Visualisation of Cytotoxic T Cells During Allograft Rejection and Tolerance

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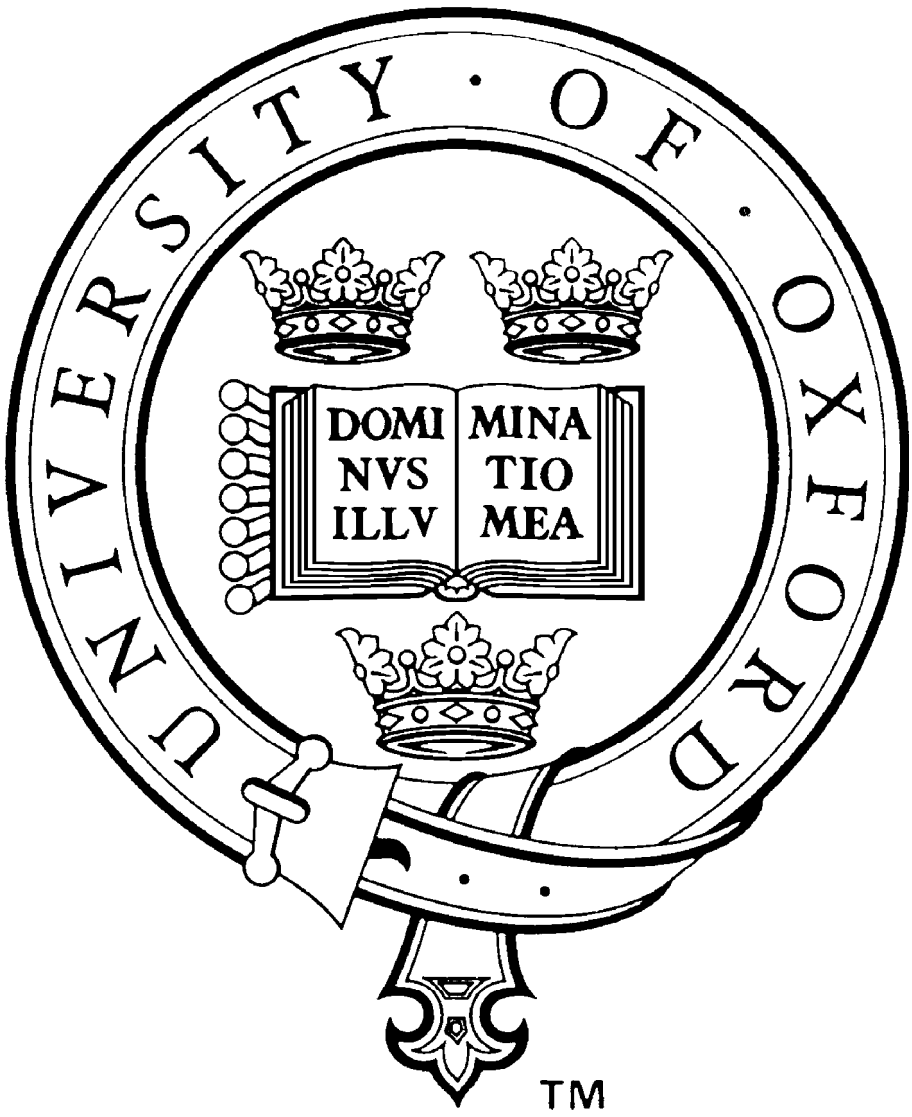
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For Christina

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Visualisation of Cytotoxic T Cells During Allograft Rejection and Tolerance

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Abstract

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Over the past century organ transplantation has developed from a rarely successful experimental procedure into an effective and ethical therapy for end-stage organ failure. However, investigators continue to search for strategies that will allow for the survival of allografts without long-term non-specific immunosuppression. Specifically disabling the immune response against transplanted tissues while leaving other immune responses against micro-organisms and neoplasms intact would reduce the unwanted morbidity associated with immunosuppression. Despite the existence of working experimental tolerance induction protocols, none have been successfully implemented in the clinic.

We hope that by improving our understanding of T cell responses to transplantation antigens we will one day achieve this goal. In this thesis, an experimental system was developed allowing the in vivo visualisation of donor reactive cytotoxic T cells responding to a cardiac allograft. Utilising normal mice (CBA/Ca, H-2^k) adoptively transferred with 2 x 10^6 CFSE labelled syngeneic $CD8^+$ TCR transgenic T cells (DES, H-2^k) specific for H-$2K^b$, we studied the role of cytotoxic T cells in acute rejection as well as their response to two tolerance induction protocols permitting the indefinite survival of fully allogeneic H-$2K^{b+}$ (CBK x BALB/c F1) cardiac allografts.

In order to establish a baseline, we studied acute rejection of H-$2K^{b+}$ (CBK x BALB/c F1) cardiac allografts. We found that H-$2K^b$ specific $CD8^+DES^+$ T cells specifically proliferated and produced inflammatory Tc1 cytokines (IL-2 and interferon-γ), but not regulatory Tc2 cytokines (IL-4 and IL-10). $CD8^+DES^+$ T cells that infiltrated H-$2K^{b+}$ allografts were distinct from those that remained in the secondary lymphoid tissues of recipients. Graft infiltrating $CD8^+DES^+$ T cells possessed an activated/effector surface phenotype in contrast to $CD8^+DES^+$ T cells present in the spleens of the same animals that retained a naïve surface phenotype. Furthermore, the conditions within H-$2K^{b+}$ allografts appeared to vigorously promote the proliferation and maturation of donor specific T cells.

H-$2K^{b+}$ bone marrow cells from a transgenic strain of CBA/Ca mice, CBK (H-2^k + H-$2K^b$), infused intravenously into CBA/Ca mice induced indefinite survival of fully allogeneic H-$2K^{b+}$ (CBK x BALB/c F1) cardiac allografts in naïve and adoptively transferred CBA/Ca mice. 50 x 10^6 CBK bone marrow cells infused 14 days prior to transplantation induced a vigorous immune response among adoptively transferred $CD8^+DES^+$ T cells resulting in clonal expansion, production of Tc1 cytokines and the development of a memory surface phenotype in the spleens of pretreated mice followed by significant peripheral clonal deletion reducing the total absolute number of donor specific T cells to a fraction of that in control mice. Interestingly, infusion of 5 x 10^6 CBK bone marrow cells under cover of depleting anti-CD4 monoclonal antibody (YTA 3.1) 27 days before transplantation did not cause peripheral clonal deletion of $CD8^+DES^+$ T cells but significantly impaired their ability to infiltrate (CBK x BALB/c F1) cardiac allografts. $CD8^+DES^+$ T cells which infiltrated (CBK x BALB/c F1) allografts in both protocols had proliferated extensively, possessed an activated/effector surface phenotype and only produced Tc1 cytokines.

The results presented in this study should improve our understanding of the response of T cells to alloantigens so that transplanted patients and their allografts may be better monitored. Additionally, we hope that the experimental system and the results obtained may aide in the development of clinically applicable tolerance induction protocols.

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Abbreviations

2C	Anti-H-2L^d T cell receptor transgenic mouse derived from 2C clone
APC	Antigen presenting cell
ATCC®	American type culture collection
BM3.3	Anti-H-2K^b T cell receptor transgenic mouse derived from BM3 clone
BMC	Bone marrow cell
CBK	Transgenic CBA/Ca expressing H-2K^b
CD	Cluster of differentiation
CFSE	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE)
CPM	Counts per million
CTL	Cytotoxic T lymphocyte
DES	Anti-H-2K^b T cell receptor transgenic mouse derived from KB5C20 clone
DTH	Delayed-type hypersensitivity
FACS®	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GIC	Graft infiltrating cells
H-2	Mouse histocompatibility system 2
HLA	Human leukocyte associated antigens
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
i.v.	Intravenous
LU	Linked unresponsiveness
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MLC	Mixed lymphocyte culture
mRNA	Messenger ribonucleic acid
MST	Median survival time
PBS	Phosphate buffered saline
PE	R-Phycoerythrin
R^0	Simple RPMI 1640
R^{10}	Complete RPMI 1640
RAG	Recombination activation genes
SD	Standard deviation
SEM	Standard error of the mean
TBAC	Tris-Buffered Ammonium Chloride
Tc1	T cytotoxic type-1, similar to Th1
Tc2	T cytotoxic type-2, similar to Th2
TCR	T Cell Receptor
TGF-β	Tumour growth factor-β

Chapter 1 - Introduction

1.1 History of Transplantation

Fludd in 1631 told the story of the *Sympathetic Slave* whom, in exchange for freedom, donated skin for the reconstruction of his owner's nose lost in battle[1]. Almost surely, plastic and reconstructive surgeons were the first to realise how tissue grafts could restore form and function to ill and injured patients. With the development of surgical techniques by Carrel in 1902[2], it became possible to transplant vascularised vital organs such as kidney and liver. Worldwide, many kidney allografts and several xenografts were transplanted into human beings over the first fifty years with little success. Groups in Paris and Boston accumulated significant numbers of patients towards the middle of the century. However, the availability of the artificial kidney[3,4] allowed surgeons in Boston to publish two well-documented series[5,6] of human renal homografts that set the stage for clinical transplantation, as we know it. They stated that "permanent function of a transplanted organ is possible (with identical twins)" and that "tissue homografts may be expected to fail --- for immunologic reasons." The community of surgeons realised that attention had to be turned to the mechanisms of rejection in order to make transplantation an ethical therapy for end organ failure.

1.2 Clinical Transplantation

Today, many organs are routinely transplanted with good results throughout the world. Key advances which made this possible are: (1) the availability of skilled

surgeons, (2) networks to procure organs, (3) HLA matching of donors and recipients[7,8] and (4) the use of potent immunosuppressive agents[9]. Immunosuppression is most responsible for the long-term graft survival that we enjoy today.

Immunosuppressive therapy needed to maintain allograft function is usually a combination of azathioprine, corticosteroids and cyclosporine. Multi-drug therapy targets different arms of the immune response, results in more effective immune suppression and permits lower dosing of individual agents. Nonetheless, non-specific immunosuppression is associated with an increased risk of opportunistic infection and malignancy. Interestingly, recent work indicates that cyclosporine may directly promote neoplasm mediated through TGF-β[10]. There is hope that widespread use of newer agents such as FK-506, rapamycin, MMF and others will be even more effective and less toxic.

A number of challenges must still be overcome for transplantation to become commonplace. Pharmacological immunosuppression is costly, difficult to manage in practice, and does not prevent chronic rejection. The incidence of chronic rejection has not changed in the recent past and continues to claim 5% of renal allografts per year and 30% of long-term surviving cardiac allografts[11-13]. We have no effective therapies against this form of graft loss. Other challenges include the lack of sufficient organs, the possibility of recurrent autoimmune disease and the need of supportive therapies for patients awaiting hearts and livers similar to dialysis or insulin. However, the final goal of all those involved in transplantation is the achievement of donor-specific immunosuppression or tolerance.

1.3 Mechanisms of Rejection

Many factors contribute to the initiation of allograft rejection. The importance of non-specific signals secondary to the physical trauma and inflammation of surgery should not be underestimated. However, the immunologic basis of rejection was established more than a half century ago. Recognition of the allograft as 'foreign' by the recipient immune system is the first event. Differences between individuals at the MHC locus were recognised as the trigger for the initiation of allograft rejection[14]. It was shown that the pivotal cell in the immune response against allografts is the small lymphocyte[15]. Some time after, it became clear that T cells interact with MHC molecules[16,17] on the surface of self[18] and foreign[19] cells. This was clearly proven by the observation that mice lacking T cells were unable to reject either xeno or allografts[20-22].

T cells normally circulate in a naïve and resting state until donor alloantigen is encountered. Host T cells encounter donor MHC molecules expressed by the allograft. However, resident within transplanted organs are donor bone marrow derived cells known as passenger leukocytes, or donor antigen presenting cells. These dendritic cells express high levels of donor MHC antigens, and are highly specialised at activating recipient T cells[23]. They emigrate from allografts presenting donor alloantigens to host T cells in the secondary lymphoid tissues[24]. These antigen presenting cells play a significant role in the presentation of donor MHC molecules to recipient T cells. With depletion of these cells from allografts prior to transplantation, survival is often prolonged[25-31].

When $CD4^+$ and $CD8^+$ T cells interact with donor MHC molecules, they become activated and differentiate into helper and cytotoxic T cells respectively. Both subsets play a role in rejection, but differ in their function. The widely accepted framework for understanding their distinct roles in rejection proposes that activated $CD4^+$ helper T cells produce cytokines or "help" mainly in the form of IL-2, IL-4 and interferon-γ secretion. This help is required by precursors of other cells of the immune system for differentiation into effector cells. These effectors include $CD8^+$ T cells that develop cytotoxicity, B cells that produce alloantibodies and non-specific arms of the innate immune system such as macrophages, monocytes and natural killer cells. Studies where adoptive transfer of $CD4^+$ but not $CD8^+$ T cells were able to restore rejection of skin grafts in T cell deficient animals support the belief that helper T cells play a central role in rejection, and suggest that $CD8^+$ T cells play a secondary role in allograft rejection[32,33]. What has become apparent is that helper function and cytotoxicity do not lie exclusively within particular subsets. Therefore, the conceptual segregation of function with co-receptor expression is perhaps too simplistic[34,35].

In certain models, $CD8^+$ T cells alone have been shown to restore rejection[36,37]. Perhaps a better interpretation of the literature is one where both helper and cytotoxic function are required to bring about rejection regardless of which subset they are generated by[38]. $CD4^+$ T cells may not be absolutely required per se, but rather the availability of "help" in association with cytotoxic effector mechanisms.

Normally, $CD8^+$ T cells provide antigen specific cytotoxicity, however activated cells of the monocyte-macrophage lineages may be sufficient to bring about rejection as in the proposed type-IV hypersensitivity (DTH) like mechanism thought to play a role in rejection[39]. The presence of graft damaging macrophages, monocytes and natural killer cells may explain the apparent redundancy of $CD8^+$ T cells. On the other hand, the existence of $CD8^+$ T cells which produce helper cytokines[35,40-42] may obviate the need for $CD4^+$ helper T cells.

T cells play an important role in recruiting other cells of the immune system. Cytokines produced by helper T cells promote the activation, proliferation and differentiation of numerous effector cells thought to contribute to rejection. Antigen specific cytotoxic T cells, activated macrophages (DTH reactions), natural killer cells, antibody-dependent cell-mediated cytotoxicity and complement-dependent antibody-mediated cytotoxicity are all able to cause significant graft damage. These effector mechanisms are somewhat redundant. However, $CD8^+$ T cells play a significant role in maintaining the kinetics of graft rejection[43]. In general, the literature suggests that "cytotoxic" $CD8^+$ T cells play an active, but hardly exclusive, role as cytotoxic effectors in the rejection process.

1.3.1 MHC

The MHC has a very high degree of polymorphism within a given species. The large variety of possible combinations of MHC antigens expressible offers the species as a whole protection against infectious micro-organisms, but makes it very unlikely that any two unrelated individuals will bear the same MHC antigens on their cells. The

MHC genes and their products are grouped into 2 classes on the basis of their structural and biological properties. The two classes of MHC proteins have a similar three-dimensional structure with subtle functional differences. MHC class I molecules are made up of one heavy chain (45 kD) and one light chain called ß2-microglobulin (12 kD) that contributes to the overall structure of the protein. MHC class II molecules consist of one α and one ß chain of similar size (34 and 30 kD). In mouse, these genes encode for MHC Class I (H-2-K, -D & -L), and MHC Class II (H-2-IA & -IE). In human they are known as Class I (HLA-A, -B & -C) and Class II (HLA-DP, -DQ & -DR)[44].

Class I molecules are expressed on virtually all nucleated cells in mouse and man[45]. In contrast, MHC class II expression is much more restricted[46]. Generally, class II is expressed by antigen presenting B cells, macrophages, and dendritic cells although expression can be induced by interferon-γ on a number of cell types including epithelium, and T cells[47]. Importantly, MHC class II expression on endothelium does not occur in mouse, but does in man[48].

The structure of both classes of MHC molecules forms a groove that accommodates a peptide[17]. There are distinct pathways of peptide loading. Class I molecules bind peptides 8 to 10 residues long. Class II peptides are longer varying between 12 and 28 residues. Peptides destined for class I molecules are derived from the endogenous pathway of antigen processing through cleavage of intracellular proteins by proteasomes[49]. Peptides loaded onto class II molecules come from extracellular proteins[50] usually endocytosed by professional antigen presenting cells. The MHC-peptide complex is the ligand for the T cell receptor.

1.3.2 The T Cell Receptor

The antigen specific nature of T cell responses is conferred by the TCR that "sees" foreign or antigenic peptides in the context of MHC molecules[51,52]. T cells may be divided into two subgroups expressing either CD4 or CD8 co-receptor in association with the TCR. The $CD4^+$ helper subset is specialised to recognise intact MHC Class II molecules[53] while $CD8^+$ cytotoxic T cells recognise intact MHC Class I molecules[54]. The T cell receptor consists of two subunits, α and β, covalently joined by disulphide bridges and is associated with the CD3 complex which along with CD4 or CD8 transmit an activating intracellular signal when the TCR is occupied by the MHC-peptide complex for which it is specific[55,56].

The thymus is the site of T cell maturation and is where the peripheral T cell receptor repertoire is chosen[57]. Through the rearrangement of variable (V), diversity (D) and joining (J) TCR genes it has been estimated that a diversity of 10^{16} different $\alpha\beta$ TCR specificities can be generated[58]. One of the most important functions of the thymus is to ensure that while there are T cells that are capable of reacting to a whole variety of antigenic peptides, all those which would be strongly self-reactive are eliminated.

A second lineage of T cells does exist known as $\gamma\delta$ T cells[59]. They rearrange their genes to produce a large diversity of specificities, associate with CD4 or CD8 and represent a small subset in the periphery, although very little is known about their

function. However, cells bearing the γδ T cell receptor are scarcely found in transplanted organs[60].

1.3.3 Allorecognition

The peripheral T cell repertoire includes $CD4^+$ and $CD8^+$ αβ T cells with random specificity. These T cells are self-tolerant and self-restricted, however the structure of certain TCR's permits a degree of cross-reactivity with intact allogeneic MHC molecules[19,61]. $CD8^+$ T cells cross-react with foreign class I molecules while $CD4^+$ T cells interact with foreign class II molecules. This pathway of allorecognition is known as direct allorecognition. A second pathway does however exist and is known to be important in $CD4^+$ T cells, but has not been very well studied in $CD8^+$ T cells. Recipient MHC class II positive antigen presenting cells endocytose allogeneic MHC molecules shed from the graft and present cleaved peptides on their cell surface in the context of self MHC class II molecules. This has been termed indirect allorecognition[62,63].

1.4 T Cell Activation

A critical event in the generation of immune responses is the clonal activation and expansion of T cells. When T cells encounter an appropriate peptide-MHC complex on the surface of an antigen presenting cell[23], a series of biochemical events follows resulting in T cell activation, proliferation and differentiation into effector populations.

Two signals are required for the successful activation of naïve T cells as similarly observed for B cells[64,65]. The TCR-peptide/MHC interaction (signal 1) confers antigen specificity, but does not suffice[27,66]. Signal 2 is also delivered by the same antigen presenting cell and can be one, or a combination, of a number of receptor-ligand interactions between the T cell and the antigen presenting cell. Additionally, these signals are potentiated by binding of CD4 and CD8 to non-polymorphic regions of MHC molecules[67].

CD28-(CD80/CD86) interaction provides the major co-stimulatory signal for augmenting and sustaining T cell responses. However, several other pairs of surface molecules have also been proposed to play a role in T cell activation/costimulation; CD40/CD154(CD40L)[68], LFA-1/ICAM-1[69], CD70[70], HSA (CD24)[71,72], and their ligands. Their modes of action have not yet been fully characterised.

Signal 2 is quantitatively distinct from signal 1. In contrast to signal 1, signal 2 is not antigen specific, but it is only available from activated antigen presenting cells that are initiating immune responses. These two signals function in a complementary fashion triggering T cell proliferation, production of cytokines and differentiation into effector cells[55].

The nature of signal 2 is one that promotes T cell activation and survival where signal 1 confers antigen specificity. Signal 1 alone leads to ineffective immune responses where proliferation is less vigorous and this often results in T cell anergy[73]. Signalling through CD28 induces the production of growth-promoting cytokines such as IL-2[74] and intracellular survival factors such as Bcl-X_L[75,76]. This

intracellular protein and other similar molecules, such as Bcl-2, are survival factors that allow T cells to progress successfully through activation to an effector state without undergoing apoptosis[77,78].

Since costimulation promotes IL-2 production, it is therefore implicated in the provision of help to naïve $CD8^+$ T cells undergoing activation. However, the importance of direct costimulation of $CD8^+$ cytotoxic T cells has also been shown. Blocking the CD28-(CD80/CD86) interaction inhibits the in vitro activation of alloreactive and antigen specific cytotoxic T cells[79]. Furthermore, surface expression of CD80 confers immunogenicity to tumour cells leading to the activation of cytotoxic T cells in vivo[80-82] and in vitro[83]. These results however may simply have represented the requirement of CD28-CD80 costimulation for the production of IL-2 that is necessary for the clonal expansion of cytotoxic T cells.

The independent requirement of costimulation for the development of cytotoxicity was suggested by Azuma and Lanier et al. [84,85] and was shown by Guerder et al.[86]. These investigators showed that CD28-CD80 interaction is necessary not only for IL-2 production by $CD8^+$ T cells, but also for the generation of cytotoxic effectors. Signalling through this pathway is required for the accumulation of mRNA encoding for granzyme B. Functionally, $CD8^+$ T cells activated in the presence of CTLA-4-Ig (which blocks CD28-CD80 interaction) demonstrated significantly reduced specific lysis of targets. Interestingly, costimulation was not required for the production of interferon-γ by $CD8^+$ T cells. Importantly, these observations could not be reversed by exogenous IL-2 exposure.

Azuma et al.[87] have also implicated the LFA-1/ICAM-1 pathway in the generation of effector cytotoxic T cells from resting T cells, but the HSA pathway was not shown to be important[86]. It appears that depending on the stage of differentiation of responder T cells, different costimulatory pathways may exert variable costimulatory function[88].

The activation of $CD8^+$ T cells normally requires "help" from $CD4^+$ T cells, in addition to signal 1 and signal 2 (costimulation) from activated antigen presenting cells[89,90]. However, under certain circumstances, it has been demonstrated that activation of $CD8^+$ T cells by antigen presenting cells can occur in the absence of $CD4^+$ cells[91]. Further highlighting the obscure nature of this interaction is the observation that certain viruses are able to induce anti-viral $CD8^+$ T cell responses in the absence of $CD4^+$ helper T cells[92,93].

Recent data from three groups has demonstrated that it is not necessary for cytotoxic and helper T cells to interact simultaneously with the same antigen presenting cell[94-96]. However, interaction between $CD4^+$ helper T cells and antigen presenting cells conditions the antigen presenting cells to acquire cytotoxic T cell activating abilities. This appears to be induced by the CD40-CD40L interaction.

CD40 is expressed on antigen presenting cells, while CD40L is expressed on activated $CD4^+$ helper T cells[97]. Blockade of CD40L prevented conditioning of the APC whereas signalling through CD40 restored it. This interaction can be co-stimulatory on its own in some cases[68]. However, it is known to converge with the CD28-CD80 pathway. CD80 also known as B7-1, is expressed by activated antigen

presenting cells and is the ligand for CD28 that is expressed by the majority of T cells[74]. CD80 expression on antigen presenting cells is up-regulated by signalling through CD40[98,99]. This flexibility on the part of the antigen presenting cell and the activated $CD4^+$ T cell has partially clarified the mechanism for the activation of cytotoxic T cells.

1.5 Differentiation of $CD8^+$ T cells

Cytotoxic T lymphocytes (CTL) represent the main effector phenotype of $CD8^+$ T cells. They kill their targets through contact dependent mechanisms, as well as by the secretion of effector cytokines such as interferon-γ. Interferon-γ is a key cytokine in rejection although it is not exclusive to cytotoxic T cells. It recruits and activates macrophages, activates endothelial cells, increases MHC expression and antigen processing, and has potent anti-viral effects. However, when activated cytotoxic T cells make direct contact with appropriate allogeneic targets, virus-infected cells or tumour cells, the antigen specific TCR-MHC/peptide interaction ensures that only cells displaying antigenic peptides are killed. Activated cytotoxic T cells can function, without the help of other cells, serially killing targets while remaining viable. Target killing can occur through either calcium-dependent or calcium-independent pathways. Acquisition of this cytotoxic ability is tightly regulated, and results from activation as previously discussed.

The contact-dependent calcium-dependent cytotoxic pathway relies on the secretion of two classes of proteins. Activated $CD8^+$ T cells have granules containing at least two classes of molecules known as perforins[100] and granzymes[101]. After an

activated $CD8^+$ T cell recognises its target via the TCR, a tight junction is formed between it and the target. The contents of granules are released onto the target cell surface at the site of contact.

The calcium-independent pathway is also contact-dependent, but employs the Fas receptor[102] (CD95) – a member of the TNF family of death receptors – and FasL (CD95L) its ligand. CD95L is synthesised within several hours after TCR stimulation while CD95 is expressed on the surface of many target cells. The CD95 molecule, when stimulated, induces an intracellular signal cascade resulting in apoptosis, or programmed cell death.

The cytoxins (perforins and granzymes) work together to result in apoptosis. Perforins polymerise to form transmembrane pores in target cell membranes[100]. These molecules resemble the membrane attack complex of complement in function and structure. Granzymes comprise at least three proteases that belong to the serine protease family[103]. The currently held model of perforin/granzyme mediated killing proposes that perforin polymerises in the presence of calcium forming pores through which granzymes pass. Once in the cytoplasm, granzymes may act on cytosolic death substrates, but the main mechanism is directed at the nucleus. In the nucleus, they cause the degradation of nuclear DNA leading to classical apoptosis[104,105].

The development of perforin, granzyme, CD95 and CD95L knockout mice have helped us understand the relative importance of these complicated pathways to target cell death. Perforin deficient mice have no demonstrable defect in T cell activation and a normal phenotype, however their cytotoxic T cell killing activity is impaired

but not abolished[106-108]. Granzyme A or B deficient mice possess a normal phenotype, but also have a impaired ability to kill their targets[102,109,110]. Since granzymes cannot be efficiently delivered to the cytoplasm of the target cell in the absence of perforin, perforin deficient mice are a functionally pan-granzyme deficient. CD95 (lpr/lpr)[111] and CD95L (gld/gld)[112] knockout mice have similar phenotypes, both gradually accumulate $CD4^-CD8^-$ T cells in the periphery and develop splenomegaly, lymphadenopathy and autoimmunity.

The perforin/granzyme mechanism accounts for the vast majority of cytotoxicity attributable to $CD8^+$ T cells. The CD95L/CD95 dependent pathway is somewhat redundant in $CD8^+$ T cells but plays a more significant role in a delayed fashion. The relative importance of these distinct pathways of cytotoxicity has been evaluated using a model of acute graft-versus-host disease originally developed by Sprent and Korngold[113]. In their model that is dependent on a MHC class I mismatch, lethally irradiated recipient mice expressing the mutant class I molecule $H\text{-}2K^{bm1}$ were reconstituted with syngeneic bone marrow cells along with allogeneic wild-type $CD8^+$ T cells. These wild type ($H\text{-}2K^{bm1-}$) allogeneic $CD8^+$ T cells recognised the mutant $H\text{-}2K^{bm1+}$ bone marrow cells and killed them resulting in death of the recipient mice within 3-4 weeks due to marrow aplasia. When wild-type ($H\text{-}2K^{bm1-}$) $CD8^+$ T cells deficient in perforin were infused instead, greater than 90% of recipients survived, with essentially normal blood counts[114,115]. This suggested that $CD8^+$ T cell cytotoxicity is nearly entirely dependent on the perforin/granzyme pathway. When granzyme-deficient perforin-competent $CD8^+$ T cells were used instead, approximately 30% of recipients survived. $CD8^+$ T cells with a defect in CD95L displayed no deficit in this model.

It has been shown that early and late forms of cytotoxicity exist in $CD8^+$ T cells. Two assay systems have been useful in dissecting the mechanisms. They are the ^{51}Cr release assay that indicates cell membrane damage and the [^{125}I]-iododeoxyuridine (IUdR)-labelled DNA release assay that indicates DNA fragmentation (apoptosis). Perforin-deficient $CD8^+$ T cells had an absolute defect in their ability to cause membrane damage and leakage of ^{51}Cr from target cells in vitro[106-108,116]. ^{51}Cr release normally occurs within 2-4 hours with wild-type $CD8^+$ T cells. Similarly, there was a deficit in [^{125}I]-IUdR release showing that apoptosis did not occur at early time points without perforin[102]. However, with prolonged incubation, perforin deficient $CD8^+$ T cells regained their ability to mediate both ^{51}Cr release and [^{125}I]-IUdR release. This late form of cytotoxicity is mediated by the CD95L pathway since $CD8^+$ T cells deficient in both perforin and CD95L do not recover cytotoxicity despite long periods of incubation[102,117]. $CD8^+$ T cells deficient only in CD95L have no demonstrable defect of early cytotoxicity in vitro.

In vitro, $CD8^+$ T cells deficient in granzyme A and B induce the early release of ^{51}Cr to the same degree as wild-type CTL[104,118]. There is however, an absolute defect in their ability to induce the early release of [^{125}I]-IUdR although perforin is fully active in these cytotoxic T cells. Similar to before, the CD95L pathway is able to restore [^{125}I]-IUdR release after prolonged incubation in vitro.

