characterise endogenous antigen-specific T cell responses and are now increasingly used[402, 407, 412]. Not only can peptide-MHC tetramers be used to identify and quantify antigen-specific T cells, they also permit the opportunity to examine their phenotype. To date, peptide-MHC tetramers have not been utilised to study alloimmune CD4 T cell responses against donor antigens in experimental transplantation.

6.2 Aims

The purpose of the research described in this chapter is to extend the findings using TCR transgenic CD4 T cells reported earlier and utilise peptide-MHC class II tetramers to characterise the endogenous indirect-pathway CD4 T cell alloresponses in C57BL/6 recipients of bm12.Kd.IE cardiac allografts.

The aims are:

1) To examine the potential of utilising peptide-MHC class II tetramer to identify indirect-pathway alloreactive CD4 T cells following challenge with an allograft.

2) To quantify and compare kinetics and phenotype of the endogenous antigen-specific CD4 T cell response directed against short-lived and long-lived indirect-pathway alloresponses.

6.3 Results

Data from the previous chapter suggest that in recipients of chronically rejecting cardiac allografts, ongoing indirect-pathway alloresponses are targeted against alloantigen expressed by the graft parenchyma. It is likely that these ongoing alloresponses are responsible for driving chronic rejection.

Results thus far have relied upon transfer of exogenous monoclonal populations of transgenic CD4 T cells, which raises the potential for criticism that this may not be reflective of the endogenous alloimmune response. Examination of the endogenous T cell response would be very useful to verify the findings. There have been many approaches to identify endogenous antigen-specific CD4 T cells[402, 403, 413], however the recent development of MHC class II tetramer technology has provided a novel approach that has been successfully used in models of infection[407, 412].

6.3.1 Identifying endogenous recipient alloreactive CD4 T cells with MHC class II tetramers

Recipient type MHC class II (I-A^b) tetramers loaded with peptide derived from donor MHC class I (H-$2K^d$) and class II (I-E) were kindly gifted by the National Institute of Health Core Tetramer Facility, Emory, US (Figure 6.1). The tetramers are fluorescently labelled permitting identification of antigen-specific CD4 T cells using flow cytometry.

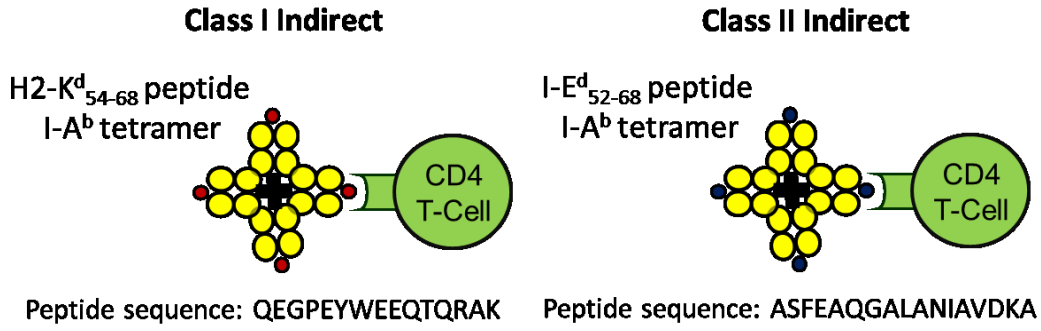


Figure 6.1 MHC class II tetramers against donor MHC class I (H-$2K^d$) and MHC class II (I-E)
Recipient MHC class II (I-A^b) tetramers loaded with donor MHC class I (H-$2K^d$ - left) and class II (I-E - right) allopeptide were gifted by the NIH Core Tetramer Facility, permitting identification of endogenous CD4 T cells with indirect-pathway allospecificity.

6.3.1.1 Enrichment step enhances ability to detect tetramer bound CD4 T cell population

Reading of the literature revealed that enrichment of tetramer bound cells has been successfully used to overcome the challenge of identifying the small antigen-specific CD4 T cell population[407]. This approach was adopted and tetramer binding to CD4 T cells was initially examined in C57BL/6 recipients two weeks following challenge with a bm12.Kd.IE cardiac allograft at which point it was anticipated an allospecific population would have developed.

Tetramer binding to other cell subsets occurs requiring 'dump-channels' to accurately exclude non-CD4 T cells. The gating strategy is demonstrated in Figure 6.2. Enrichment very effectively permits identification of the antigen-specific CD4 T cells as can be seen from 'before' and 'after' flow cytometry plots (Figure 6.3). Confirmation that binding is antigen-specific is achieved with negative-control tetramers, identical to the experimental tetramer, but loaded with peptide from the endogenous human CLIP protein.

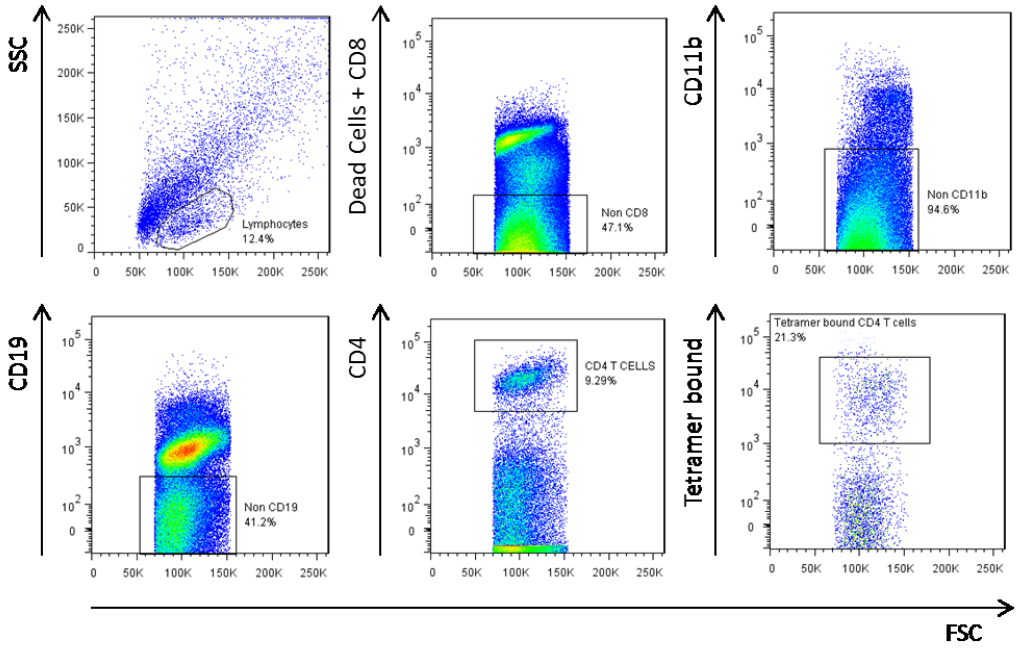


Figure 6.2 Gating strategy for identification of antigen-specific tetramer bound CD4 T cells
Single cell suspensions of experimental spleens were obtained. They were incubated at 37^{o}C for 2 hours with MHC class II tetramer at a 1/200 concentration. Subsequently enrichment was performed (see below) and surface marker staining was performed and cells analysed by flow cytometry. Tetramer bound CD4 T cells were identified as follows: lymphocytes were first gated (top left panel); dead cells and CD8 T cells were excluded (PerCP, top centre panel); CD11b^{+} macrophages and DCs were excluded (APC-Cy7, top right panel); CD19^{+} B cells were excluded (FITC, bottom left panel); CD4 T cells were positively gated from the remaining population (PE-Cy7, bottom centre panel); Tetramer bound CD4 T cells were then identified by plotting against PE (bottom right panel).

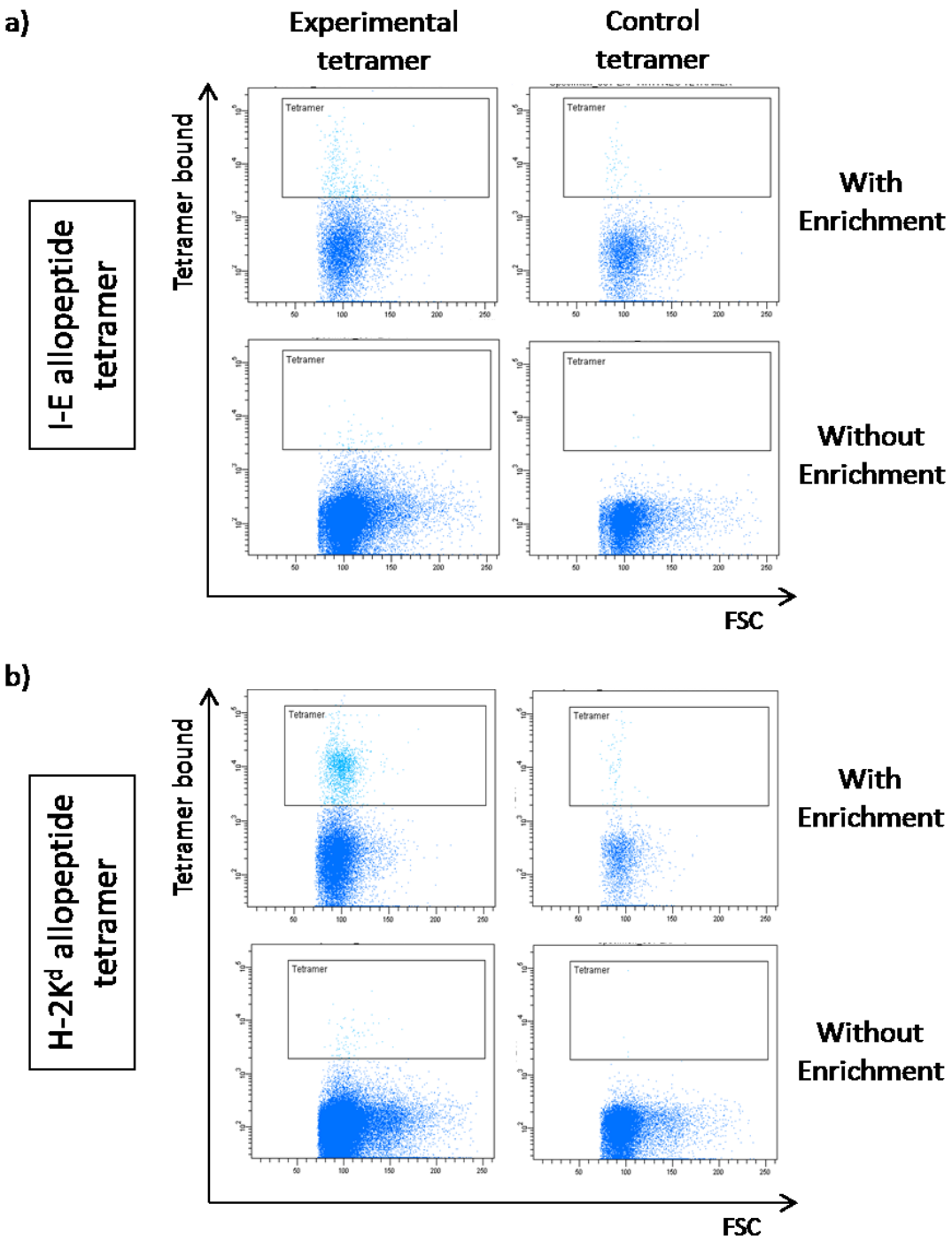


Figure 6.3 An enrichment protocol improves ability to detect tetramer bound CD4 T cells

Staining was performed as described in Figure 6.2. For enrichment, following incubation with the tetramer cells were incubated with anti-PE microbeads (the tetramer bound flurochrome) and separated with MACS following the manufacturers instruction. The positive fraction, enriched for tetramer bound cells was then stained for surface markers and processed by flow cytometry. Here, both H-2K^d (a) and I-E (b) allopeptide tetramers were used to stain C57BL/6 recipient splenocytes 2 weeks following transplantation with a bm12.Kd.IE cardiac allograft. Very little, if any, tetramer bound CD4 T cells were detected with the I-E allopeptide tetramer, despite enrichment (a); but following enrichment a definite population of H-2K^d allopeptide tetramer bound CD4 T cells was identified [(b), top left panel] – which was not identified without enrichment (bottom left panel). Antigen-specificity was confirmed by absence of binding to control tetramer loaded with human CLIP peptide (right panels).

Whilst a tetramer-bound CD4 T cell population was reproducibly identified with the H-2K^{d} peptide loaded tetramer, this was not the case for the I-E loaded tetramer. To further examine this difference, the two TCR transgenic strains: TCR75 and TEa, which possess TCRs recognising the exact epitope presented by the donor MHC class I and class II loaded I-A^{b} tetramers respectively, were employed as positive controls. Similar to the previous results, the H-2K^{d} loaded tetramer was effective at binding to TCR75 CD4 T cells, with all binding the tetramer. However, the I-E loaded tetramer did not appear to bind to TEa CD4 T cells (Figure 6.4).

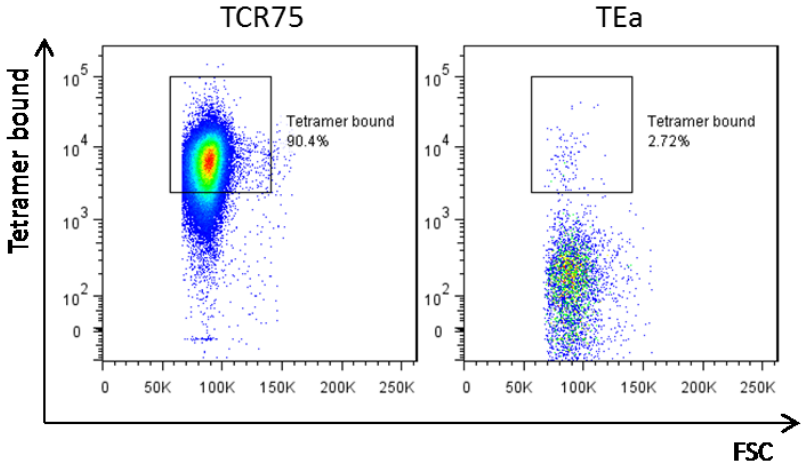


Figure 6.4 Using T cell receptor transgenic mice as positive controls for tetramer binding
Staining and enrichment were performed as described in Figure 6.3. Naïve TCR75 and TEa splenocytes were employed as positive controls for tetramer binding since their TCR epitopes are formed by the H-2K^{d} and I-E peptide loaded tetramers respectively. Whilst TCR75 CD4 T cells were all bound to tetramer (left panel), TEa CD4 T cells appeared not to bind to the I-E tetramer with similar binding to control tetramer observed (not shown). Representative flow cytometry plots from three independent experiments.

Concerned that this lack of binding was a problem with the tetramer the NIH Core Tetramer Facility was contacted to seek advice. This tetramer has been previously used to detect antigen-specific CD4 T cells following immunisation with synthetic I-E peptide, rather than following challenge with intact, conformational I-E class II antigen. In an attempt to replicate this, 100µg synthetic I-E peptide in CFA was injected subcutaneously to drive an indirect-pathway response against the MHC class II I-E. Staining with the tetramer was performed after seven days and revealed a population of antigen-specific CD4 T cells (Figure 6.5).

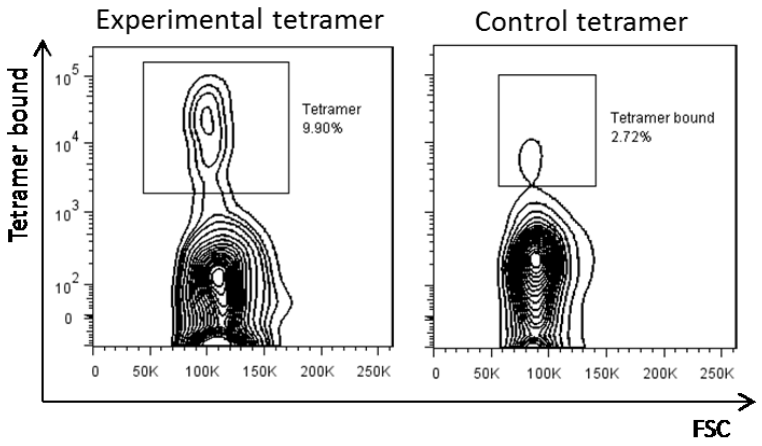


Figure 6.5 MHC class II - I-E allopeptide loaded tetramer binds CD4 T cells responding to synthetic I-E allopeptide immunisation

C57BL/6 mice were immunised with 100µg synthetic I-E peptide in CFA s.c. and after 10 days spleens were harvested (n=2). Staining and enrichment were performed as described in Figure 6.3. Recipient antigen-specific CD4 T cells binding to the I-E allopeptide loaded I-A^b tetramer were identified following this challenge (left panel) with negligible binding to the control tetramer (right panel).

Why there should be a difference between synthetic I-E peptide immunisation and exposure to endogenous I-E antigen is not clear. Perhaps this relates to absence of post-translational modification; or differences in peptides such as different lengths of peptide bound in the groove, such that although the core motif is similar, flanking regions outside the groove alter the confirmation of the central motif. These possibilities are considered further in the discussion. As a result, subsequent experiments were performed with the H-$2K^d$ peptide loaded tetramer only.

It is recognised that CD4 T cells become resident in tissues, and populations are known to reside within organs such as the heart (Figure 3.17)[306, 414]. It was therefore interesting to consider if alloantigen-specific populations of T cells specifically traffic to donor allografts. Donor and native recipient hearts were harvested two weeks following transplantation and homogenates stained with tetramer as described. Whilst CD4 T cell populations were identified within both allograft and native hearts, a significant tetramer positive population was only found within the donor allograft, suggesting that CD4 T cells have a predilection to traffic to and become concentrated within donor tissues (Figure 6.6). Perhaps this is because they are retained by infiltrating recipient APCs presenting cognate antigen, and/or are exhibiting or driving adaptive effector function from within the allograft.

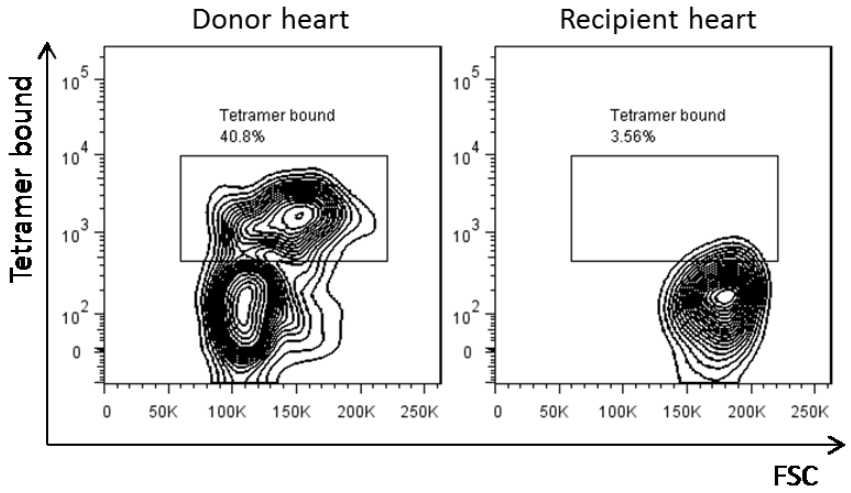


Figure 6.6 Antigen-specific CD4 T cells identified to reside within the donor allograft

Bm12.Kd.IE cardiac allografts were transplanted into C57BL/6 recipients (n=2). Fourteen days following transplantation donor and native recipient hearts were harvested and homogenised to obtain a single cell suspension. Staining and enrichment were performed as described in Figure 6.3, using the H-2K^d allopeptide loaded tetramer. Recipient antigen-specific CD4 T cells binding to the H-2K^d allopeptide loaded I-A^b tetramer were identified following this challenge within donor (left panel) but not native recipient (right panel) hearts.