1.5.1 Cytokine Secretion by Cytotoxic T Cells

Unlike murine $CD4^+$ T cells, which differentiate readily into either Th1 or Th2 cells[119], naïve $CD8^+$ T cells show a strong preference for differentiating into a Tc1 (Th1-like) cytokine production pattern in vivo[120]. Tc1 cells are defined as $CD8^+$ T cells that secrete interferon-γ but not IL-4 or IL-5 and Tc2 cells are $CD8^+$ cells that secrete IL-4 or IL-5 but not interferon-γ[121]. As for $CD4^+$ T cells, exposure to interferon-γ and IL-12 promote differentiation to Tc1 cells. On the other hand, Tc2 cells can be generated by exposure to IL-4 and blocking antibodies to interferon-γ[119]. Both subsets are cytotoxic via the perforin/granzyme and Fas pathways. However, whether they are able to provide help to other cells (e.g. B cells) is debatable since they have a strong ability to kill their targets, including antigen presenting cells. However, in limited circumstance, IL-4 producing Tc2 $CD8^+$ T cells can be induced to express CD40 and do provide "help" to B cells[122,123].

1.5.2 Monitoring T Cell Phenotype

The assessment of T cell phenotype and activation state through direct functional assays can be limited for reasons of inadequate sample size or cell numbers. Furthermore, functional in vitro studies yield information about bulk populations of T cells, but cannot assign a particular phenotype to individual T cells. Fortunately, T cells often up-regulate or down-regulate the expression of certain cell surface molecules with the occurrence of cellular events that mark changes in their activation state and function. Through careful observation, investigators have provided us with monoclonal antibodies specific for some of these stably expressed cell surface

markers which have made possible the description of a cell's true phenotype by examining its surface phenotype. These cell surface molecules are sometimes known to participate in cellular activation, adhesion and homing, however their exact function is not always known.

Both $CD4^+$ and $CD8^+$ T cells clonally expand as they progress from small resting T cells to effector T cells to memory T cells. A number of events occur during this process and likely initiate subsequent events somewhat[124,125]. After the complex series of intracellular biochemical changes that occur after a T cell receives signal 1 and signal 2 it takes only a short time before T cells become committed. T cells will continue to become activated after this stage despite withdrawal of signal 1 and/or signal 2. Within minutes of activation, two proto-oncogenes (c-myc and c-fos) are transcribed. Within hours, IL-2, and CD25 genes are switched on. DNA synthesis required for clonal expansion does not begin until about 24 hours later. In $CD8^+$ T cells, transcription of the genes for perforins and granzymes begins approximately 3 days after initial activation. To be able to assign a T cell to a particular activation state, effector state, or memory phenotype based on its cell surface phenotype would be very advantageous.

Naïve resting T cells are $CD69^{low}CD62L^{high}CD45RB^{high}CD44^{low}$[126-130]. The detection of T cells early after activation remains difficult, however up-regulation of CD69 expression has been shown to occur within one hour of activation[131]. CD69 (also known as very early activation antigen) is an 85-kDa disulphide-linked homodimer of differentially glycosylated subunits. CD69 expression indicates a state of early activation[130] and is up-regulated in both naïve and memory cells after

activation[132]. Although the fraction of $CD8^+CD69^{high}$ T cells that possess perforin granules is increased, expression of CD69 does not without doubt indicate cytotoxic effector function[133]. The fact that up-regulation of CD69 precedes transcription of genes for perforin and granzymes is the most likely explanation for this. Certainly, most $CD8^+$ T cells recovered from rejecting allografts are $CD69^{high}$[60,134].

CD62L (also known as L-Selectin, LECAM-1 and Ly-22) is another surface that is modulated with activation and is useful in the study of T cells. This member of the selectin adhesion molecule family plays a significant role in the homing of naïve lymphocytes to peripheral lymph nodes through binding of its ligand expressed on high endothelial venules[135,136]. Expression of CD62L is rapidly down-regulated upon activation[128]. Cytotoxic T cells recovered from rejecting cardiac allografts do not express CD62L, but do express genes coding for serine esterases (granzymes) that confer cytotoxicity to $CD8^+$ T cells[137].

CD45 has been widely used as a marker of memory, with the higher molecular weight isoforms defining the pool containing naïve T cells and the low molecular weight isoform indicating a memory phenotype. In fact, multiple CD45 isoforms are expressed on the cell surface in various combinations[138]. Empirically, the presence of any of the three epitopes A, B, or C is often taken as evidence that the high molecular weight naïve form is present[139]. In the mouse, no antibody specific for the low molecular weight form CD45RO has been generated and therefore the CD45RB isoform alone has been used to classify T cells as naïve ($CD45RB^{high}$) or memory ($CD45RB^{low}$). The level of CD45RB expression has been shown to decrease as T lymphocytes progress from naïve to memory cells[140,141].

CD44 (also known as Pgp-1, Ly-24) is a polymorphic glycoprotein that is expressed as multiple isoforms. The standard form of CD44, lacking variable exons and referred to as CD44H or CD44S, is widely expressed on haematopoietic and non-haematopoietic cells[142-144]. CD44 isoforms encoded by variable exons are expressed on epithelial cells, but only at low levels on most lymphocytes[145]. This surface molecule is a cell adhesion receptor, and its ligand, hyaluronate, is a common component of extracellular matrices[146]. In the periphery, the level of CD44 expression increases upon activation of B cells, $CD4^+$ T cells and $CD8^+$ T cells and is stably expressed on memory T cells[126,147].

Recognition of antigen by the immune system evokes a number of changes in lymphocytes and lymphocytes subsets that allow the immune system to clear the antigen and to respond more rapidly and appropriately after renewed antigen encounter, a process referred to as immunological memory. Within the $CD8^+$ T cell compartment, unprimed (or naïve), effector T cells (possessing cytotoxic abilities), and memory T cells can be distinguished from one another. Functionally, memory T cells have a number of characteristics that differ from naïve T cells. They respond efficiently to recall antigens with less stringent requirements for activation and have the potential to secrete a more extensive set of cytokines[148-150].

In addition, memory and naïve T cells differ in the surface expression of certain surface markers as previously discussed. Based on these markers though, the distinction between effector and memory cells remains difficult. Both CD62L and CD45RB are expressed at high levels on naïve murine T cells, are down-regulated on

activation and are thought of as surface memory markers. However, whether down-regulation of these two molecules can be looked upon as true markers of memory rather than a reflection of cellular activation is debatable[151,152]. Although CD45 isoform expression is reasonably reliable for human T cells, most would argue that these phenotypes were less reliable in mouse. CD62L expression is lost upon activation[153], however some memory cells (especially $CD8^+$ T cell) regain expression of CD62L, which limits its usefulness as a marker of memory[154].

In a murine model of lymphocytic choriomeningitis virus infection studied via the adoptive transfer of antigen specific $CD8^+$ T cells, Zimmermann et al.[155] demonstrated phenotypic distinctions between naïve, effector and memory T cells confirming previous reports[128,156,157] stating that compared to naïve $CD8^+$ T cells effectors have strongly down-regulated CD62L while CD44 surface expression is up-regulated. In contrast to effector $CD8^+$ T cells, memory $CD8^+$ T cells were heterogeneous with respect to CD62L expression. The CD45RB isoform expression could not be used as a reliable marker for memory $CD8^+$ T cells[155].

Since both CD45RB and CD62L are not ideal markers of memory $CD8^+$ T cells on their own, investigators have also followed the surface expression of CD44 that increases with activation and is stably expressed over the long-term. Unfortunately, activated effector T cells have already down-regulated CD62L expression and elevated CD44 expression[158]. CD69 and CD25 (true early activation markers) are useful in making the distinction between effector and memory $CD8^+$ T cells. CD69 is not expressed on memory $CD8^+$ T cells and is up-regulated on re-activation during a recall response[132]. In order to discriminate between naïve, effector, and memory

$CD8^+$ T cells multiple criteria are applied. Thus, $CD44^{high}$ T cells low in the expression of activation markers such as CD25 and CD69 are classified as memory cells. $CD44^{high}$ T cells also expressing CD69 are thought of as activated effector T cells. In general, this appears to be the case[154].

1.6 Mechanisms of Tolerance

In 1940, Ray Owen demonstrated chimerism in the peripheral blood of dizygotic twin freemartin cattle[159]. This was explained by the earlier finding that dizygotic cattle twins develop a fusion of placental circulation in utero[160]. He then introduced the concept of immunological tolerance when he noted that one twin did not generate a cytotoxic antibody response against the other twin's red blood cells and vice-versa. Billingham, Brent and Medawar later demonstrated that skin grafts could be exchanged without rejection in these twins, contradicting what they had predicted based on the allogeneic mismatch[161].

These have proven to be important observations in transplantation immunology that motivated many investigators to successfully reproduce this tolerance experimentally. Encouraged by their unexpected observation with skin grafts in dizygotic twins Billingham, Brent and Medawar worked towards a model that would also allow for the deliberate induction of tolerance to tissue allografts. They approached this by injecting foetal and neonatal mice with foreign cells. These mice readily accepted skin grafts of the same strain as the cell donors and proved that tolerance could be induced experimentally[162].

The benefits of tolerance induction to the clinic are clear, and efforts over the past 50 years have produced many protocols that work in the laboratory, but none that reliably work in the clinic. However, there is hope that tolerance can also be induced in human beings. There have been selected reports of patients accepting transplanted organs without long-term immunosuppression[163-165]. Current immunosuppressive therapy must also be re-examined as we attempt tolerance induction because cyclosporine, for example, has been shown to interfere with the induction of tolerance[166].

Immunological tolerance is not simply a laboratory observation, but is a phenomenon that the intact immune system must be able to generate. As T cells are critical to the rejection of allografts, they are also prime targets of tolerance induction. Likewise, they also participate in many types of autoimmunity and pathological immune responses and therefore the body maintains mechanisms that silence T cell responses against these autoantigens.

T cell tolerance to self-antigens primarily occurs via clonal deletion of immature thymocytes in the thymus. The vast majority of T cells that are produced are deleted in the thymus either because they are not self-restricted[167] or because they are self-reactive[168]. Hence, the thymus is an organ with a major role for the establishment of self-tolerance. However, not all self-antigens are expressed within the thymus or at a level high enough to ensure complete deletion of possibly self-reactive T cells.

Various mechanisms of peripheral T cell tolerance have been described, which can ensure unresponsiveness to autoantigens maintaining self-tolerance in the periphery.

These mechanisms are known as clonal deletion, anergy, regulation, and ignorance and are often what we attempt to capitalise on to induce tolerance to transplantation antigens. Admittedly, it is difficult to study T cell tolerance in the periphery however the development of TCR transgenic mice and clonotypic monoclonal antibodies have helped partially uncover the underlying mechanisms of self-tolerance. It should be noted that these mechanisms do not function in isolation of one another and that often two or more co-operate to produce tolerance.

1.6.1 Clonal Deletion

It has become apparent that the T cell repertoire is also moulded in the periphery. Self-reactive T cells which survive selection in the thymus can be controlled by clonal deletion. This was demonstrated by the use of TCR transgenic mice crossed onto mice that express the nominal antigen constitutively, or by the adoptive transfer of transgenic T cells into mice expressing the nominal antigen[169-172]. In these studies, self-reactive TCR transgenic T cells mounted an initial powerful immune response noted by massive clonal expansion followed by massive clonal deletion. A residual population was left behind, but these were subsequently non-reactive or anergic. The nature of the antigen presentation is undoubtedly exhaustive and likely to be defective in that proper costimulation is not available. This lead to the majority of cells being clonally deleted.

1.6.2 Anergy

Anergy has been defined as the functional unresponsiveness of viable T cells[173]. This is an intrinsic property of the cell and should not be confused with regulation or

suppression where another cell prevents a productive immune response. This distinction is difficult to make experimentally. This was originally proposed as "functional deletion" as applied to B cells tolerance by Bretscher and Cohn[64]. They proposed that two separate signals are necessary for the activation of lymphocytes. Lafferty et al. later expanded this observation to include T cells; the two signal hypothesis[27,66]. They showed that T cells not only required recognition of antigen, but also a signal from an antigen presenting cell in order to become fully activated (signal 1 and signal 2 were discussed previously). Non-functional antigen presenting cells, such as fixed B cells, induce anergy or tolerance in T cells[65] both in vitro and in vivo. After exposure to these defective antigen presenting cells in vitro, T cells clones were subsequently found to be unresponsive to attempted activation with fully functional antigen presenting cells. These cells do not proliferate or produce IL-2 in response to antigen, but if exogenous IL-2 is added, their reactivity can be regained[174]. The dominant second signal necessary to prevent this state of anergy, was found to be signalling through the CD28 surface molecule on T cells[175]. The most reliable way to generate anergic T cells has been through defective antigen presentation[173].

1.6.3 Ignorance

It has been shown that self-reactive T cells are present in the peripheral T cell repertoire without signs of autoimmunity[176,177]. It is also possible that T cells remain unaware of the presence of their specific antigen in vivo. The concept of T cell ignorance, as it is called, was first introduced by Ohashi et al. In their experiments, a glycoprotein from the lymphocytic choriomeningitis virus (LCMV)

was expressed as a transgene on pancreatic β cells of mice with a tissue specific promoter[178]. These mice were then crossed with a TCR transgenic mouse specific for the same glycoprotein presented by H-2D^b. The glycoprotein specific T cells in these mice were self-reactive, but were "ignorant" to the antigen with no demonstrable signs of tolerance or activation. However, upon deliberate LCMV infection of the dual transgenic mice LCMV-specific TCR transgenic T cells became activated and resulted in the destruction of the glycoprotein expressing β cells (diabetes). In this experimental system, T cells were apparently unaware or "ignorant" of the presence of their nominal antigen in the periphery.

1.6.4 Regulation/Suppression

Gershon and Kondo in 1971 first introduced the term "infectious immunological tolerance" when they described, using a complicated experimental system, the adoptive transfer of tolerance from mice tolerised to sheep red blood cells to naïve mice[179]. Their use of the term precedes Qin et al.'s introduction of "infectious tolerance", a related concept with wider implications[180]. Gershon and Kondo demonstrated that adoptively transferred tolerant T cells subsequently prevented naïve mice from producing antibodies in response to subsequent challenge with sheep red blood cells. This provided some of the first support for the concept of suppressor T cells. The work of Gershon and Kondo identified a basic immunoregulatory mechanism that could explain how T cells specific for one alloantigen could suppress the response of naïve T cells.

Since then, more evidence that T cells can regulate the reactivity of other T cells has come from adoptive transfer experiments[181]. Adoptive transfer of cells from animals which have long-term surviving allografts transferred to a syngeneic animal has shown that tolerance can often be transferred to naïve animals, in most cases by $CD4^+$ T cells[180,182-186]. It has also been shown that cells from tolerant animals can prevent the proliferation or generation of cytotoxic responses specific for donor alloantigens[187]. Examples of regulation also exist in the prevention or control of autoimmunity[188-191].

1.7 Strategies of Tolerance Induction Using Allogeneic Cells

True donor specific tolerance remains the goal of transplantation immunology. We hope, one day, that by harnessing the mechanisms which produce self-tolerance we will achieve this goal. Delivery of donor alloantigens into the periphery prior to transplantation has been used for several decades to induce experimental tolerance[162]. However, most of the tolerogenic strategies described have been studied in rodents and it remains to be seen whether they can be implemented for use in human beings. We will focus on those relevant to bone marrow infusion prior to transplantation. Generally, allogeneic cells administered prior to transplantation may induce tolerance by a number of mechanisms. Clonal deletion, anergy and regulatory mechanisms have all been proposed.

Bone marrow has been suggested as an ideal vehicle for delivering alloantigens in tolerance induction protocols and is being clinically investigated[192-194] for the

following reasons. With relative ease, it can be harvested from organ donors at the same time as organs for transplantation. Secondly, because of the potential to establish chimerism and express alloantigens for extended periods.

Donor bone marrow cells express alloantigens on their surface and are recognised by recipient T cells. Under the appropriate conditions, haematopoietic stem cells present in donor bone marrow may engraft and establish chimerism in the recipient, similar to what was observed by Owen[159]. Chimerism is thought to be an important factor associated with the induction and maintenance of tolerance[195], but this view has been questioned. Others have suggested that it is merely an epiphenomenon unrelated to acute or chronic rejection[196,197]. By establishing chimerism, bone marrow is able to provide a potentially long-lived and permanent source of donor type allogeneic cells that reach the periphery. Essentially, donor antigens could be expressed constitutively by the recipient. Haematopoietic stem cells have also been proposed to contribute to central mechanisms of tolerance. Donor type cells home to the thymus of recipients where they reside and present donor alloantigens to maturing T cells resulting in clonal deletion of donor reactive recipient T cells[198,199].

In another capacity, bone marrow cells act as non-professional antigen presenting cells providing cognate TCR-alloantigen interactions without costimulation. Hence, they provide signal one without signal two. Incomplete activation of T cells in this manner has been shown to yield T cells that are anergic as previously mentioned. Bone marrow, when administered in high doses has also been associated with

peripheral clonal deletion of recipient donor-specific T cells[198]. Indeed both of these mechanisms represent potential routes to tolerance.

1.8 Visualisation of Antigen Specific T Cells

The major problem in the study of T cell responses is that of tracking T cells of a given specificity. Physical detection of lymphocytes specific for any particular antigen in vivo has been technically impossible, due to their exceedingly low frequency. Consequently, the study of T cell responses has relied on short-term polyclonal cell cultures, cloned lymphocyte lines or hybridomas in vitro. Others have exploited natural correlations between the expression of a particular V_β gene segment and T-cell receptor specificity in vivo[200], but this does not identify antigen specific cells with certainty confounding molecular or biochemical analysis of the antigen specific T cells. These methods have and still do yield a great deal of information. By their nature though, they do not allow the direct study of antigen specific T cells in an intact immune system. An alternative approach was taken[201-203]. Transgenic mice expressing $\alpha\beta$ antigen receptors derived from alloreactive T cell clones were generated. These mice produced large numbers of T cells that were alloreactive against targets with the same specificity as the original clones.

T cell receptor transgenic animals offer an excellent source of antigen specific T cells that can be monitored in vivo or in vitro for the study of antigen specific T cell responses. However, the peripheral T cell repertoire of intact transgenic mice is dominated by a single specificity often lacking either $CD4^+$ or $CD8^+$ T cells. Several strategies have been developed that in part solve these problems, but we have chosen

to use an experimental system first published by Moskophidis et al.[204] and later used by Kearney, et al.[205] The experimental system employed normal CBA/Ca mice seeded with trace numbers of $CD8^+$ TCR transgenic T cells specific for an MHC class I alloantigen. These TCR transgenic cells were identified with a clonotypic monoclonal antibody recognising the transgenic αβ TCR.

1.8.1 TCR Transgenic Mice

TCR transgenic mice are generated by injecting the already productively re-arranged TCR α and β genes DNA constructs into the fertilised oocytes of mice. This ensures that the majority of T cells generated in these animals bear the transgenic TCR. Endogenous TCR β chain rearrangement is prevented by the injected genes through allelic exclusion[206]. However, α allelic exclusion is not as complete permitting endogenous α TCR chains to associate with transgenic β chains[207,208]. This allows T cells to be produced that express small numbers of other αβ TCR's on their surface in addition to the dominant TCR[209]. Each has a different specificity and both are potentially functional[210,211]. Dual α chain expression has not prevented conclusions being drawn from transgenic systems, but to overcome this problem workers have crossed TCR transgenic mice onto mice lacking the recombination-activating gene ($RAG^{-/-}$ knockout). In these mice transgenic TCR-expressing T cells are the only lymphocytes present. No endogenous TCR genes are expressed. The possibility of dual α chain expression must be kept in mind when using interpreting results obtained from experimental systems utilising TCR transgenic mice.

1.8.2 CFSE

Labelling of TCR transgenic cells prior to adoptive transfer is a complimentary technique which provides valuable information about division history. CFSE is a non-toxic fluorescein-based molecule that, in its native form, is uncharged and able to traverse cell membranes[212]. It is then cleaved by esterases within the cytoplasm resulting in a charged molecule trapped within the cell. CFSE then binds covalently to cytoplasmic proteins further trapping itself within the cell. Consequently, it remains within viable cells for several weeks. CFSE fluorescence is detectable by flow cytometry in the FL-1 channel allowing measurement of fluorescence intensity within individual cells. As labelled T cells divide, CFSE is equally distributed between daughter cells and therefore each daughter cell fluoresces exactly half as much as the maternal cell[213]. This characteristic allows the resolution of multiple successive divisions of CFSE labelled cells by flow cytometry.

1.8.3 Premise for Employing an Adoptive Transfer System

We have chosen an adoptive transfer system for the experiments described in this thesis because it allows for the tracking of antigen specific T cells in vivo. However, it is important to understand that certain assumptions were made in order to interpret the results obtained. We believe that adoptive transfer of a trace number of identifiable antigen specific cells returns them to a normal environment where $CD4^+$ T cell "help" is abundant hopefully restoring normal activation pathways and mechanisms. Secondly, this small trace population will not grossly alter the balance of the peripheral T cell repertoire. Thirdly, that this population will not alter the

course of normal immune responses resulting in either immunity or rejection although we hope though that they will reflect the behaviour of normal alloreactive $CD8^+$ T cells during an immune response against transplantation alloantigens. With these assumptions we set forth to study cytotoxic T cells under a number of conditions previously only studied by indirect means.

1.9 General Aims of the Thesis

The induction of donor specific unresponsiveness to an organ allograft remains the principal goal of transplantation immunologists and clinicians the world over. Although this can be predictably achieved in the laboratory, we continue to await the successful transfer of such protocols to the clinic. Hopefully, an improved understanding of the mechanisms underlying both allograft rejection and tolerance induction in these experimental protocols will permit us to achieve this goal.

We have previously shown that pretreatment with a single MHC class I donor alloantigen ($H\text{-}2K^b$) can result in operational tolerance to a fully allogeneic cardiac allograft[214-218]. The spreading of tolerance from a single "key" alloantigen to potentially multiple "bystander" alloantigens co-expressed by a fully allogeneic allograft has been confirmed by other investigators[219-223] and has wide implications for the development of tolerance induction protocols for the clinic.

The general aim of this thesis is to investigate the role of alloreactive cytotoxic T cells in allograft rejection and during the establishment of tolerance. Using $H\text{-}2K^b$ specific $CD8^+$ TCR transgenic T cells derived from DES mice[203] we developed an adoptive

transfer model[204,205] to study the response of alloreactive cytotoxic T cells in vivo. With this experimental system we tracked and phenotyped antigen specific T cells present in the secondary lymphoid tissues as well as within transplanted allografts. We established the role of $CD8^+$ T cells in acute rejection. Next, adoptively transferred $CD8^+DES^+$ T cells were studied following pretreatment with syngeneic bone marrow cells (CBK) expressing the single MHC class I alloantigen $H\text{-}2K^b$ with the aim of identifying which mechanisms of tolerance might be operational in these protocols.

We hope that the experimental system that we have developed and the studies presented in this thesis will add to the body of knowledge already produced by other investigators around the world with the final aim of developing clinically applicable tolerance inducing protocols.

Chapter 2 - Methods & Materials

2.1 Animal Related Methods

2.1.1 Mice

DES, 2C, BM3.3, CBK, CBA/Ca, BALB/c, (CBA/Ca x BALB/c F1), (CBK x BALB/c F1), (CBA/Ca x C57BL/10 F1), C57BL/6, C57BL/10 and NZW mice[224] were bred and housed in the Biomedical Services Unit (BMSU) of the John Radcliffe Hospital, Headington, UK in accordance with the Animals (Scientific Procedure) Act 1986 of the United Kingdom[225]. DES, 2C, BM3.3 and CBK mice were kind gifts of Dr. Andrew L. Mellor, Augusta, Georgia, USA.

Strain	K	I-A	I-E	D	L
DES[203]	k	k	k	k	k
2C[201]	k	k	k	k	k
BM3.3[202]	k	k	k	k	k
CBK[226]	k/b	k	k	k	k
CBA/Ca	k	k	k	k	k
BALB/c	d	d	d	d	d
(CBA/Ca x BALB/c F1)	k/d	k/d	k/d	k/d	k/d
(CBK x BALB/c F1)	k/b/d	k/d	k/d	k/d	k/d
(CBA/Ca x C57BL/10 F1)	k/b	k/b	k/-	k/b	k/b
C57BL/6	b	b	-	b	b
C57BL/10	b	b	-	b	b
NZW	u	u	u	z	z

DES, 2C, BM3.3 and CBK mice were transgenic strains generated by Dr. Andrew L. Mellor on a CBA/Ca (H-2^k) background by microinjecting functional gene DNA constructs into the male pronuclei of fertilised oocytes from CBA/Ca mice.

The clonotypic monoclonal antibodies Désiré-1[227], 1B2[228] and Ti98[229] recognise the αβ heterodimers of the transgenic TCR's of {DES mice/KB5.C20 clone}, {2C

mice/2C clone} and {BM3.3 mice/BM3 clone}, respectively. All experiments were performed with sex-matched mice age between 6 and 12 weeks of age.

Transgenic	Gene Product	Clone	Comment	Origin
DES[203]	H-2K^b Specific TCR[230]	KB5.C20[231]	Vα2-JαA10-Vβ2-Dβ2-Jβ2.3	B10.BR (H-2^k)
2C[201]	H-2L^d Specific TCR[232,233]	2C[228]	Vα3.1Vβ8.2	BALB/B (H-2^b)
BM3.3[202]	H-2K^b Specific TCR	BM3[231]	Vβ2-Dβ1-Jβ1.3	CBA/J (H-2^k)
CBK[226]	H-2K^b MHC Class I	-	Natural Promoter	C57BL/6

2.1.2 Pharmaceuticals

Hypnorm® (fentanyl citrate and fluanisone; Jensen Pharmaceutical Ltd., UK) and Hypnovel® (midazolam; Roche Products Ltd., UK) were used in a balanced anaesthetic regimen for recovery surgical procedures performed on mice. Sterile injection grade normal saline (0.9%) was used to dilute stock solutions to working solutions. Hypnorm® was diluted to a working solution of 10μg/ml fentanyl and 330μg/ml fluanisone. Hypnovel® was diluted to 500μg/ml. Hypnorm® (0.3ml) and Hypnovel® (0.3ml) were administered subcutaneously prior to surgical procedures. Marcaine® (bupivicaine hydrochloride; Astra, UK) diluted to 0.8mg/ml was used as a local anaesthetic. Multiparin (Heparin Sodium; Leo Laboratories Ltd., UK) was used at 18,000 IU/ml and administered intravenously prior to donor cardiac explantation.

2.1.3 Injections

Subcutaneous injections of anaesthetics were made into the dorsal thorax of awake mice. Intravenous injections of cells or other solutions were made in the dorsal

penile vein under an operating microscope using a 27 gauge needle. Mice were anaesthetised with Hypnorm® (0.3ml) and Hypnovel® (0.3ml) for penile vein injections.

2.2 Preparation of Cells

2.2.1 Leukocyte Preparation

Leukocytes from spleen, thymus and lymph nodes were all prepared in a similar manner. The donor mice were killed by cervical dislocation and the skin cleansed with 70% ethanol. Using an operating microscope, the spleen, thymus, mesenteric and axillary lymph nodes were collected into cold R^{10} and were passed through a fine metal sieve to make a single cell suspension in R^{10}.

2.2.2 Bone Marrow Cell Preparation

Animals used as bone marrow donors were sacrificed by cervical dislocation. Bone marrow cells were flushed out of femoral and tibial bones with sterile PBS. A single cell suspension was prepared by passing the bone marrow through a 19 gauge needle. Bone marrow cells were then counted by trypan blue exclusion, centrifuged at $4^{O}C$, 1200rpm for 10 minutes and re-suspended at a concentration of 100×10^6 cells/ml in PBS for immediate i.v. injection. Each donor typically yielded 30 to 80 x 10^6 cells. Each recipient received 50×10^6 bone marrow cells.

2.2.3 Isolation of Graft Infiltrating Cells

Animals were sacrificed by cervical dislocation and hearts retrieved into separate vials of cold R^{10}. GIC were prepared by collagenase digestion of transplanted hearts[234]. Retrieved cardiac grafts were minced and incubated with 1mg/ml type VII collagenase solution for 30 minutes at $37^{O}C$. A single cell suspension was prepared by teasing the collagenase-digested tissue. GIC were separated on a Ficoll gradient (Sigma-Aldrich, UK). GIC cells were counted by trypan blue exclusion and stained for flow cytometric analysis.

2.3 Adoptive Transfer

2.3.1 Enrichment of $CD8^{+}TG^{+}$ T Cells

Leukocytes from donor TCR transgenic mice (2C, DES or BM3.3: all H-2^{k}) were harvested, enriched for $CD8^{+}$ T cells by negative selection, labelled with CFSE and injected into syngeneic CBA/Ca mice to generate what will be referred to as CBA^{2C}, CBA^{DES}, $CBA^{BM3.3}$ or generically as CBA^{TG} mice.

Donor TCR transgenic mice were sacrificed by cervical dislocation, their spleens and mesenteric lymph nodes harvested into cold R^{10}, passed through a fine metal sieve in cold R^{10} and centrifuged at 1200rpm, $4^{O}C$ for 10 minutes. The pellet was resuspended in 5ml of cold TBAC for 4 minutes on ice and then quenched with 45 ml of R^{10} to lyse erythrocytes. Viable cells were then counted by trypan blue exclusion and centrifuged at 1200 rpm, $4^{O}C$ for 10 minutes before enrichment.

Cells were then labelled with unconjugated rat monoclonal antibodies (anti-H-2IEα; M5/114.15.2 and anti-CD4; YTA 3.1) which would be bound by BioMag® Goat anti-Rat IgG (H+L) labelled magnetic beads (PerSeptive Diagnostics). In a 50ml conical tube the pellet was resuspended in R^{10} and 0.75μg/million cells of both YTA 3.1 and M5/114.15.2 such that the final concentration was 40 x 10^6 cells/ml. The suspension was incubated on a rocker for 45 minutes on ice in the dark. The cells were washed three times with cold R^{10} to eliminate unbound antibody.

Up to 2 x 10^9 cells were enriched in a single 50ml tissue culture flask (greiner Labortechnik Art. Nr. 690160) using a ratio of 100 BioMag® beads per cell. Before use the required number of magnetic beads were washed three times in R^{10} to eliminate preservatives (e.g. NaN_3) in which the beads are stored.

The washed antibody bound cells were mixed with the washed beads and brought to a concentration of 40 x 10^6 cells/ml with cold R^{10}. The mixture was incubated cold for 45 minutes on a rocker in the dark. The magnet was applied to the cell/bead mixture for 15 minutes to remove $CD4^+$ and MHC Class II^+ cells from solution. The cells that remained in solution were enriched for $CD8^+$ cells and were collected for adoptive transfer. Four colour FACS® analysis was performed with anti-CD45/B220-FITC, non-competing anti-CD4-PE (RM4-5), biotinylated anti-clonotypic mAb (e.g. Désiré-1), anti-CD8-PerCP, and Streptavidin-APC™ to determine the percentage of $CD8^+TG^+$ T cells

2.3.2 CFSE Labelling

Cells enriched for $CD8^+$ T cells were centrifuged at 1200 rpm, 4^OC for 10 minutes and labelled with CFSE (Molecular Probes, Eugene, Oregon, USA) prior to adoptive transfer by resuspending the pellet in 3ml of an 8.3μM solution of CFSE in RPMI 1640 at 37^OC for 10 minutes. Labelling was quenched with 45ml of cold PBS. Cells were counted by trypan blue exclusion, and centrifuged at 1200rpm, 4^OC for 10 minutes.

2.3.3 Preparation of CBA^{TG} Mice

Enriched CFSE labelled $CD8^+$ T cells were resuspended in cold normal saline at a concentration of 4 x 10^6 $CD8^+TG^+$ T cells/ml as calculated from cell counts and FACS® analysis. 2 x 10^6 $CD8^+TG^+$ T cells in 0.5ml were injected i.v. into recipient CBA/Ca mice to make CBA^{TG} mice.

2.4 Cardiac Transplantation

The mouse heterotopic vascularised cardiac transplantation model has come into widespread use since its introduction by Drs. Robert Corry and Paul S. Russell in 1973[235].

In this model the donor ascending aorta is anastomosed end-to-side to the recipient abdominal aorta and the donor pulmonary artery is anastomosed to the recipients inferior vena cava (IVC). Hearts transplanted heterotopically behave functionally as aorto-caval fistulae. Blood enters the donor ascending aorta from the recipient

abdominal aorta and is diverted into the coronary arteries by the closed aortic valve. After the myocardium is perfused, deoxygenated blood drains into the right atrium through the coronary sinus and is pumped back into the recipients IVC by the right ventricle.

Heterotopic heart transplantation was carried out using an operating microscope (Carl Zeiss OPMI1-FC or OPMI6-CH, Germany). Operating time was approximately one hour and overall operative mortality/technical errors accounted for less than 10% of all transplants.

2.4.1 Donor Cardiac Explantation

Donor mice were anaesthetised with Hypnorm® (0.5ml) and Hypnovel® (0.5ml). At this point 1ml of cold heparin (diluted to 18,000U/ml final concentration with sterile saline) was administered via the penile vein using a 1ml syringe and a 27 gauge needle. The abdominal cavity was opened with a large transverse incision and the abdominal viscera deflected to the left side of the animal exposing the aorta and IVC. Using a 2ml syringe and a 27 gauge needle, the donor was exsanguinated via the IVC. The thoracic cavity was opened by cutting through the ribs along both sides of the thoracic spine all the way to the thoracic inlet. The anterior chest wall was deflected superiorly and held in place with a piece of adhesive tape. The IVC, azygous vein and the superior vena cava were ligated with 7-0 silk ties. The ascending aorta was divided proximal to the brachiocephalic artery. The main pulmonary artery was cut proximal to its bifurcation. The connective tissue between the ascending aorta and pulmonary artery was gently teased away at this stage. The pulmonary veins were ligated as a group with a single 7-0 silk tie. The donor heart was gently detached

from the remaining connective tissues with blunt dissection and was placed in cold saline until ready for implantation.

2.4.2 Recipient Cardiac Implantation

Recipient mice were anaesthetised with Hypnorm® (0.3ml) and Hypnovel® (0.3ml). A midline abdominal incision was made from the pubic symphysis to the xiphoid. The urinary bladder was emptied using a 27 gauge needle and 1ml syringe. A self-retaining retractor was placed to expose the abdominal contents. Using two cotton buds the small intestine was gently deflected superiorly onto a piece of moistened cotton gauze on the thoracic cavity. Testes were deflected inferiorly in the same manner in male recipients. The testes and intestines were covered with moist gauze throughout the procedure. The meso-sigmoid was divided allowing a strip of moist gauze to pass under and around the sigmoid colon. The strip of gauze was pulled to deflect the sigmoid colon to the animal's left side. Using two cotton buds the abdominal aorta and IVC were dissected free from the surrounding tissues in the retroperitoneum exposing the lumbar arteries and veins which lay directly posterior coming from the great vessels in two or three groups between the renal vessels and the bifurcations of the abdominal aorta and IVC inferiorly. The two or three groups of lumbar vessels were ligated individually with 7-0 silk ties. The testicular vessels in male recipients were mobilised from the great vessels. A Scoville-Lewis clamp (Downs Surgical Ltd., U.K.) was placed inferior to the renal vessels interrupting flow in both the aorta and IVC. Another clamp was positioned proximal to the bifurcation of the great vessels in a similar fashion.

The donor heart was placed into the recipients abdomen with the remnant of ascending aorta lying towards the left side of the animal. An aortotomy was performed in the abdominal aorta of the recipient using a 30 gauge needle. The aortotomy was extended with micro-scissors cephalad to a length of equal to or slightly smaller than the opening of the donor ascending aorta. The donor ascending aorta was anastomosed to the recipient abdominal aorta using 10-0 nylon Bear surgical suture™ on a round-bodied 4mm (3/8) needle (Kyowa Precision Instruments Corp., Tokyo, Japan). A stay suture was placed at the proximal apex bringing the donor ascending aorta and the recipient abdominal aorta together (Figure 2.2.1). The ends of the stay suture were cut to a length of approximately 2mm and 5mm. Similarly, the distal apex of the donor aorta and the recipient aorta were brought together with a 10-0 nylon suture and tied leaving a 5mm length of suture on one end and the needle on the other end. The anastomosis was then completed with the same suture in a counter-clockwise direction with a continuous running stitch (Figure 2.2.2). The distance from the distal apex to the proximal apex was covered with four stitches. The stitch was tied to the proximal stay suture. The second side of the anastomosis was completed in a similar manner continuing from the proximal apex to the distal apex and was tied to the distal stay suture (Figure 2.2.3).

The donor pulmonary artery was now anastomosed to the recipient IVC. A venotomy was performed in the same fashion as the aortotomy, but made a short distance inferior to the aortotomy leaving a space between the two of approximately 2mm (Figure 2.2.4). In contrast to the aortic anastomosis, a stay suture was first placed at the distal apex bringing together donor pulmonary artery and recipient IVC. The ends of the stay suture were tied leaving a 5mm length of suture on one end and

the needle on the other end (Figure 2.2.5). Similarly, the proximal apex of the donor pulmonary artery and the recipient IVC were brought together with a 10-0 suture. The ends of the proximal stay suture were cut to a length of approximately 2mm and 5mm (Figure 2.2.6). The anastomosis was then completed with the distal stay suture in a clockwise direction with a continuous running stitch. The distance from the distal apex to the proximal apex was covered with four throws (Figure 2.2.7). The stitch was tied to the proximal stay suture. At this point the needle was passed between the two donor vessels and the donor heart was flipped from the left side of the animal to the right (Figure 2.2.7). The second side of the anastomosis was completed in a similar manner continuing from the proximal apex to the distal apex and was tied to the distal stay suture. The anastomosis was performed similar to the aortic anastomosis except that the distal stay suture was done first and that a continuous running suture went in clockwise fashion (Figure 2.2.8).

The distal clamp was removed first to check the anastomosis of the IVC. After approximately 5 to 10 seconds the vessel filled and the anastomosis became competent. Small pieces of moistened haemostatic agent Spongostan (Johnson and Johnson) were placed around each anastomosis. After ensuring adequate haemostasis the proximal clamp was slowly removed. The transplanted heart filled with blood and began contracting. If there was no further bleeding, the intestines and testes were returned to the abdominal cavity. The abdominal incision was closed in two layers. Separately, the muscle and skin layers were closed with a continuous running stitch of 4-0 chromic catgut (Davis and Geck, U.K.)

2.4.3 Recovery from Anaesthesia

On completion of the procedure, the recipient was placed on soft dry bedding material in a warming cabinet for 8 to 12 hours with easy access to food and water.

2.4.4 Assessment of Transplanted Hearts

Mice with non-functioning grafts within two days of transplantation were considered technical failures and were excluded (<5%). The rejection of transplanted hearts was followed by abdominal palpation

0 = no palpable contraction
1 = very weak and slow cardiac contraction
2 = decreased rate, irregular rhythm or both
3 = decreased contractile force with normal rate
4 = normal rate and regular rhythm

Absence of a palpable heartbeat confirmed by ECG for at least 7 consecutive days constituted rejection[236]. In uncertain cases, exploratory laparotomy was performed to inspect the graft visually for contractions. In the absence of visible contractions, the hearts were considered rejected.

2.5 Analysis of Adoptively Transferred T Cells by Flow Cytometry

Single cell suspensions from spleens, mesenteric lymph nodes, bilateral axillary lymph nodes and GIC were prepared. Splenocytes were treated with TBAC to lyse erythrocytes. GIC were prepared by collagenase digestion of transplanted hearts[234].

GIC were counted by trypan blues exclusion while absolute cell counts for other tissues were determined using a Coulter Counter® (Coulter International Corp.).

Single cell suspensions were brought to a total volume of 5ml in polystyrene, round-bottom tubes (Falcon 2054 or 2053). Total cell counts for each tissue were obtained using a Coulter Counter® (Coulter International Corp.). 40µl of each single cell suspension was added to 20ml of FACS®Flo solution (Becton Dickson) in a universal tube. Three tubes of FACS®Flo solution without cells were also prepared as controls for background. A Coulter Counter® set to white blood cells, aperture control at 2, and threshold at 17 was used to obtain the cell counts. The mean of two passes through the Coulter Counter® was obtained for every sample as well as for background controls.

(mean counts - mean background counts) x 1000 = lymphocytes/ml

lymphocytes/ml x 5 ml = total cells

2.5.1 Design of FACS® Assay

The purpose of the FACS® assays was to determine the phenotypic state of adoptively transferred $CD8^{+}TG^{+}$ CFSE labelled T cells following experiments where adoptively transferred animals were exposed to various forms and routes of alloantigen exposure. Separate tissues from experimental animals were individually prepared, counted, stained for FACS®, and analysed. Cells stained for surface activation markers and intracellular cytokine production came from the same tissue. When there were insufficient cells from spleens or lymph nodes to perform a full

analysis, the number of cytokine or activation analyses were selectively reduced. Due to the low number of GIC isolated from individual hearts, it was occasionally necessary to pool GIC from identically treated animals.

Adoptively transferred cells were identified by positive staining for CD8 and the relevant clonotypic monoclonal antibody. $CD8^+TG^+$ T cells were gated and analysed for several parameters. The percentage of $CD8^+TG^+$ T cells of total cells was determined making possible the absolute enumeration of $CD8^+TG^+$ T cells. Gated $CD8^+TG^+$ T cells were analysed for division history by analysing the loss of CFSE (FL-1) fluorescence. Gated $CD8^+TG^+$ T cells were acquired and analysed for surface activation markers, and intracellular cytokine production. Between 250 and 10,000 $CD8^+TG^+$ T cell events were acquired and analysed for the vast majority of samples.

2.5.1.1 Surface Staining

Four colour FACS® analysis was performed. 1.5 – 3.0 x 10^6 cells were washed in PBS containing 10% (v/v) FCS and 0.1% (w/v) NaN_3 (washing buffer), centrifuged and incubated with directly conjugated monoclonal antibodies in washing buffer containing 20% mouse serum for 60 minutes at 4^OC in the dark. The cells were then washed and centrifuged once before incubation with Streptavidin-Cychrome™ in washing buffer/20% mouse serum for 15 minutes at 4^OC in the dark. Samples were then fixed in 2% (v/v) formaldehyde in washing buffer and stored at 4^OC until acquisition.

2.5.1.2 Intracellular Cytokine Staining

For intracellular cytokine evaluation[237] cell preparations from individual tissues were stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) for 4 hours at 37^{O}C. Brefeldin A (10μg/ml) was added for the final 2 hours of culture. The samples were then stained for the presence of cell surface antigens with directly conjugated anti-CD8-APC and biotinylated Désiré-1 in PBS/1% FCS (v/v) containing Brefeldin A for 30 minutes at 4^{O}C in the dark followed by Streptavidin Cy-Chrome™ for 15 minutes at 4^{O}C in the dark. Samples were fixed in 2% (v/v) formaldehyde in washing buffer/Brefeldin A and stored overnight at 4^{O}C. The following day the samples were washed in washing buffer and preincubated in permeablisation buffer (PBS/1% (v/v) FCS/0.1% (w/v) NaN3/0.5% (w/v) Saponin (SIGMA)) mixed with either anti-IL2-PE (2.5μg/ml), anti-Interferon-γ-PE (5.0μg/ml), anti-IL-4-PE (5.0μg/ml), anti-IL-10-PE (5.0μg/ml), or an appropriate isotype matched PE conjugated monoclonal antibody for 30 minutes at 4^{O}C in the dark. After two washes in permeablisation buffer, the cells were washed in PBS/1% (v/v) FCS/0.1% (w/v) NaN3 without saponin to allow membrane closure.

2.5.2 FACS® Acquisition

A FACSort® and CellQuest® software (Becton Dickinson, UK) were used to acquire either 50,000 total events or between 250 and 10,000 $CD8^{+}TG^{+}$ events for individual phenotypic analyses. Analysis was performed using either CellQuest® or WinMDI (Joseph Trotter, Scripps Institute, La Jolla, California, USA) software.

2.6 Histological Techniques

Mouse hearts, spleens or lymph nodes were harvested from anaesthetised animals and snap frozen in tissue-tek O.C.T. by submersing in liquid nitrogen. 7μm sections were cut from snap frozen tissue embedded in tissue-tek OCT (Miles Laboratories) using a cryostat (Reichart) at -30^{O}C. Cut sections were removed directly from the blade of the microtome onto glass slides at room temperature. The slides were air dried overnight, then fixed in acetone (BDH) for 10 minutes at room temperature immediately before staining. Approximately 100μl of the appropriate mAb solution was applied to each section and was incubated for 60 minutes in the dark. Sections were then incubated with avidin biotin peroxidase complex from Vector Labs, LTD for 45 minutes. This was followed by the DAB reagent from Vector labs, Ltd. The sections were then stained with Gills Haematoxylin No 2 (BDH) and rinsed in tap water followed by dehydration in several ethanol baths. The sections were then treated with xylene before DPX and a cover slip were applied.

2.7 Mixed Lymphocyte Culture

This assay was used to measure the proliferative response of T cells to allogeneic splenic stimulators. Responder and stimulators cells were prepared in R^{10} with 2.5 x 10^{-5} M 2-mercaptoethanol. 1 x 10^5 mesenteric lymph node cell responders in 100 μl were cultured with 2 x 10^5 irradiated (3000 rad, ^{137}Caesium source) splenocyte stimulators in 100μl in 96 well U bottom plates. T cell proliferative responses were measured by ^{3}H-thymidine incorporation (0.5μCi/μl into each well) on the indicated days. Cells were harvested after 18 hours onto a glass fibre printed filtermat A and

the incorporated ^{3}H-thymidine measured using a betaplate counter. The results are expressed as mean counts per minute (CPM) values ±SEM of triplicate wells.

2.8 Materials

2.8.1 Monoclonal Antibodies

Monoclonal antibodies used in this thesis were purchased in conjugated form or produced in the NDS tissue culture facility. Hybridomas were grown as spent tissue culture supernatants in R[10]: RPMI 1640 (GIBCO) media supplemented with 10% (v/v) FCS (GIBCO), 2mM L-glutamine, 45μg/ml penicillin (GIBCO), 45μg/ml streptomycin sulphate (GIBCO), 90μg/ml kanamycin sulphate (GIBCO). These hybridomas were expanded at a constant concentration of 2.0 x 10^5 cells/ml until the desired volume of supernatant had been reached. The cultures were allowed to grow to exhaustion that was defined by less than 80% cell viability. The supernatants were centrifuged (1200 rpm for 7 minutes) prior to filtration through a 22μm filter (Cat No. GSWP 04700, Millipore Ltd. Watford, U.K.) and purification[238].

Hybridomas were obtained from the ATCC® (Rockville, Maryland, USA) or as kind gifts from Professor Bernd Arnold (Clone: B20.2.2), Dr. Andrew L. Mellor (Clones: 1B2, Désiré-1 and Ti98) and Professor Herman Waldmann (Clones: YTA 3.1, YTS 177.9, YTS 191.1 and YTS 169.4).

2.8.1.1 Purification of Monoclonal Antibodies

Monoclonal antibody preparations were purified on a Beckman high performance liquid chromatography system using a weak anion exchange column (Sperogel TSK DEAE-5PW). Absorbance was measured at 280nm using a Beckman 160 Absorbance Detector. 200µl samples were run at 1.0ml/min over a gradient between solvent A (0.02M Tris pH 8.0) and solvent B (1.0M NaCl) such that %B went from 0% to 40% in 20 minutes. The antibody peak, usually fraction 2 was collected and concentrated to 1mg/ml and stored at $-20^{O}C$.

2.8.1.2 Biotinylation of Monoclonal Antibodies

Biotin is water-soluble and can be easily conjugated to proteins if derivatized using succinamide ester. The compound N-hydroxy-succinamidobiotin was used for biotinylation of purified monoclonal antibodies.

Purified monoclonal antibodies were extensively dialysed in 0.1 M $NaHCO_3$ 4 to 7 days prior to use and the protein concentration was adjusted to 1mg/ml with $NaHCO_3$. The biotin succinamide ester was dissolved at 1mg/ml in DMSO and 120µl added per ml of protein. The mixture was mixed thoroughly and left at room temperature for 4 hours. Biotinylated protein was dialysed overnight against PBS.

2.8.1.3 Monoclonal Antibodies Used

Specificity	Clone	Species	Source	Conjugate
2C.TCR[239]	1B2	Mouse IgG_1	NDS	Biotin
DES.TCR[227]	Désiré-1	Mouse IgG_{2a}, κ	NDS	Biotin
DES.TCR[240]	B20.2.2	Rat IgG_{2b}	NDS	Ø
(BM3.3).TCR[229]	Ti98	Mouse	NDS	Biotin
CD4[241]	RM4-5	Rat IgG_{2a}, κ	Pharmingen	PE
CD4[242]	YTA 3.1	Rat IgG_{2b}	NDS	Ø
CD8[243]	53-6.72	Rat IgG_{2a}, κ	Pharmingen	APC/PE/PerCP
CD25[244]	3C7	Rat IgG_{2b}, κ	Pharmingen	PE
CD25[245]	PC61	Rat IgG_{2b}, κ	Pharmingen	PE
CD44[246]	IM7.8.1	Rat IgG_{2b}, κ	Pharmingen	PE
CD45/B220[247]	RA3-6B2	Rat IgG_{2a}, κ	Pharmingen	FITC
CD45RB[248]	C363.16A	Rat IgG_{2a}, κ	Pharmingen	PE
CD62L[135]	MEL-14	Rat IgG_{2a}, κ	Pharmingen	PE
CD69[131]	H1.2F3	Hamster IgG	Pharmingen	PE
CD95L[249]	MFL3	Hamster IgG, group 1, κ	Pharmingen	Ø
H-2I-$A^{b,d,q}$ H-2I-$E^{d,k}$[250]	M5/114.15.2	Rat IgG_{2b}, k	NDS	Ø
H-$2K^b$[251]	AF6-88.5.3	Mouse IgG_{2a}, k	Pharmingen	PE
H-$2L^d$[252]	28-14-8S	Mouse IgG_{2a}, κ	NDS	Biotin
Interferon-γ[253]	XMG1.2	Rat IgG_1	Pharmingen	PE
Isotype (IgG_1)[241]	R3-34	Rat IgG_1	Pharmingen	PE
Isotype (IgG_{2b})[241]	R35-38	Rat IgG_{2b}, κ	Pharmingen	PE
IL-2[254]	JES6-5H4	Rat IgG_{2b}	Pharmingen	PE
IL-4[255]	11B11	Rat IgG_1	Pharmingen	PE
IL-10[256]	JES5-16E3	Rat IgG_{2b}	Pharmingen	PE
Mouse IgG_1 (H)[257]	LO-MG1-2	Rat IgG_1	Serotec MCA336B	Biotin
Mouse IgG_{2a} (H)[257]	Ø	Rat	SerotecMCA421B	Biotin
Rat IgG (H+L)[258]	Ø	Mouse	Jackson 212-095-082	HRP

2.8.2 Solutions

FACS® washing buffer

PBS was the base of FACS® buffer to which 2.0% (v/v) heat inactivated foetal calf serum was added and 1.0% (w/v) sodium azide (NaN_3)

Phosphate buffered saline (PBS)

Phosphate buffered saline solution, pH 7.3 was made by adding ten tablets of Dulbecco's A (Oxoid Ltd., U.K.) in each 1000ml of distilled water.

Sodium chloride	8.0g/L
Potassium chloride	0.2g/L
Disodium hydrogen phosphate	1.15g/L
Potassium dihydrogen phosphate	0.1g/L

Tris-buffered ammonium chloride (TBAC)

TBAC was used for the lysis of erythrocytes. 90ml of 0.16M NH_4Cl was added to 10ml of 0.17M Tris base and adjusted pH to 7.2 using 1M HCl.

Tris buffered saline (TBS)

30.28 g of Tris and 8.77 g of NaCl were added to 1 litre ddH_2O. The final solution was 0.25M Tris and 0.15 M NaCl.

^{3}H-thymidine

Stock ^{3}H-thymidine (1mCi/ml) (Amersham Ltd., U.K.) was diluted with PBS to 0.5μCi/ml. 1μl of this solution (0.5μCi) was added to each tissue culture well 96-well U-bottom plates (Life Technologies) in mixed leukocyte cultures (MLC).

Trypan Blue

A 0.05% solution of trypan blue (Sigma) was prepared and maintained by Mr. Gareth Plant and was diluted to 0.025% for use in cell counting. This vital stain is excluded by viable cells, stains dead cells and was used for the assessment of cell viability

2.8.3 Cell Media

R^0 RPMI 1640 (Roswell Park Memorial Institute 1640 medium)

R^{10} RPMI 1640 (GIBCO - 22511 - 026)

L-glutamine (BDH) 2mM
Penicillin (Sigma - P3032) 45μg/ml
Streptomycin Sulphate (GIBCO - 11860 - 038) 45μg/ml
Kanamycin Sulphate (GIBCO - 11815 - 032) 90μg/ml
Foetal calf serum (GIBCO - 10106 - 151) 10%

Antibiotics

Streptomycin sulphate, penicillin G and kanamycin sulphate (GIBCO) were added in tissue culture media at final concentrations of 45μg/ml, 75μU/ml and 90μg/ml, respectively. They were stored at –20^{O}C in a 1000x stock solution.

Foetal Calf Serum (FCS) Heat Inactivated

Virus and mycoplasma screened FCS (GIBCO - 10106 - 151) was thawed, and incubated at 56^{O}C for 30 minutes in order to inactivate the complement components of the serum. Inactivated serum was stored at -20^{O}C until use.

L-glutamine

L-glutamine (BDH) was added in tissue culture media at final concentration of 2mM. It was stored at -20^{O}C in a 100x stock solution.

Figure 2.1 Explantation of the Donor Heart

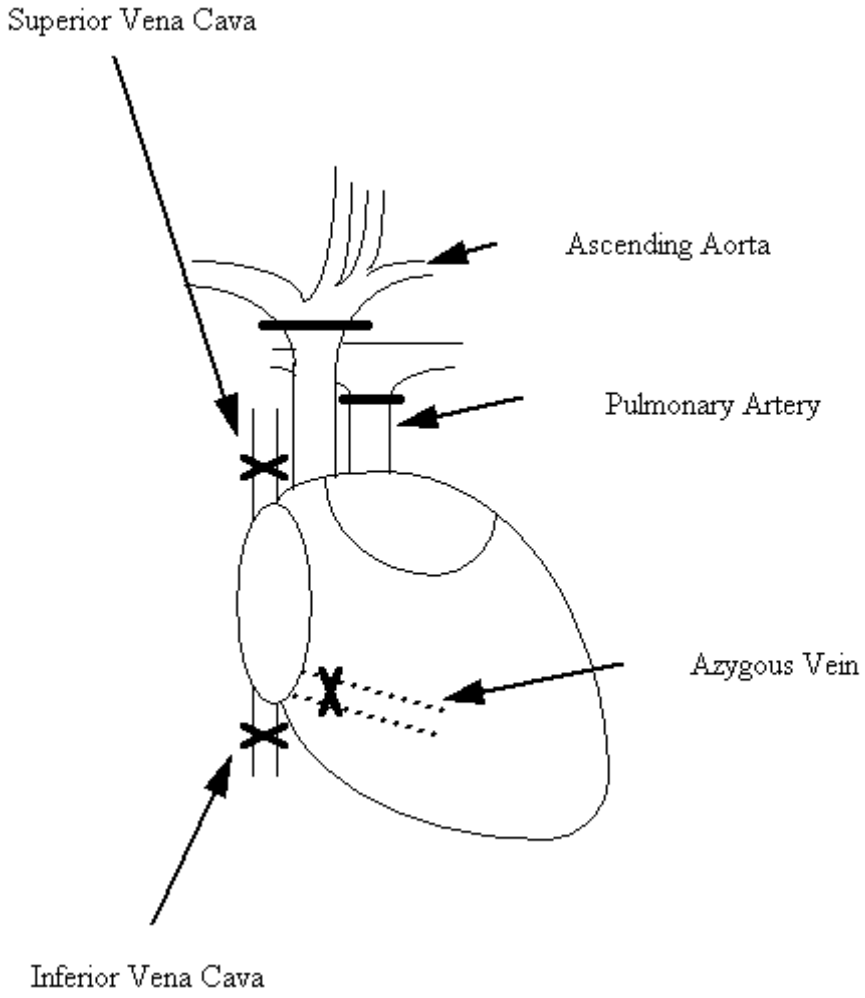


Figure 2.1 Following heparinisation and exsanguination of the donor the azygous vein and the superior & inferior vena cava and pulmonary veins (not shown) were ligated and divided. The ascending aorta and pulmonary artery were simply divided at a length appropriate for subsequent anastamosis in the recipient.

Figure 2.2 Implantation of the Donor Heart in the Recipient

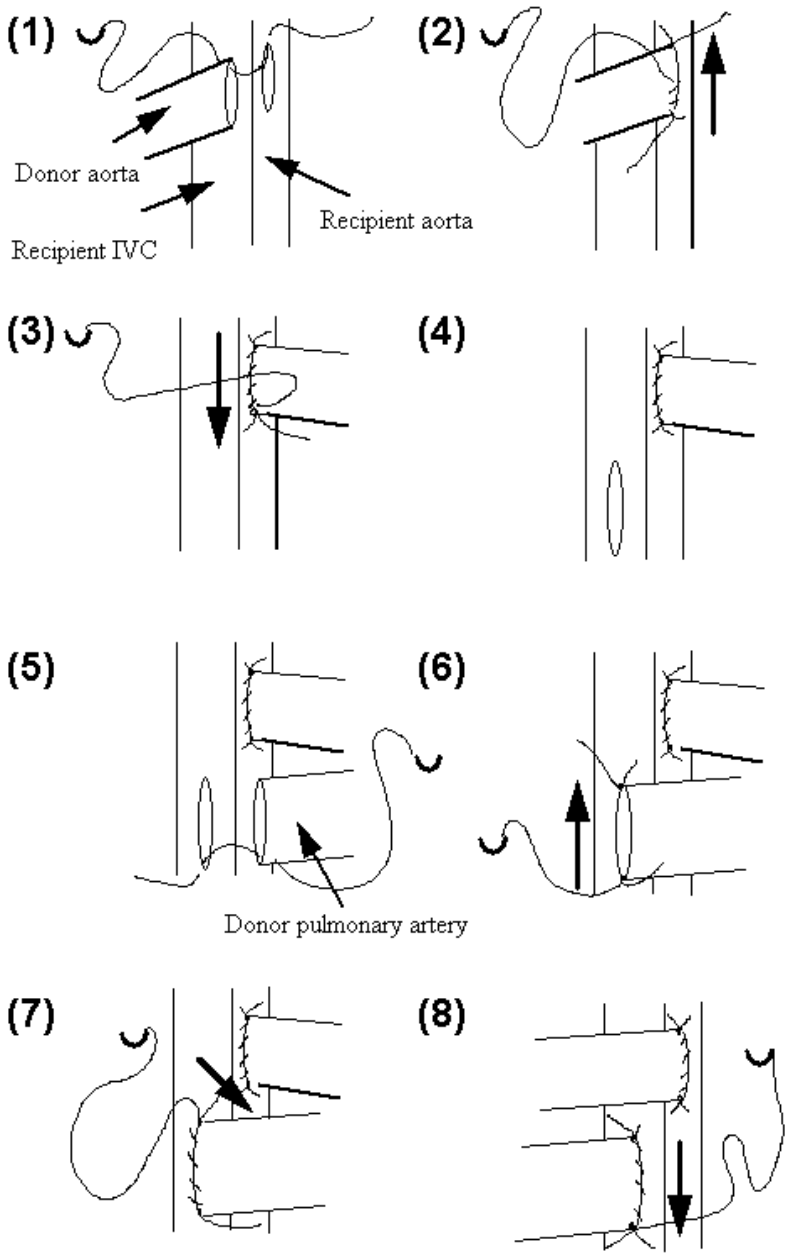


Figure 2.2 Following placement of the proximal and distal clamps occluding the recipient abdominal aorta and vena cava, an aortotomy was performed to which the donor ascending aorta was anastamosed. A venotomy was then performed to which the donor pulmonary artery was anastamosed. The distal and the proximal cross clamp were released sequentially, the donor heart filled with blood and began to contract.