6.3.2 Quantifying the endogenous indirect-pathway donor class I alloreactive CD4 T cell population

In a similar manner to that described for CD4 T cell responses against conventional protein antigens, the endogenous donor H-2K^d specific indirect-pathway response to a bm12.Kd.IE cardiac allograft was next quantified and mapped over time[407]. Spleens from recipient mice were harvested at weekly intervals following transplantation and the number of antigen-specific CD4 T cells estimated as described in Figure 6.7[407, 412].

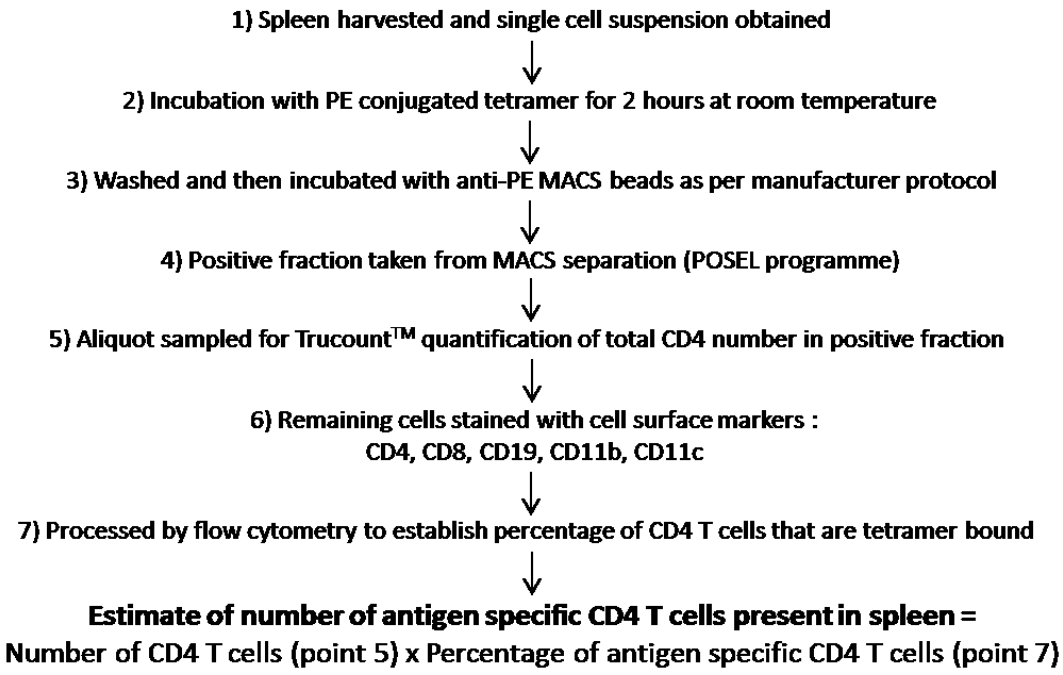


Figure 6.7 Stages in quantification of tetramer bound cells within a recipient spleen
A step-by-step description of how an estimate of the number of tetramer bound CD4 T cells in a recipient spleen was quantified. Adapted from the work of M Jenkins (University of Minnesota, US)[407, 412]

This revealed that H-2K^d allopeptide-specific CD4 T cells undergo typical expansion, contraction and memory phases following transplantation with bm12.Kd.IE donor cardiac allografts (Figure 6.8a). In comparison, when recipient mice were challenged with an acutely rejecting BALB/c cardiac allograft the expansion phase was much less marked and furthermore, in the order of 10,000 fold fewer cells persisted into the memory phase. This finding is despite similar levels of H-2K^d alloantigen being expressed on BALB/c and bm12.Kd.IE cardiac myocytes (Figure 6.8b,c).

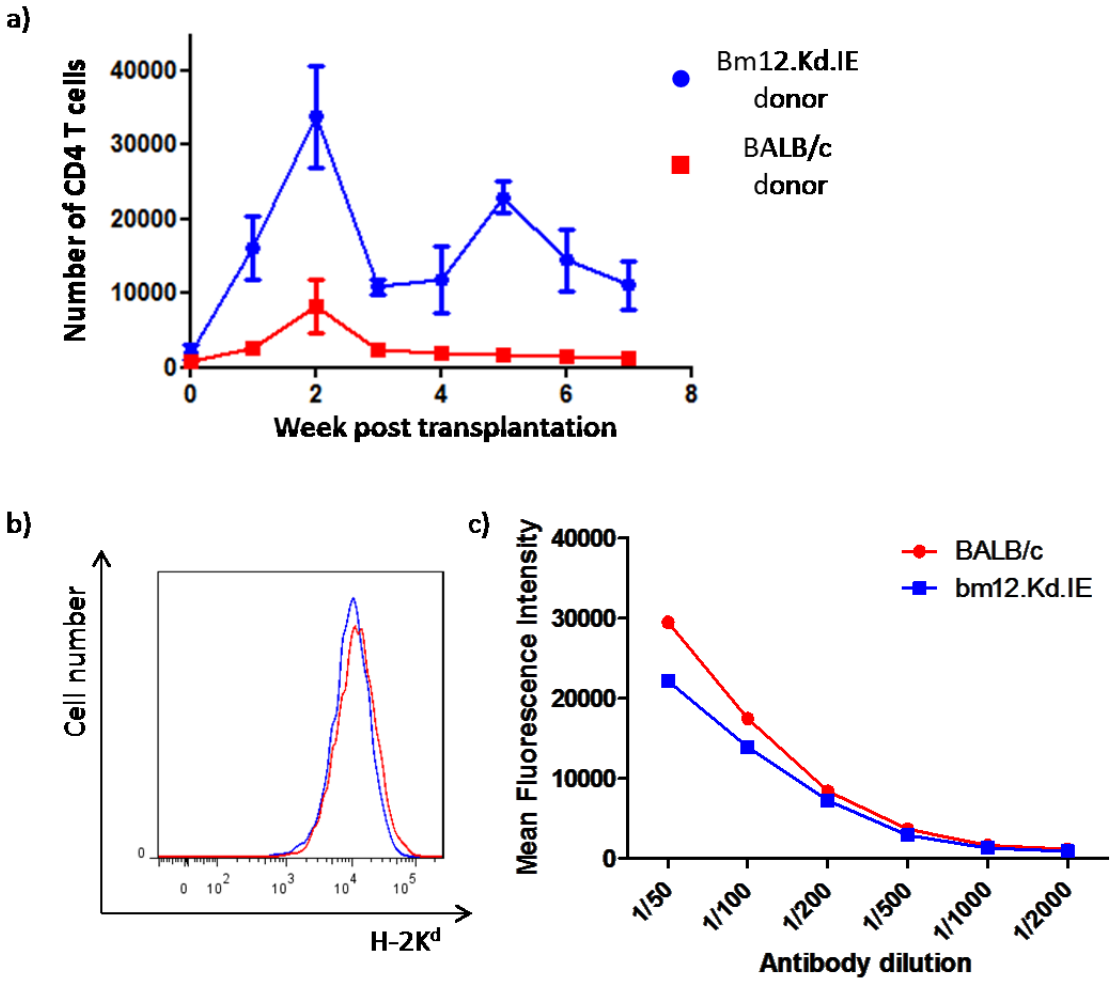


Figure 6.8 Quantification of the donor MHC class I indirect-pathway CD4 T cell alloresponse
(a) Bm12.Kd.IE, or acutely rejecting BALB/c, cardiac allografts were transplanted into C57BL/6 recipients. At weekly intervals following transplantation, recipient spleens were harvested. Staining and enrichment using the H-2K^d allopeptide loaded tetramer were performed as described in Figure 6.3, and number of tetramer bound cells quantified as described in Figure 6.7. Each time point n=3. Expression level of H-2K^d by naïve bm12.Kd.IE (blue lines) and BALB/c (red lines) cardiac myocytes was examined by homogenising hearts to obtain a single-cell suspension. (b) Flow cytometry histogram of BALB/c and bm12.Kd.IE H-2K^d expression with anti-H-2K^d antibody at 1/100 dilution. (c) Mean fluorescence intensity H-2K^d staining of cardiac myocytes with a series of antibody dilutions.

The increased number of H-2K^d-specific CD4 T cells following challenge with a bm12.Kd.IE cardiac allograft is presumably a consequence of ongoing allopeptide presentation. This hypothesis was tested using co-stimulation blockade therapy to abrogate acute rejection of BALB/c allografts[375]. Administration of anti-CD154 monoclonal antibody around the time of transplantation (which interferes with the CD40-CD40L co-stimulation pathway disrupting the activation of CD4 T cells) prolongs the survival of BALB/c allografts, which are rejected chronically (Figure 6.9a)[375]. There is also a marked reduction in the anti-H-2K^d alloantibody response in treated recipients (Figure 6.9b). In keeping with anti-CD154 treatment prolonging persistence of alloantigen, proliferation of the TCR transgenic TCR75 CD4 T cells was observed at the late time point following transplantation. This is in contrast to untreated recipients of BALB/c allografts where no such TCR75 proliferation is seen (Figure 6.9c).

In keeping with these findings, a less profound contraction in the H-2K^d alloantigen-specific CD4 T cell population was observed in recipients of BALB/c cardiac allografts that received anti-CD154 antibody, with a significantly greater population of tetramer-bound CD4 T cells quantified six weeks following transplantation (Figure 6.10a).

Persistence of alloantigen correlates with proliferation of adoptively transferred naïve indirect-pathway CD4 T cells. Tetramer staining provides the opportunity to examine whether endogenous CD4 T cells are similarly undergoing proliferation at these late time-points. This was achieved by staining for Ki-67, an intracellular protein whose expression is strictly associated with cellular proliferation[415, 416]. Ki-67 staining of tetramer-bound CD4 T cells revealed that expansion of the late endogenous alloreactive T cell population in recipients of chronically rejecting allografts correlates with ongoing proliferation. The percentage of Ki-67 positive, H-2K^d allopeptide-specific CD4 T cells was higher at 6 weeks in unmodified recipients of bm12.kd.IE or anti-CD154-treated recipients of BALB/c grafts than in C57BL/6 recipients of acutely-rejecting BALB/c heart allografts (Figure 6.10b,c).

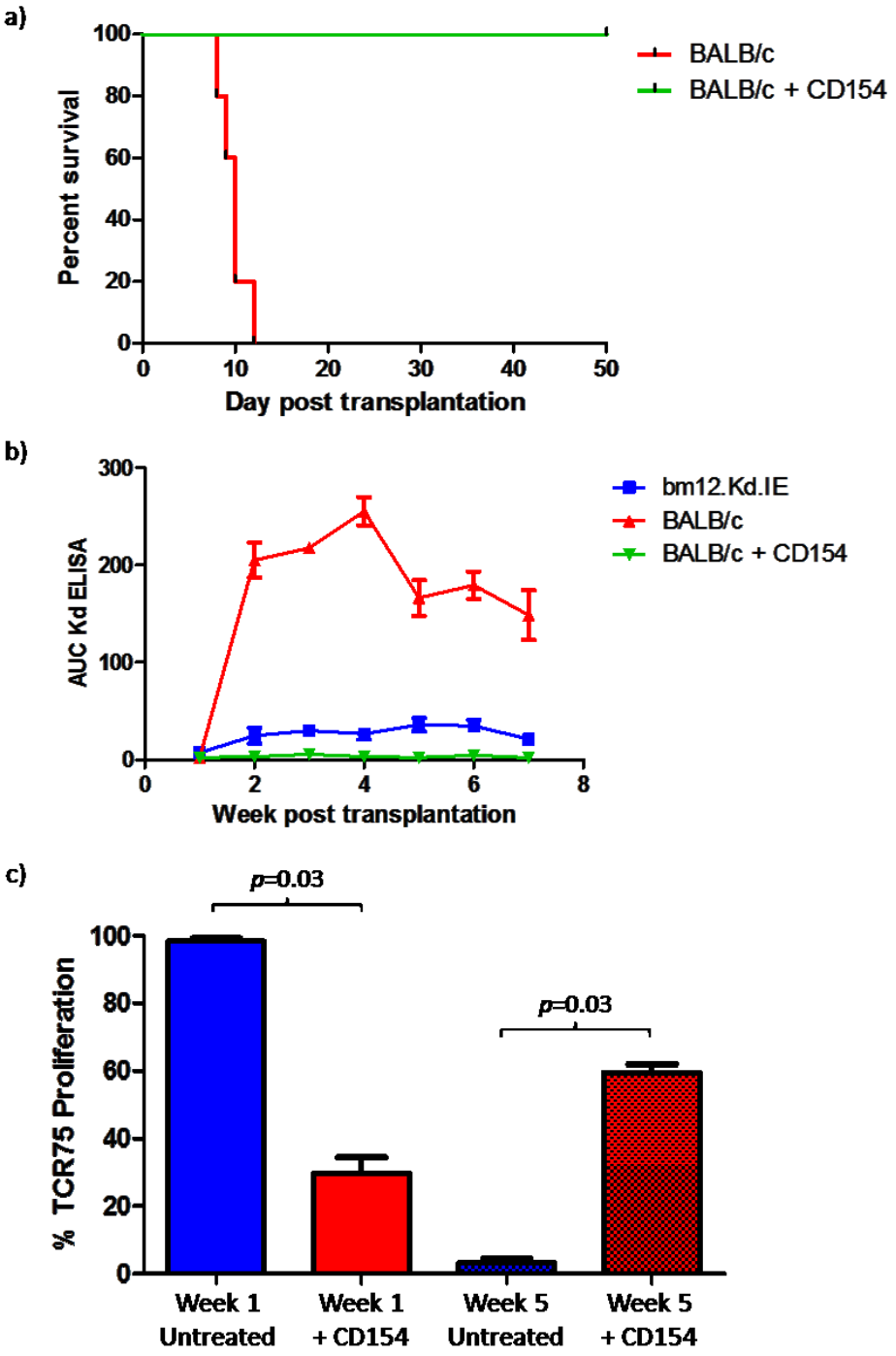


Figure 6.9 Co-stimulation blockade delays allograft rejection and prolongs indirect allorecognition
BALB/c cardiac allografts were transplanted into C57BL/6 recipients +/- administration of anti-CD154 co-stimulation blockade (1mg i.p. days -2, 0, +2, +4 in relation to transplantation). (a) Abdominal palpation of heartbeat demonstrated that co-stimulation blockade abrogated acute allograft rejection (n=5). (b) Weekly serum was collected and anti-H-2K^{d} alloantibody quantified by ELISA. A substantial reduction in the level of alloantibody occurred following treatment with anti-CD154. (c) Following transplantation 4-6x10^{6} CFSE labelled TCR75 splenocytes were adoptively transferred either on the day of transplant or four weeks later, and extent of proliferation quantified after 6 days. Proliferation was attenuated in CD154 treated recipients at week 1. However, the indirect-pathway response against donor MHC class I was prolonged in CD154 treated recipients, with robust proliferation observed at the late time point. *P* values using Mann Whitney test.

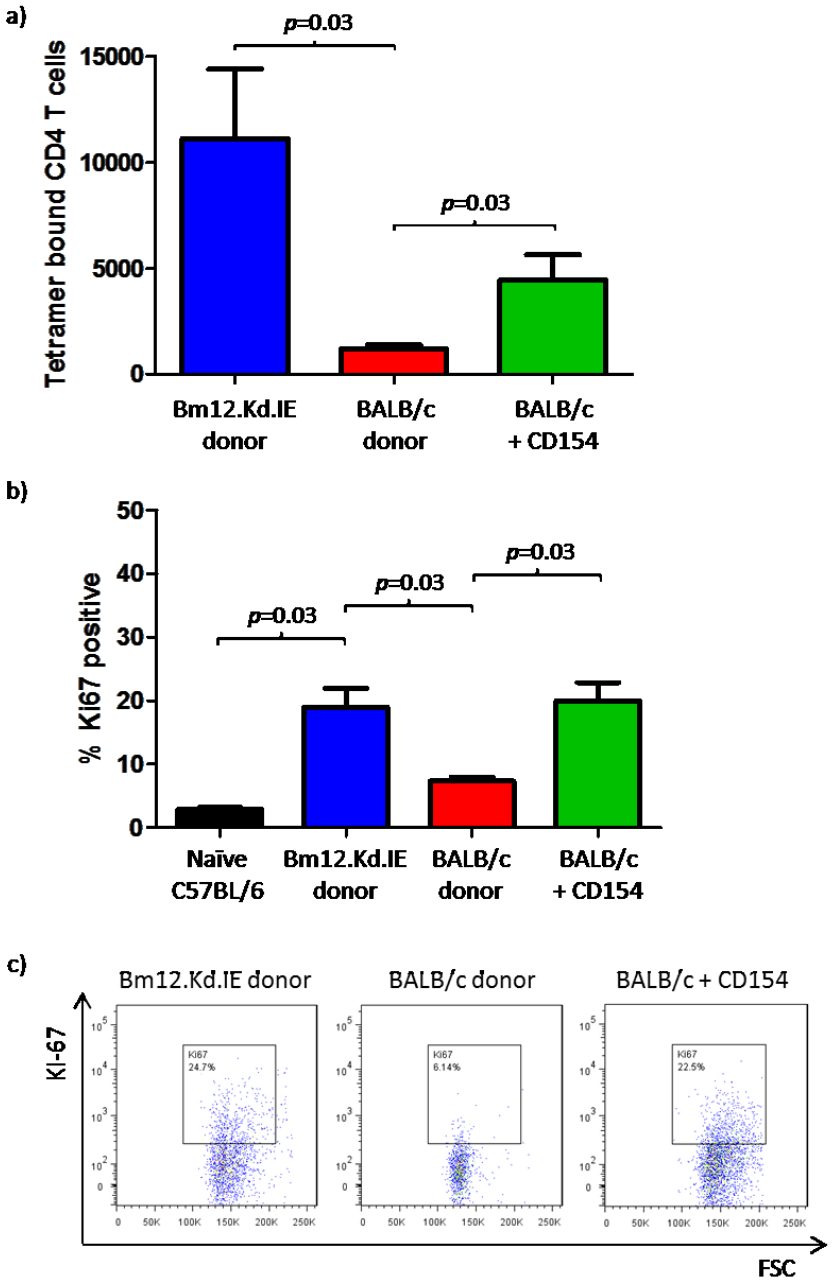


Figure 6.10 Persistent indirect-pathway responses are characterised by expanded populations of antigen-specific CD4 cells that continue to proliferate

Bm12.Kd.IE, or acutely rejecting BALB/c cardiac allografts +/- anti-CD154 treatment (1mg i.p. days -2, 0, +2, +4 in relation to transplantation), were transplanted into C57BL/6 recipients. At 4 weeks following transplantation, recipient spleens were harvested. Staining and enrichment using the H-2K^d allopeptide loaded tetramer were performed as described in Figure 6.3, Each group n=3. (a) The number of tetramer bound cells was quantified as described in Figure 6.7. In keeping with prolongation of the indirect – pathway alloresponse, a significantly greater number of tetramer bound cells was observed in recipients of BALB/c cardiac allografts following treatment with anti-CD154. Separately, following enrichment, cells were additionally stained for expression of intracellular protein Ki-67, a marker of recent proliferation and the proportion of tetramer bound cells expressing Ki-67 quantified. The expanded population of tetramer bound cells observed in recipients of bm12.Kd.IE and BALB/c with anti-CD154 treatment are actively undergoing proliferation, unlike the contracted population following BALB/c acute rejection (b). Representative flow cytometry plots demonstrating Ki-67 staining. *P* values using Mann Whitney test.