Chapter 3 - Establishment of the Adoptive Transfer Model

3.1 Introduction

In the study of T cell responses to alloantigens, investigators up to this point have hoped for more physiologic models that can better show how T cells respond in vivo. Allogeneic mixed lymphocyte cultures and cytotoxicity assays have been used reliably to dissect T cell biology. However, the unnatural "milieu" of tissue culture medium and the conditions required to initiate T cell activation in vitro have been viewed as artificial. The generation of techniques permitting sustainable clones of helper and cytotoxic alloreactive T cells to be developed facilitated the examination of many aspects of T cell responses. Furthermore, the isolation of rearranged TCR genes[230,232,233] from these well characterised clones resulted in production of TCR transgenic mice[201-203] that were again of great use to transplant as well as other immunologists. These TCR transgenic animals, however, have a T cell repertoire dominated by a single specificity often accompanied by a paucity of effective $CD4^+$ or $CD8^+$ effector T cell function. The responses shown by these animals have often been questioned due to the extraordinarily high precursor frequencies that they possess. No doubt, investigators have long desired techniques that would allow the identification of T cells with known specificities as they responded in vivo in normal animals. Identification would allow for the tracking and phenotyping of these cells.

Adoptive transfer strategies can achieve this goal by making use of T cells from TCR transgenic mice specific for certain alloantigens. The advantages to such an approach are:

(1) There is a large body of experience using TCR transgenic mice and the original T cell clones from which their TCR's were derived.

(2) The TCR's themselves have been well characterised.

(3) Monoclonal antibodies against the clonotypic TCR's exist.

(4) X-ray crystal structures exist for some of these TCR's[52]

By returning TCR transgenic T cells to normal mice, a normal "milieu" for T cell interactions can be recreated. Here the single specificity that the transgenic T cells represent makes up a trace population among a diverse peripheral T cell repertoire, including $CD4^+$ helper T cells, where they must compete and co-operate with the other cells of the immune system to become activated and develop effector function.

Adoptive transfer was established in our laboratory as a new technique. Our strategy was based upon the models developed by Moskophidis et al.[204], Kearney et al.[205] and subsequently used by many others[77,78,132,155,205,259-286]. What we have done differently is to use this strategy to examine T cell responses against a vascularised cardiac allograft model in a MHC class I mismatch situation. We were fortunate in having several $CD8^+$ TCR transgenic strains specific for two different class I alloantigens. These mice were on a background syngeneic with our most common transplant recipient, CBA/Ca ($H\text{-}2^k$). The model also allowed us to examine of the role of cytotoxic T cells in a variety of transplant models that were already established in our laboratory[214-216]. With these considerations in mind, it

was left for us to show that the adoptive transfer model, the TCR transgenic strains, and the transplantation protocols could function together in this new experimental system to yield interpretable observations.

3.2 Results

3.2.1 Confirmation of H-2K^b and H-2L^d Expression

Our intention was to study CD8$^+$ cytotoxic T cell interactions with specific MHC class I alloantigens expressed by a number of different strains. Therefore, closer examination of the source of alloreactive cytotoxic T cells (DES, 2C & BM3.3 mice) and the mouse strains expressing the alloantigens to which they respond (H-2K^b & H-2L^d) was warranted.

First, we confirmed alloantigen expression by the strains of mice we proposed to use in our experiments. Spleen, mesenteric and bone marrow cells from different mouse strains were stained with monoclonal antibodies specific for H-2K^b (AF6-88.5.3) and H-2L^d (28-12-8S) and analysed by FACS® on 2 separate occasions. Strains known to be H-2K^{b+} (C57BL/10 and the transgenic line CBK), H-2L^{d+} (BALB/c), H-2K^{b-} H-2L^{d-} (CBA/Ca & NZW) as well as F1 mice specifically bred in our facilities for these experiments (CBA/Ca x BALB/c F1) & (CBK x BALB/c F1) were analysed. Figure 3.1 shows representative results for splenocytes. Analysis of mesenteric lymph node cells and bone marrow cells gave similar results. H-2K^b expression was entirely consistent with expectations. However, staining with (28-12-8S) specific for H-2L^d was more difficult to interpret due to the known cross reactivity of the monoclonal antibody (28-12-8S) with a C57BL/10 (H-2^b) alloantigen, but not with NZW (H-2^z) alloantigens[252].

3.2.2 Characterisation of TCR Transgenic Mice

We investigated how introduction of rearranged TCR α and β gene constructs into the germline modified the T cell receptoire of CBA/Ca mice by determining (1) which T cell subsets expressed the clonotypic TCR and (2) in what way the peripheral T cell repertoire had been changed? The T cell repertoire of intact DES, 2C and BM3.3 mice was evaluated by FACS® and compared to that of normal CBA/Ca mice.

We first confirmed that the monoclonal antibodies (Désiré-1, 1B2 and Ti98) specific for the clonotypic TCR's of DES, 2C and BM3.3 mice respectively did not stain cells in naïve CBA/Ca mice (Figure 3.2). Non-specific staining of T cells in the spleens of CBA/Ca mice was below 0.05% of total cells when gating on either $CD4^+$ or $CD8^+$ T cells. Next splenocytes from age- and sex-matched DES, 2C and BM3.3 mice were stained with anti-CD4 (RM4-5), anti-CD8 (53-6.72) and the appropriate anti-clonotypic monoclonal antibody. FACS® analysis revealed that the three TCR transgenic strains had quite different T cell repertoires. We first observed that DES & BM3.3 mice produced roughly equal numbers of $CD4^+$ and $CD8^+$ T cells. However, 2C mice had 3 times more $CD4^+$ T cells than $CD8^+$ T cells (Figure 3.2).

$CD4^+$ T cells from TCR transgenic mice also expressed the clonotypic TCR to varying degrees. BM3.3, DES and 2C mice had approximately 1.5%, 3% and 7% $CD4^+$ clonotype positive T cells in their spleens respectively. The same mice had 14%, 7% and 2.5% $CD8^+TG^+$ T cells of total leukocytes respectively. These results suggested that BM3.3 mice provided the most abundant source of transgenic T cells for enrichment while 2C mice would be the least abundant.

To complement the above results we then asked whether we could show that T cells from intact TCR transgenic mice were specifically reactive to their relevant alloantigens in vitro. Mixed lymphocyte cultures were performed on two occasions using mesenteric lymph node cells from DES, 2C and BM3.3 mice as responders. Irradiated splenocytes from strains known to be H-2K^{b+} or H-2L^{d+} were used as stimulators. Figure 3.3 shows that DES cells proliferated to varying degrees in response to H-2K^{b+} stimulators in the following order CBK < (CBK x BALB/c F1) < C57BL/10. BM3.3 cells proliferated vigorously against all H-2K^{b+} strains. Neither DES nor BM3.3 showed proliferative responses against control CBA/Ca, H-2K^{b-} or H-2L^{d+} strains. In contrast, 2C cells proliferated much less strongly to H-2L^{d+} stimulators than either population of H-2K^{b} reactive transgenic T cells. 2C cells also showed a significant degree of cross reactivity against C57BL/10 splenocytes, but not against CBK (H-2^{k} + H-2K^{b}) cells indicating that this cross reactivity is not due to responsiveness against H-2K^{b}. In vivo results discussed later confirm these data and showed that CD4^{+}1B2^{+}, but not CD8^{+}1B2^{+} T cells proliferated and expanded in response to C57BL/10 alloantigens, (Figure 3.12).

3.2.3 Adoptive Transfer of CD8^{+}TCR Transgenic T Cells

The focus of all experiments performed in this thesis examined alloreactive CD8^{+} T cells that respond to MHC Class I alloantigens (H-2K^{b} or H-2L^{d}). However, the DES, 2C and BM3.3 TCR transgenic mice used as donors of CD8^{+}TG^{+} cytotoxic T cells for adoptive transfer also generated CD4^{+} T cells bearing the clonotypic TCR (Figure 3.2). In order to prevent difficulties in analysing and interpreting results, we chose to deplete bulk T cells from TCR transgenic mice of CD4^{+} T cells before

adoptive transfer. We achieved this goal by labelling T cells with a rat anti-mouse CD4 monoclonal antibody (YTA 3.1) before removing them with goat anti-rat Ig conjugated magnetic beads, as described in Chapter 2.

FACS® analysis was performed to determine the percentage of $CD8^{+}TG^{+}$ T cells and to assess depletion of $CD4^{+}TG^{+}$ T cells for every adoptive transfer experiment presented in this thesis. Figure 3.4 illustrates the FACS® of one such adoptive transfer. Each sample was stained with anti-CD4 (RM4-5, non-competing with YTA 3.1), anti-CD8 (53-6.72) and the monoclonal antibody specific for the clonotypic TCR (Désiré-1 in this case). Plots of pooled spleen and mesenteric lymph node cells from CBA/Ca, DES and CD4 depleted DES cells prepared for adoptive transfer are shown. Normal CBA/Ca mice have no T cells staining positive for Désiré-1, 1B2, or Ti98 and served as negative controls. Depletion of bulk DES cells with YTA 3.1 and magnetic beads successfully removed virtually all $CD4^{+}$ T cells leaving an average of 0.4% $CD4^{+}DES^{+}$ T cells in the adoptive transfer population. Based on the percentage of $CD8^{+}DES^{+}$ T cells determined by FACS® a known number of CFSE labelled $CD8^{+}DES^{+}$ T cells could then be adoptively transferred into recipient CBA/Ca mice.

3.2.4 Detection of Adoptively Transferred TCR Transgenic T Cells

2×10^{6} $CD8^{+}TG^{+}$ T cells were injected i.v. into each CBA/Ca mouse thus generating what we have termed CBA^{TG} (i.e., CBA^{DES}, CBA^{2C}, or $CBA^{BM3.3}$) mice. Next, we demonstrated that adoptively transferred T cells could be detected and gated upon for analysis among the normal and diverse T cell repertoire of naive CBA^{TG} mice. We found that by staining for CD8 and the clonotypic monoclonal antibody we

could consistently detect adoptively transferred T cells by FACS® in the spleens of CBA^{TG} mice (Figure 3.5). Five days after adoptive transfer of 2 x 10^6 $CD8^+DES^+$ T cells a discrete population of $CD8^+DES^+$ T cells could be detected in the spleens of CBA^{DES} mice. Not surprisingly, $CD4^+DES^+$ T cells could be identified. These findings confirmed that ex vivo depletion of $CD4^+$ T cells was complete and did not harm $CD8^+TG^+$ T cells that were adoptively transferred. To show that adoptively transferred cells were long-lived, we rested CBA^{DES} mice for 5, 30 & 100 days following adoptive transfer, harvested and analysed them by FACS®. DES cells were easily detectable for up to 100 days after adoptive transfer.

3.2.4.1 Enumeration of Adoptively Transferred TCR Transgenic T Cells

Satisfied that we were able to detect adoptively transferred $CD8^+TG^+$ T cells for extended periods of time, we developed a technique to enumerate the number of adoptively transferred T cells in each separately prepared tissue. At the time of tissue harvest, total cell counts were determined. The percentage of $CD8^+TG^+$ T cells of total cells was determined by FACS® analysis. The product of the percentage and the total cell count gave the absolute number of $CD8^+TG^+$ T cells detected in a given tissue. The mean absolute number of $CD8^+DES^+$ T cells detected 5 (n=12), 30 (n=4) and 100 (n=3) days after adoptive transfer to naïve CBA/Ca mice is shown in (Figure 3.6).

3.2.5 Homing of Adoptively Transferred T Cells to Secondary Lymphoid Tissues

We were interested in the homing behaviour of adoptively transferred $CD8^+DES^+$ T cells. The mean absolute number of $CD8^+DES^+$ T cells present in the various primary and secondary lymphoid tissues was therefore determined 5 days following adoptive transfer (n=7 CBA^{DES} mice). Figure 3.7 demonstrates that $CD8^+DES^+$ T cells had homed to the secondary lymphoid tissues and that virtually none homed to the thymus or to the bone marrow compartment at this time point.

3.2.6 Assessment of Phenotype

Thus far, we have focused on the enumeration and localisation of adoptively transferred $CD8^+TG^+$ T cells, however our primary objective in developing this model was visualising the different states of T cell activation following interaction with alloantigens expressed by a cardiac allograft. Consequently, we designed a FACS® assay that allowed evaluation of cell division and the cell surface and intracellular phenotype of adoptively transferred cells. In each assay, cells were stained for CD8 and with the appropriate clonotype monoclonal antibody specific for the transgenic TCR to enable the adoptively transferred $CD8^+TG^+$ T cells to be identified as shown in figure 3.8.

TCR transgenic cells were labelled with CFSE prior to adoptive transfer permitting the evaluation of cell division within the population of adoptively transferred T cells in CBA^{TG} mice. CFSE is a fluorescein-based molecule that when excited at the appropriate wavelength emits fluorescence detectable in the "green" FL-1 channel of a flow cytometer. It is a useful tool for tracking immune cells in vivo. After cell

labelling, CFSE molecules become trapped within cells due to their positive charge following cleavage by cytoplasmic esterases and their covalent binding to cytoplasmic proteins. These two properties ensure that CFSE remains within viable cells. However, CFSE is most useful in that it provides detailed information about the number of cell divisions that a particular cell has undergone. Since the cytoplasm of dividing cells is equally distributed between two daughter cells, so is the amount of CFSE. Thus each daughter cell has a "green" FL-1 fluorescence intensity equal to exactly half that of the original cell.

The approaches for determining the surface phenotype and cytokine production of the adoptively transferred $CD8^{+}TG^{+}$ T cells were similar although the actual staining techniques were different (Chapter 2). $CD8^{+}TG^{+}$ T cells were stained with monoclonal antibodies against surface activation markers (CD62L, CD69, CD45RB and CD44) and intracellular cytokines (interferon-γ, IL-2, IL-4 and IL-10) were specifically gated and acquired (Figure 3.8). Appropriate isotype controls were used.

3.2.7 $CD8^{+}TG^{+}$ T Cells Retain a Naïve Phenotype Following Adoptive Transfer

Five days after adoptive transfer the spleens of CBA^{DES} mice were harvested and analysed by FACS®. Each histogram in Figure 3.9 shows either a surface phenotypic marker, or cytokine production. The relevant isotype control monoclonal antibody is shown as an empty overlay for each characteristic determined.

The results indicated that adoptively transferred T cells remained in a resting state when transferred into naïve CBA/Ca mice.

3.2.8 $CD8^+TG^+$ T Cells Are Responsive to Alloantigens In Vivo

Having previously shown in vitro that TCR transgenic cells proliferated in response to alloantigens, it was left for us to demonstrate that adoptively transferred T cells were able to mount a measurable response to alloantigens in vivo.

Increasing doses of H-$2K^{b+}$ splenocytes were administered intravenously to CBA^{DES} mice. Five days later, $CD8^+DES^+$ T cells present in the spleens of these mice were analysed for division by determining the loss of CFSE fluorescence. Figure 3.10 clearly shows that the percentage of $CD8^+DES^+$ T cells that have divided once or more was directly related to the dose of allogeneic splenocytes administered. Secondly, that doses ranging from 0 to 1 x 10^7 H-$2K^{b+}$ splenocytes resulted in the full range of possible responses from virtually none to virtually all $CD8^+DES^+$ T cells having divided at least once.

In figure 3.11, a clear demonstration of clonal expansion by the adoptively transferred cells is shown. 4 x 10^6 CD4 depleted CFSE labelled $CD8^+DES^+$ T cells were transferred into H-$2K^{b-}$ (CBA/Ca x BALB/c F1) or H-$2K^{b+}$ (CBK x BALB/c F1) mice rather than normal CBA/Ca mice. Both of these strains are syngeneic with DES mice for all CBA/Ca alloantigens such that adoptively transferred cells are not rejected (see chapter 2). We hypothesised that maximal stimulation of adoptively transferred $CD8^+DES^+$ T cells would occur in (CBK x BALB/c F1) mice. This was found to be the case (Figure 3.11). Three days after adoptive transfer, the difference

between H-2K^{b+} and H-2K^{b-} hosts was striking. There was an eleven-fold increase in the absolute number of CD8$^+$DES$^+$ T cells present in the spleens of (CBK x BALB/c F1) mice (n=4) when compared to those adoptively transferred to (CBA/Ca x BALB/c F1) mice (n=4).

A similar experiment was performed with non-CD4 depleted CFSE labelled 1B2$^+$ T cells from H-2L^d specific 2C mice. 4 x 10^6 CD8$^+$1B2$^+$ and 7.1 x 10^6 CD4$^+$1B2$^+$ cells were transferred to H-2L^{d-} CBA/Ca (n=3), H-2L^{d+} (CBA/Ca x BALB/c F1) (n=3) and H-2L^{d-} (CBA/CA x C57BL/10 F1) (n=3). We chose not to CD4 deplete the adoptively transferred cells in this experiment to allow us to investigate the response of CD4$^+$1B2$^+$ T cells under conditions of maximal stimulation. The spleens were harvested three days after adoptive transfer and 1B2$^+$ T cells were analysed for proliferation and enumerated to determine clonal expansion. As for CD8$^+$DES$^+$ T cells there was significant clonal expansion (about 14 fold) of CD8$^+$1B2$^+$ T cells only when H-2L^d was expressed by the host (Figure 3.12). We have previously shown that 2C mice generate large numbers of CD4$^+$1B2$^+$ T cells (Figure 3.2) and that in standard allogeneic mixed leukocyte cultures bulk 2C T cells appear to mount a proliferative response against C57BL/10 alloantigens (Figure 3.3). CD4$^+$1B2$^+$ T cells transferred to CBA/Ca mice did not proliferate as indicated by analysis of CFSE profiles. However virtually identical division (85% dividing > once) and clonal expansion (approximately 5-6 fold) was observed when the cells were transferred into H-2L^{d+} (CBA/Ca x BALB/c F1) or H-2L^{d-} (CBA/Ca x C57BL/10 F1) hosts (Figure 3.13). This convincingly shows that CD4$^+$1B2$^+$ T cells are also reactive to at least one unknown C57BL/10 (H-2^b) alloantigen, most likely the MHC class II H-2IA^b, as well as to one or more BALB/c (H-2^d) alloantigens. The candidate BALB/c alloantigens could be the MHC class I H-2L^d (via CD8 independent recognition) or

another undefined MHC class II alloantigen. It is also possible that $CD4^+1B2^+$ T cells also recognise H-$2D^b$ independently of CD8 engagement. Despite the cross reactivity of $CD4^+1B2^+$ T cells, $CD8^+1B2^+$ T cells appeared to respond specifically against H-$2L^d$. Taken together with the previous in vitro data (Figure 3.3), it suggests that for the purposes of a third party control for 2C T cells that NZW would be a much better choice than C57BL/10.

Our main conclusion from these in vivo results is that $CD8^+TG^+$ T cells transferred into naïve host with a diverse T cell repertoire remain functional. We have been able to show that the adoptively transferred $CD8^+TG^+$ T cells can proliferate in response to antigen specifically and that the population of transferred cells can undergo massive clonal expansion under conditions of maximal stimulation. Taken together, these data confirm that adoptively transferred TCR transgenic T cells are fully responsive.

3.3 Discussion

In this chapter we have developed and assessed the suitability of an adoptive transfer model to examine the responses of alloantigen specific cytotoxic T cells in vivo during cardiac allograft rejection. We studied $CD8^+$ cytotoxic T cells derived from the TCR transgenic lines; DES, 2C and BM3.3 used in the generation of CBA^{DES}, CBA^{2C}, $CBA^{BM3.3}$ mice, generically termed CBA^{TG} mice. These mice were to be used in an attempt to answer questions about the role of cytotoxic T cells in already established models of allograft rejection and tolerance. Thus, we defined the reactivity of TCR transgenic cells precisely as previously unrecognised responses would prevent clear interpretation of the data we had obtained.

We have presented evidence that the model we have developed is sound. $CD8^+TG^+$ T cells were shown to be specific for their relevant alloantigens (Figure 3.3). They did not respond to the other alloantigens to which they will be exposed in the course of our experiments. $CD8^+TG^+$ T cells were long-lived in vivo despite the trauma of ex vivo CD4 depletion, CFSE labelling, and intravenous injection (Figure 3.6). We were able to detect, enumerate and characterise the adoptively transferred $CD8^+TG^+$ T cells, they homed to the secondary lymphoid tissues (Figure 3.7) and remained in a resting state as determined by their expression of surface activation markers and the absence of cytokine production (Figure 3.9). A graded proliferative response (Figure 3.10) to increasing doses of intravenously administered allogeneic splenocytes was observed and massive clonal expansion (Figure 3.11) in vivo confirmed the functional status of adoptively transferred $CD8^+DES^+$ and $CD8^+1B2^+$ T cells.

We were fortunate in having two H-2K^b specific TCR transgenic strains to choose from for our experiments. DES and BM3.3 mice differ not only in that they were derived from different H-2K^b specific cytotoxic T cell clones, but also in their functional characteristics. DES TCR transgenic cells have been shown to be CD8 dependent while BM3.3 cells have been shown to be CD8 independent[287] referring to the need for simultaneous interaction between CD8 on the T cell and H-2K^b on the target cell. Although this interaction is not an absolute requirement for the activation of CD8$^+$ T cells, it is accepted that it represents a necessary physiologic interaction that can be thought of as enhancing the interactions between TCR and MHC resulting in T cell activation. However, BM3.3 cells become activated in the absence of this interaction, suggesting that the affinity of the BM3.3 clonotypic TCR's for H-2K^b is higher. DES cells on the other hand require this interaction for effective activation. In the diverse repertoire of normal CBA/Ca mice, it is likely that there exists a random collection of CD8$^+$ T cells specific for H-2K^b constructed of many different V_α and V_β subunits with a spectrum of affinities for H-2K^b. DES and BM3.3 T cells lie at different points along this spectrum. This diversity of TCR's specific for a single alloantigen must in part be considered when designing an adoptive transfer model to be used in a transplantation model.

The in vitro experiments showed that BM3.3 and DES cells proliferated specifically against H-2K^{b+} strains (Figure 3.3). However, the responses were not identical. DES cells displayed a wide spectrum of proliferative responses, the lowest being against CBK which only expressed H-2K^b, to the greatest response against the fully allogeneic C57BL/10 (4.5 fold greater than CBK) also expressing other C57BL/10 alloantigens in addition to H-2K^b. BM3.3 cells showed a much narrower spectrum of responsiveness and proliferated vigorously against all H-2K^{b+} strains. This

disparity between DES and BM3.3 may be explained by their differential requirements for CD8 engagement, affinities for H-2K^{b}, and requirement for CD4^{+} T cell help[288]. Nonetheless, our observations would suggest that BM3.3 cells lie at one extreme of the spectrum of natural T cells specific for H-2K^{b}. Therefore, DES cells that are able to respond to different degrees under different conditions may potentially be more representative of the responses of the diverse population of H-2K^{b} specific T cells in normal animals as a whole.

We did not have a choice of TCR transgenic lines specific for H-2L^{d}. 2C TCR transgenic mice turned out to be the poorest source of CD8^{+}TG^{+} T cells for adoptive transfer due to the large number of CD4^{+}1B2^{+} and the small number of CD8^{+}1B2^{+} T cells that were generated on a CBA/Ca (H-2^{k}) background. 2C mice used by the majority of investigators are on a C57BL/6 (H-2^{b}) background and this difference may explain our observation. The haplotype of the TCR transgenic mice has a great effect on the eventual T cell repertoire. It is known that the 2C TCR positively selects well on H-2K^{b}[289], which is absent on a CBA/Ca (H-2^{k}) background. Hence, positive selection must occur on different H-2^{k} alloantigens. The generation of large numbers of CD4^{+}1B2^{+} T cells suggests that positive selection of 1B2^{+} T cells in the thymus occurs efficiently on a MHC class II molecule in CBA/Ca mice, either H-2IA^{k} or H-2IE^{k}. However, positive selection leading to CD8^{+} H-2L^{d} specific cells must be much less efficient (Figure 3.2).

The known affinity of the 2C TCR for H-2K^{b} was a concern. The 2C TCR recognises four known peptides (p2Ca, QL9, SL9, and P1A)[290-294] presented by H-2L^{d} and dEV8[295] presented by H-2K^{b} and H-2K^{bm3}. All of these peptides are

derived from ubiquitous mitochondrial proteins[290,291,295,296]. The initial allogeneic mixed lymphocyte cultures showed that bulk 2C cells responded to at least one C57BL/10 alloantigen in addition to H-2L^{d}, an observation that warranted further investigation. Experiments where non-CD4 depleted 2C cells were adoptively transferred in H-2L^{d+} or H-2L^{d-} mice helped clarify the situation (Figure 3.13). CD4^{+}1B2^{+} T cells were shown to be responding to C57BL/10 alloantigens. However, CD8^{+}1B2^{+} T cells did not proliferate or expand to C57BL/10 antigens. This observation may be explained by the fact that the 2C TCR may be CD8 dependent in the context of C57BL/10 alloantigens as are CD8^{+}DES^{+} cells in their interactions with H-2K^{b}.

Despite the lower proliferative responses, less favourable CBA/Ca (H-2^{k}) background, and large numbers of CD4^{+}1B2^{+} T cells we could not show that CD8^{+}1B2^{+} T cells responded to H-2K^{b}. However, it has been shown that the dEV8 peptide binds effectively to H-2K^{b} and at high concentration, this complex induces strong cytolytic activity by CD8^{+}1B2^{+} T cells[297]. We did however uncover an interesting and potentially useful property of CD4^{+}1B2^{+} T cells. Further characterisation would be required, but they may be useful as a H-2^{b} class II specific TCR transgenic in future experiments.

From the experience gained in developing this adoptive transfer model we realised that biological and technical variability in the generation and analysis of CBA^{TG} might interfere with comparing data between experiments. Therefore, each experiment was designed carefully to minimise this potential problem as follows:

1. Each individual experiment was designed such that naïve CBA^{TG} mice were included as a control group providing a direct comparison for treated/experimental CBA^{TG} mice.

2. All recipient CBA/Ca mice receiving $CD8^{+}TG^{+}$ T cells were closely age-matched within each experiment.

3. CD4 depletion and adoptive transfer was performed on the same day for all CBA^{TG} animals to be used in a given experiment.

4. Any treatments or transplants performed on CBA^{TG} mice for a given experiment were all performed on the same day with age-matched donors or with reagents prepared at the same time.

5. Harvest and FACS® staining of all CBA^{TG} mice in a given experiment were performed on the same.

6. Appropriate isotype control antibodies were used for every tissue in the FACS® analyses in every experiment.

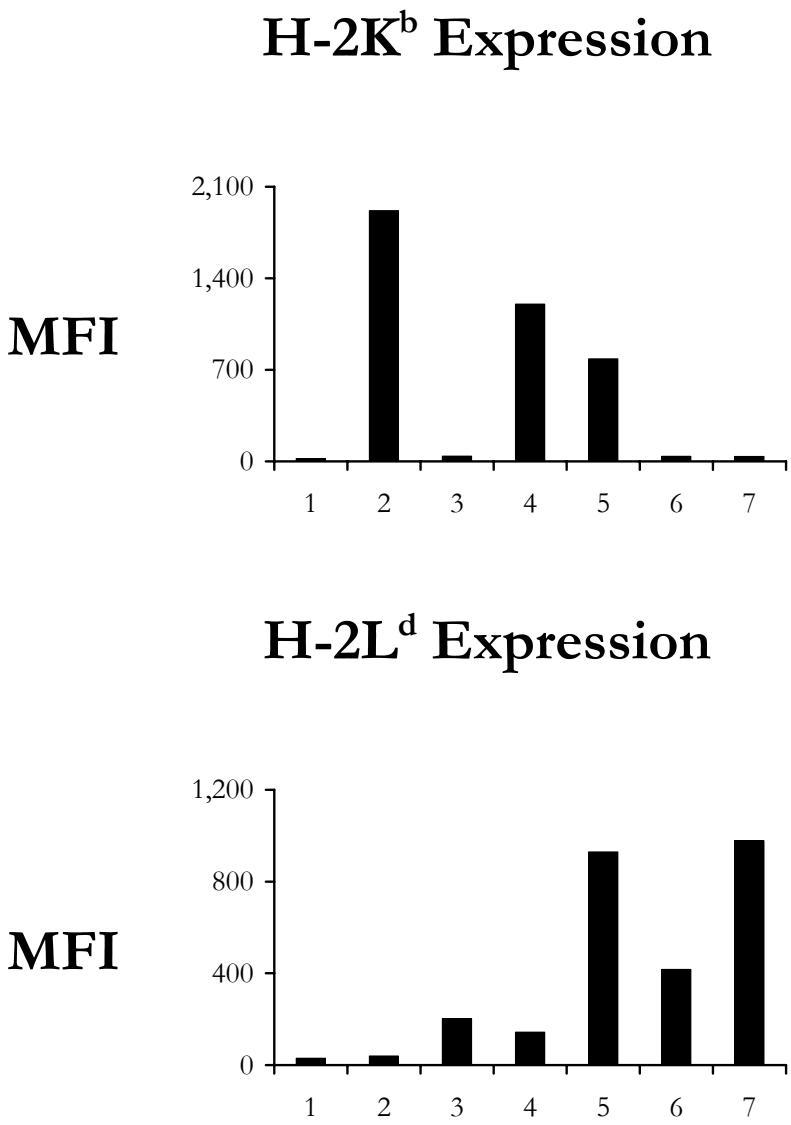
7. The entire FACS® acquisition for all samples from a given experiment were performed at one sitting on the FACS® machine.