6.3.3 Phenotypic characterisation of endogenous H-2K^d specific indirect CD4 T cells

In addition to quantifying the size of the endogenous CD4 T cell response to an alloantigen, tetramer staining provides the opportunity to examine additional details of the phenotype of endogenous antigen-specific CD4 T cells.

6.3.3.1 Assessing memory status of antigen-experienced CD4 T cells

Following population expansion a significant proportion of CD4 T cells undergo apoptosis during the contraction phase[306, 417, 418]. Some effector T cells though, develop into memory T cells that persist as heterogeneous populations in lymphoid and mucosal sites. Two subsets of memory CD4 T cells are commonly described: 'effector memory' Tem ($CD44^+$ $CD62L^{lo}$ $CCR7^{lo}$) and 'central memory' Tcm ($CD44^+$ $CD62L^+$ $CCR7^+$)[418-421].

The memory status of antigen-specific tetramer bound CD4 T cells following a transplant challenge was next examined. Of interest was if there is any difference in the characteristics of antigen-specific CD4 T cell memory status following challenge with an acutely or a chronically rejecting allograft.

BALB/c and bm12.Kd.IE cardiac allografts were transplanted into C57BL/6 recipients and 6 weeks later the memory phenotype of tetramer bound cells was examined. The percentage of antigen-experienced $CD44^{hi}$ CD4 T cells was comparable after transplantation with BALB/c or bm12.Kd.IE cardiac allografts, but the antigen experienced CD4 T cells in recipients of a bm12.Kd.IE heart graft were predominantly $CD44^{hi}CCR7^{lo}CD62L^{lo}$ effector memory, while those in BALB/c heart-grafted recipients were more skewed towards $CD44^{hi}CCR7^{hi}CD62L^{hi}$ central memory (Figure 6.11).

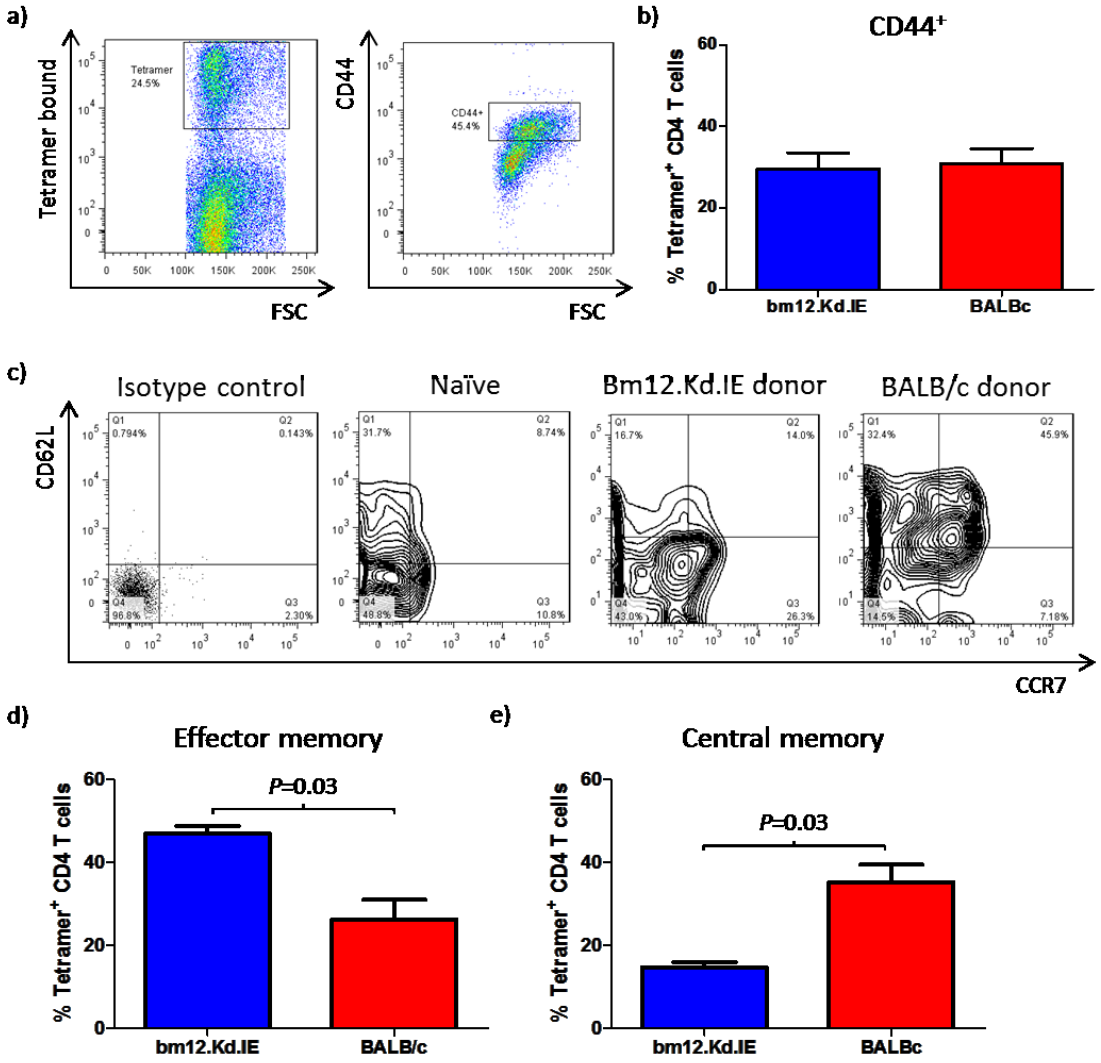


Figure 6.11 Memory phenotype of tetramer bound CD4 T cells

Bm12.Kd.IE, or acutely rejecting BALB/c cardiac allografts were transplanted into C57BL/6 recipients. At 6 weeks following transplantation, recipient spleens were harvested. Staining and enrichment using the H-2K^{d} allopeptide loaded tetramer were performed as described in Figure 6.3, Each group n=4. a) Representative flow cytometry plots showing tetramer bound CD4 T cell population (left panel) and gating strategy for identifying CD44^{+} antigen experienced cells. b) Comparison of proportion of tetramer bound CD4 T cells that are CD44^{+} following transplantation with bm12.Kd.IE or BALB/c allografts. c) Representative flow cytometry plots of CD44^{+} CD4 T cells from a naïve C57BL/6 mouse (left 2 panels) and tetramer bound CD4 T cells from recipients of bm12.Kd.IE or BALB/c allografts (right 2 panels) with CD62L plotted against CCR7 (Far left panel is with isotype control antibodies). Proportion of CD44^{+} tetramer bound CD4 T cells that were d) CD44^{hi}CCR7^{lo}CD62L^{lo} effector memory or e) CD44^{hi}CCR7^{hi}CD62L^{hi} central memory. *P* values using Mann Whitney test.

To assess the functional consequences of this difference in memory phenotype, T cell-deficient TCRKO (*Tcrbd*$^{-/-}$) C57BL/6 mice were reconstituted with CD4 T cells purified from recipients of bm12.Kd.IE or BALB/c heart grafts 6 weeks following transplantation, and the reconstituted TCRKO mice then challenged with a C57BL/6 donor heart that expressed H-2K^{d} as a transgene (B6.K^{d}) while simultaneously receiving co-stimulation blockade treatment with anti-CD154 mAb (Figure 6.12a).

It was anticipated that only established H-2K^{d}-specific memory CD4 T cells would be able to provide co-stimulation-independent help for development of anti-H-2K^{d} alloantibody responses against the B6.K^{d} graft[382]. Whereas anti-H-2K^{d} alloantibody was not observed in TCRKO recipients reconstituted with CD4 T cells from bm12.Kd-IE grafted mice, weak, but consistent, anti-H-2K^{d} alloantibody responses developed in TCRKO recipients reconstituted with CD4 T cells from BALB/c grafted mice (Figure 6.12b), despite the vastly reduced number of H-2K^{d} allopeptide-specific CD4 T cells present in this latter group at late time points after transplantation (Figure 6.10a). Thus, chronic allopeptide presentation drives continual division of the responding alloreactive CD4 T cell population, with persistence of a greatly expanded population, but with functional evidence of altered memory development.

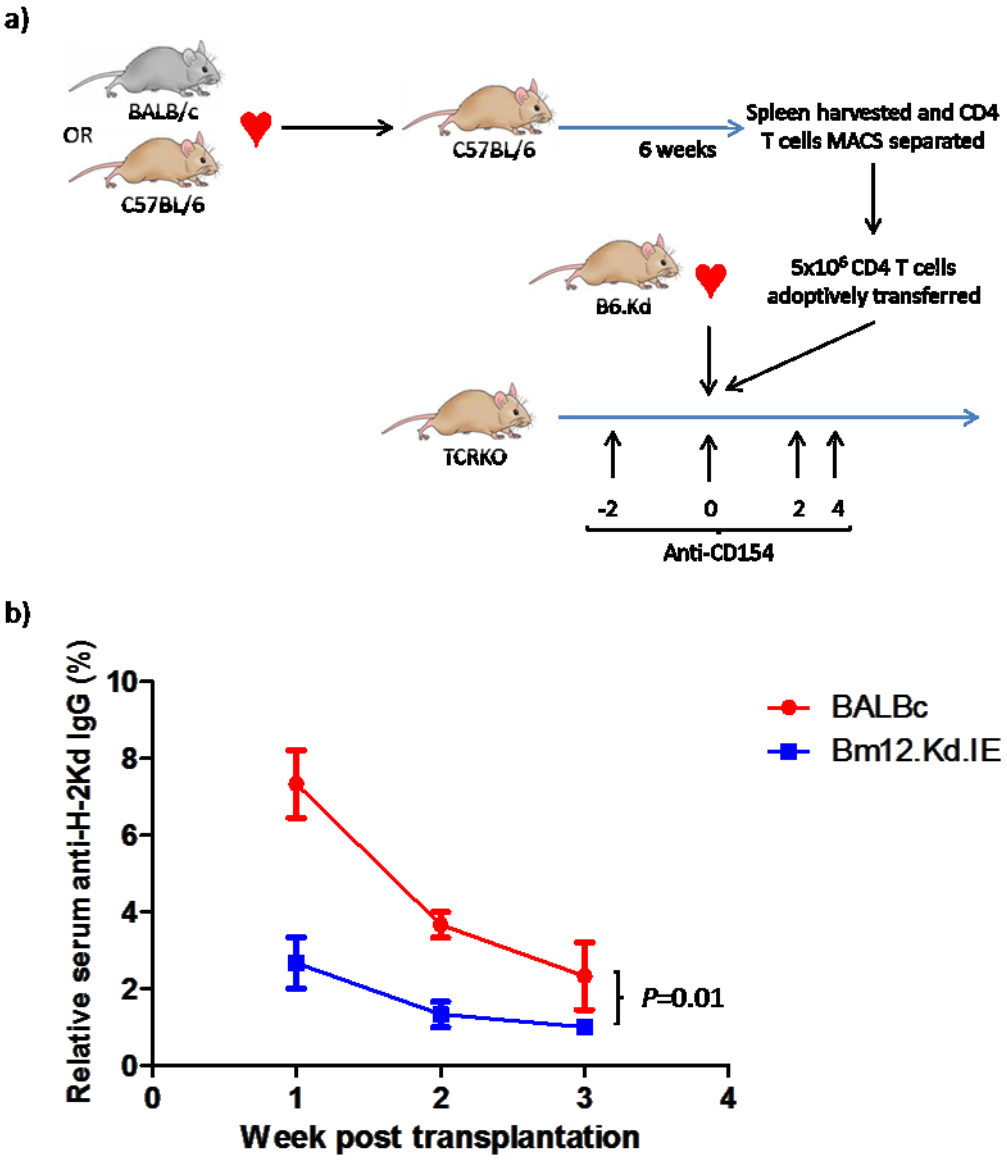


Figure 6.12 Assessing functional relevance of CD4 T cell memory cell phenotype differences

a) BALB/c or bm12.Kd.IE cardiac allografts were transplanted into C57BL/6 recipients. After 6 weeks, recipient spleens were harvested and CD4 T cells purified using MACS separation (using anti-CD4 MicroBeads). $5x10^6$ CD4 T cells from recipients of either bm12.Kd.IE or BALB/c allografts were adoptively transferred i.v. into T cell deficient TCRKO mice that were recipients of B6.Kd cardiac allografts (each group n=3). The TCRKO recipients were treated with anti-CD154 500µg i.p. on days -2, 0, +2, +4. b) Weekly serum samples were collected from TCRKO recipients and anti-H-$2K^d$ alloantibody was quantified using ELISA. *P* value using two-way ANOVA

6.4 Discussion

This chapter has examined the endogenous indirect-pathway alloresponses targeted against donor MHC class I H-2K^{d} in recipients following cardiac transplantation. The results have confirmed peptide-loaded MHC class II tetramer to be an effective tool to examine alloantigen-specific CD4 T cells. To date, MHC class II tetramer use in human transplantation to study alloresponses has been limited, perhaps due to a lack of knowledge of the immunogenic peptides for all HLA molecules, and the requirement to generate different peptide loaded tetramers for each donor/recipient HLA combination. However, tetramers have been used more widely in studies of infection, where immunodominant peptides have been characterised for many organisms. One such study was performed in human transplant recipients, utilising MHC class I tetramers loaded with immunodominant peptide from CMV in order to examine CMV specific CD8 T cell responses[422]. Experimentally, MHC class I tetramers have also been recently reported as a tool to examine alloreactive recipient B cells[423].

In recipients of acutely rejecting BALB/c allografts, the alloantigen-specific CD4 T cell population undergoes typical expansion followed by contraction phases. By contrast, in recipients of chronically rejecting bm12.Kd.IE allografts the expansion phase was much more marked, and approximately 10,000 fold greater cells persisted into the memory phase, despite similar levels of H-2K^{d} alloantigen expression. In support that this reflects ongoing alloantigen availability, a less profound contraction was observed in recipients of BALB/c allografts treated with anti-CD154 co-stimulation blockade. Furthermore, ongoing alloantigen presence correlated with continued division of the H-2K^{d} alloantigen-specific CD4 T cell population. In addition to these differences in kinetics, there were also phenotypic differences between the acute and chronically rejecting models. The antigen experienced CD4 T cells in recipients of a bm12.Kd.IE heart graft were predominantly CD44^{hi}CCR7^{lo}CD62L^{lo} effector memory, while those in BALB/c heart-grafted recipients were more skewed towards CD44^{hi}CCR7^{hi}CD62L^{hi} central memory, with functional evidence of altered memory development.

MHC class II tetramers have been successfully used to examine CD4 T cell responses following infection, with much of this work performed by the group of Marc Jenkins[412]. Adoption of the enrichment technique they describe permitted successful evaluation of alloreactive CD4 T cells in this work[407]. It was disappointing that the donor MHC class II peptide-loaded tetramer was unable to bind to I-E allopeptide-specific CD4 T cells responding to a cardiac allograft, as this would have provided interesting insight into the dynamics of the CD4 T cell alloresponses

against MHC class II alloantigen. It is interesting to consider why there should be differential recognition of CD4 T cells with this tetramer following challenge with identical synthetic peptide and endogenous protein. There are however two possible explanations for this observation. Otvos *et al*. have demonstrated using rabies virus peptides that post-translational side-chain modifications can affect the MHC binding ability and conformation of the T cell epitopes[424]. It may be that post-translational modification of the I-E molecule leads to processed allopeptide adopting a slightly different confirmation within the tetramer that does not correspond to the epitope presented on APCs. Alternatively, Viner *et al*. have observed, using natural protein and synthetic peptides of hen egg lysozyme, that the interaction of synthetic free peptides with MHC class II molecules can generate complexes that are antigenically dissimilar to those resulting from endosomal processing of intact antigen[425]. Thus, the I-E peptide may be able to adopt different confirmations within the I-A^b peptide binding groove and use of synthetic peptide to generate the tetramer create an epitope not recognised by the endogenous alloreactive CD4 T cells.

To overcome the limitation of not using the I-E-peptide loaded tetramer, the indirect-pathway responses following short-lived and ongoing alloantigen exposure were examined using acute and chronic rejection models, using the H-$2K^d$-loaded tetramer in both cases. Most notably, the expansion of the H-$2K^d$ specific CD4 T cell population was much more modest in acute, than in chronic, rejection. This may simply reflect that the multiple allogeneic epitopes against which the CD4 T cell population is responding in the acute BALB/c to C57BL/6 model results in the response against each epitope being proportionally smaller. Alternatively, it has been reported that polyclonal CD4 T cell populations expand in proportion to the frequency of their naïve progenitors[412]. Thus, this difference could be also explained by the presence of a higher precursor frequency specific for a different epitope presented by BALB/c allografts that proliferate preferentially, or other factors that have been proposed to affect the magnitude of CD4 T cell responses such as antigen abundance, efficiency of antigen processing by APCs, MHC binding affinity and stability of the peptide-MHC complexes[412].

Comparison of antigen-specific CD4 T cell responses using MHC class II tetramers has also been made in acute vs chronic infection models. Acute infections include lymphocytic choriomeningitis virus and *Listeria monocytogenes*[426, 427]. Following exposure to these infections there is a substantial expansion of the antigen-specific CD4 T cells with the population peaking at one week. Subsequently ~90% of effector cells die leaving a memory population which continues to decline in number after the infection has been cleared since their death rate is greater than their low rate of homeostatic proliferation[427, 428]. In

comparison, chronic infection has been studied using *Salmonella* species[406]. Following infection, the typical expansion phase was observed; however, less than 80% of the population was cleared and the population stabilised at an augmented level and was maintained long-term. Furthermore, the authors observed increased proliferation of these cells, thought to account for the maintenance of an augmented population. The authors were able to confirm that ongoing antigen presentation was responsible for population maintenance since treatment of the mice with antibiotics to clear the infection resulted in a significant reduction in antigen-specific CD4 T cells. A similar finding was also observed in work using TCR transgenic CD4 T cells – Obst *et al.* reported a significantly reduced proliferation rate (and therefore contraction of population size) following early withdrawal of antigen, reaffirming that antigen persistence is required throughout the maintenance phase to ensure a stable population size[429].