With these measures in place, we were able to limit the complexity and variability of these experiments such that we could make valid comparisons between experiments. This permitted us to draw conclusions from a large number of different studies.

The data presented in this chapter established that the goals of the thesis were feasible. Others have successfully used similar models to answer different questions, however we are among the first to use this type of adoptive transfer model in transplantation. Adoptive transfer will allow us to add to the body of knowledge already generated by in vitro assays and to study T cell alloresponses in vivo while avoiding the peculiar T cell repertoire of intact TCR transgenic mice dominated by a single clonotypic TCR. Taken together, the results presented in this chapter were encouraging, they did highlight important issues that enabled us to make decisions about the experimental design of the later part of this study. In conclusion, we have developed an experimental model where the immune response of alloreactive $CD8^{+}$

T cells can be visualised directly and studied in vivo. We have explored the limits of the capabilities of the experimental system and have shown that our goals are feasible and the model sound.

Figure 3.1 Determination of H-2K^b and H-2L^d Surface Expression in Spleen



LABEL	STRAIN
1	CBA/Ca
2	CBK (H-2K^{b+})
3	(CBA/Ca x BALB/c F1) (H-2L^{d+})
4	(CBK x BALB/c F1) (H-2K^{b+}/H-2L^{d+})
5	C57BL/10 (H-2K^{b+})
6	BALB/c (H-2L^{d+})
7	NZW

Figure 3.1 Spleen, mesenteric lymph node and bone marrow cells from the indicated strains were stained with anti-H-2K^b and anti-H-2L^d monoclonal antibodies and analysed by FACS®. Surface expression of H-2K^b and H-2L^d was represented as the mean fluoresence intensity. The data from splenocytes are shown and are representative of the surface expression of bone marrow and mesenteric lymph nodes cells. The analysis was repeated twice yielding similar results.

Figure 3.2 Phenotype of TCR Transgenic Mice

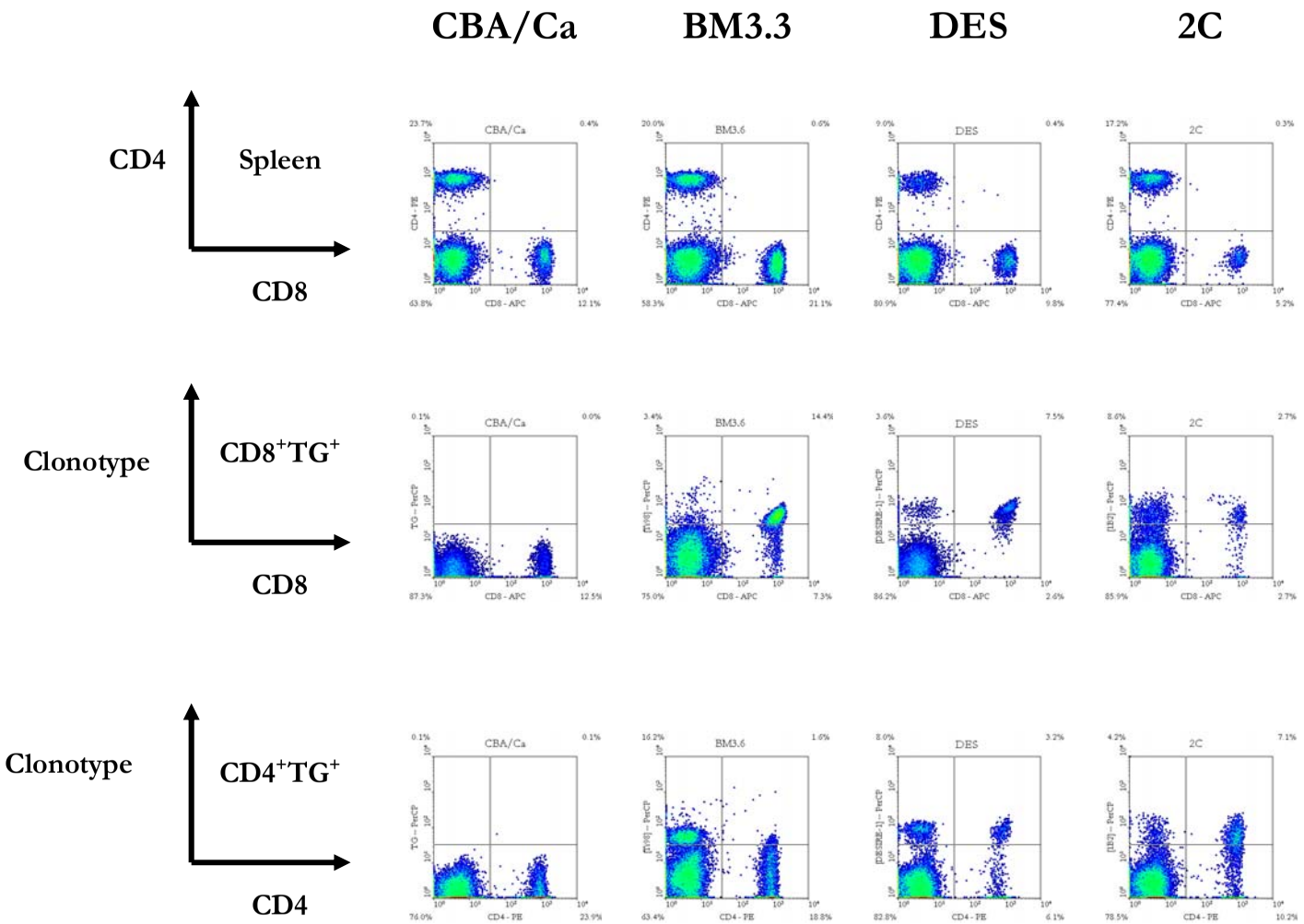
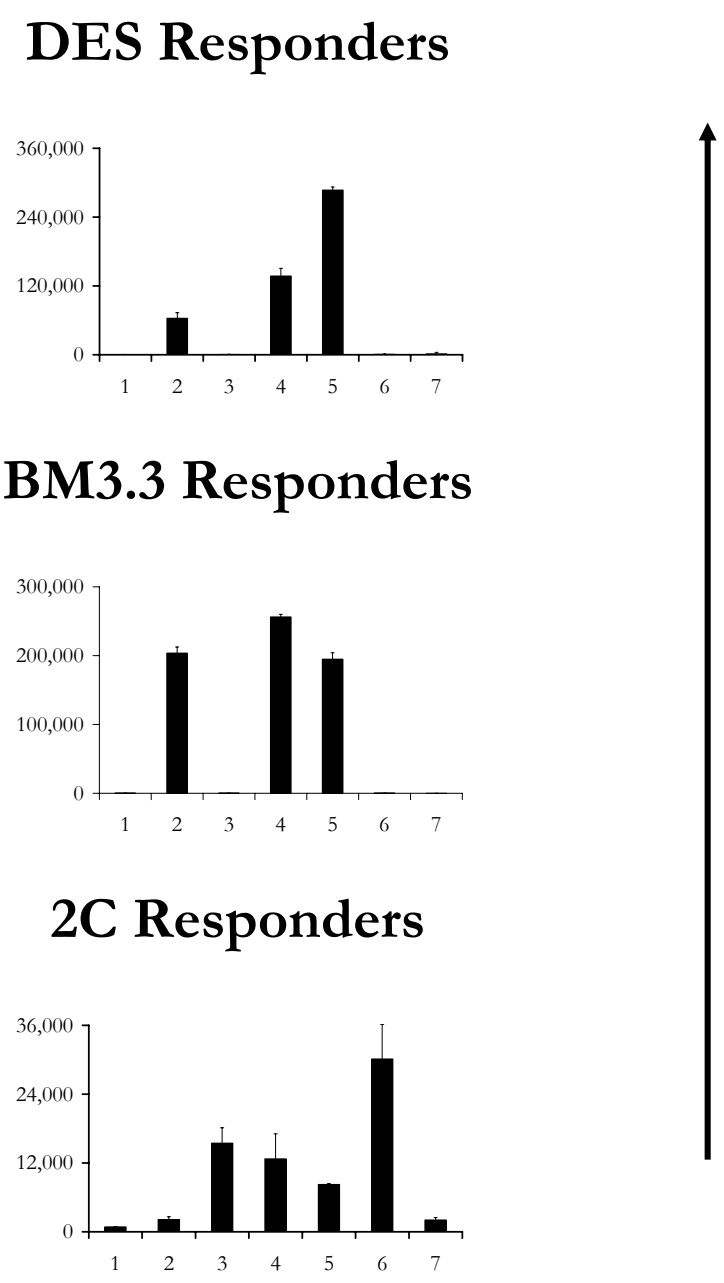


Figure 3.2 The peripheral T cell repertoires of TCR transgenic mice were compared to that of age- and sex-matched CBA/Ca mice. Splenocytes from the indicated strains were analysed by FACS® for the surface expression of their respective transgenic TCR's. Splenocytes from CBA/Ca, BM3.3, DES, and 2C mice were stained with anti-CD4, anti-CD8 and anti-clonotypic antibody and were analysed by three colour FACS® analysis. The percentage of total cells in each quadrant is shown. The TCR transgenic strains possessed a varying percentage of $CD8^+TG^+$ T cells among spleen cells. However, $CD4^+TG^+$ T cells were also present. The data shown are representative of 3 BM3.3, and >3 2C and DES analyses.

Figure 3.3 In Vitro Reactivity Against H-2K^{b+} and H-2L^{d+} Stimulators



	LABEL	STRAIN
Stimulators	1	CBA/Ca
	2	CBK (H-2K^{b+})
	3	(CBA/Ca x BALB/c F1) (H-2L^{d+})
	4	(CBK x BALB/c F1) (H-2K^{b+}/H-2L^{d+})
	5	C57BL/10 (H-2K^{b+})
	6	BALB/c (H-2L^{d+})
	7	NZW

Figure 3.3 In vitro proliferative responses of DES, BM3.3 and 2C responder mesenteric lymph node cells (1 x 10^5 cells/well) against 3000 rad irradiated splenocyte stimulators (2 x 10^5 cells/well) from the indicated strains are shown. T cell proliferative responses were measured by ^{3}H-thymidine incorporation on the 4th day of culture. The results represent the mean CPM values +SEM of triplicate wells. The MLC were performed on two occasions with similar results.

Figure 3.4 Negative Sorting of CD8$^+$ T Cells

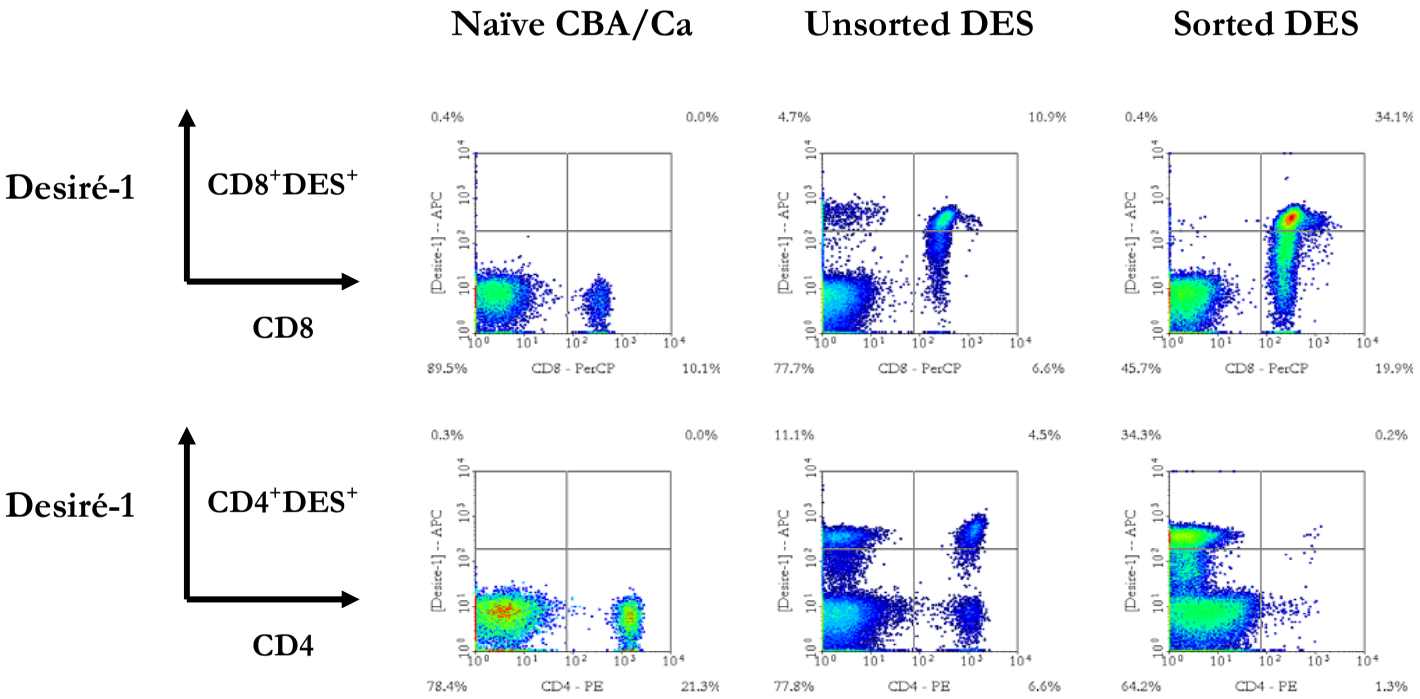


Figure 3.4 Pooled spleen and mesenteric lymph node cells from TCR transgenic mice were negatively sorted with goat anti-rat BioMag® magnetic beads and purified anti-CD4 monoclonal antibody. FACS® analysis was performed on every occasion prior to adoptive transfer in order to confirm depletion of CD4$^+$DES$^+$ T cells and to count the number of CD8$^+$DES$^+$ T cells being transferred. A representative example is shown of a negative sort of DES lymphocytes. The percentage of cells in each quadrant is shown. Depletion of CD4$^+$ T cells was nearly complete preventing the adoptive transfer of CD4$^+$DES$^+$ T cells.

Figure 3.5 Detection of Adoptively Transferred Cells in CBA^{DES} Mice

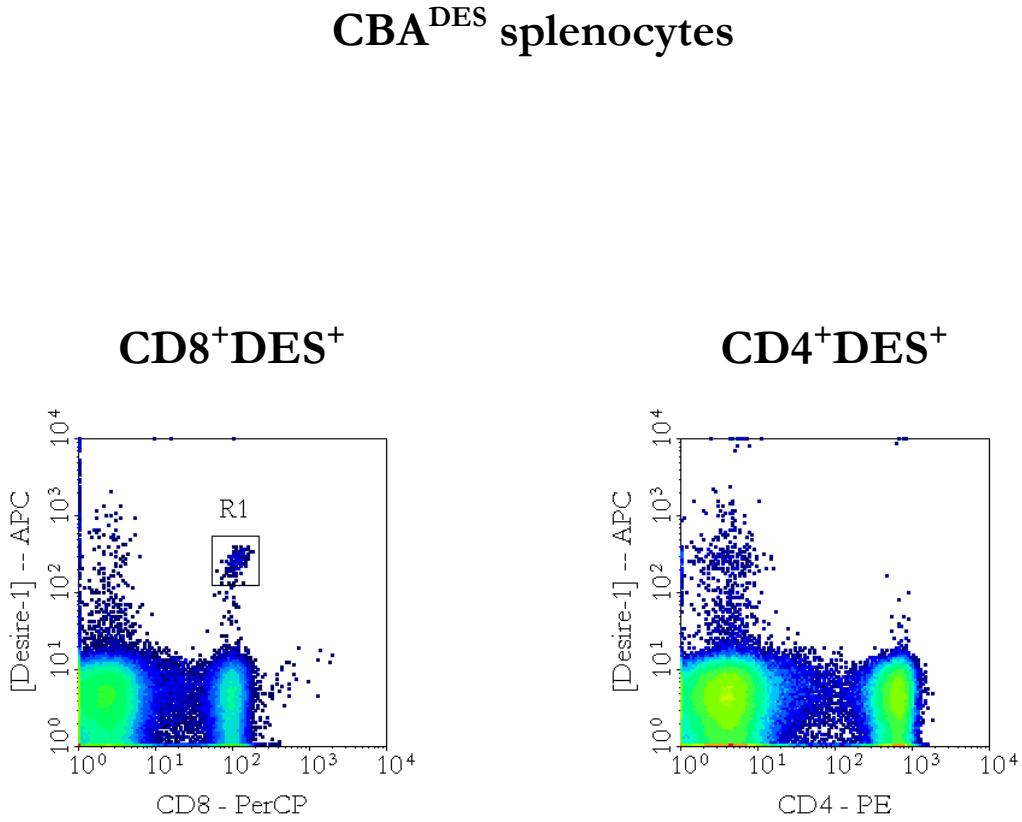


Figure 3.5 Five days following adoptive transfer of 2 x 10^6 $CD8^+DES^+$ T cells into CBA/Ca mice, splenocytes from CBA^{DES} mice were anlaysed by FACS®. A population of $CD8^+DES^+$ T cells between 0.25 to 0.45% was detectable using an anti-clonotype monoclonal antibidy in combination with anti-CD8 monoclonal antibody. $CD4^+DES^+$ T cells were not detected in the spleens of CBA^{DES} mice.

Figure 3.6 Longevity of Adoptively Transferred $CD8^+DES^+$ T Cells

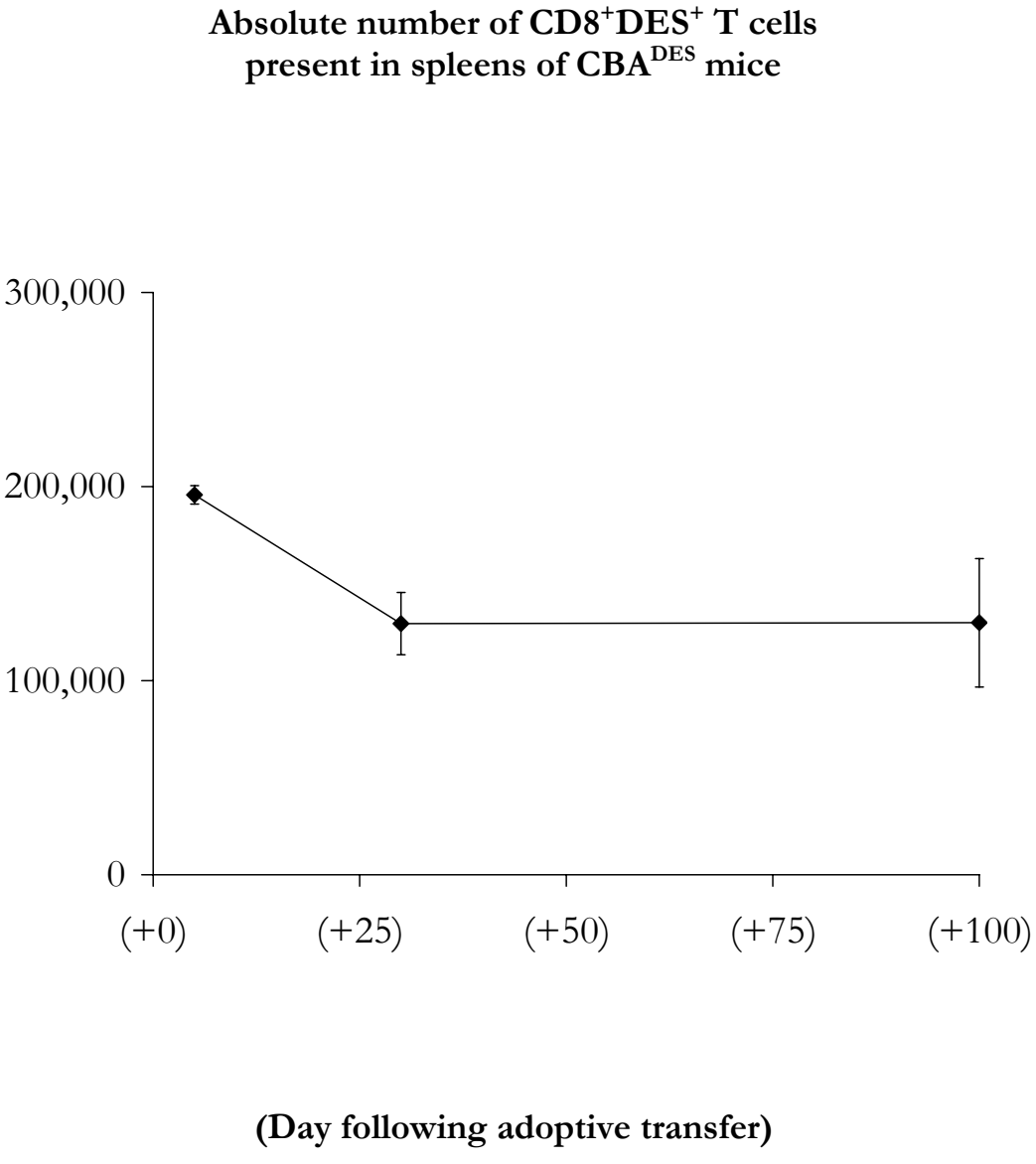


Figure 3.6 The longevity of adoptively transferred cells was demonstrated by enumerating the number of $CD8^+DES^+$ T cells present in the spleens of CBA^{DES} mice at 5 days (n=12), 30 days (n=4) and 100 days (n=3) days after adoptively transferring 2 x 10^6 $CD8^+DES^+$ T cells into CBA/Ca mice. The absolute number of $CD8^+DES^+$ T cells was determined from total cell counts and FACS® analysis. The mean absolute number ±SEM is shown for each time point.

Figure 3.7 Trafficking of Adoptively Transferred $CD8^+DES^+$ T Cells

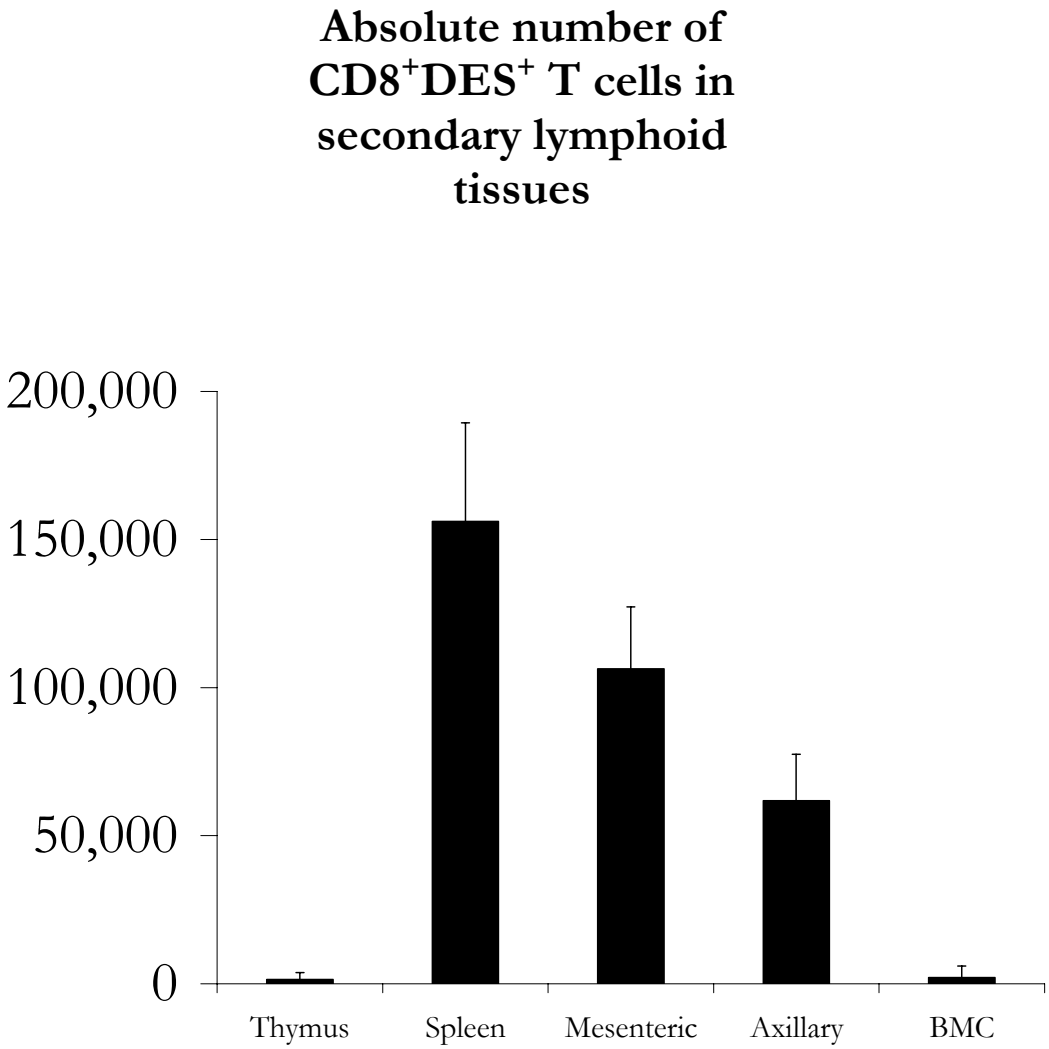


Figure 3.7 The mean absolute counts +SEM of $CD8^+DES^+$ T cells in the lymphoid tissues of CBA^{DES} mice (n=7) five days after adoptive transfer are shown. Resting $CD8^+DES^+$ T cells homed only to the secondary lymphoid tissues and not to the thymic or bone marrow compartments.

Figure 3.8 Phenotyping of $CD8^+DES^+$ T Cells

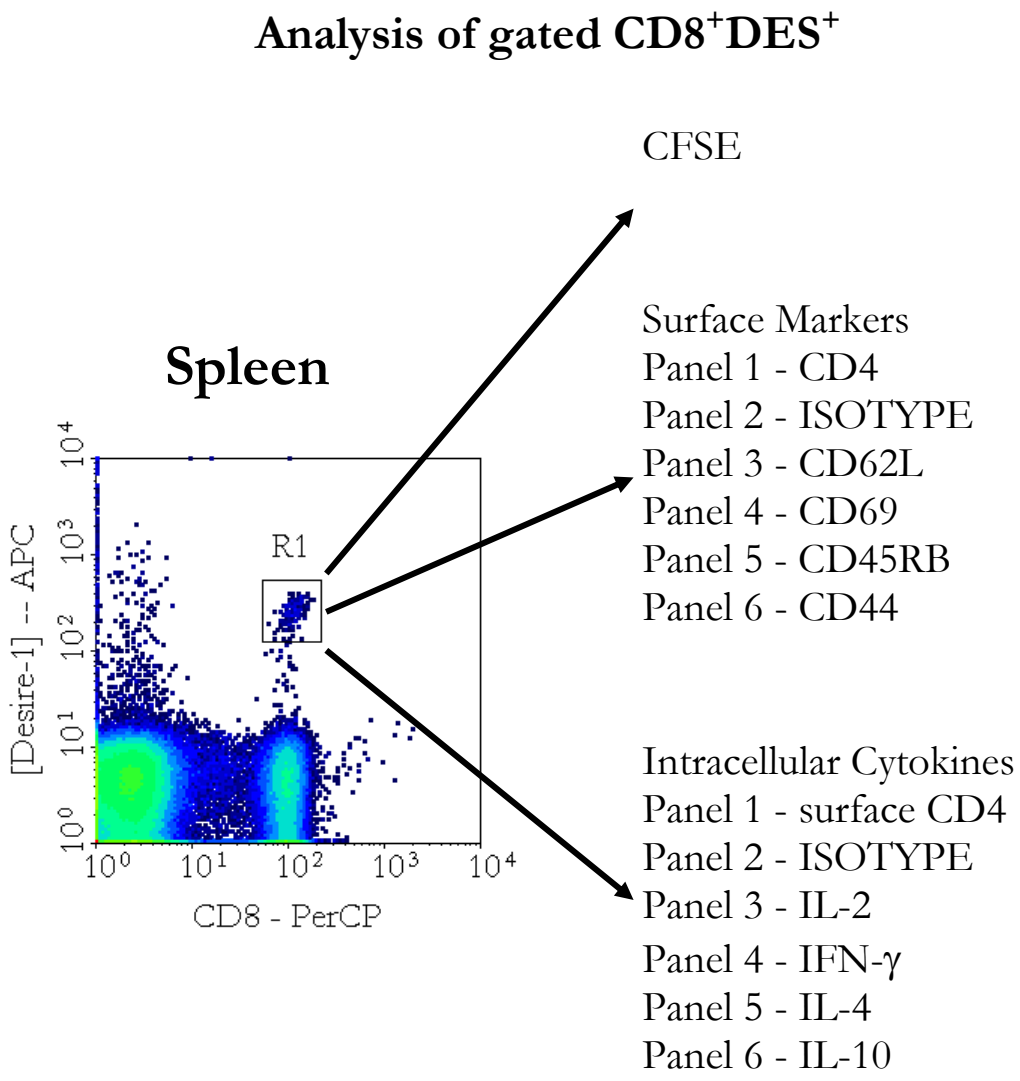


Figure 3.8 Adoptively transferred cells were analysed by four colour flow cytometry. Between 250 and 10,000 gated events were collected and analysed for CFSE (FL-1) intensity and expression of surface activation markers (FL-2) or intracellular production of cytokines (FL-2) in each experiment.