These results, in models of infection, are consistent with the results presented in this chapter. The use of a single tetramer in both the acute and chronic rejection models in this work help to control for any differences that there may be between infection models, such as in tetramer-TCR affinity and precursor CD4 T cell frequency, and allows for a more robust comparison to be made.

The differences in population kinetics observed between acute and chronic rejection models in the maintenance phases is of interest when considering the development of CD4 T cell memory. Much work has been performed examining CD8 T cell memory, and it is well established that persistent stimulation by their peptide-MHC class I epitope results in functional exhaustion[430-432]. For CD8 T cells it is observed that a single encounter with their epitope is sufficient to initiate a program to drive cells through multiple rounds of division as well as acquiring effector and memory functions, without requiring further antigenic stimulation – described as being on 'autopilot'[433-435]. This appears not to be the case for CD4 T cells. The data described above, and findings in this chapter seem to suggest that there is not a similar programme initiated within CD4 T cells, but rather CD4 T cell population dynamics are intimately related to availability of antigen – not only for their expansion, but also for the manifestation of their effector functions[429]. It has been highlighted that this perhaps makes sense considering the central helper role of CD4 T cells within the adaptive immune response. Their requirement for antigen exists as an additional control checkpoint on their activity[429].

Despite this apparent requirement for on-going antigen persistence to maintain an antigen-specific CD4 T cell population, there is also some evidence that a lengthened period of

antigenic stimulation during the primary response may be deleterious to memory generation, similar to that observed in CD8 T cells[417, 436]. However, in that study description of memory generation was simply based upon number of surviving adoptively transferred CD4 T cells and no phenotypic examination was performed to confirm memory status. Nevertheless, it is conceivable that ineffective memory generation occurs whilst antigen expression persists. In support of this possibility, in the chronic rejection model described in this chapter, a significantly higher proportion of the H-2K^{d}-specific CD4 T cells stained positive for Ki-67, indicating recent proliferation, than in the acute rejection model. A recent study, similarly using MHC class II tetramers to examine memory CD4 T cell responses in infection, suggested that antigen-specific CD4 T cells divide at a rate of <1% per day[415]. The higher proliferation rate observed here may indicate that memory CD4 T cell development is dysfunctional in the setting of ongoing antigen presentation.

The use of peptide-MHC class II tetramer to identify endogenous alloantigen-specific CD4 T cells has the advantage over other techniques of identifying these cells, as it provides opportunity to examine their phenotype. There are two functionally distinct subsets of CD4 T cell memory: CD44^{hi}CCR7^{lo}CD62L^{lo} effector memory (Tem) described to reside in the blood, spleen and non-lymphoid tissues that will rapidly respond to antigen by producing effector molecules and CD44^{hi}CCR7^{hi}CD62L^{hi} central memory (Tcm) found in lymph nodes, spleen and blood (but not in non-lymphoid tissues) that are slower in making cytokines or becoming effector cells[420, 437]. Examination of the H-2K^{d}-specific CD4 T cells revealed a phenotypic difference in the acute and chronic models. In the acute model, there was a predominance of Tcm, whilst in the chronic model there was a predominance of Tem. There is some evidence that CD8 T cells appear to follow a linear differentiation pathway from naïve → effector → Tem → Tcm, with progressive acquisition of memory characteristics[437]. There is some data suggesting that this may also be true for CD4 T cells. The predominance of a Tem population in the presence of ongoing antigen presentation may then reflect incomplete memory development in this setting.

Other data though suggests that Tem and Tcm develop in parallel – with Tem developing from Th1, Th2 and Th17 effectors, whilst Tcm develop independently of Tem from alternative precursors, possibly Tfh cells[427], that become quiescent following termination of GC reactions[438]. If this model proves to be correct, then the lack of Tcm in the chronic model observed in this chapter, could be accounted for by the on-going nature of the adaptive alloimmune responses (as described in Chapter 3), retaining Tfh within the GCs.

The functional relevance of phenotypic differences between memory CD4 T cells in the acute and chronic model was highlighted by adoptive transfer of CD4 T cells, from recipients at 6 weeks following transplantation, into T cell deficient recipients of H-2K^{d} expressing cardiac allografts (Figure 6.12). In the presence of anti-CD154 co-stimulation blockade (which would be expected to prevent naïve but not memory CD4 T cell alloresponses[382]), anti-H-2K^{d} alloantibody was only observed following transfer of CD4 T cells from recipients of acutely rejected BALB/c donors. That is despite the significantly smaller proportion of H-2K^{d}-specific CD4 T cells remaining in these recipients. These results suggest that persistence of antigen, although supporting augmented antigen-specific CD4 T cell populations, may lead to the development of altered, perhaps dysfunctional, memory responses.

Work in this chapter demonstrates the potential of utilising peptide-MHC class II tetramers in examining the alloimmune response following transplantation. The supply of tetramer limited further examination, but future work with this tetramer could include characterising other aspects of the H-2K^{d}-specific CD4 T cell alloresponse such as comparing the Th1/Th2/Th17 phenotype between the acute and chronic models and characterising the Tfh population. Additionally, having defined the normal alloimmune response tetramers, could be used as tools to more precisely examine the impact of immunosuppressive therapies on the CD4 T cell alloresponse in these models.

Furthermore, although not considered in this work thus far, donor MHC class I tetramers can be used to examine B cell alloresponses, since they form the conformational epitope[423, 439-441]. H-2K^{d} tetramer could be utilised to quantify and compare the population kinetics and phenotypic characteristics of alloreactive B cells corresponding to the CD4 T cell alloresponses characterised with the H-2K^{d} peptide-loaded I-A^{b} tetramer providing further insight into the anti-H-2K^{d} alloimmune response generated within these recipients.

7 Knowledge of allorecognition pathways can inform development of effective immune regulatory cellular therapies

7.1 Introduction

Developing regulatory cellular immunotherapy has become one of the main goals of current transplant immunological research. At the forefront of this are regulatory T cells (Treg) as the most widely characterised and studied regulatory cell. Work in animal models has demonstrated the efficacy of Treg therapy at preventing allograft rejection and clinical translation has already begun[209, 250]. There is evidence that antigen-specific Treg may be more effective than polyclonal Treg at preventing allograft rejection[165, 224]. It seems likely that understanding of allorecognition pathways will be important to understanding how best to utilise these regulatory cells therapeutically.

7.1.1 Alloantigen specific regulatory T cells

A variety of techniques have been adopted to induce and expand polyclonal and alloantigen-specific Treg *in vitro* (Table 7.1). These techniques have permitted examination and comparison of the efficacy of polyclonal and alloantigen-specific Treg in murine models of transplantation, including humanised models (where severely immunocompromised murine strains are irradiated and reconstituted with human bone marrow cells and are then transplanted with human tissues[225, 442]).

Paper	Treg type	Culture conditions	Origin
Chen 2003[443]	Polyclonal	nTreg isolated ($CD4^+$ $CD25^+$) and cultured with plate-bound anti-CD3, IL-2, soluble anti-CD28, TGF-β +/- IL-10	Murine
Nadig 2010[444]	Polyclonal	nTreg isolated ($CD4^+$ $CD25^+$ $CD127^-$) and cultured with anti-CD3/anti-CD28 coated beads and IL-2	Human
Scotta 2013[445]	Polyclonal	nTreg isolated ($CD4^+$ $CD25^+$) and cultured with anti-CD3/anti-CD28 coated beads, IL-2 +/-rapamycin +/-ATRA	Human
Takasato 2014[446]	Polyclonal	Naïve CD4 T cells isolated and cultured with anti-CD3, anti-CD28, IL-2, TGF-β and ATRA	Murine
Huang 2013[447]	TCR transgenic antigen specific	CD4 T cells isolated and cultured with IL-2, plate-bound anti-CD3 and TGF-β	Murine
Jiang 2006[448]	Indirect-pathway antigen specific	nTreg isolated ($CD4^+$ $CD25^+$) and cultured with self DCs from peripheral blood, pulsed with allopeptide and IL-2	Human
Tsang 2008[449]	Indirect-pathway antigen specific	nTreg isolated ($CD4^+$ $CD25^+$) were transduced with transgenic TCRα and β genes specific for a self-MHC restricted allopeptide	Murine
Veerapathran 2011[450]	Indirect-pathway antigen specific	nTreg isolated ($CD4^+$ $CD25^+$ $CD127^-$) and cultured with irradiated donor cell lysate loaded self DCs and IL-2, 1L-15 and rapamycin	Human
Takasato 2014[446]	Indirect-pathway antigen specific	Naïve CD4 T cells isolated and cultured with syngeneic BMDC pulsed with allopeptide, anti-IFNγ, anti-IL-4, IL-2, TGF-β and ATRA	Murine
Tsang 2008[449]	Direct-pathway antigen specific	nTreg isolated ($CD4^+$ $CD25^+$) and cultured with immature allogeneic DCs, IL-2	Murine
Nouze 2011[451]	Direct-pathway antigen specific	nTreg isolated ($CD4^+$ $CD25^+$) and cultured with irradiated allogeneic splenocytes and IL-2	Murine
Sagoo 2011[224]	Direct-pathway antigen specific	nTreg isolated ($CD4^+$ $CD25^+$ $CD127^-$) and cultured with irradiated mature $CD1c^+$ DCs from peripheral blood or skin with IL-2	Human
Veerapathran 2011[450]	Direct-pathway antigen specific	nTreg isolated ($CD4^+$ $CD25^+$ $CD127^-$) and cultured with irradiated allogeneic DCs or CD4 depleted splenocytes and IL-2, 1L-15 and rapamycin	Human
Putnam 2013[452]	Direct-pathway antigen specific	nTreg isolated ($CD4^+$ $CD25^+$ $CD127^-$) and cultured with allogeneic CD40L stimulated B cells	Human
Landwehr-Kenzel 2014[453]	Direct-pathway antigen specific	nTreg isolated ($CD4^+$ $CD25^+$) and cultured with allogeneic B cells, IL-2 and rapamycin	Human
Takasato 2014[446]	Direct-pathway antigen specific	Naïve CD4 T cells isolated and cultured with allogeneic BMDC, anti-IFNγ, anti-IL-4, IL-2, TGF-β and ATRA	Murine

Table 7-1 *In vitro* culture conditions for generating regulatory CD4 T cells

7.1.2 Direct vs Indirect-pathway alloantigen specific regulatory T cells

Both direct and indirect-pathway CD4 T cell alloresponses are thought likely to play a role in allograft rejection and it could be predicted that to achieve tolerance would require Treg of both direct and indirect allospecificity. However, results from Chapter 4 highlight that direct-pathway alloresponses are short-lived and may only play a role early after transplantation, raising the possibility that indirect-pathway specific Treg may be most effective. Several studies have utilised direct and/or indirect-pathway Treg and compared their efficacy in transplant models.

A study by Sanchez-Fueyo *et al.* in 2007 demonstrated that although Treg activation *in vivo* can occur through both direct and indirect-pathways, their immunoregulatory function was significantly pronounced when allorecognition was restricted to the indirect-pathway[454]. They found that directly presented alloantigens elicited a significantly weaker Treg immunoregulatory response. This finding was in keeping with other early reports suggesting that Treg induced immune regulation was mediated primarily against indirect alloresponses[455, 456]. This finding has also been demonstrated in human clinical transplantation. It has been shown that depleting Treg in stable renal transplant recipients reveals significant indirect-pathway alloresponses, highlighting the importance of Treg in suppressing indirect-pathway allorecognition[457]. In contrast, depleting Treg did not lead to enhanced direct alloresponses, suggesting that Treg are not responsible for the observed direct-pathway hypo-responsiveness in these stable human kidney transplant recipients[458]. One possible explanation for this is the observation that the naturally occurring Treg TCR repertoire appears to be heavily biased towards high affinity recognition of self-MHC peptide complexes – perhaps relating to their role in suppressing autoreactivity[454]. Indeed it is observed that a smaller frequency of Treg cross-react with allogeneic MHC molecules *in vitro*, compared to effector T cells[50].

Further understanding of the relative importance of direct and indirect-pathway Treg was provided by Joffre *et al.* who generated Treg specific for directly, and Treg specific for directly and indirectly presented alloantigen[222]. They demonstrated that direct-pathway Treg alone could abrogate acute rejection of skin and cardiac allografts, but could not prevent the development of the vascular changes of chronic rejection. This required administration of direct and indirect-pathway Treg – emphasising the role of indirect-pathway Treg in preventing the development of AV. This was also the finding of Tsang *et al.*, who used Treg with direct allospecificity, and Treg with dual direct and indirect allospecificity (the indirect allospecificity

being conferred by TCR gene transfer)[449]. They demonstrated that Treg with both direct and indirect specificity showed superior protection compared with Treg with direct specificity in a semi-mismatched heart transplant model. In a fully mismatched strain combination, Treg with direct specificity were also able to induce indefinite survival in some animals. However, histological analysis of the heart grafts from mice treated with Treg that only had direct-pathway anti-donor allospecificity revealed clear signs of rejection which was completely absent in those treated with dual Tregs. However, in neither of these studies was there comparison of direct and indirect-pathway Treg used alone. This was the focus of a recent publication by Takasato *et al*. who generated indirect-pathway Treg using allopeptide pulsed recipient DCs[446]. They observed that indirect-pathway induced Treg (iTreg) were significantly more effective then direct-pathway Treg at prolonging graft survival and suppressing AV and chronic rejection. The efficacy of indirect-pathway Treg alone to confer long term graft survival has also been demonstrated by Tsang *et al.*in a different study across a complete mismatch combined with short-term immunosuppression[223].

It is apparent considering these findings that indirect-pathway Treg are likely to be a key component of successful Treg cellular therapy. However, the results presented in Chapter 5 report that the indirect-pathway is a heterogeneous collection of responses against all mismatched alloantigens and that the responses have variable longevity. It is conceivable that this may have implications for effectiveness of indirect-pathway Treg of different antigen specificities, and this will be examined in this chapter.

7.2 Aims

The purpose of the research described in this chapter is to extend the findings from earlier chapters and investigate the relevance of the finding that indirect-pathway responses against MHC class I are long-lived and against MHC class II are short-lived, to the development of regulatory T cell therapy in the bm12.Kd.IE to C57BL/6 model of transplantation.

The aims are:

1. To develop an *in vitro* culture protocol for the generation of induced polyclonal and indirect-pathway antigen specific regulatory CD4 T cells.

2. Examine the relative effectiveness of polyclonal and MHC class I and MHC class II alloantigen-specific indirect-pathway regulatory CD4 T cells in the abrogation of chronic rejection in the bm12.Kd.IE to C57BL/6 model of chronic allograft rejection.

7.3 Results

7.3.1 In vitro induction of regulatory T cells

Evidence from murine models is mounting that antigen specific Treg may be more effective at abrogating allograft rejection than polyclonal non antigen-specific Treg. In the bm12.Kd.IE to C57BL/6 model results previously presented revealed that the indirect-pathway response against donor MHC class I is long-lived and therefore likely responsible for the development of chronic rejection, whilst the response against donor MHC class II, and similarly the direct-pathway, is short-lived. This raises the possibility that Treg specific for different donor antigens may be variably effective due to the duration of the response against the particular antigen of interest. This knowledge will be useful in guiding ongoing development of Treg immunotherapy.

To investigate this, Treg were induced *in vitro* (iTreg) using the protocol of Huang *et al.*[447], whereby CD4 T cells are cultured in the presence of plate-bound anti-CD3, the interleukin Il-2 and the inhibitory cytokine TGF-β. This protocol proved to be very effective at inducing Treg from naïve CD4 T cells (C57BL/6) with the vast majority of live CD4 T cells exhibiting the $CD25^+$ $FOXP3^+$ phenotype (Figure 7.1a). Despite the relatively straight forward nature of the induction protocol the iTreg proved effective at inhibiting polyclonal CD4 T cell proliferation (induced with anti-CD3/anti-CD28 Dynabeads™ *in vitro* as further evidence of their regulatory phenotype (Section 2.6.6; Figure 7.1b).

One outstanding question in the literature relates to the stability of *in vitro* iTreg phenotype upon transfer *in vivo* with the concern that they may revert to an effector phenotype and exacerbate the alloimmune response. To explore this, fluorescently labelled polyclonal C57BL/6 iTreg (expressing the CD45.1 congenic marker) were adoptively transferred into C57BL/6 recipients of bm12.Kd.IE cardiac allografts on the day of transplantation and examined after seven days for evidence of proliferation and loss of regulatory phenotype. The iTreg had undergone only a small degree of proliferation and reassuringly retained their $CD25^+$ $FOXP3^+$ phenotype (Figure 7.1c).