Figure 3.9 Resting State of CD8$^+$DES$^+$ T Cells after Adoptive Transfer

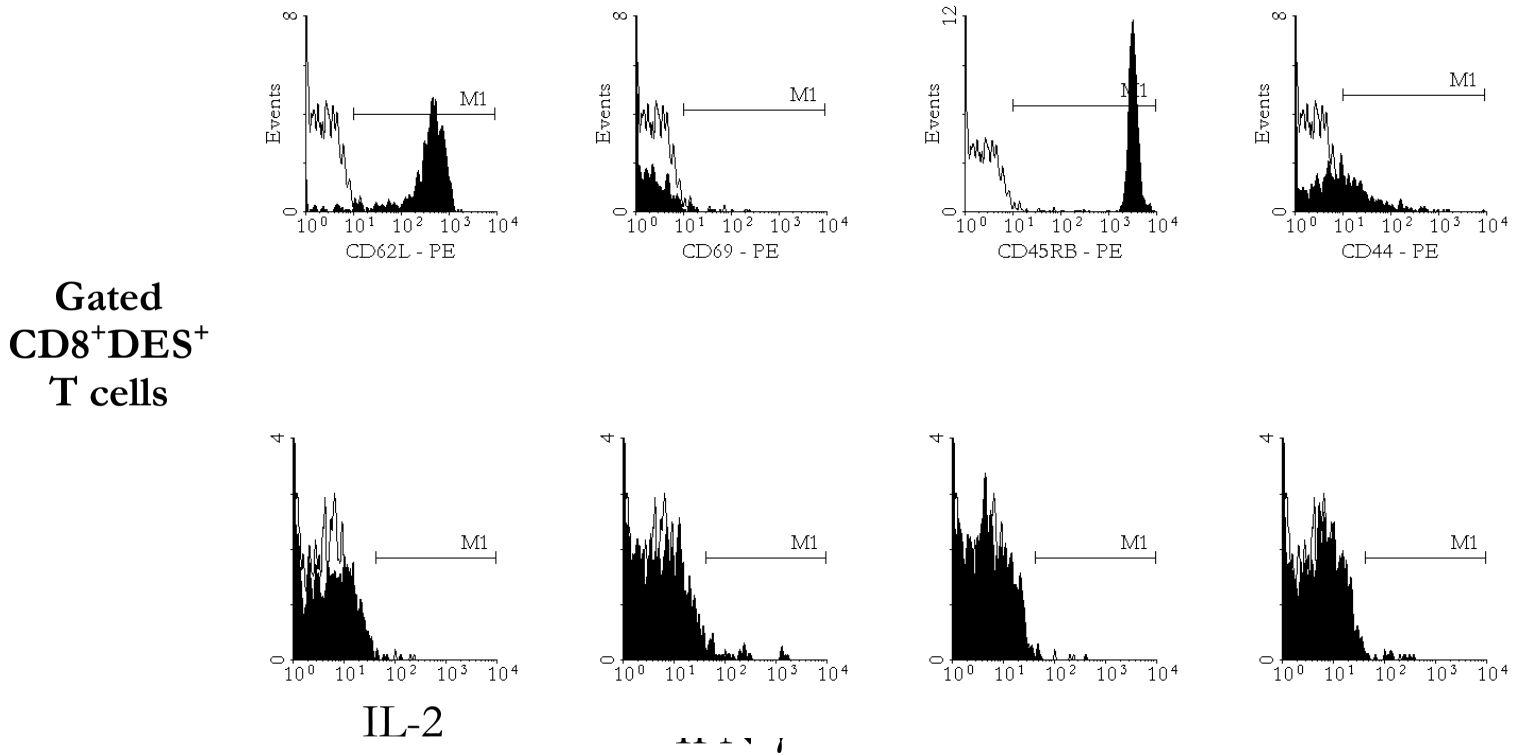


Figure 3.9 Gated CD8$^+$DES$^+$ T cells present in the spleens of CBA^{DES} mice 5 days following adoptive transfer were analysed for the surface expression of activation markers and the production of Tc1 and Tc2 cytokines. Representative histograms are shown from naïve CBA^{DES} mice. The empty overlays show the background staining obtained with an appropriate PE-labelled isotype control monoclonal antibody. These results indicate that in the absence of exposure to alloantigens that CD8$^+$DES$^+$ T cells remain in a resting state. The data are representative of all analyses performed on naive CBA^{DES} mice which were performed with every experiment (n>3).

Figure 3.10 Detection of Cell Division with CFSE

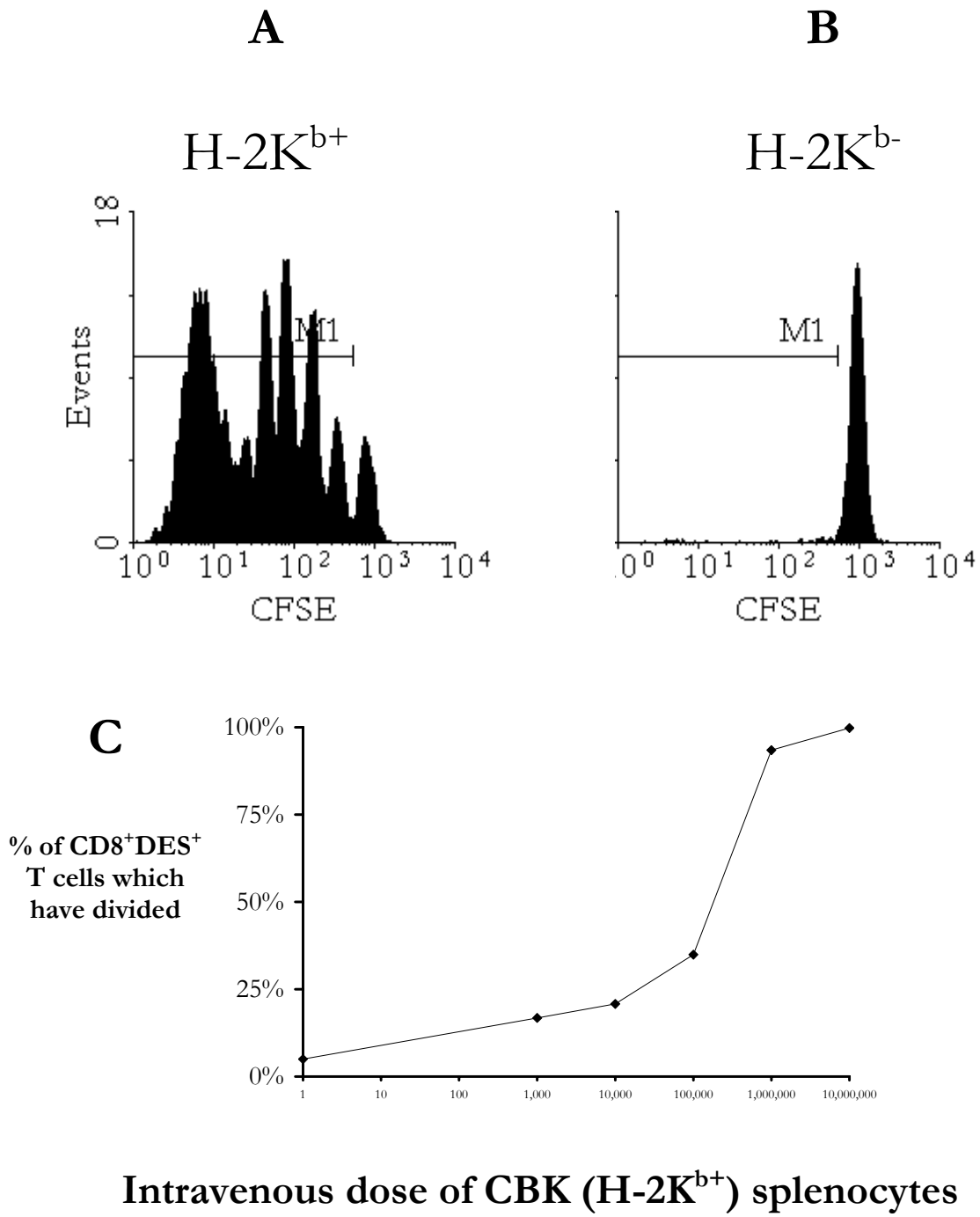


Figure 3.10 (A) Five days following intravenous infusion of 1 x 10^6 CBK splenocytes into CBA^{DES} mice, a mean of 93% of $CD8^+DES^+$ T cells had divided (n=3) while (B) control mice (n=3) infused with the same dose of H-$2K^{b-}$ NZW splenocytes did not divide. (C) With increasing doses of CBK bone marrow cells, the percentage of $CD8^+DES^+$ T cells which had divided increased in a titratable fashion. This experiment was performed once with only 1 CBA^{DES} mouse per dose indicated.

Figure 3.11 Clonal Expansion of Adoptively Transferred $CD8^+DES^+$ T Cells

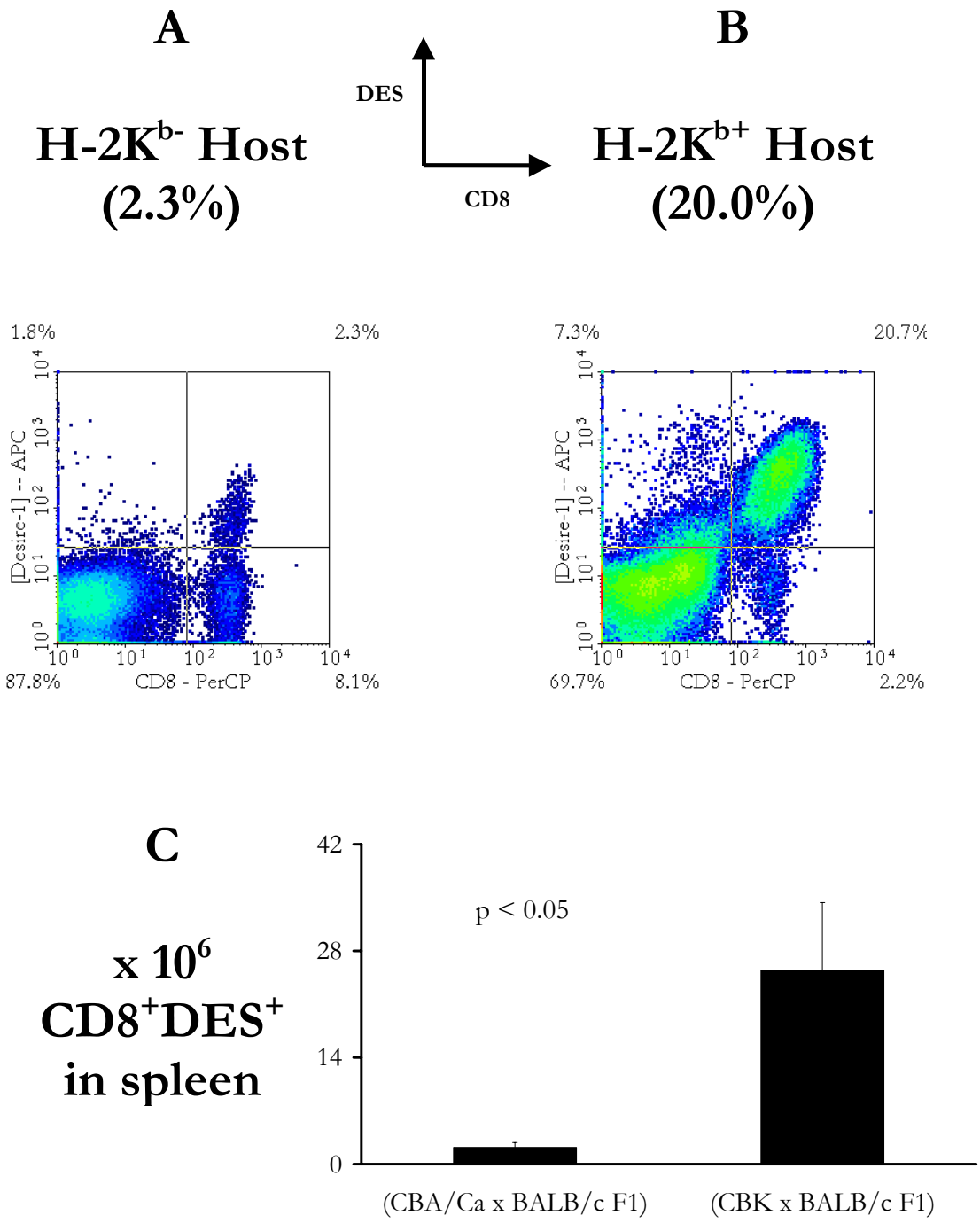


Figure 3.11 4 x 10^6 CD4 depleted CFSE labelled $CD8^+DES^+$ T cells were transferred into (A) H-$2K^{b-}$ (CBA/Ca x BALB/c F1) (n=4) or (B) H-$2K^{b+}$ (CBK x BALB/c F1) (n=4) hosts. Clonal expansion of $CD8^+DES^+$ T cells was apparent; the mean percentage of $CD8^+DES^+$ T cells in the spleens increased from 2.3% to 20.0% at five days after adoptive transfer. (C) The mean absolute number of $CD8^+DES^+$ T cells present in the spleens of the recipients three days later was enumerated and is shown +SEM.

Figure 3.12 Specifitiy of $CD8^+1B2^+$ T Cells

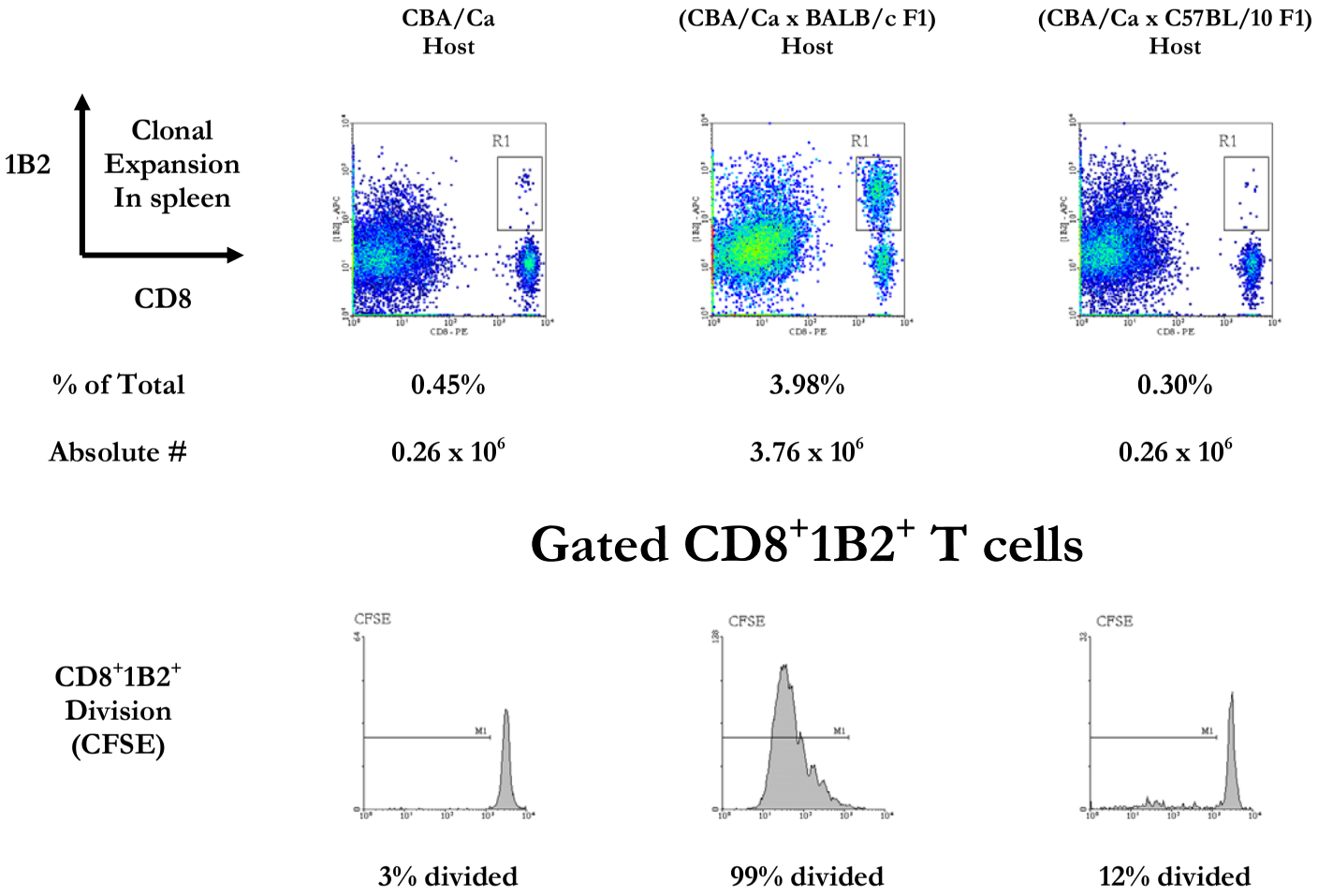


Figure 3.12 Non-CD4 depleted CFSE labelled H-$2L^d$ specific 2C T cells were adoptively transferred into H-$2L^{d+}$ (CBA/Ca x BALB/c F1) (n=3) or H-$2L^{d-}$ (CBA/Ca x C57BL/10 F1) (n=3) hosts. Three days later, gated $CD8^+1B2^+$ T cells were analysed by FACS®. $CD8^+1B2^+$ T cells were specific for H-$2L^d$. $CD8^+1B2^+$ T cells proliferated and clonally expanded only in response to a H-$2L^{d+}$ host.

Figure 3.13 Specifitiy of $CD4^+1B2^+$ T Cells

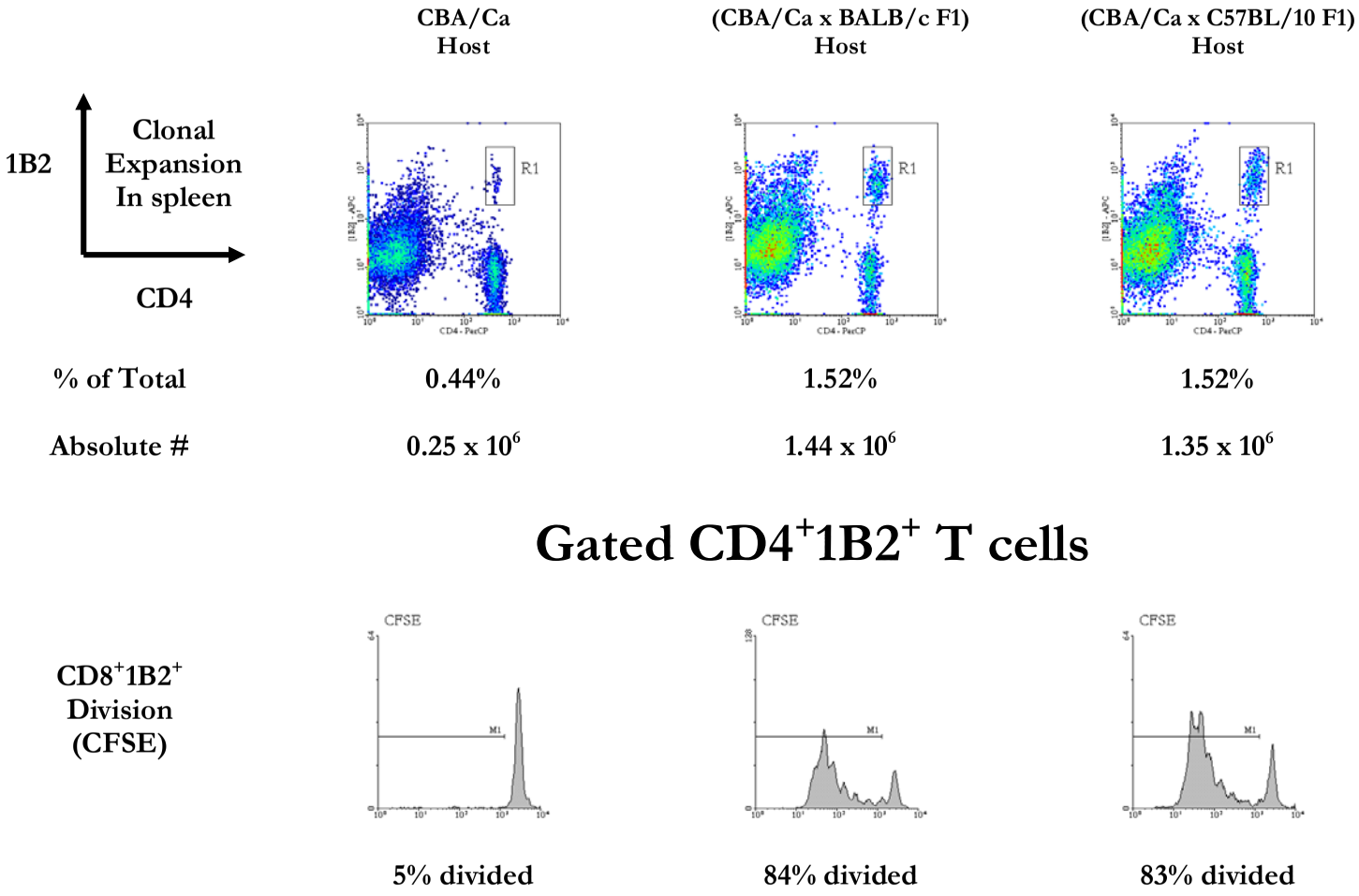


Figure 3.13 Non-CD4 depleted CFSE labelled H-$2L^d$ specific 2C T cells were adoptively transferred into H-$2L^{d+}$ (CBA/Ca x BALB/c F1) (n=3) or H-$2L^{d-}$ (CBK x C57BL/10 F1) (n=3) hosts. Three days later, gated $CD4^+1B2^+$ T cells were analysed by FACS®. $CD4^+1B2^+$ T cells were reactive to an undetermined H-2^d and at least one H-2^b alloantigen.

Figure 3.14 Augmented In Vitro Reactivity of CBA^{TG} Mice

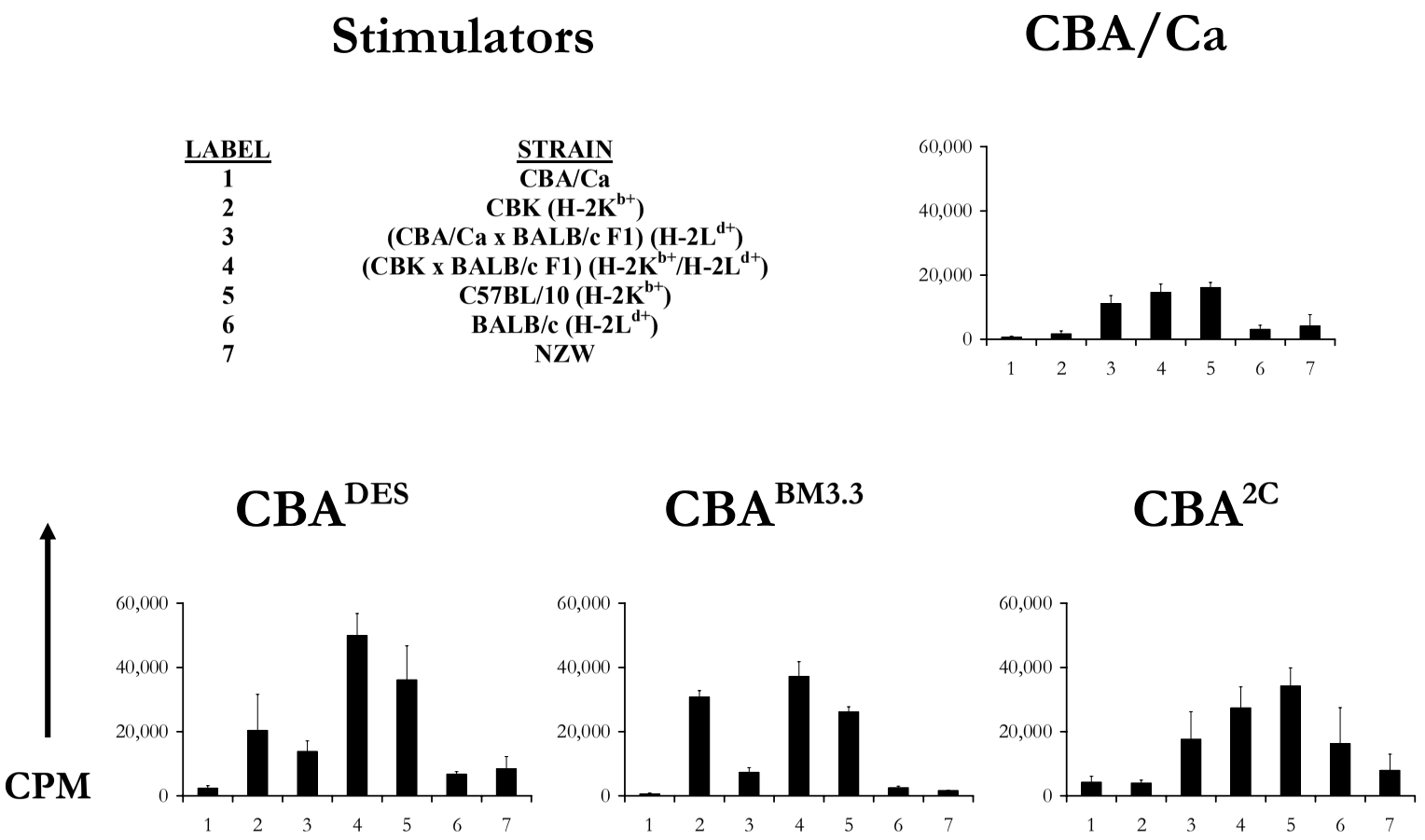


Figure 3.14 In vitro proliferative responses of CBA/Ca, CBA^{DES}, CBA$^{BM3.3}$ and CBA^{2C} responder mesenteric lymph node cells (1 x 10^5 cells/well) against 3000 rad irradiated splenocyte stimulators (2 x 10^5 cells/well) from the indicated strains are shown. T cell proliferative responses were measured by ^{3}H-thymidine incorporation on the 4th day of culture. The results represent the mean CPM values +SEM of triplicate wells. The proliferative response against H-2K^{b+}/H-2L^{d+} (CBK x BALB/c F1) is augmented in all three CBA^{TG} mice. This experiment was not repeated.

Chapter 4 - In Vivo Generation of Effector and Memory $CD8^+DES^+$ T Cells

4.1 Introduction

In this study we have chosen to investigate the fate of alloreactive $CD8^+$ T cells that recognise intact donor MHC class I molecules. In the design of the experiments described in this chapter, $CD8^+DES^+$ T cells were only able to recognise MHC class I alloantigens expressed by parenchymal cells or passenger leukocytes in cardiac allografts. This response to class I alloantigen via the direct pathway is more straightforward, in that $CD8^+DES^+$ T cells recognise native alloantigen with their bound peptides, while presentation of donor alloantigens on host antigen presenting cells to naïve $CD8^+DES^+$ T cells cannot play a direct role in their activation. Thus, the location and source of intact alloantigen can be focused upon in the study of $CD8^+DES^+$ T cell responses for the purposes of this thesis.

Like the majority of naturally occurring alloreactive $CD8^+$ T cells, alloreactive $CD8^+DES^+$ T cells directly recognise foreign MHC class I molecules and develop cytotoxic effector function. However, in most cases $CD8^+$ T cells require help from other T cells, usually $CD4^+$, before they can clonally expand and develop into cytotoxic effector T cells that participate in the destruction of allografts[89]. 2×10^6 $CD8^+DES^+$ T cells were transferred into syngeneic CBA/Ca recipients with diverse T cell repertoires. Both $CD4^+$ T cells that recognise the mismatched donor MHC class I molecules indirectly as well as $CD8^+$ T cells that recognise foreign MHC class I molecules via the direct pathway are present in CBA^{DES} mice. The diverse T cell

repertoire recreated a "normal" milieu for $CD8^+DES^+$ T cell interactions. Normal T cells in CBA^{DES} mice will be able to produce cytokines to assist in the activation of alloreactive $CD8^+DES^+$ T cells.

The experiments described in this chapter were designed to shed light on the role of $CD8^+$ T cells during allograft rejection in the context of an abundance of $CD4^+$ T cell "help". In the rejection of allografts, questions persist concerning the site where graft specific T cells are activated and the development of immunological memory. It has always been possible to go to the graft, both experimentally and in the clinical setting, in order to isolate what are presumed to be activated graft specific T cells[298] and to determine their immunological status[299]. However, finding graft specific T cells in the periphery has proven impossible until recently[204,205]. This model allows such cells to be identified greatly facilitating the study of alloreactive $CD8^+$ T cells and permitting us to study aspects of antigenic stimulation, proliferation, differentiation, trafficking and effector function.