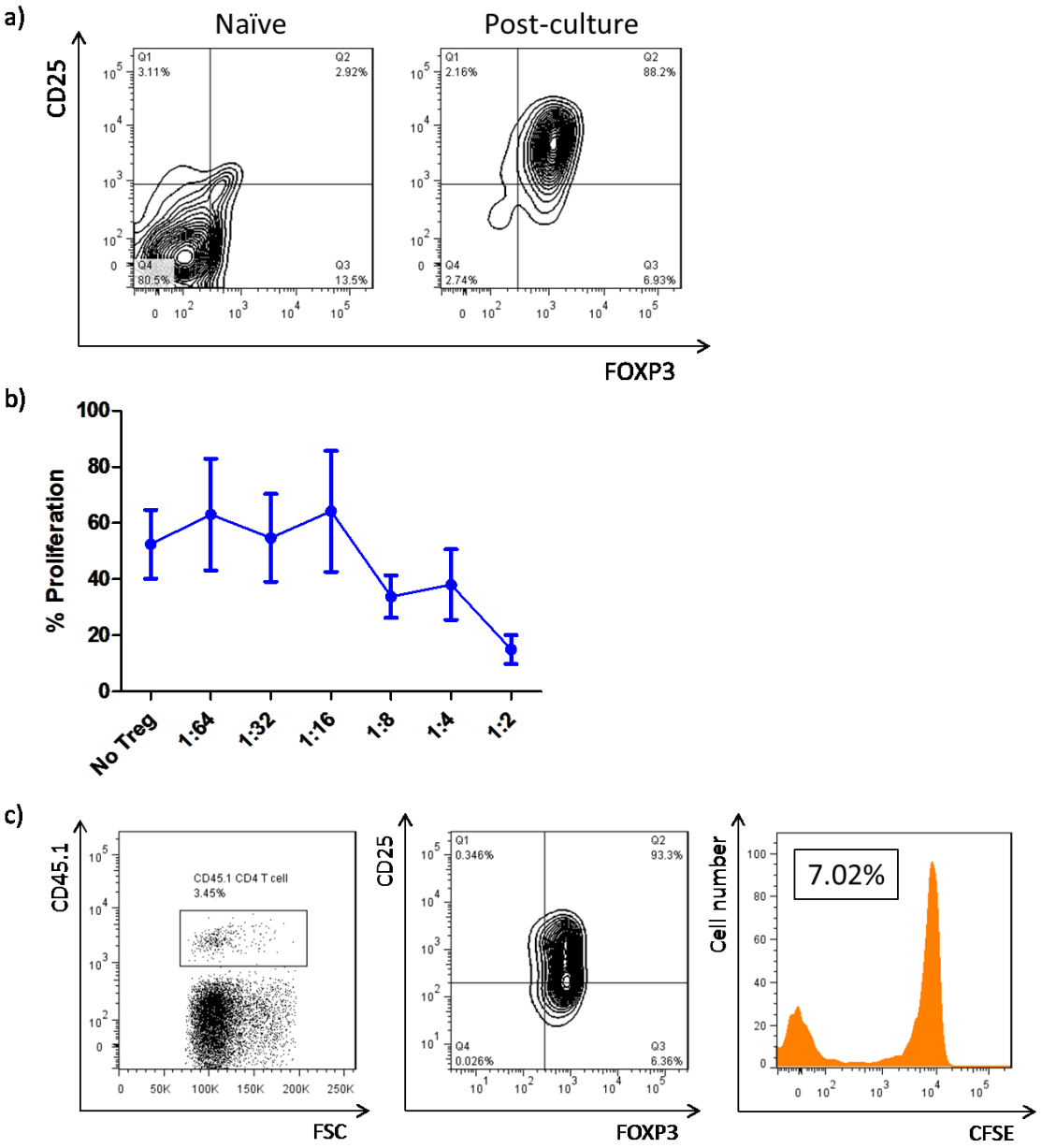


Figure 7.1 Regulatory CD4 T cells can be induced *in vitro* and exhibit inhibitory properties
C57BL/6 CD4 T cells were purified (MACS separated with anti-CD4 microbeads) and subjected to a 5 day period of *in vitro* culture in the presence of plate-bound anti-CD3, IL-2 and TGF-β[447]. (a) Analysis by flow cytometry, before and after culture, confirmed effective conversion to a $CD25^+$ $FOXP3^+$ Treg phenotype. (b) To further confirm a regulatory phenotype Treg were used in a suppression assay, whereby they were co-cultured with CFSE labelled naïve C57BL/6 CD4 T cells at different ratios, in the presence of anti-CD3/CD28 Dynabeads™ to drive naïve CD4 T cell proliferation[276]. The extent of proliferation was quantified and displayed graphically, confirming the ability of Treg to suppress CD4 T cell proliferation. (Assay performed on 3 independent occasions) (c) To evaluate the behaviour of the Treg *in vivo*, CFSE labelled congenically marked Treg (from CD45.1-C57BL/6 mice) were adoptively transferred into C57BL/6 recipients of a bm12.Kd.IE cardiac allograft on the day of transplant. Six days later, recipient spleens were analysed by flow cytometry. The transferred Treg were readily identifiable with anti-CD45.1 (left panel). Reassuringly the Treg retained their $CD25^+$ $FOXP3^+$ phenotype (centre panel) and had not undergone significant proliferation (right panel).

7.3.2 Generation of antigen-specific indirect-pathway regulatory T cells

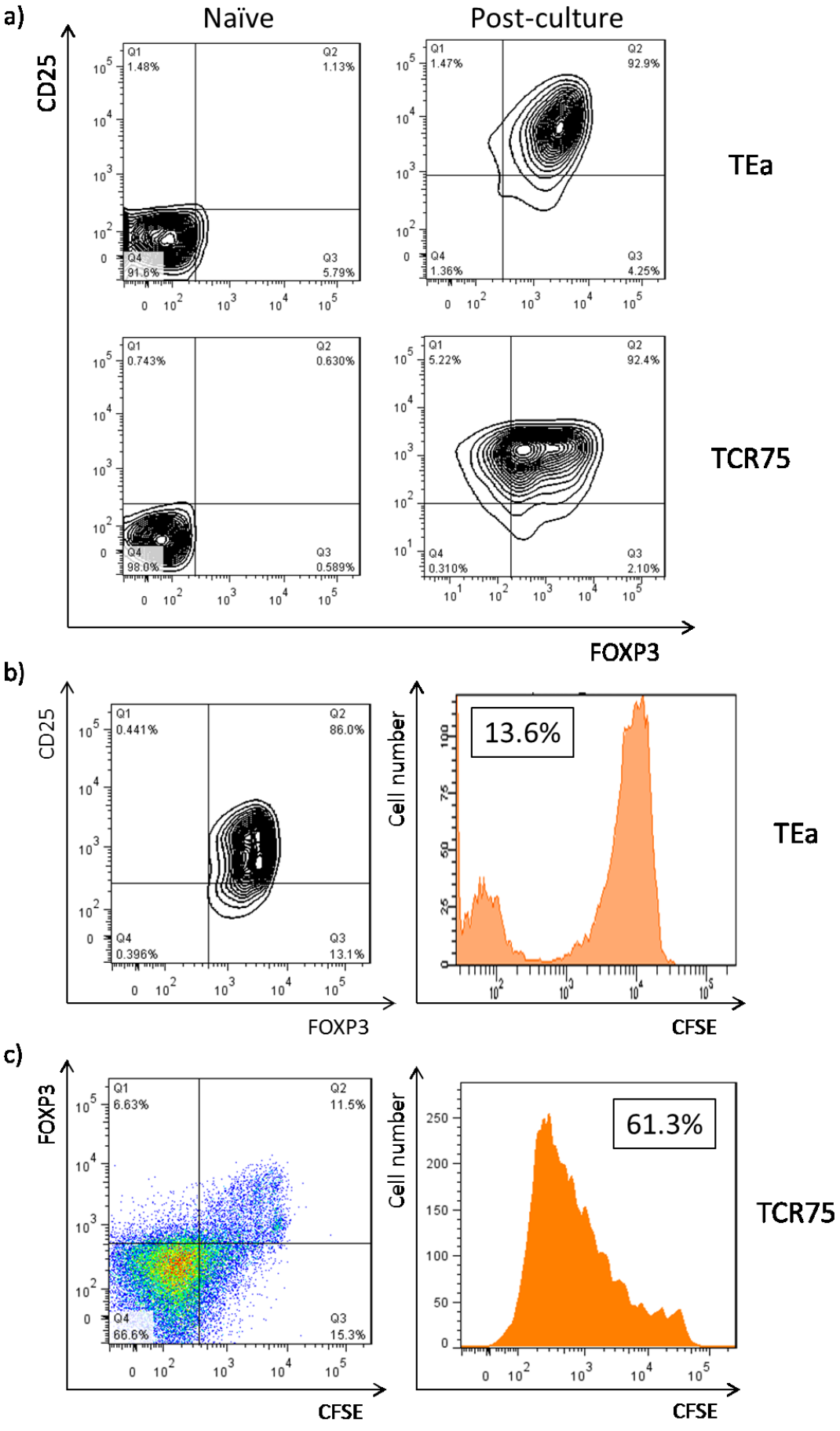
An attempt was next made to obtain indirect-pathway antigen-specific iTreg using the TCR transgenic strains: TCR75 (H-2K^{d} allopeptide specific) and TEa (I-E allopeptide specific). Within the CD4 T cell population wildtype mice possess a small population (2-5%) of natural Treg (nTreg). However, it is recognised that TCR transgenic mice lack populations of nTreg [459-462], raising the possibility that Treg induction would not be so straightforward since in some protocols iTreg generation may not represent true induction of undifferentiated CD4 T cells to a Treg phenotype, but rather selective expansion of the nTreg population.

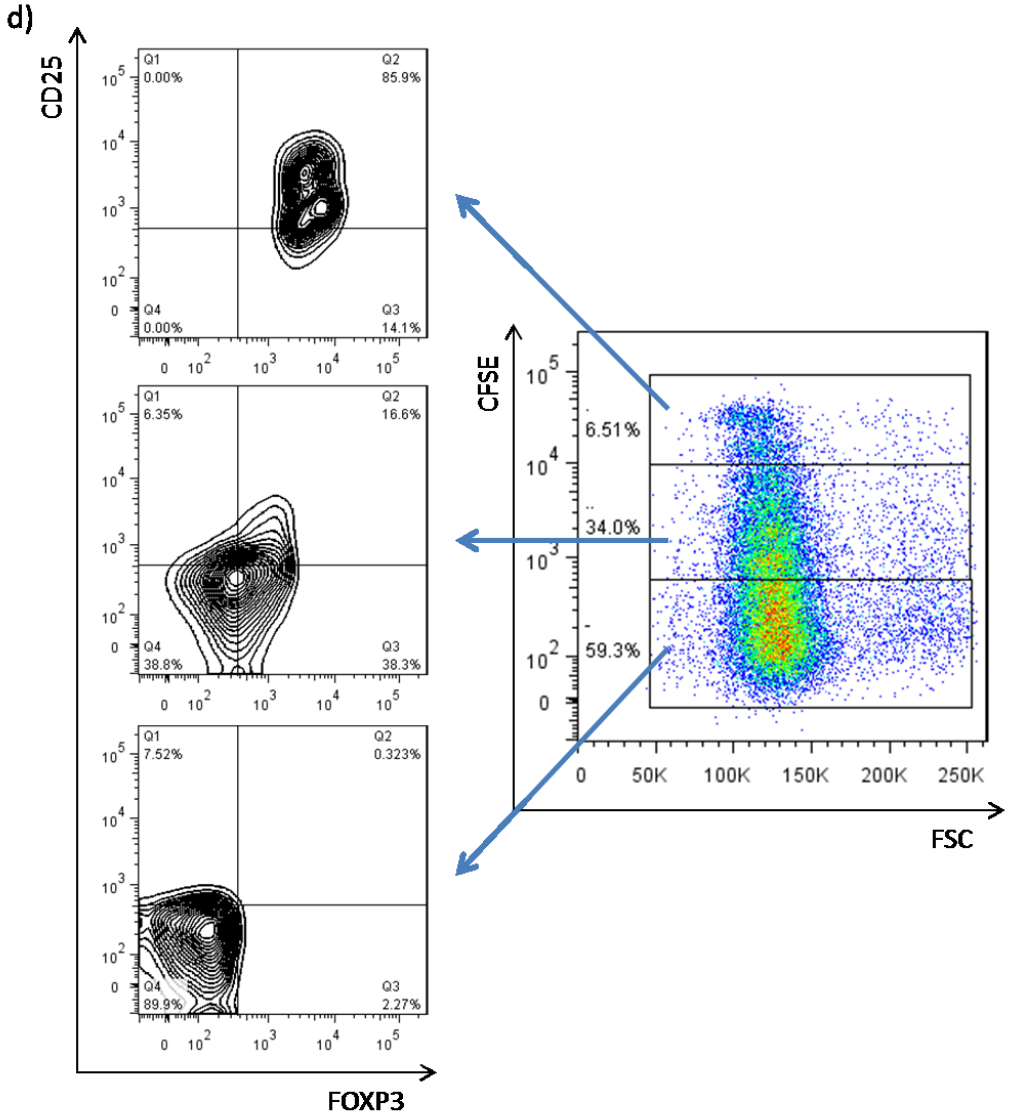
TCR75 and TEa CD4 T cells were therefore subjected to the same *in vitro* culture conditions as above. There was effective induction of a CD25^{+} *FOXP3*$^{+}$ Treg phenotype resulting in monoclonal populations of antigen-specific indirect-pathway CD4 iTreg being generated (Figure 7.2a). It is apparent from the profiles however, that there is some variation in Treg conversion between the two transgenic cells – for example an apparently higher proportion of CD25^{+} FOXP3^{-} cells with TCR75. Why this should be is not clear but perhaps reflects an intrinsic difference between these two transgenic CD4 T cells and could be explored further.

When CFSE labelled TEa CD4 Treg were transferred *in vivo* into a C57BL/6 recipient of a bm12.Kd.IE cardiac allograft, as with polyclonal iTreg, there was minimal proliferation of the TEa CD4 T cells one week later and the transferred CD4 T cells retained the CD25^{+} *FOXP3*$^{+}$ phenotype (Figure 7.2b).

However, in the case of TCR75 iTreg there was a significant degree of proliferation observed, with associated loss of *FOXP3* expression, following transfer into recipients of bm12.Kd.IE cardiac allografts. This suggests that at least a proportion of the population reverted to a non-regulatory phenotype (or that a residual population of transferred non-iTreg – perhaps the CD25^{+} FOXP3^{-} cells alluded to above - were undergoing significant expansion) (Figure 7.2c). Further analysis revealed that the greater the number of divisions, the lower the level of *FOXP3* expression (Figure 7.2d). To exclude that this proliferation was spontaneous and non-antigen specific, CFSE labelled TCR75 iTreg were similarly transferred to naïve C57BL/6 mice. In this setting no proliferation was observed and iTreg phenotype was retained, suggesting that the findings in the transplanted recipients represent antigen-specific proliferation, of at least a proportion, of the transferred population (Figure 7.2e). It was unclear why this difference between TCR75 and TEa iTreg was observed since both naive TCR transgenic populations are

found to undergo proliferation when transferred early following transplantation (Chapter 5). Perhaps this relates to the slightly different CD25/FOXP3 profiles observed following culture.


a)
Naïve
Post-culture
CD25
FOXP3
TEa
TCR75
Q1 1.48%
Q2 1.13%
Q4 91.6%
Q3 5.79%
Q1 1.47%
Q2 92.9%
Q4 1.36%
Q3 4.25%
Q1 0.743%
Q2 0.630%
Q4 98.0%
Q3 0.589%
Q1 5.22%
Q2 92.4%
Q4 0.310%
Q3 2.10%
b)
CD25
FOXP3
Q1 0.441%
Q2 86.0%
Q4 0.396%
Q3 13.1%
Cell number
13.6%
CFSE
TEa
c)
FOXP3
CFSE
Q1 6.63%
Q2 11.5%
Q4 66.6%
Q3 15.3%
Cell number
61.3%
CFSE
TCR75


d)
CD25
FOXP3
Q1
0.00%
Q2
85.9%
Q4
0.00%
Q3
14.1%
Q1
6.35%
Q2
16.6%
Q4
38.8%
Q3
38.3%
Q1
7.52%
Q2
0.323%
Q4
89.9%
Q3
2.27%
CFSE
FSC
6.51%
34.0%
59.3%
0
50K
100K
150K
200K
250K