What is the role of the allograft in the activation of $CD8^+$ T cells? Where do $CD8^+$ T cells clonally expand and differentiate? Are graft specific T cells in the periphery a reflection of the immune status within an allograft? This final point may provide a route to the non-invasive monitoring of the immune status within an allograft that would be of great benefit clinically and for the further development of tolerance inducing protocols.

Many in vivo studies have analysed the state of $CD8^+$ T cells in relation to phenotype and effector function in the secondary lymphoid tissues or the site of antigen[128,300].

However, the role of lymphoid organs in relation to the allograft for the activation of $CD8^+$ T cells remains incompletely resolved. Similar adoptive transfer systems have been useful in studying in vivo activation of $CD8^+$ T cells specific for peptides presented by self[132,155,204] and allogeneic[265,276] MHC class I molecules. However, we have done this in a vascularised transplantation model where MHC class I alloantigen is present on the allograft endothelial cells, the allograft parenchyma and on donor antigen presenting cells.

In this chapter, we study graft specific T cells early in the course of acute rejection in the hope that analysis may offer valuable predictive information. We chose the time point of 5 days after transplantation since allografts were in the midst of an established rejection crisis, but were still functioning. We have described the stages of the response of $CD8^+DES^+$ T cells following transplantation of H-$2K^{b+}$ hearts and compared this to transplantation of H-$2K^{b-}$ hearts. The results presented for CBA^{DES} mice are drawn from a great number of CBA^{DES} mice (>100) transplanted with H-$2K^{b+}$ and H-$2K^{b-}$ cardiac allografts. The data presented are representative of all the experiments carried out.

4.2 Results

4.2.1 Cardiac Allograft Rejection in Naïve Recipients

Naïve CBA/Ca mice rejected H-2K^{b+} C57BL/10 cardiac allografts with a median survival time of 8 days (n=9) while naïve DES mice rejected C57BL/10 cardiac allografts with a median survival time of 17.5 days (n=10) (Figure 4.1a). CBA^{DES} mice rejected C57BL/10 cardiac allografts with a median survival time of 9 days (n=9). Similar results were observed with (CBK x BALB/c F1) cardiac allografts which were rejected by CBA/Ca (n=5), DES (n=9) and CBA^{DES} (n=17) with median survival times of 8, 17 and 7.5 days respectively (Figure 4.1b).

4.2.2 T Cell Infiltration of Cardiac Allografts During Acute Rejection

A time course was performed to determine the kinetics of T cell infiltration into (CBK x BALB/c F1) cardiac allograft (Figure 4.2). (CBK x BALB/c F1) hearts were transplanted into CBA/Ca mice (n=3 per day) and 1, 3, 4, 5, 7, 9 and 11 days after transplantation the heart allografts were removed for isolation of graft infiltrating cells. Total graft infiltrating cells were counted and stained with anti-CD4 (RM-4.5) and anti-CD8 (53-6.7) for FACS® analysis. CD4$^+$ and CD8$^+$ T cells were quantified (Figure 4.2). We observed that CD4$^+$ and CD8$^+$ T cells infiltrate (CBK x BALB/c F1) allografts with similar kinetics. Both T cell subsets began infiltrating cardiac allografts three days after transplantation. The absolute number in each subset peaked at 7 days, after which time they declined. After rejection, it became technically difficult to recover adequate numbers of graft infiltrating cells for analysis.

4.2.3 Alloantigen Specific Proliferation of $CD8^+DES^+$ T Cells

CBA^{DES} mice transplanted with H-$2K^{b+}$ (CBK x BALB/c F1) hearts (n=3) and H-$2K^{b-}$ (CBA/Ca x BALB/c F1) hearts (n=3) were compared with untransplanted CBA^{DES} mice (n=3) (Figure 4.3). Splenocytes and graft infiltrating cells from these mice were stained with anti-CD8 (53-6.7) and anti-clonotypic TCR (Désiré-1) monoclonal antibodies in order to identify adoptively transferred cells by FACS®.

CFSE analysis revealed that gated $CD8^+DES^+$ T cells present in the spleen five days after transplantation proliferated only when H-$2K^b$ was expressed by the cardiac allograft (CBK x BALB/c F1). Figure 4.4 shows that approximately 40-50% (mean 41%) of gated $CD8^+DES^+$ T cells present in the spleens of CBA^{DES} mice transplanted with H-$2K^{b+}$ hearts had divided more than once however very few had divided greater than three times. When H-$2K^{b-}$ (CBA/Ca x BALB/c F1) grafts were transplanted there was no detectable division. The same was observed when CBA/Ca, NZW, or BALB/c hearts were transplanted. Gated $CD8^+DES^+$ T cells in the spleens of untransplanted CBA^{DES} mice had not divided either.

4.2.4 $CD8^+DES^+$ T Cells Infiltrate H-$2K^{b+}$ but not H-$2K^{b-}$ Cardiac Allografts

Next, we focused on leukocytes infiltrating the cardiac allografts. Graft infiltrating cells were analysed by FACS® five days after transplantation. We found that H-$2K^b$ specific $CD8^+DES^+$ T cells had infiltrated the graft following transplantation of H-$2K^{b+}$ (CBK x BALB/c F1) but not H-$2K^{b-}$ (CBA/Ca x BALB/c F1) cardiac allografts (Figure 4.5). These findings were confirmed by immunohistology (Figure 4.6).

Gated $CD8^+DES^+$ T cells were then analysed for proliferation. We found that $CD8^+DES^+$ T cells infiltrating H-$2K^{b+}$ cardiac allografts had a division history (CFSE profile) distinct from the $CD8^+DES^+$ T cells in the spleens of the same mice. The striking difference between graft infiltrating leukocytes and those in the periphery was that the overwhelming majority (>80%) of $CD8^+DES^+$ T cells present in H-$2K^{b+}$ rejecting hearts had undergone a minimum of three rounds of division (Figure 4.7).

4.2.5 Graft Infiltrating $CD8^+DES^+$ T Cells Possess an Activated Surface Phenotype

Splenocytes and graft infiltrating cells from CBA^{DES} mice transplanted with H-$2K^{b+}$ (CBK x BALB/c F1) cardiac allografts were harvested and stained with Désiré-1 and CD8 (53-6.72) for identification of $CD8^+DES^+$ T cells together with either an appropriate isotype control, CD62L, CD69, CD45RB, or CD44. $CD8^+DES^+$ T cells present in the spleens were compared with those that had infiltrated the H-$2K^{b+}$ cardiac allografts.

$CD8^+DES^+$ T cells that infiltrated the cardiac allografts possessed a surface phenotype that was strikingly different from $CD8^+DES^+$ T cells that remained in the periphery. $CD8^+DES^+$ T cells present in rejecting H-$2K^{b+}$ cardiac allografts had down-regulated surface expression of CD62L, CD45RB and up-regulated expression of CD69 and CD44 (Figure 4.8). All of these observations are consistent with an activated/effector phenotype. In the periphery, a resting surface phenotype was maintained. $CD8^+DES^+$ T cells in CBA^{DES} mice transplanted with H-$2K^{b-}$ (CBA/Ca

x BALB/c F1) cardiac allografts remained naïve in the spleen and did not home to the H-2K^{b-} cardiac allograft.

4.2.6 Allograft Rejection Induces CD8$^+$DES$^+$ T Cells to Produce IL-2 and Interferon-γ

Analogous to the situation among CD4$^+$ "helper" T cells, CD8$^+$ "cytotoxic" T cells can develop into cells secreting different cytokines depending on the conditions prevailing at the time of activation. IL-2 and interferon-γ are considered Tc1 cytokines while IL-4 and IL-10 are considered Tc2 cytokines[120,121]. CD8$^+$DES$^+$ T cells in CBA^{DES} mice transplanted with H-2K^{b-} and H-2K^{b+} cardiac allografts were analysed for cytokine production by intracellular FACS® staining.

CD8$^+$DES$^+$ T cells present in the spleens of mice transplanted with a H-2K^{b+} heart produced Tc1 cytokines (a mean of 4.6% IL-2 & 8.3% interferon-γ). No evidence for the production of Tc2 cytokines was obtained (Figure 4.9). When CD8$^+$DES$^+$ T cells infiltrating rejecting H-2K^{b+} cardiac allografts were analysed at the same time point, five days after transplantation, we found that 30% of CD8$^+$DES$^+$ T cells produced interferon-γ, but fewer were producing IL-2 (Figure 4.9b). As expected, CD8$^+$DES$^+$ T cells in CBA^{DES} mice transplanted with H-2K^{b-} hearts showed no proliferation, no change in surface phenotype, no homing to H-2K^{b-} cardiac allografts and no detectable production of either Tc1 or Tc2 cytokines.

4.2.7 $CD8^+DES^+$ Memory T Cells are Detectable 100 Days after Transplantation

All previous analyses were performed 5 days after transplantation for the reasons stated previously. We next wanted to examine the long-term fate of antigen experienced $CD8^+DES^+$ T cells following rejection of H-$2K^{b+}$ cardiac allografts. CBA^{DES} mice were transplanted with H-$2K^{b+}$ hearts and harvested up to 100 days after for FACS® analysis. All hearts had been rejected at this time point (Figure 4.1) and were atrophied, thus it was not possible to perform FACS® analysis on $CD8^+DES^+$ T cells present in the cardiac allografts. Analysis of spleens revealed that $CD8^+DES^+$ T cells were present up to 100 days after transplantation and that unlike 5 days after transplantation a large percentage of gated $CD8^+DES^+$ T cells had divided such that their CFSE fluorescence was very low (Figure 4.10). $CD8^+DES^+$ T cells in mice transplanted with H-$2K^{b-}$ cardiac allografts had not divided significantly.

The surface and cytokine phenotype of these cells was determined as described previously. At day 100, we observed that $CD8^+DES^+$ T cells present in spleen had up-regulated CD44 and produced interferon-γ while CD45RB and CD62L expression were heterogeneous. These memory cells also displayed a $CD69^{low}$ surface phenotype. This phenotype was biased towards $CD8^+DES^+$ T cells that had lost nearly all CFSE fluorescence (Figure 4.11). This suggested to us that antigen experienced $CD8^+DES^+$ T cells persisted for significant periods in CBA^{DES} mice that had rejected H-$2K^{b+}$ cardiac allografts. Secondly, that "memory" $CD8^+DES^+$ T cells potentially originated from among $CD8^+DES^+$ T cells which were the most antigen experienced, as indicated by the number of divisions they had undergone. From these data, it cannot be determined whether "memory" $CD8^+DES^+$ T cells differentiated directly from naïve $CD8^+DES^+$ T cells that had responded acutely via

an independent pathway, or whether they were selected from those $CD8^+DES^+$ T cells which first developed an effector phenotype.

4.3 Discussion

Both $CD4^+$ and $CD8^+$ T cells play a significant role in rejection, but the relative importance of each subset and their pathway of recognition is incompletely understood and may be critically dependent on the precise nature of the alloantigen disparity as well as the immune status of the recipient[38]. In the majority of situations $CD4^+$ T cells have been shown to be an absolute requirement for allograft rejection to occur[32,33]. Therefore, much transplantation research has focused on $CD4^+$ T cells. $CD8^+$ T cells are however pivotal in certain situations[36,37] and are nonetheless required to maintain the normal kinetics[43] of rejection. Much of what is known about $CD8^+$ T cell biology comes from studying anti-virus and anti-tumour T cell responses where $CD8^+$ cytotoxic T cell responses are critical for resolution. In an attempt to broaden our knowledge of the role of $CD8^+$ T cells in allograft rejection we have performed the studies described in this chapter.

In summary, we show that, CBA^{DES} mice reject H-$2K^{b+}$ cardiac allografts with the same tempo as CBA/Ca mice and go on to determine the kinetics with which these grafts are infiltrated by non-transgenic $CD4^+$ and $CD8^+$ T cells. Secondly, we demonstrate that MHC class I alloantigens expressed on allografts induce the proliferation, cytokine production, homing, and generation of memory cells in an alloantigen specific manner. We then demonstrate that graft specific $CD8^+DES^+$ T cells possess different phenotypes in the periphery and within the allograft, suggesting that the allograft may drive activation, proliferation and differentiation to a large degree. Experiments not described using CBA^{2C} mice as recipients showed results consistent with these findings. Furthermore antigen experienced $CD8^+DES^+$

T cells that had proliferated extensively and that possessed a memory phenotype were present in the spleen long after allograft rejection had occurred. The ability to visualise particular graft specific $CD8^+$ T cells through all of these events in the periphery and in the allograft is in itself novel and significant. Individually, our observations are interesting and taken together they may offer new insights as to how cytotoxic T cells become activated and differentiate.

Transplantation of H-$2K^{b+}$ cardiac allografts revealed that intact DES TCR transgenic mice rejected both C57BL/10 and (CBK x BALB/c F1) hearts in a moderately delayed fashion (Figure 4.1a & Figure 4.1b). This was an unexpected finding since intact transgenic mice had the highest precursor frequency of H-$2K^b$ specific T cells. Delayed rejection can potentially be explained by the lack of a diverse repertoire of effective $CD4^+$ helper T cells in TCR transgenic mice. This again suggested that intact transgenic mice did not always behave as unmanipulated naïve mice with an intact T cell repertoire. We believe that $CD8^+DES^+$ T cells placed in a normal T cell repertoire, as in this adoptive transfer system, receive abundant $CD4^+$ T cell help. We believe that the resulting CBA^{DES} mice behaved similarly to normal CBA/Ca mice. In support of this, we observed that CBA^{DES} mice rejected H-$2K^{b+}$ cardiac allografts with essentially identical kinetics to unmanipulated CBA/Ca mice.

Identification of the site of T cell activation in immune responses has always been sought. We clearly demonstrated that graft specific $CD8^+DES^+$ T cells present in the periphery remain in a resting state, as determined by the surface phenotype (Figure 4.8). Graft infiltrating $CD8^+DES^+$ T cells clearly differed in that they were activated when assessed by CD69, CD62L, CD45RB and CD44 expression (Figure 4.8).

These activated graft infiltrating $CD8^+DES^+$ T cells were heavily biased towards cells that had proliferated greater than three times (Figure 4.7). As activated $CD8^+DES^+$ T cells were only present in the graft during the rejection crisis, one interpretation would be that graft specific cells, were stimulated to proliferate and differentiate within the allograft. Cells that had divided more than three times were rarely seen in the periphery acutely. We have also investigated cells present in the mesenteric lymph node, axillary lymph node and para-aortic lymph nodes observing the same thing that had been seen in the spleen (i.e. the vast majority of cells having divided fewer than three times with a resting surface phenotype). These are by no means conclusive but the results are worthy of discussion.

The long held and widely accepted belief is that activation of T cells occurs in the draining lymph nodes or secondary lymphoid tissues of the animal being challenged[205,278,301]. However, cardiac allografts pose some difficulty because the lymphatic drainage of the heart has been disrupted by transplantation. There is no universally accepted draining lymph node for transplanted hearts, but most would agree that the spleen is an appropriate secondary lymphoid organ to study. The observations in this chapter suggest that host secondary lymphoid tissues are not the exclusive site of T cell activation. CFSE proved very useful and provided support for these ideas. Division history could be assigned to $CD8^+DES^+$ T cells in different locations aiding in the interpretation of the data. Proliferation was found to be intimately tied to differentiation and the development of effector and memory phenotypes[284,302-306]. Extensive division coincident with the adoption of an activated/effector phenotype within the allograft provides strong support for an activating role of the allograft. Certainly in this case, the spleen did not appear to be the main site of T cell division.

Two explanations are plausible. The first, that donor antigen presenting cells emigrate to the spleen where they activated $CD8^+DES^+$ T cells. However, these donor antigen presenting cells are short lived (1 to 3 days)[24] limiting their activating potential over time possibly resulting in full activation of only a small fraction of the 2×10^6 $CD8^+DES^+$ T cells transferred. Alternatively, the graft may have a significant potential to recruit and activate naïve $CD8^+DES^+$ T cells for the following reasons; (1) expression of MHC class I on graft endothelium and parenchyma, and (2) the ability of activated endothelial cells to act as antigen presenting cells through the expression of CD80/CD86 and other adhesion molecules with costimulatory capabilities[307,308]. We favour the idea that the abundance of alloantigen in the allograft plays a role in driving $CD8^+DES^+$ T cells to become effector cells that later were potentially the source of memory cells.

Due to our inability to identify and follow a particular graft specific clone, it has not been possible to address such questions easily in the past. Kedl et al. have shown similar results in their adoptive transfer model[265]. Although the experimental system employed was similar in that H-$2L^{d+}$ P815 mastocytoma cells were "transplanted" into the peritoneal cavity and recognised by $CD8^+1B2^+$ T cells, it did differ on two points. Firstly, no antigen presenting cells were transferred and secondly that there was no H-$2L^{d+}$ endothelium in contact with the recipients circulation. They observed that activated ($CD25^{high}CD44^{high}CD62L^{low}VLA\text{-}4^{high}FSC^{high}$) $CD8^+1B2^+$ T cells were only present within the peritoneal cavity and not in the spleen or lymph nodes acutely. These findings were interpreted to indicate that full activation and clonal expansion of tumour specific T cells occurred at the site of antigen expression rather than in lymph nodes or spleen. Their results were

even more surprising since they were able to demonstrate H-2L^{d+} P815 tumour cells in the "draining" lymph nodes and spleen.

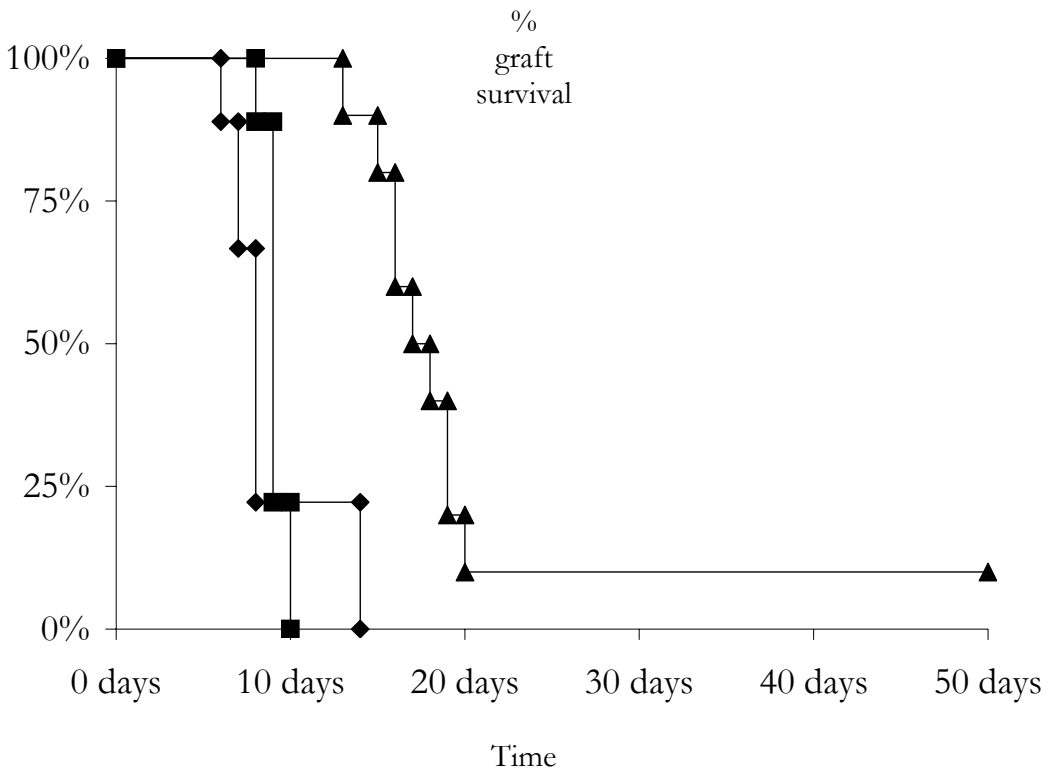
We do not propose that the allograft played an exclusive role in the activation of CD8$^+$DES$^+$ T cells. The proliferation demonstrated in the periphery would suggest the contrary, however, the observations of Kedl et al. are consistent with our own and together they suggest (1) that donor antigen presenting cells are not an absolute requirement, (2) that donor endothelium may potentially substitute as an antigen presenting cell, and (3) that the allograft itself offers a strongly stimulating milieu that drives graft specific CD8$^+$DES$^+$ T cells to full activation and later towards a memory phenotype.

The second important implication of our observations is that graft specific CD8$^+$DES$^+$ T cells in the periphery do not entirely reflect the true activation state of CD8$^+$DES$^+$ T cells infiltrating the allograft. What this suggests, at least for alloreactive CD8$^+$ T cells, is that even if donor reactive CD8$^+$ T cells could be identified in the periphery of transplanted individuals, that they would probably not be reflective or predictive of the immune status within the allograft. Essentially, what we have done in our experimental system is analogous to an allograft biopsy that is often done for clinical diagnostic purposes. These results were then compared with graft specific cells in the peripheral lymphoid organs; presumably, graft specific cells in the blood stream would be similar, but this was not confirmed. Although graft specific cells in the periphery could be shown to have proliferated (the majority less than 3 times) with a fraction producing IL-2 and interferon-γ their surface phenotype remained in a resting state. Limited proliferation in the periphery also supported the conclusion that these cells were not full effector cells.

Following rejection, a persistent population of $CD8^+DES^+$ T cells could be identified with a phenotype consistent with a "memory" phenotype. "Memory" $CD8^+DES^+$ T cells were $CFSE^{low}$, produced interferon-γ but not IL-2 and had a surface phenotype accepted as a memory phenotype ($CD44^{high}CD45RB^{low}CD62L^{high}CD69^{low}$). A question still unanswered today is whether there is an independent pathway to the generation of "memory" T cells or whether T cells must first become effector cells before differentiating into "memory" cells. The observation that "memory" $CD8^+DES^+$ T cells were $CFSE^{low}$ similar to graft infiltrating cells suggests that "memory" cells are selected from previous effector cells which have reverted to a $CD69^{low}$ phenotype.

In summary, we have described a model where $CD8^+$ T cell responses to foreign tissue grafts can be followed in vivo. We show that peripheral MHC class I specific T cells proliferate and become capable of producing IL-2 and interferon-γ following transplantation of an MHC class I mismatched cardiac allograft. We found that a proportion of the MHC class I specific T cells that had proliferated extensively home to the graft where they possess an activated surface phenotype and cytokine profile that is consistent with terminally differentiated effector cells. This adoptive transfer system should permit more precise analysis of immune responses to alloantigens under both immunogenic and tolerogenic conditions.

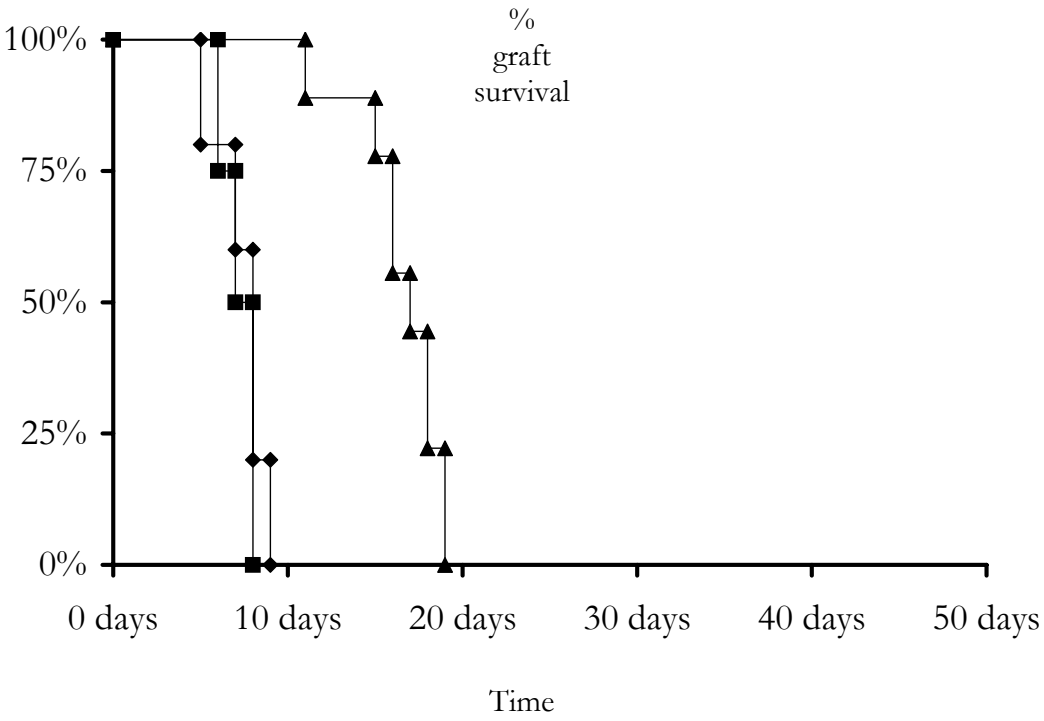
Figure 4.1a Rejection of C57BL/10 Heart Allografts



Label	Recipient	Donor	(n=)	(mst=)
◆	CBA/Ca	C57BL/10	9	8 days
■	CBA^{DES}	C57BL/10	9	9 days
▲	DES	C57BL/10	10	17.5 days

Figure 4.1a The rejection of H-$2K^{b+}$ C57BL/10 cardiac allografts by adult CBA/Ca, CBA^{DES} and DES mice was compared. CBA^{DES} mice rejected their allografts acutely with the same tempo as naïve CBA/Ca mice.

Figure 4.1b Rejection of (CBK x BALB/c F1) Heart Allografts



Label	Recipient	Donor	(n=)	(mst=)
◆	CBA/Ca	(CBK x BALB/c F1)	5	8 days
■	CBA^{DES}	(CBK x BALB/c F1)	17	7.5 days
▲	DES	(CBK x BALB/c F1)	9	17 days

Figure 4.1b The rejection of H-$2K^{b+}$ (CBK x BALB/c F1) cardiac allografts by adult CBA/Ca, CBA^{DES} and DES mice was compared. CBA^{DES} mice rejected (CBK x BALB/c F1) allografts acutely with the same tempo as naïve CBA/Ca mice. This was comparable to rejection of C57BL/10 hearts.

Figure 4.2 Infiltration of H-2K^{b+} Hearts by T Cells

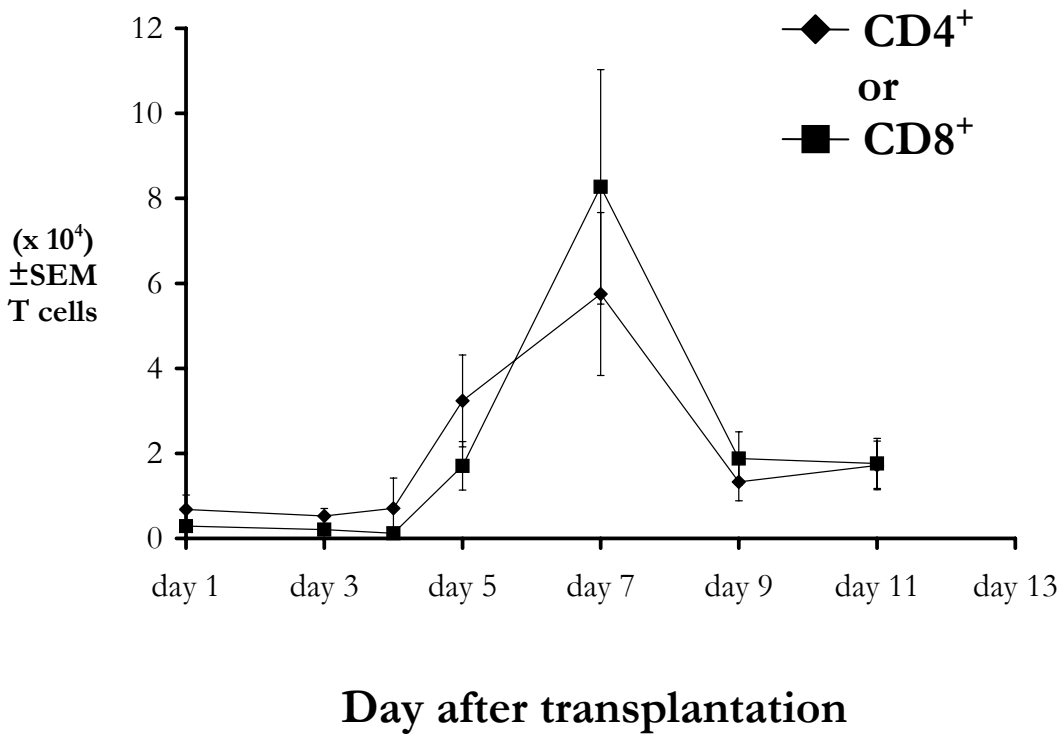


Figure 4.2 Age- and sex-matched H-2K^{b+} (CBK x BALB/c F1) cardiac allografts were transplanted into age- and sex-matched naïve CBA/Ca mice. Graft infiltrating cells were isolated from transplanted hearts (n=3 at each time point) and analysed by FACS® for the presence of CD4$^+$ or CD8$^+$ T cells. The absolute number of T cells was calculated from the total cell counts and percentages determined by FACS®. The mean absolute number ±SEM of CD4$^+$ and CD8$^+$ T cells found within rejecting allografts on the indicated days is shown. Peak infiltration of the cardiac allograft for both subsets was at 7 days.