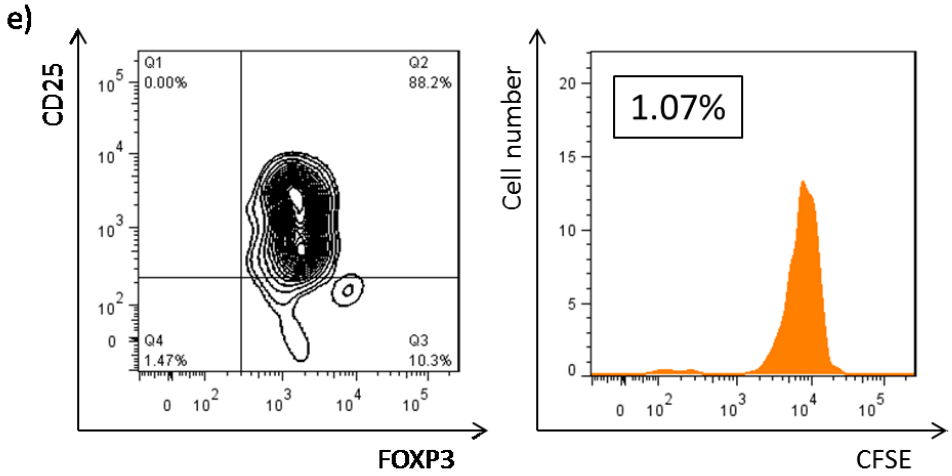

e)
CD25
FOXP3
Q1
0.00%
Q2
88.2%
Q4
1.47%
Q3
10.3%
Cell number
CFSE
1.07%

Figure 7.2 T cell receptor transgenic induced regulatory T cells

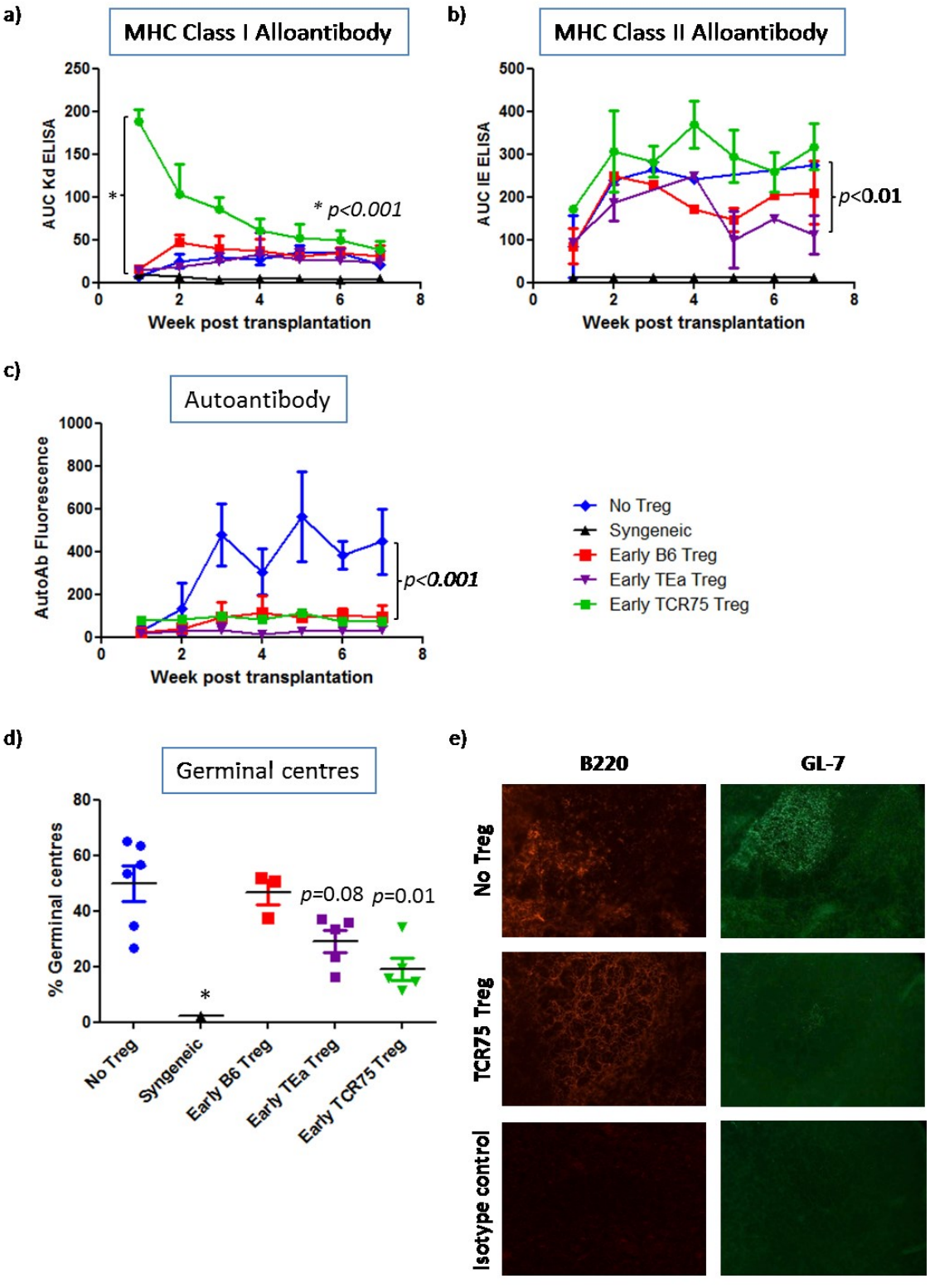
(a) TEa and TCR75 CD4 T cells were purified (MACS separated with anti-CD4 microbeads) and subjected to a 5 day period of *in vitro* culture in the presence of plate-bound anti-CD3, IL-2 and TGF-β[447]. Analysis by flow cytometry, before and after culture, confirmed effective conversion to a $CD25^+$ $FOXP3^+$ Treg phenotype for both TEa (top panels) and TCR75 (bottom panels). To examine their behaviour *in vivo*, $1x10^6$ CFSE labelled TEa (b) and TCR75 (c) iTreg were adoptively transferred into C57BL/6 recipients of a bm12.Kd.IE cardiac allograft on the day of transplant. Six days later, recipient spleens were analysed by flow cytometry. TEa iTreg (b) retained their $CD25^+$ $FOXP3^+$ phenotype (left panel) and had not undergone significant proliferation (right panel). In contrast, TCR75 iTreg (c) had undergone significant proliferation (right panel) and loss of their $CD25^+$ $FOXP3^+$ phenotype was suggested (left panel). (d) To examine the TCR75 iTreg following transfer to transplant recipients in more detail, the identified iTreg were divided into three populations based upon CFSE dilution (right panel). Presenting CD25 vs *FOXP3* for these gated populations revealed that the cells which had not undergone any proliferation (top left panel) retained their $CD25^+$ $FOXP3^+$ phenotype, whilst those undergoing the most proliferation (bottom left panel) had completely lost their $CD25^+$ $FOXP3^+$ phenotype, suggesting that with proliferation there was a loss of iTreg phenotype. (e) $1x10^6$ CFSE labelled TCR75 iTreg were adoptively transferred into naïve C57BL/6 recipients and examined six days later by flow cytometry. In naïve recipients the TCR75 iTreg did not undergo proliferation (right panel) and retained their $CD25^+$ $FOXP3^+$ phenotype.

7.3.3 In vivo effectiveness of iTreg at abrogating chronic rejection

The effectiveness of iTreg at abrogating chronic rejection in the bm12.Kd.IE to C57BL/6 model was next evaluated. Initially $1x10^6$ iTreg were adoptively transferred to recipients on the day of transplantation, a similar number as has been reported previously in murine studies[446, 447]. The impact of C57BL/6 polyclonal and TCR75 and TEa monoclonal iTreg on the alloimmune response was evaluated by quantifying anti-class I and class II alloantibody and autoantibody, examining for germinal centre (GC) activity, and quantifying the degree of allograft vasculopathy (AV) at 50 days following transplantation.

Polyclonal and TEa iTreg had a similar impact on antibody responses. There was minimal impact on generation of MHC class I alloantibody although TEa iTreg appeared effective at reducing class II alloantibody. However, a significant reduction in autoantibody generation was observed with both iTreg (Figure 7.3a-c). Neither iTreg population significantly reduced day 50 GC activity, although TEa iTreg treated mice typically possessed phenotypically smaller GCs (Figure 7.3d,e). Although TCR75 iTreg administration similarly abrogated autoantibody and had little impact on MHC class II alloantibody, it markedly augmented the early MHC class I alloantibody response; perhaps a consequence of proliferation of the TCR75 iTreg population following intravenous administration, as described above (Figure 7.3a-c). Despite this, there was a significant reduction in day 50 GC activity (Figure 7.3d,e).

Examining the cardiac allografts revealed that in keeping with recent reports, alloantigen specific iTreg were more effective at reducing the development of AV, with a significant reduction observed only for TEa and TCR75 iTreg (Figure 7.3f). In addition to the reduced AV, the parenchyma was notably more preserved with only minimal evidence of cardiomyocyte drop-out and fibrous replacement. This suggests that although a proportion of TCR75 iTreg revert to an effector phenotype, enough retain their Treg phenotype to prevent the development of GC responses which are responsible for the development of high affinity, class-switched allo- and autoantibody that appear dominant in driving the development of AV in this model. The augmented anti-H-$2K^d$ alloantibody may then reflect extrafollicular antibody production. Allograft survival was not considered in these experiments since rejection is chronic in this model and typically occurs beyond 50 days.


a)
MHC Class I Alloantibody
AUC Kd ELISA
250
200
150
100
50
0
*
* p<0.001
0
2
4
6
8
Week post transplantation
b)
MHC Class II Alloantibody
AUC IE ELISA
500
400
300
200
100
0
p<0.01
0
2
4
6
8
Week post transplantation
c)
Autoantibody
AutoAb Fluorescence
1000
800
600
400
200
0
p<0.001
0
2
4
6
8
Week post transplantation
No Treg
Syngeneic
Early B6 Treg
Early TEa Treg
Early TCR75 Treg
d)
Germinal centres
% Germinal centres
80
60
40
20
0
*
p=0.08
p=0.01
No Treg
Syngeneic
Early B6 Treg
Early TEa Treg
Early TCR75 Treg
e)
B220
GL-7
No Treg
TCR75 Treg
Isotype control

f)

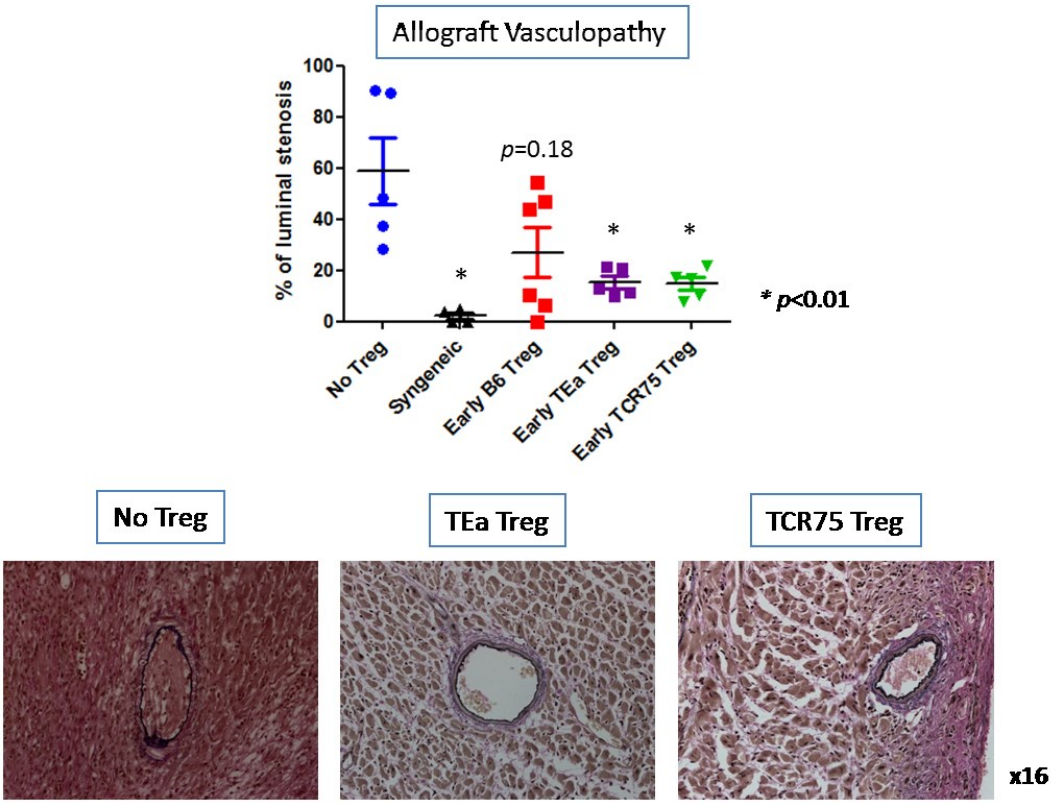


Figure 7.3 *In vivo* effectiveness of iTreg when transferred early following transplantation
Polyclonal C57BL/6 (n=6) and TCR transgenic TEa (n=5) and TCR75 (n=5) CD4 T cells were purified (MACS separated with anti-CD4 microbeads) and subjected to a 5 day period of *in vitro* culture in the presence of plate-bound anti-CD3, IL-2 and TGF-β[447]. Analysis by flow cytometry prior to use confirmed effective conversion to a $CD25^+$ $FOXP3^+$ Treg phenotype. $1x10^6$ iTreg were transferred to C57BL/6 recipients of bm12.Kd.IE cardiac allografts on the day of transplantation. Weekly blood samples were taken and tissue samples were harvested at 50 days following transplantation. From weekly serum samples, anti-H-$2K^d$ alloantibody [(a), ELISA], anti-I-E alloantibody [(b), flow cytometric labelling of I-E expressing BMDCs] and antinuclear autoantibody [(c), HEp-2 indirect immunofluorescence] was quantified. (d) The proportion of splenic follicles demonstrating germinal centre (GC) activity was quantified on immunofluorescence stained cryostat sections of recipient spleen, 50 days following transplantation (B220 and GL-7). (e) Representative immunofluorescence images of splenic follicle (B220 stain, left panels) and GC B cells (GL-7 stain, right panels), demonstrating the smaller nature of GCs observed in TCR75 and TEa iTreg treated recipients (bottom panels) compared with untreated recipients (upper panels). (f) The extent of allograft vasculopathy (AV) was quantified on elastin van Gieson stained paraffin sections of donor heart allografts 50 days following transplantation. Representative images are presented highlighting the AV and fibrous change typical in untreated recipient allografts (left panel). In recipients treated with TEa (middle panel) and TCR75 (right panel) iTreg, negligible AV is observed in the majority of vessels and parenchyma is well preserved. *P* values using two-way ANOVA (a,b,c) and Mann Whitney test (d,f) * = $p<0.01$.

7.3.4 Effectiveness of antigen-specific regulatory T cells administered late after transplantation requires insight into duration of alloresponses

One of the challenges facing adoption of cellular immunotherapies in transplantation is the ability to generate antigen-specific Treg for administration at the time of transplant where, other than in the case of living donor transplants, the donor is unknown until shortly prior to transplantation. An alternative option would be to administer Treg at some point after transplantation – perhaps even to treat episodes of rejection. However, identification that the longevity of direct and indirect-pathway alloresponses varies according to target alloantigen raises the possibility that antigen-specific Treg will not be equally effective when administered late after transplantation.

Given that donor MHC class II indirect alloresponses are short-lived and MHC class I long-lived in this model, I hypothesised that TCR75 iTreg may be more effective at abrogating chronic rejection than TEa iTreg when administered beyond the first week following transplantation. Therefore, TCR75 or TEa iTreg were adoptively transferred 3 weeks following transplantation and responses examined.

Late administration of either iTreg had no impact on MHC class I alloantibody (Figure 7.4a). There was no augmentation of the response following administration of TCR75 iTreg as was observed when they were administered early. TCR75 iTreg were more effective at attenuating autoantibody responses than TEa iTreg (Figure 7.4b). This was also the case when examining GC responses and AV, where TEa iTreg were inferior to TCR75 iTreg (Figure 7.4c). With late administration of TCR75 iTreg, the majority of parenchymal vessels displayed no evidence of AV and the parenchyma was markedly better preserved than seen with TEa iTreg (Figure 7.4d-f). In both cases, as expected, early administration of iTreg proved more efficacious at attenuating AV progression than late administration.

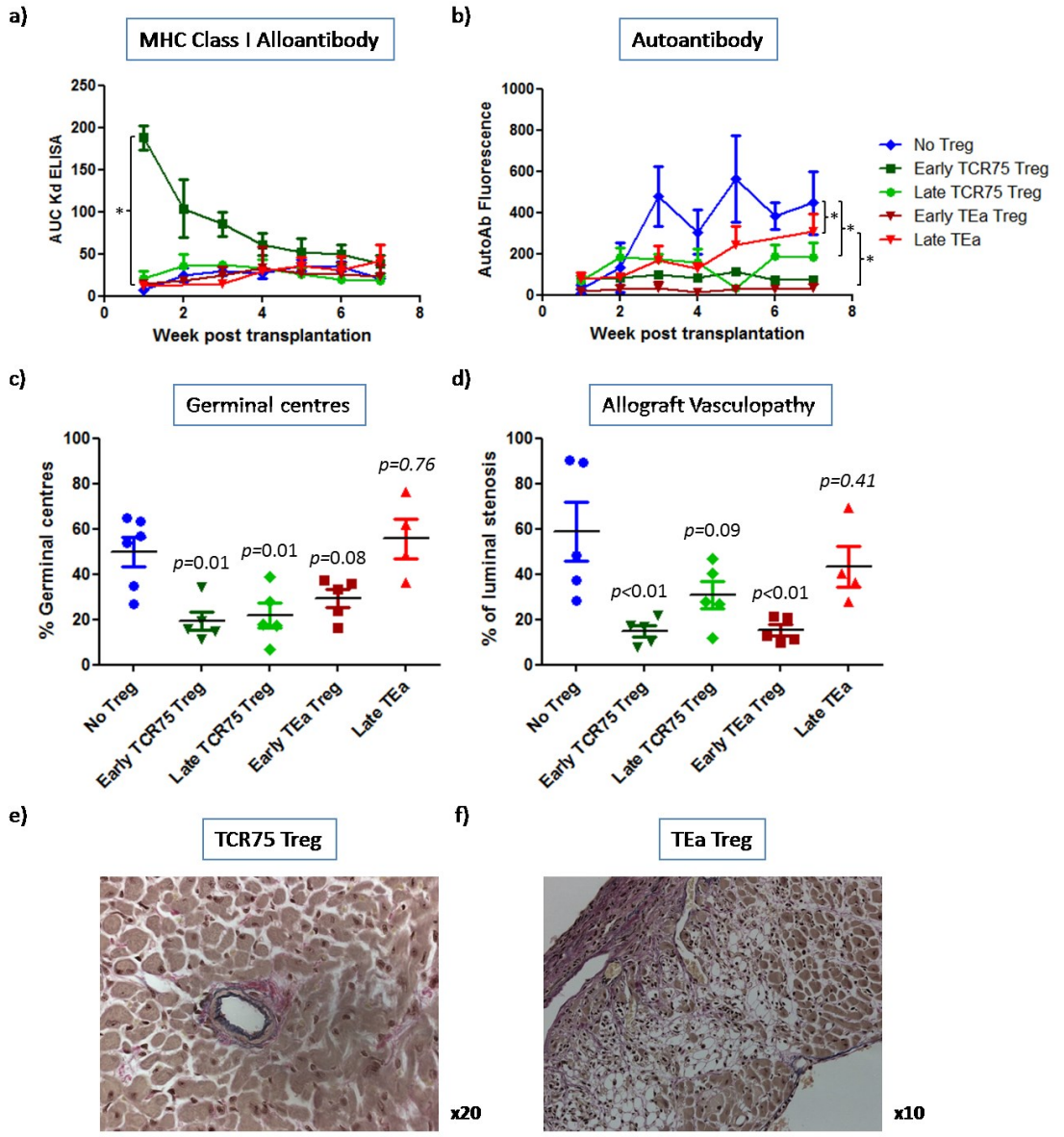


Figure 7.4 Effectiveness of TCR75 and TEa iTreg administered three weeks following transplantation
TCR transgenic TEa (n=5) and TCR75 (n=5) CD4 T cells were purified (MACS separated with anti-CD4 microbeads) and subjected to a 5 day period of *in vitro* culture in the presence of plate-bound anti-CD3, IL-2 and TGF-β[447]. Analysis by flow cytometry prior to use confirmed effective conversion to a $CD25^+$ $FOXP3^+$ Treg phenotype. $1x10^6$ iTreg were transferred to C57BL/6 recipients of bm12.Kd.IE cardiac allografts 3 weeks following transplantation. Weekly blood samples were taken and tissue samples were harvested at 50 days following transplantation. From weekly serum samples, anti-H-$2K^d$ alloantibody [(a), ELISA] and antinuclear autoantibody [(b), HEp-2 indirect immunofluorescence] was quantified. (c) The proportion of splenic follicles demonstrating germinal centre (GC) activity was quantified on immunofluorescence stained cryostat sections of recipient spleen, 50 days following transplantation (B220 and GL-7). (d) The extent of allograft vasculopathy (AV) was quantified on elastin van Gieson stained paraffin sections of donor heart allografts 50 days following transplantation. Representative images are presented highlighting the preserved parenchyma and absence of AV typical in TCR75 iTreg treated recipients (e) and the presence of myocyte dropout and fibrous regeneration observed in TEa iTreg treated recipients (f). *P* values using two-way ANOVA (a,b) and Mann Whitney test (c,d) in comparison to 'no-Treg' group * = $p<0.01$. Data in absence of Treg and with TCR75 and TEa iTreg administered on the day of transplantation are included to permit comparison.

To extend these observations, and exclude any functional difference between TCR75 and TEa iTreg accounting for the differences observed, the bm12.IE x bm12.Kd.IE bone marrow chimeric mice were once again employed (Section 2.8.1.2). In these mice expression of MHC class I H-2K^{d} is restricted to the haematopoietic lineage. Previously it was observed that whilst TCR75 CD4 T cells proliferated in recipients early, they did not proliferate upon late transfer (Section 5.3.3.2). Thus, following the previous finding, it was hypothesised that TCR75 iTreg would only be effective at attenuating progression of chronic rejection when transferred early after transplantation of these chimeric donors.

Bm12.IE x bm12.Kd.IE chimeric donor cardiac allografts were therefore transplanted into C57BL/6 recipients and TCR75 iTreg transferred on the day of transplantation or after three weeks. TCR75 iTreg were equally effective at abrogating autoantibody responses, GC activity and AV when transferred on the day of transplantation, as previously observed in recipients of wildtype donors. Again, an augmentation of the early H-2K^{d} alloantibody response was observed. However, when TCR75 iTreg were administered three weeks following transplantation allo- and autoantibody responses and GC activity were unaffected. In keeping with these observations, there was no amelioration of AV (Figure 7.5).

Together, these results suggest that identifying the alloimmune pathways active in a transplant recipient is essential for the rational development of effective antigen-specific Treg therapy following transplantation.

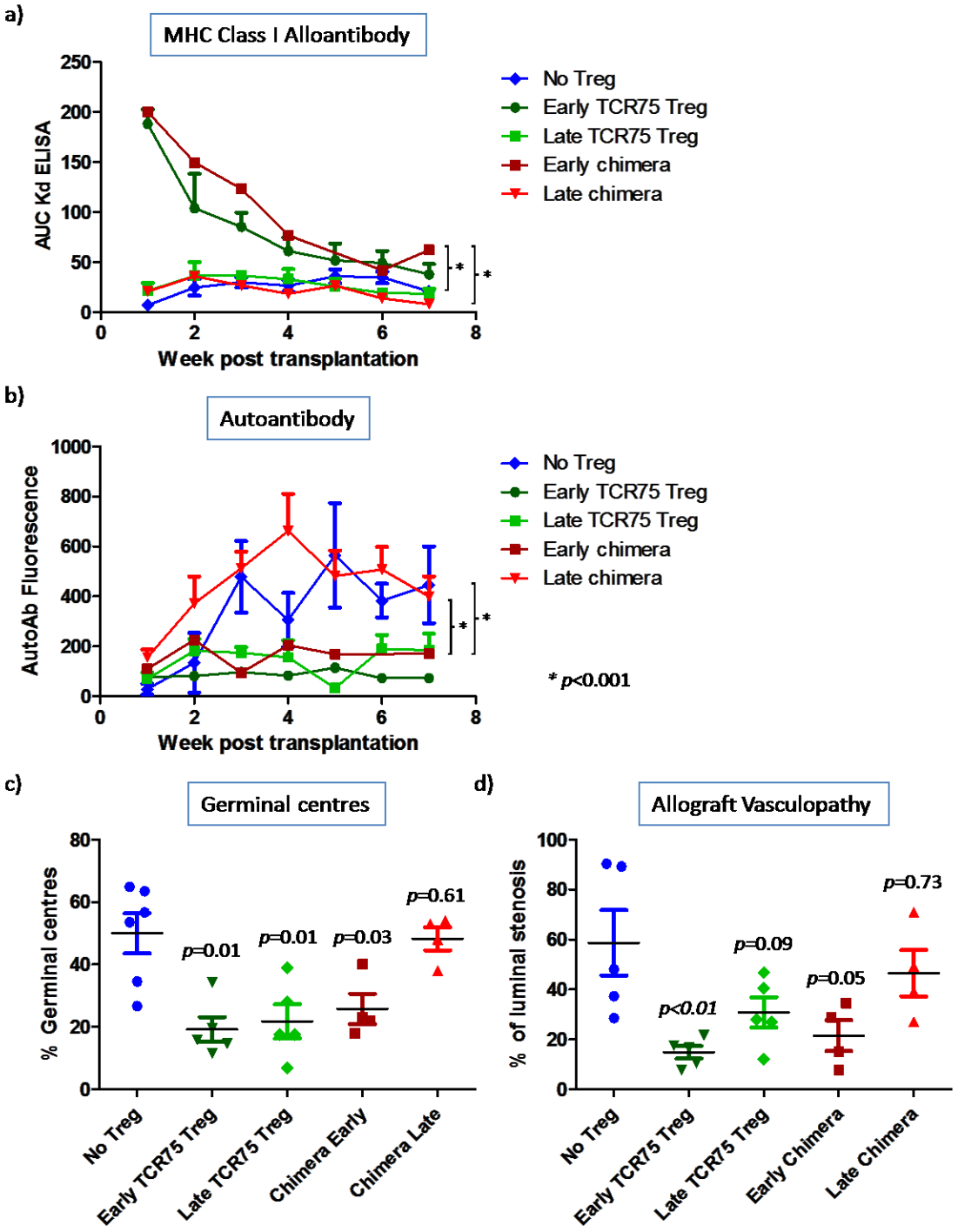

a)
MHC Class I Alloantibody
AUC Kd ELISA
250
200
150
100
50
0
0
2
4
6
8
Week post transplantation
No Treg
Early TCR75 Treg
Late TCR75 Treg
Early chimera
Late chimera
*
*
b)
Autoantibody
AutoAb Fluorescence
1000
800
600
400
200
0
0
2
4
6
8
Week post transplantation
No Treg
Early TCR75 Treg
Late TCR75 Treg
Early chimera
Late chimera
* p<0.001
c)
Germinal centres
% Germinal centres
80
60
40
20
0
p=0.01
p=0.01
p=0.03
p=0.61
No Treg
Early TCR75 Treg
Late TCR75 Treg
Chimera Early
Chimera Late
d)
Allograft Vasculopathy
% of luminal stenosis
100
80
60
40
20
0
p<0.01
p=0.09
p=0.05
p=0.73
No Treg
Early TCR75 Treg
Late TCR75 Treg
Early Chimera
Late Chimera

Figure 7.5 Effectiveness of TCR75 iTreg in recipients of bone-marrow chimeric donor allografts

TCR transgenic TCR75 CD4 T cells were purified (MACS separated with anti-CD4 microbeads) and subjected to a 5 day period of *in vitro* culture in the presence of plate-bound anti-CD3, IL-2 and TGF-β[447]. Analysis by flow cytometry prior to use confirmed effective conversion to a $CD25^+$ $FOXP3^+$ Treg phenotype. $1x10^6$ iTreg were transferred to C57BL/6 recipients of bm12.IE x bm12.Kd.IE bone-marrow chimeric cardiac allografts on the day of transplantation or 3 weeks later (n=4). Weekly blood samples were taken and tissue samples were harvested at 50 days following transplantation. From weekly serum samples, anti-$H\text{-}2K^d$ alloantibody [(a), ELISA] and antinuclear autoantibody [(b), HEp-2 indirect immunofluorescence] was quantified. (c) The proportion of splenic follicles demonstrating germinal centre (GC) activity was quantified on immunofluorescence stained cryostat sections of recipient spleen, 50 days following transplantation (B220 and GL-7). (d) The extent of allograft vasculopathy (AV) was quantified on elastin van Gieson stained paraffin sections of donor heart allografts 50 days following transplantation. *P* values using two-way ANOVA (a,b) and Mann Whitney test (c,d) in comparison to 'no Treg' group * = $p<0.01$. Data in absence of Treg and with TCR75 iTreg administered on the day of transplantation or 3 weeks later into recipients of bm12.Kd.IE allografts are included to permit comparison.

7.4 Discussion

This chapter has examined the effectiveness of Treg in abrogating chronic rejection in C57BL/6 recipients of bm12.Kd.IE cardiac allografts. Polyclonal Treg were least effective at abrogating chronic rejection as has previously been reported[250]. When administered on the day of transplantation, both MHC class I and MHC class II indirect-pathway allospecific Treg were effective at delaying the progression of chronic rejection, confirming that indirect-pathway specific Treg are able to ameliorate the development of chronic rejection alone, without direct-pathway Treg. However, when administered after 3 weeks following transplantation, only MHC class I antigen specific Treg were effective at abrogating chronic rejection. These results suggest that antigen-specific Treg therapy may only be effective when their allospecific responses are active in a recipient and perhaps explain the observation that in general, most studies have recognised indirect-pathway antigen-specific Treg to be more effective than direct-pathway antigen-specific Treg.

Many of the protocols to obtain alloantigen specific Treg begin with obtaining enriched populations of nTreg (Table 7.1). There is some evidence to suggest that nTreg are more stable than iTreg which is the basis of this preference. In one study, Koenecke *et al.* demonstrated that whilst nTreg were effective at abrogating development of GVHD in their model, iTreg were not, and underwent significantly greater proliferation which was associated with loss of *FOXP3* expression[463]. However, in another study in which nTreg and iTreg of the same specificity were generated, iTreg performed equivalently, if not slightly better than nTreg, and no concern over iTreg reversion was raised[447].

This is relevant because it is recognised that TCR transgenic strains on a RAGKO background typically lack nTreg[459-462]. There are several factors which are proposed to account for this. nTreg are generated in the thymus after high affinity interaction with peptide-MHC, of affinity/avidity on the brink of resulting in clonal deletion[464]. In the case of TCR transgenic strains, no 'agonist' epitope is available for the TCR in the thymus for such selection to take place[465]. The absence of endogenous TCR chain rearrangement on the RAGKO background therefore prevents potential nTreg clones from developing[465].

For this work it was therefore necessary to generate iTreg from the TCR75 and TEa strains rather than being able to isolate nTreg. This was achieved using the method of Huang *et al.* who similarly developed iTreg from TCR transgenic strains[447]. As with many protocols, this involves incubating CD4 T cells with inhibitory cytokine TGF-β which is thought to induce Treg

through the induction of *FOXP3* and down-regulation of Smad7 signalling[466]. As alluded to above, stability of Treg *in vivo* is clearly an important issue that needs to be addressed prior to their usage in human clinical transplantation[215, 467]. As has been recognised previously, iTreg induced with TGF-β have the potential to lose *FOXP3* expression and regulatory phenotype[463, 468, 469]. In my work, TCR75 iTreg underwent significant proliferation when transferred to transplant recipients and this was associated with loss of *FOXP3* expression. Furthermore, there was augmentation of the anti-H-2K^{d} alloantibody response acting as further evidence that some of these cells convert to an effector phenotype. It should be stressed that this augmentation in alloantibody was not sustained and the kinetics of the alloantibody response are in keeping with an initially rapid burst of alloantibody production that is switched off almost immediately, with the decay phase simply reflecting the half-life of antibody. Certainly, subsequent GC activity was barely above naïve background, inferring that the escaped helper CD4 T cells are either incapable of full differentiation to follicular helper cells, or are prevented from doing so by the residual *FOXP3*-expressing Tregs. Hence the net effect was still profound inhibition of host alloimmunity and prolonged allograft survival. Nevertheless, this is clearly a concern when considering transferring antigen-specific Treg to a human transplant recipient due to the potential for accelerating rejection.

It is worth considering that rather than an issue of stability, this reflects transfer of non-Treg (CD25^{+} FOXP3^{-} cells). To avoid transfer of non-Treg following *in vitro* culture would require an ability to sort cells. Unfortunately, as an intracellular marker, *FOXP3* expression cannot be used as a basis of sorting. Human Treg are sorted on the basis of CD25 and CD127 expression[442] and similar reliable cell surface markers may be discovered for murine Treg making this possible in murine experimental work.

One strategy that has reported to improve the stability of iTreg is addition of rapamycin and/or all-*trans* retinoic acid (ATRA) to the culture medium[445]. Rapamycin, an mTOR kinase inhibitor, is an immunosuppressive drug that inhibits effector T cell proliferation and activation and appears to selectively promote Treg expansion[470]. ATRA is a vitamin A metabolite that is thought to stabilise *FOXP3* expression[471]. Treg induced in the presence of rapamycin and ATRA were found to be functionally stable *in vivo* even in the presence of a severe systemic inflammatory response suggesting that this approach should be considered in future development of iTreg[445].

More recently it has been recognised that the *FOXP3* locus is subject to epigenetic control. Specifically, an element termed 'Treg-specific demethylated region' (TSDR) has been identified

in the 5' untranslated region of the *FOXP3* locus[472]. Demethylation of CpG motifs within the TSDR element is observed only in stable Treg populations. TGF-β-induced Treg have been reported to display only weakly demethylated CpG motifs and that this is not heritable, likely accounting for loss of *FOXP3* expression upon proliferation[466, 473]. This is informative since determination of TSDR methylation status may prove to be an effective quality control check for iTreg prior to their clinical use.

Despite the apparent conversion of a proportion of the TCR75 iTreg when administered early, in the context of the acute inflammatory milieu post-transplantation, they proved effective at abrogating the development of GC antibody responses and progression of chronic rejection. Indeed, the augmented anti-H-2K^{d} alloantibody was short-lived, suggesting that the early response was not sustained. This likely indicates that sufficient cells retain a regulatory phenotype to tip the balance towards regulation. Treg are believed to regulate by acting both in recipient secondary lymphoid tissue and directly in the allograft[219]. A study by Takasato *et al* investigated the impact of their indirect-pathway antigen-specific iTreg following transplantation[446]. They observed that presence of iTreg resulted in DCs exhibiting an immature phenotype, as demonstrated by down-regulated co-stimulation. They also demonstrated significantly greater numbers of *recipient* Treg within the cardiac allografts, suggestive of 'infectious tolerance'. Furthermore, their transferred iTreg did not survive long-term, suggesting that ongoing regulation was dependent upon the generation of recipient Tregs.

The idea of 'infectious tolerance' was elegantly demonstrated in a study by Pasquet *et al*. who developed mice which express the diphtheria toxin receptor (DTR) gene under the *FOXP3* promoter[474]. This renders Treg susceptible to deletion by diphtheria toxin. In this study the authors were able to demonstrate that after 2 weeks following transfer, the iTreg were no longer required for maintenance of tolerance, and that their tolerogenic role had been transferred to recipient Treg. They postulated that this was due to the transferred iTreg inducing tolerogenic APCs that would subsequently favour conventional recipient T cell differentiation into Treg .

This may therefore, explain why TCR75 iTreg were effective in regulating allograft survival, despite a significant proportion reverting to non-regulatory phenotypes. As long as sufficient iTreg are present to induce 'infectious tolerance', the recipient Treg will then ensure ongoing regulation, and not require long-term survival of the administered iTreg population.

One of the key observations from this chapter is that iTreg transfer could be effective at abrogating progression of allograft rejection, even when transferred late following transplantation. These are clearly preliminary studies, and effectiveness at the late time point was not uniform, however these experiments are at least proof of principal that iTreg therapy administration may be considered beyond the early phase following transplantation. Current efforts to develop Treg therapy are focusing on living-donor transplantation where the surgery is performed on an elective basis and the donor is known in advance of the transplant[228]. The majority of transplants though, are performed using cadaveric donors and the donor HLA type will be known only a few hours prior to transplantation providing insufficient time to prepare an antigen-specific cellular therapy. The results in this chapter demonstrate that cellular immunotherapy may be effective even when administered after transplantation. It could even be used as a treatment for episodes of acute rejection for example.

The results also highlight that efficacy of Treg is dependent upon ongoing alloresponses against the antigen to which the Treg are directed. This suggests that TCR signalling is important for the function of these iTreg. This is in agreement with a recent publication by Levine *et al.* who developed a system in which they could induce ablation of the TCR[475]. They demonstrated that continuous engagement of the TCR on Treg is essential for driving the transcriptional programme that maintains the immunosuppressive function of Treg. This helps to explain why iTreg were effective at impacting upon allograft rejection only when their TCR epitope continued to be present, in this chapter. This likely also accounts for why indirect-pathway Treg appear more efficacious than direct-pathway Treg at abrogating chronic rejection in transplant models. The short-lived nature of intact donor MHC class II likely limits the opportunity for TCR ligation to the period immediately following transplantation[48]. If they have been unable to induce effective infectious tolerance by the time the antigen is cleared, then the regulation may be less effective. In this regard it is also worth highlighting that previous success with monoclonal indirect-pathway iTreg have both been targeted against donor MHC class I allopeptide[446, 448]. Had the authors of these studies generated indirect-pathway Treg directed against donor MHC class II allopeptide, the results may have been different.

One of the notable impacts of Treg in this chapter was abrogation of GC responses. GCs are specialised microenvironments that form in B-cell follicles within secondary lymphoid organs upon immunisation with T-dependent antigens. The outputs of GCs are long-lived, high-affinity antibody secreting cells and memory B cells[316]. When Treg are transferred at the time of transplantation, it is likely that they act principally by inhibiting initiation of the GC response.

When Treg are transferred after transplantation, it is likely that the GC responses will have already developed[316]. However, the TCR75 iTreg were effective at limiting the GC responses even when transferred three weeks following transplantation, suggesting that regulation of GCs by Treg can occur after they have developed.

FOXP3^{+} T cells were first identified within GCs a decade ago, and are now referred to as follicular regulatory T cells (Tfr), a subset of Treg[476]. To date Tfr's are not well characterised, however they are thought to express the transcription factor bcl-6, and cell surface molecules ICOS and SAP which facilitate T-B cell interactions, adopting features similar to follicular helper T cells which are responsible for driving GC responses, enabling them to migrate into GCs and exert their regulatory effect[477]. There is evidence that Tfr's are able to limit the size of GC responses and may control production of antigen-specific antibody, however the mechanism and targets of their suppression remain controversial[477, 478]. It could be postulated therefore, that a proportion of the iTreg generated in this chapter differentiate into a Tfr phenotype permitting them access to GCs and effectively limiting the response. It could alternatively be that Tfr are of recipient origin and develop through infectious tolerance. This could be examined by incorporating SAP knock-out mice[317]. SAP (signaling lymphocyte activation molecule-associated protein) expression on T cells is found to be essential for stable T-B cell interactions in GCs and thought to be important for Tfr differentiation. It would be anticipated that SAP deficient iTreg would be unable to differentiate into Tfr's and the impact of this on GC responses and allograft rejection could be examined. This would provide evidence for a role for Tfr's in transplantation tolerance that has not been examined to date.

Clinical translation of Treg immunotherapy has already begun with some phase-1 trials demonstrating safety and some efficacy[226-228, 479]. Over the last few years several studies have been published describing use of human Treg therapy in humanised mouse models of human skin[224, 225], islet[442] and arterial[444] transplantation. Although many hurdles to translation have been overcome through these studies, such as optimising culture conditions for expanding human Tregs, there are still several questions outstanding[209, 215]. Perhaps most pertinent is the concern about Treg stability described above. Another outstanding issue is the effect of immunosuppression on Tregs. Certainly initially, cellular immunotherapy will be administered with conventional immunosuppression whilst the efficacy of Treg therapy is evaluated. Calcineurin inhibitors such as tacrolimus are the mainstay of current immunosuppression but there is some evidence that they may adversely affect Treg suppressor activity[480, 481], although there are some conflicting results[482]. Rapamycin on the other hand has been observed to have beneficial effects on Treg function, and is thought to be the least deleterious

immunosuppressive drug for Tregs[483]. Similarly, the exact Treg product that will be most effective has yet to be identified. The multicentre, international ONE study aims to test the safety of several regulatory cellular therapies including polyclonal Treg and alloantigen-driven Treg[228]. However, current efforts in developing alloantigen-specific Treg are focusing on direct-pathway antigen specificity using allogenic B cells as target alloantigen[452, 453]. The results in this chapter have some significance for clinical translation of Treg immunotherapy and would suggest that more effective immune regulation may be achieved with indirect-pathway alloantigen-specific Treg and that direct-pathway alloantigen-specific Treg will be effective only if transferred immediately following transplantation. Certainly if Treg therapy is to be administered after the immediate post-operative period it seems likely that indirect-pathway Treg will be most effective and these results would suggest that efforts should also be focused on developing techniques to induce and expand human indirect-pathway antigen-specific Treg.

8 Final Discussion

Solid organ transplantation is now an established and effective treatment option for end-stage organ failure. Whilst early outcomes have improved significantly over recent decades, longer-term outcomes have changed little. Despite advances in immunosuppression, most transplanted organs suffer an inevitable decline in function. The decline in function is likely multifactorial with 'alloimmune' chronic rejection at least partly responsible. The alloimmune response remains incompletely characterised. Crucially, despite its description several decades ago, the precise contributions that the direct (recognition of intact allogeneic MHC) and indirect (recognition of self-MHC restricted allopeptide) pathways make to allograft rejection remain incompletely understood[48]. In this thesis, murine models of heterotopic cardiac transplantation have been utilised to analyse these pathways. The key findings are discussed below:

1) Direct-pathway CD4 T cell allorecognition is restricted to the immediate post transplantation period. Donor APCs are the major source of MHC class II for direct-pathway priming and are cleared rapidly by both innate and adaptive responses of the recipient, effectively limiting the duration of direct-pathway allorecognition.

Although it is commonly held that direct-pathway allorecognition is most important early following transplantation[48], the duration of direct-pathway CD4 T cell allorecognition has not been definitively demonstrated. In Chapter 4 the results confirm that direct-pathway CD4 T cell allorecognition is indeed short-lived and limited by both innate and adaptive alloimmune-mediated elimination of donor APCs. The use of TCR transgenic CD4 T cell transfer does not rule out the possibility that CD4 T cells activated early following transplantation go on to play an important role late after transplantation as memory cells. Against this, however, human clinical studies have reported general direct-pathway hypo-responsiveness in recipients late after transplantation[77, 339]. Moreover, the work with tetramer staining presented in Chapter 6 suggests that, at least for indirect-pathway CD4 T cells, a rapid contraction of the antigen-specific T cell population occurs following loss of target antigen.

Whilst these findings question the exact role of recipient CD4 T cell direct-pathway responses in allograft rejection, due to its surprisingly short duration, findings from Chapter 3 have revealed a potentially more important, previously unrecognised, role for direct-pathway allorecognition in the alloresponse – but for *donor* CD4 T cells.

2) If able to evade NK cell killing, passenger donor CD4 T cells can make cognate, direct-pathway, interactions with recipient B cells. This interaction results in augmentation of all arms of the alloimmune response and acceleration of allograft rejection.

The role of passenger leukocytes in solid organ transplantation is unclear. Previous work from our laboratory revealed a role for donor CD4 T cells in driving the development of autoantibody in the limited-mismatch bm12 to C57BL/6 model through interaction via the direct-pathway with intact allogeneic MHC class II on recipient B cells[274]. Work in this thesis has extended this observation to reveal that passenger CD4 T cells can additionally, if able to survive long enough, significantly augment alloimmune responses, accelerating allograft rejection. It was identified that ability to evade NK cell killing was critical to their ability to mediate this augmentation, supporting a previously undescribed mechanism by which NK cells could play a role in dampening the alloimmune response following transplantation. The precise role played by NK cells in the alloimmune response remains incompletely understood[197]. Nonetheless, this work strongly suggest that they play an important immune regulatory function.

Although the findings have been confirmed in two independent models there are potential criticisms of both. In the bm12.Kd.IE to C57BL/6 model, allografts are rejected very slowly without any immunosuppression which is very different to that observed in clinical transplantation. The BALB/c to TCRKO (reconstituted with TCR75 CD4 T cell) model is also unlike the clinical situation in that there is a monoclonal population of recipient CD4 T cells. As such, wider acceptance of this novel finding may be achieved with further work seeking to verify the role of passenger donor CD4 T cells in a model more aligned to the clinic.

In clinical transplantation, recipients typically receive induction therapies at the time of transplantation with the intention of rendering them immunosuppressed. It is plausible that these treatments will also affect passenger donor CD4 T cells, especially since therapies, such as anti-thymocyte globulin, are T cell ablative. Anecdotally, a colleague who has been studying donor CD4 T cell chimerism in human cardiothoracic transplantation has observed frequent chimerism in lung transplant recipients, but none in cardiac transplant recipients (O. Gjorgjimajkoska, manuscript in preparation). Although this may relate to differences such as the presence of greater lymphoid tissue in lung allografts, it is notable that cardiac transplant recipients receive anti-thymocyte globulin as part of their induction immunosuppression at the

studied centre. Therefore, it would be important to examine the impact of donor CD4 T cells in the setting of immunosuppression. One proposed experiment to investigate this is to administer anti-CD154 costimulation blockade to C57BL/6 recipients of BALB/c cardiac allografts, which is known to attenuate acute rejection in this model[375]. Were recipient NK depletion to result in accelerated rejection in this experiment, it would add weight to the clinical relevance of the findings in this thesis.

As discussed in Chapter 3, the relevance of these findings to solid organ transplantation could also be verified by clinical studies. Specifically, inferior outcomes would be predicted in donor-recipient pairs in which there are HLA mismatches, but NK cell receptors are matched such that donor cells evade NK cell killing. The immediate challenge with this approach, however, is the lack of a complete understanding of NK cell receptors[179]. Yet, if proven to be the case, this novel role for NK cells in the alloimmune response may have implications for matching of donors and recipients in clinical transplantation.

Donor T cell direct-pathway interaction with recipient APCs is not restricted to solid organ transplantation. It is also of significant importance in bone-marrow transplantation where donor CD4 T cells are responsible for the development of graft-versus-host disease (GVHD). In a similar manner to that proposed in this thesis, in GVHD mature donor CD4 and CD8 T cells within the graft can be activated by recognising alloantigen on recipient APCs that have themselves been activated to upregulate antigen presentation by the bone-marrow transplant conditioning regimen. When interacting with mismatched MHC molecules, the recognition is by the direct-pathway, explaining the magnitude of the response observed. Following activation, a cascade of cellular mediators including CD8 T cells and NK cells, in combination with soluble inflammatory mediators results in inflammation and target tissue destruction[484]. The difference in bone-marrow transplantation is the absence of recipient lymphocytes and adaptive immunity to limit the longevity of donor T cell survival, resulting in the significant morbidity and mortality associated with GVHD in this setting[309, 484].

Results in this thesis (Chapter 3) highlight the important role for recipient NK cells in clearing donor CD4 T cells, limiting their impact on the alloimmune response following solid organ transplantation. In bone-marrow transplantation the situation is more complex since the NK cells will also be of donor origin since recipients are typically subject to a myeloablative conditioning regimen (total body irradiation with additional administration of a chemotherapeutic agent) which acts to both clear disease and suppress immune reactions to the transplant. Additionally, bone-marrow transplant recipients and donors are usually very

well matched since the severity and frequency of GVHD is directly related to the degree of HLA mismatch[484, 485]. Despite HLA matching, other factors contribute to the development of GVHD such as the presence of minor histocompatibility mismatches. Despite well matched donors and recipients, NK cells seem to have a similarly important impact on outcomes in bone-marrow transplantation, perhaps due to the fact that donor NK cell recognition of recipient also depends on the presence of matched NK receptors, which can be independent of HLA matching[189]. There is evidence that donor NK cells have a role in limiting GVHD[203]. To achieve this, NK cells are thought to act both by lysing chronically activated self-T cells and by deleting residual recipient DCs that are thought to be central to driving GVHD[486, 487].

In order to prevent GVHD, strategies to deplete mature T cells from bone-marrow transplants have been utilised. Substantial reductions in GVHD have been observed with this approach, supporting the central role of donor CD4 T cells[488-490]. However, the benefit of reduced GVHD was offset by high rates of graft failure, infections and loss of 'graft-versus-leukaemia' effect with high rates of malignant disease relapse highlighting that donor CD4 T cells do not simply have deleterious effects and that their role in bone-marrow transplantation is much more complex[484]. Currently, pharmacological manipulation of CD4 T cells in the recipient with calcineurin inhibitors such as cyclosporine is the mainstay of GVHD prophylaxis, although newer therapies such as Treg immunotherapy are undergoing investigation and clinical assessment[485]. In one study, for example, expanded populations of nTreg were used to treat two patients who developed GVHD following bone-marrow transplantation, and clinical improvement was seen in both patients[479]. Together, these findings have revealed the role played by donor CD4 T cells following bone-marrow transplantation to be particularly complex, which is unlikely to be the case in solid organ transplantation in which although impaired, the recipient's immune response remains intact. If the findings in this thesis relating to donor CD4 T cell augmentation of alloimmunity prove to be clinically significant, then the greater understanding of GVHD in bone-marrow transplantation will be useful in guiding management of transplant recipients in whom it is predicted donor CD4 T cells may have a deleterious impact on outcome.

3) The duration of indirect-pathway responses against different alloantigens is variable, limited by availability of donor antigen. Expression of donor MHC class II is restricted to APCs and possibly endothelium (where expression is transient) limiting the duration of indirect-pathway allorecognition against MHC class II alloantigen. In response to

continual presentation of target epitope, indirect-pathway CD4 T cell responses against parenchymal expressed alloantigen are long-lived, and can provide help for generating alloantibody against different MHC alloantigens. The continual division of these cells results in greatly increased numbers of alloantigen-specific CD4 T cells in the chronic phase of the response, but despite this, memory responses are impaired.

The role of indirect-pathway allorecognition in allograft rejection has been increasingly appreciated[48]. Despite this, the intricacies of the pathway remain incompletely understood, and the heterogeneity in duration of responses against different alloantigens reported in this thesis – although perhaps intuitive – has not been previously demonstrated. The findings in Chapters 4-7 suggest that the driving force for development of progressive AV is continued presentation of immunogenic MHC class I allopeptide by the indirect-pathway, although this concept has not been addressed formally. By extension, this suggests that MHC class II indirect-pathway responses are stimulated early, provoke a memory response, but do not influence chronic rejection. There is indirect evidence supporting this: i) in the BALB/c to C57BL/6 acute rejection model the tetramer staining CD4 T cell population rapidly contracts following clearance of alloantigen and exhibit a quiescent memory phenotype; and ii) a lack of impact of MHC class II indirect-pathway Treg when administered late following transplantation. Nevertheless, it would be important to demonstrate this formally, for example by utilising tetramers to deplete MHC class I indirect-pathway CD4 T cells and observing the impact on progression of AV to confirm their dominant role in the pathophysiology of chronic rejection. As alluded to throughout, caution must be exercised when considering the extrapolation of these findings to human transplantation. There is an important difference in MHC class II expression between humans and mice which may lead to observations in this work not being relevant in human transplantation[254]. It is conceivable that constitutive MHC class II expression in humans will provide a source of MHC class II long-term and therefore indirect-pathway responses will continue. It would be interesting to examine this in human transplant recipients. To date studies examining alloresponses have looked at global 'direct' and 'indirect' alloreactivity, perhaps this could be refined to examine responses against individual alloantigens in order to compare findings in humans to those in this research.

Perhaps the most unexpected finding of this work, described in Chapter 5, was that MHC class I specific indirect-pathway CD4 T cells can provide help for the generation of late MHC class II alloantibody, a time when MHC class II indirect-pathway specific CD4 T cells could not – apparently contravening the central tenet of 'linked' help[97]. This phenomenon of 'un-linked'

help is likely restricted to the unique circumstances of transplantation, since in a conventional antigenic challenge such as an infection, internalised adjacent membrane bound antigens of infected cells, including MHC molecules, will be of self-origin to which the host will be tolerant. Previous work from our laboratory describing the concept of 'un-linked' help in transplantation whereby a B cell acquires neighbouring membrane bound alloantigen whilst internalising its target epitope provides a possible explanation[382]. However, as discussed in Chapter 5 this would suggest that an MHC class II specific B cell will continue to present MHC class I long after expression of its target epitope by the allograft has ceased. This is an aspect that requires further work to elucidate the mechanisms involved. One approach being considered is the creation of mice that express green fluorescent protein (GFP) linked to MHC class I protein. Hence B cells that acquire MHC class I alloantigen could be identified on the basis of GFP expression. This strain could then be utilised to definitively confirm the ability of B cells to acquire accessory alloantigens and potentially to examine the sequestration and processing pathways utilised for the accessory alloantigens that may help explain the observations in this thesis.

MHC class II tetramers have been invaluable to permitting comprehensive evaluation of the endogenous CD4 T cell indirect-pathway alloresponse and have provided a novel insight into the impact of chronic antigen exposure on memory development. The findings in this thesis reassuringly mirror observations following challenges with infection - the finding that antigen-specific CD4 T cell memory declines following antigen clearance has also been seen in previous studies of chronic infection. A report from Marc Jenkins laboratory observed a profile of antigen-specific CD4 T cell population dynamics following acute (*Listeria monocytogenes*) and chronic (*Salmonella* species) infections concordant with those observed targeted against cleared and persisting alloantigen[406]. Together, these findings suggest that maintenance of memory CD4 T cells requires ongoing TCR stimulation – in stark contrast to the case identified in CD8 T cells where antigen persistence is associated with exhaustion[430, 431].

Future work with MHC class II tetramers aims to further explore the phenotype of indirect-pathway CD4 T cells, examining T helper subset differentiation and specifically examining T follicular helper cell differentiation. Furthermore, as alluded to in Chapter 6, equally important findings are expected to be obtained utilising tetramers to examine B cell alloresponses, quantifying antigen specific B cell populations that develop following transplantation and how they relate to the indirect-pathway CD4 T cell responses providing their help.

4) Generating indirect-pathway regulatory T cells specific for parenchymal expressed alloantigen appears to be the most effective strategy to ameliorating chronic rejection.

The clinical significance of understanding intricacies of the pathways of allorecognition is most apparent when considering the findings in Chapter 7. Regulatory cellular immunotherapies are being widely studied with clinical translation of Treg on the horizon, however there are many unanswered questions, such as which type of Treg product is most effective, how many Tregs to transfer and how often[215]. It is now appreciated that 'antigen-specific' Treg are more effective than polyclonal Treg, and that direct-pathway Treg generally perform inferiorly to indirect-pathway Treg[48]. Work in this thesis provides insight into why this may be and highlights that antigen specificity must relate to epitopes being presented in an immunogenic fashion at the time of Treg administration. Whilst this may have limited bearing on the specificities of Treg administered at the time of transplantation, it is of importance when Treg are administered later – as will likely be the case for cadaveric organ transplant recipients where donors are not known until only hours before the transplant providing insufficient time to generate a Treg product. The role regulatory cellular immunotherapies will have in transplantation is yet to be defined, but it is conceivable that they may be utilised as a rescue therapy to treat episodes of rejection. In this setting, results in Chapter 7 would suggest that the most effective Treg product may be generated by first establishing the specificity of the on-going alloimmune response in that recipient – something that may become easier with the recent development of HLA monomers as a technology to examine indirect-pathway CD4 T cell specificity in transplant recipients[391].

Regulatory T cell therapy is not only relevant to transplantation and there may be wider relevance of these findings. There is now a body of work examining the potential of Treg to treat autoimmune diseases, in which adaptive immune responses are directed against self-antigens. It is worth highlighting that lack of functional *FOXP3* expression results in severe autoimmune phenotypes (scurfy in mice and 'immune dysregulation, polyendocrinopathy, eneteropathy X-linked' in humans)[216]. In keeping with this, several reports have observed reduced numbers of Treg in patients with various autoimmune diseases and that the degree of deficit was associated with disease activity[491]. Furthermore, in a murine model of collagen-induced arthritis deletion of Treg, with administration of a depleting anti-CD25 mAb, significantly augmented and accelerated the onset of disease[492]. In addition to a reduced frequency of Treg, it has also been reported that autoimmune disease can alter the functional activity of Tregs. Tregs isolated from active rheumatoid arthritis (RA) patient's demonstrated

significantly impaired suppressive activity than disease-free controls[493]. It may similarly be the case that with ongoing inflammatory alloimmune responses, Treg in transplant recipients demonstrate impaired regulatory function.

A result of these observations is a move to utilising Treg therapy against autoimmune disease. Work in this thesis, and the transplantation literature, has highlighted the greater efficacy of antigen-specific Treg. Similar findings have also been observed in autoimmunity. Whilst polyclonal Treg were able to prevent the onset of diabetes in NOD mice, they were unable to alter the progression of disease once established[494-497]. Similar findings have been observed in models of autoimmune gastritis[498], collagen induced arthritis[492, 499] and multiple sclerosis[500]. In these reports, TCR transgenic iTreg have been generated *in vitro* and demonstrated significantly greater efficacy than polyclonal nTreg[501].

In this thesis (Chapter 7) it has been observed that the most effective iTreg were those specific for targets of on-going alloimmune responses. Unlike in transplantation where the HLA mismatch is known, identification of the target antigens in autoimmunity is not so straightforward. In RA for example, the autoantigens remain incompletely characterised[502]. Although several putative autoantigens have been identified by examination of patient serum – such as collagen type II and human chondrocyte glycoprotein 39, there is little evidence implicating most of them in the disease pathogenesis[503]. Furthermore, a feature of chronic immune responses is epitope diversification – both inter and intra-molecular, potentially complicating identification further since the dominant determinant driving the autoimmune response may change over the course of the disease[132, 319, 504]. One proposal to aid the generation of antigen-specific Treg in autoimmunity has been to isolate nTreg from patients with autoimmune disease and expand these *in vitro* with the aim of addressing the imbalance between effector and regulatory cells that has been described[491]. This may be problematic though due to the impaired function of nTreg in autoimmune disease that has been reported[493].

In the final chapter it was demonstrated that allograft rejection in the bm12.Kd.IE to C57BL/6 model can be abrogated by iTreg specific for single alloantigens expressed by the allograft, despite expression of additional alloantigens, suggesting that linked-suppression occurs. This raises the possibility that iTreg targeted against one tissue-specific autoantigen may be effective at attenuating disease progression even if it is not the main target of the autoimmune response – therefore avoiding the need to define the precise specificity of pathogenic autoreactive T cells in each patient. In support of this, a study revealed that generating iTreg

specific to an antigen expressed in synovial joints, in a model of autoimmune arthritis, significantly attenuated disease progression even though the model was engineered such that pathogenic T cells were targeted against a different antigen[505]. This may be due to Treg accumulation in the target organ permitting the linked-suppression of pathogenic T cells irrespective of their antigen specificity.

Several outstanding questions must be answered before Treg immunotherapy becomes a clinical reality in both transplantation and autoimmunity. There remains the real concern that antigen-specific iTreg may revert to an effector phenotype following transfer into a pro-inflammatory environment and exacerbate the disease they are designed to prevent. Much work is underway attempting to define the optimal *in vitro* culture conditions and attempting to identify markers of Treg phenotype stability – such as methylation status of the 'Treg specific demethylated region'[472]. Furthermore, the effective human 'dose' of Treg is not yet appreciated. There may be many problems with simply extrapolating from murine studies due to differences between murine and human immune systems[254]. Nevertheless, significant *in vitro* expansion will be required with the inherent concern that Treg phenotype may be altered during this process. Additionally, whilst single administrations of Treg are often effective in murine models, it is not clear that this is necessarily the case in human patients. These questions are the focus of much on-going research throughout the world that will be informative to the development of regulatory immunotherapy in both transplantation and autoimmunity.

9 References

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