Figure 4.3 Protocol for Studying Acute Cardiac Allograft Rejection

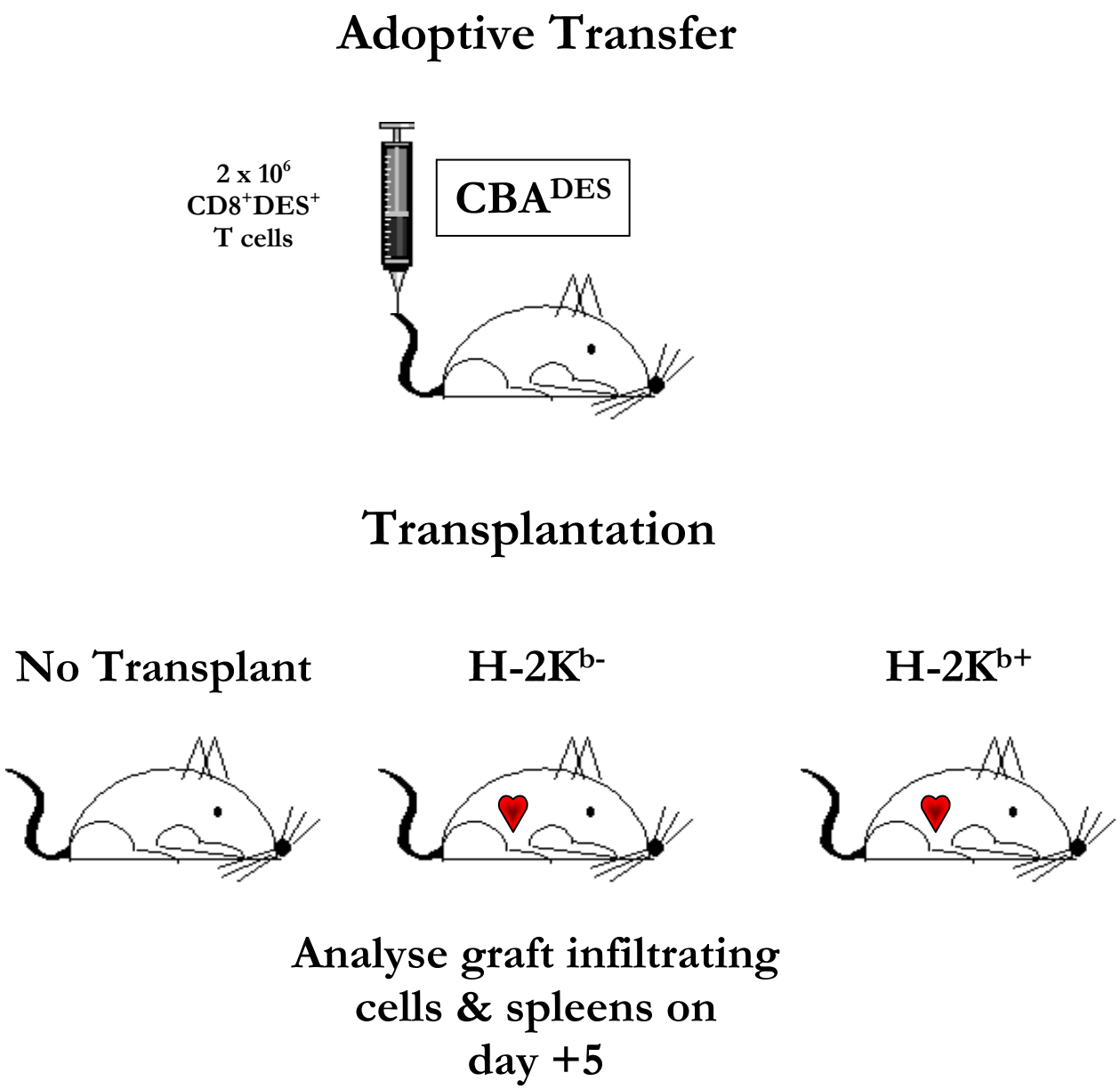


Figure 4.3 The role of H-2K^b specific CD8$^+$DES$^+$ T cells was investigated in vivo. CBA^{DES} mice were transplanted with either H-2K^{b+}, H-2K^{b-} cardiac allografts or not transplanted. Five days following transplantation, during acute rejection, CD8$^+$DES$^+$ T cells present among graft infiltrating cells and splenocytes were analysed by FACS®. The division history, surface phenotype and intracellular cytokine production were compared between the groups. There were a minimum of three CBA^{DES} animals per group in each experiment.

Figure 4.4 Proliferation of $CD8^+DES^+$ T Cells Induced by H-$2K^{b+}$ Heart Allograft Transplantation

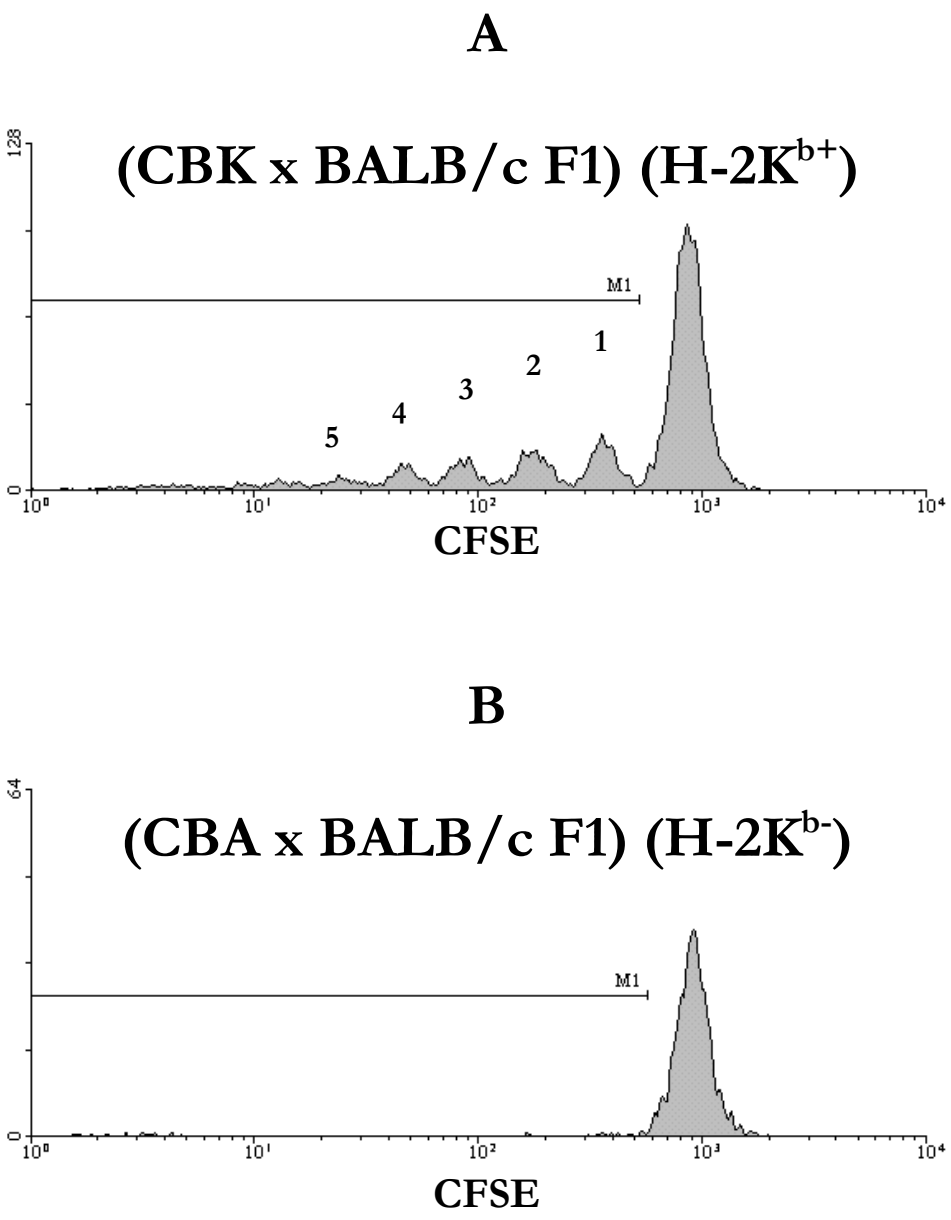


Figure 4.4 2 x 10^6 CD4 depleted $CD8^+DES^+$ T cells were adoptively transferred into CBA/Ca mice (CBA^{DES}). Five days following transplantation of H-$2K^{b+}$ cardiac allografts (n=3) (A) adoptively transferred $CD8^+DES^+$ T cells had undergone several rounds of division. A representative histogram of gated $CD8^+DES^+$ T cells is shown clearly demonstrating four discrete peaks of CFSE flouresence intensity corresponding to the number of divisions which they have undergone. (B) Control CBA^{DES} mice (n=3) transplanted with H-$2K^{b-}$ third party allografts analysed at 5 days did not result in division of adoptively transferred $CD8^+DES^+$ T cells.

Figure 4.5 H-2K^{b} Specific Infiltration of H-2K^{b+} Cardiac Allografts by CD8^{+}DES^{+} T Cells Five Days Following Transplantation

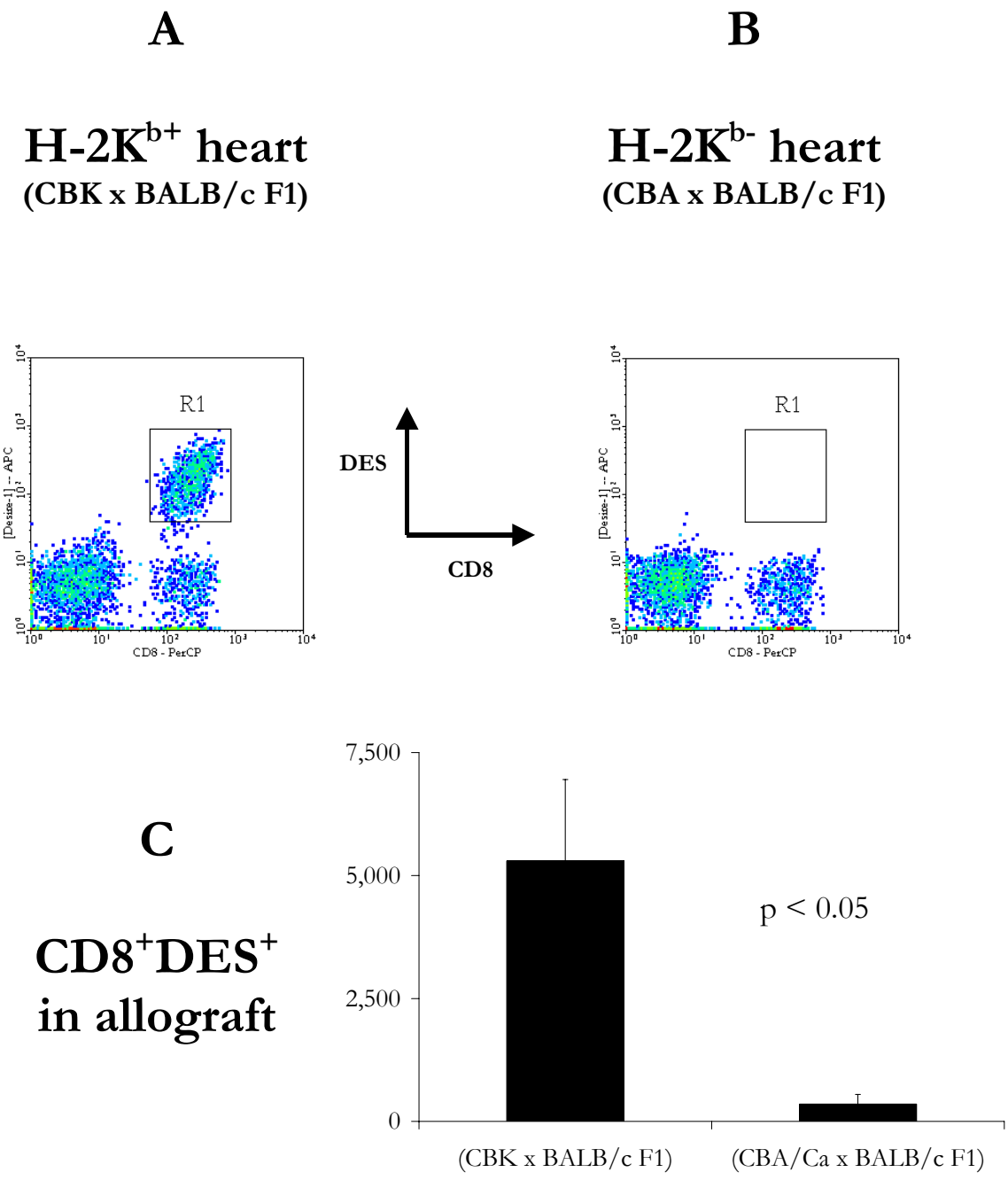


Figure 4.5 Five days following transplantation of (A) H-2K^{b+} (n=3) or (B) H-2K^{b-} (n=3) hearts into CBA^{DES} mice, graft infiltrating cells from transplanted hearts were isolated and analysed by FACS®. Adoptively transferred CD8^{+}DES^{+} T cells were only present in H-2K^{b+} hearts and not in allogeneic H-2K^{b-} hearts. (C) The mean absolute number +SEM (n=3/group) of CD8^{+}DES^{+} T cells present in rejecting allografts at day five is shown. CD8^{+}DES^{+} T cells also infiltrated H-2K^{b+} hearts (C57BL/10 & CBK) but did not infiltrate H-2K^{b-} hearts (NZW, BALB/c & CBA).

Figure 4.6 Infiltration of the Allograft by H-2K^b Specific T Cells Five Days Following Transplantation

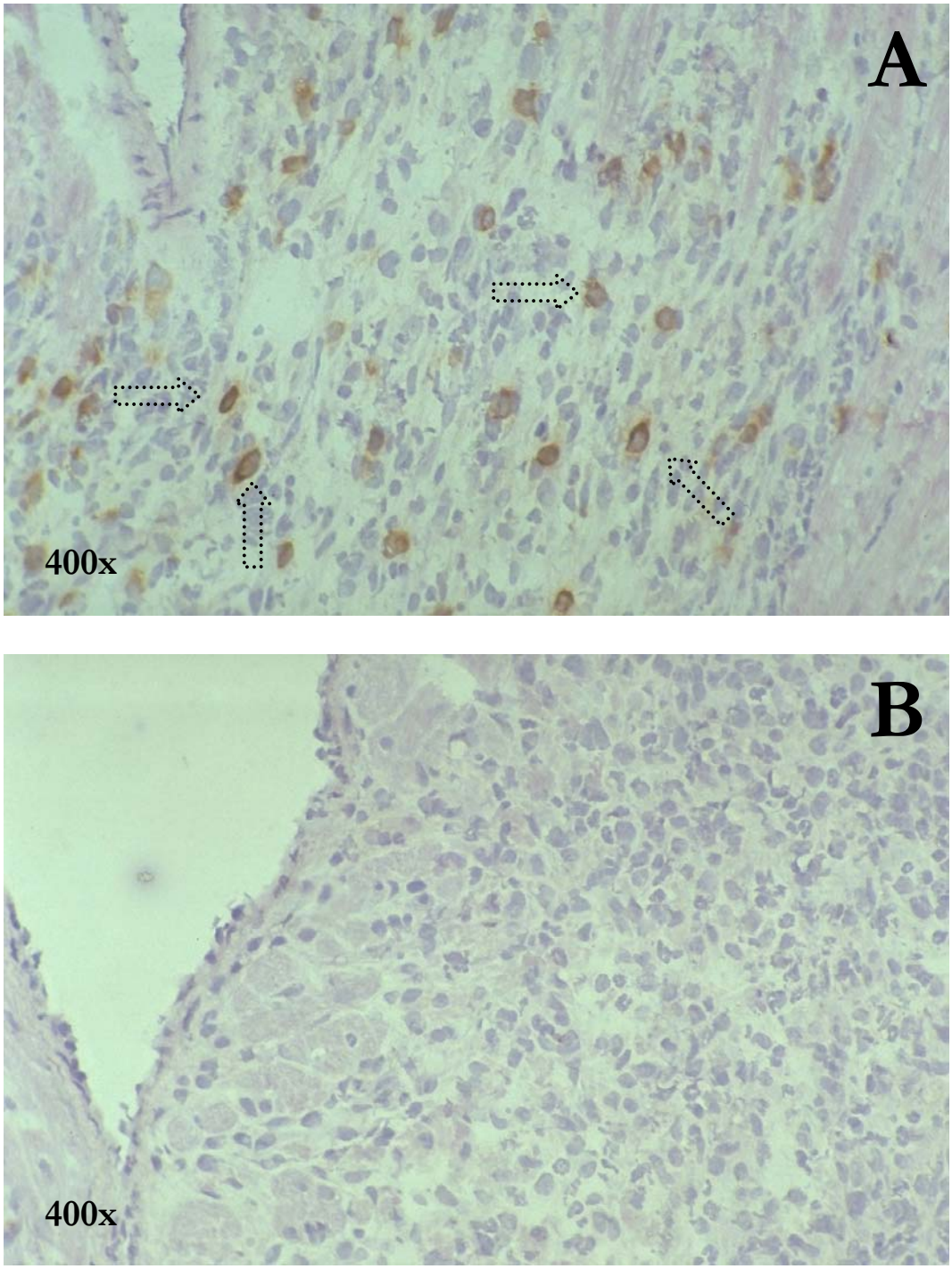


Figure 4.6 (A) H-2K^{b+} (CBK x BALB/c F1) or (B) H-2K^{b-} (CBA/Ca x BALB/c F1) hearts were transplanted into CBA^{DES} mice prepared in the usual fashion. Frozen sections of heart allografts five days after transplantation were stained with the clonotypic monoclonal antibody B20.2.2 specific for the DES transgenic TCR. Both sections were heavily infiltrated with lymphocytes however only H-2K^{b+} hearts were infiltrated by H-2K^b specific DES$^+$ T cells. (magnification 10x40)

Figure 4.7 Proliferation of $CD8^+DES^+$ T Cells Five Days Following Transplantation Is More Profound Within The Allograft than in the Spleen

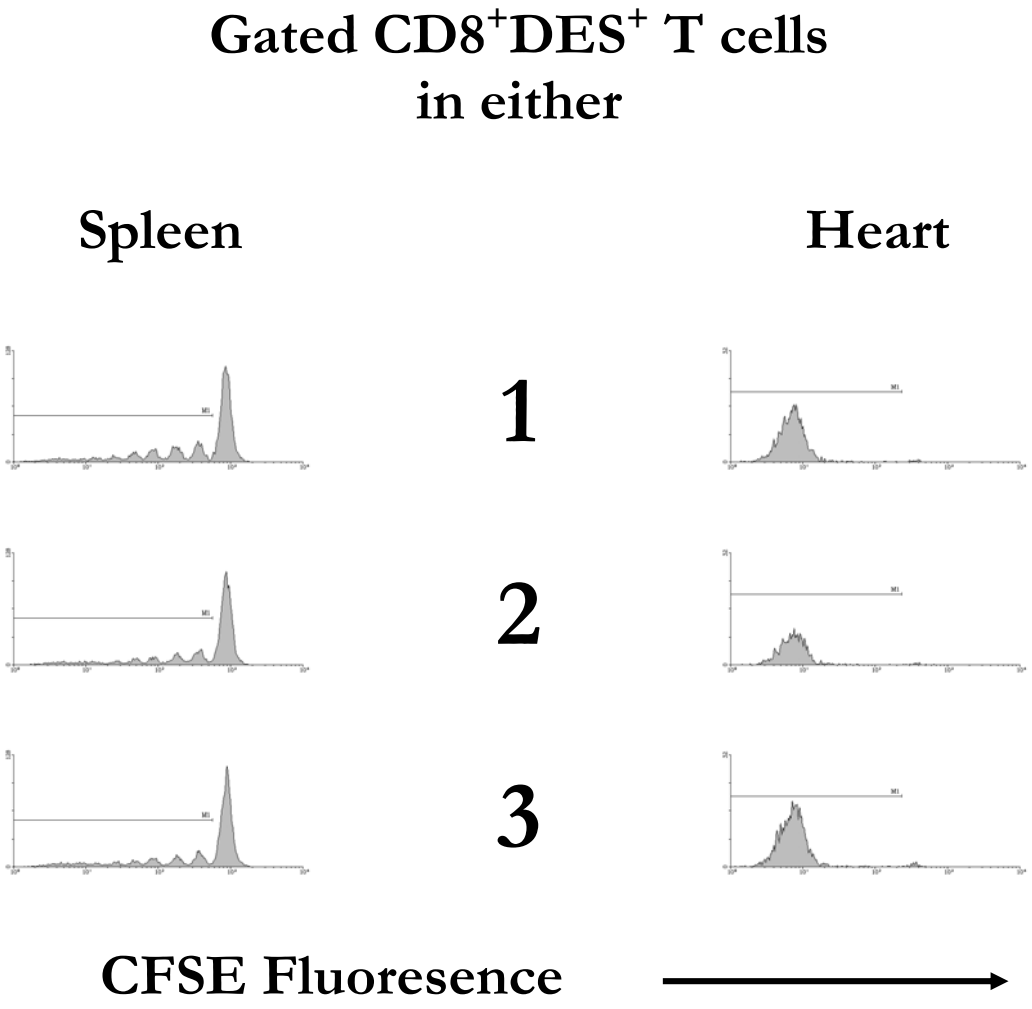
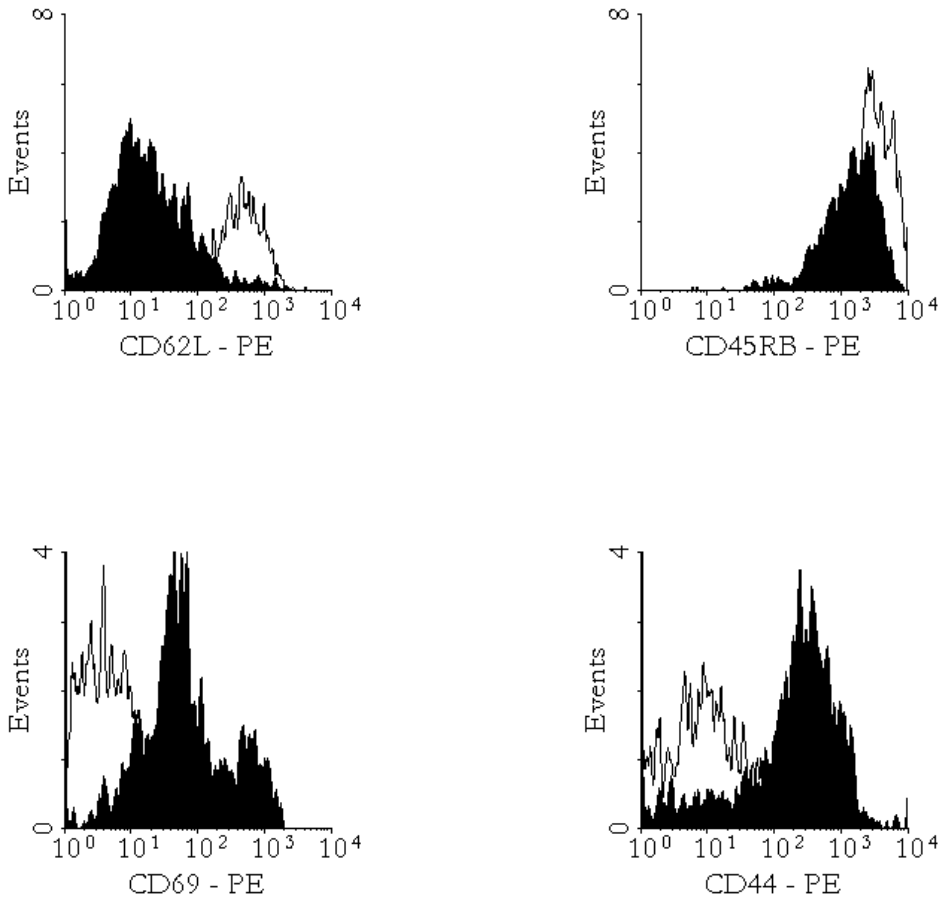


Figure 4.7 Five days after transplatation of (CBK x BALB/c F1) hearts into CBA^{DES} mice prepared in the usual fashion. The division history (CFSE) of $CD8^+DES^+$ T cells remaining in the spleen and those infiltrating the allograft were compared. $CD8^+DES^+$ T cells infiltrating H-$2K^{b+}$ cardiac allografts had clearly divided more extensively. CFSE histograms of $CD8^+DES^+$ T cells present in spleen and allografts from three separate CBA^{DES} mice are shown.

Figure 4.8 $CD8^+DES^+$ T Cells Infiltrating H-$2K^{b+}$ Hearts Possess an Activated Surface Phenotype



Filled – graft infiltrating cells
Empty overlay - spleen

Figure 4.8 Gated $CD8^+DES^+$ T cells present in rejecting allografts (CBK x BALB/c F1) had a surface phenotype consistent with an activated state. $CD8^+DES^+$ T cells in the spleens of the same animals possessed a resting surface phenotype. Representative histograms comparing adoptively transferred $CD8^+DES^+$ T cells present in spleens and rejecting hearts five days after transplatation are shown. Three CBA^{DES} mice were included in each group in this experiment. These results are representative of all the experiments performed.

Figure 4.9 Graft Infiltrating CD8$^+$DES$^+$ T Cells Possess a Tc1 Phenotype

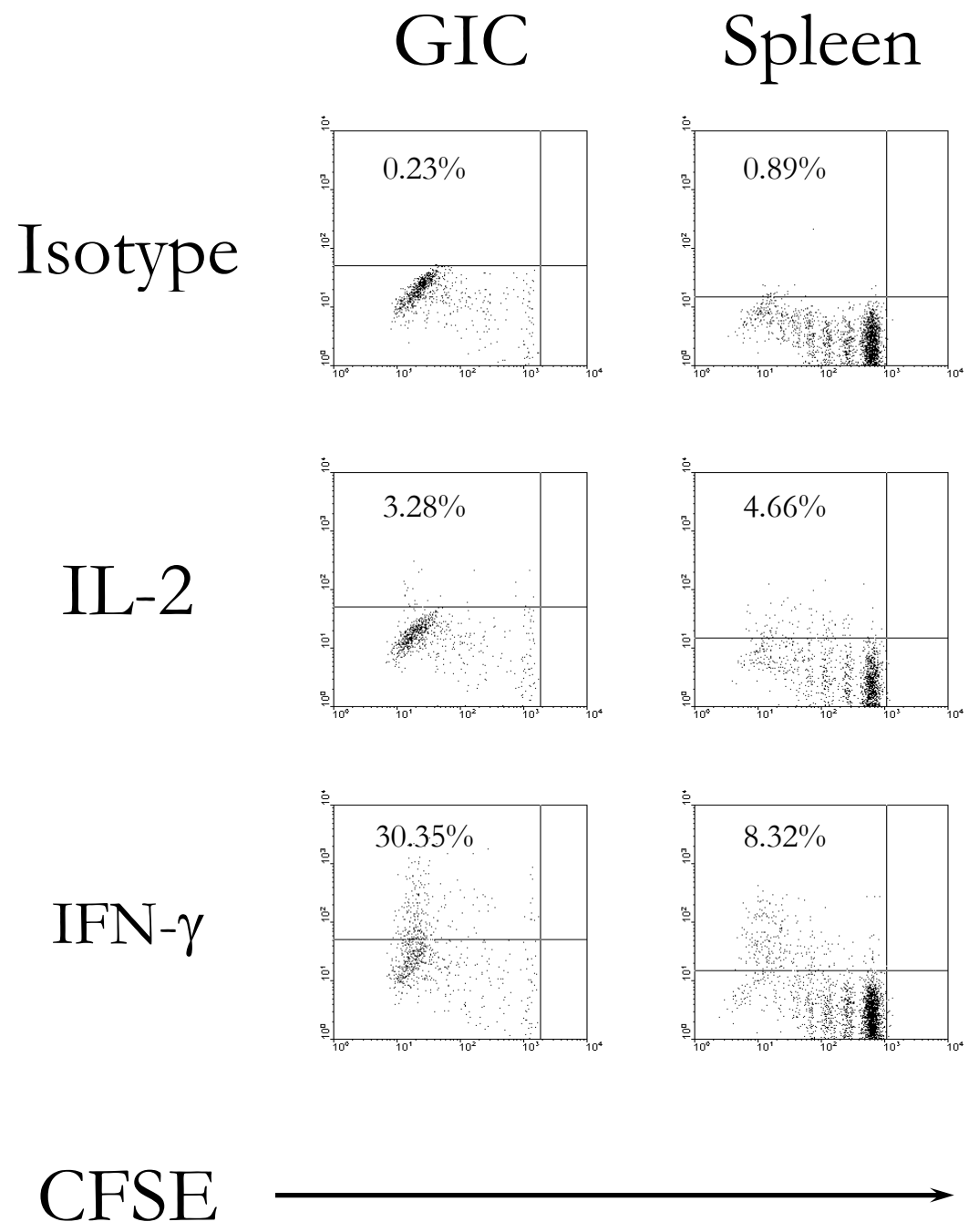


Figure 4.9 Gated CD8$^+$DES$^+$ T cells present in CBA^{DES} mice rejecting H-2K^{b+} cardiac allografts (CBK x BALB/c F1) (n=3) were analysed by FACS® for the production of Tc1 (IL-2 & IFN-γ) and Tc2 (IL-4 & IL-10) cytokines. The mean percentage of CD8$^+$DES$^+$ T cells producing Tc1 cytokines is shown. Tc2 cytokines were not produced following transplantation of H-2K^{b+} or H-2K^{b-} hearts. Three CBA^{DES} mice were included in each group in this experiment. The results are representative of all the experiments performed.

Figure 4.10 $CD8^+DES^+$ T Cells Persisted in the Spleen Long after Acute Rejection and Produced Interferon-γ

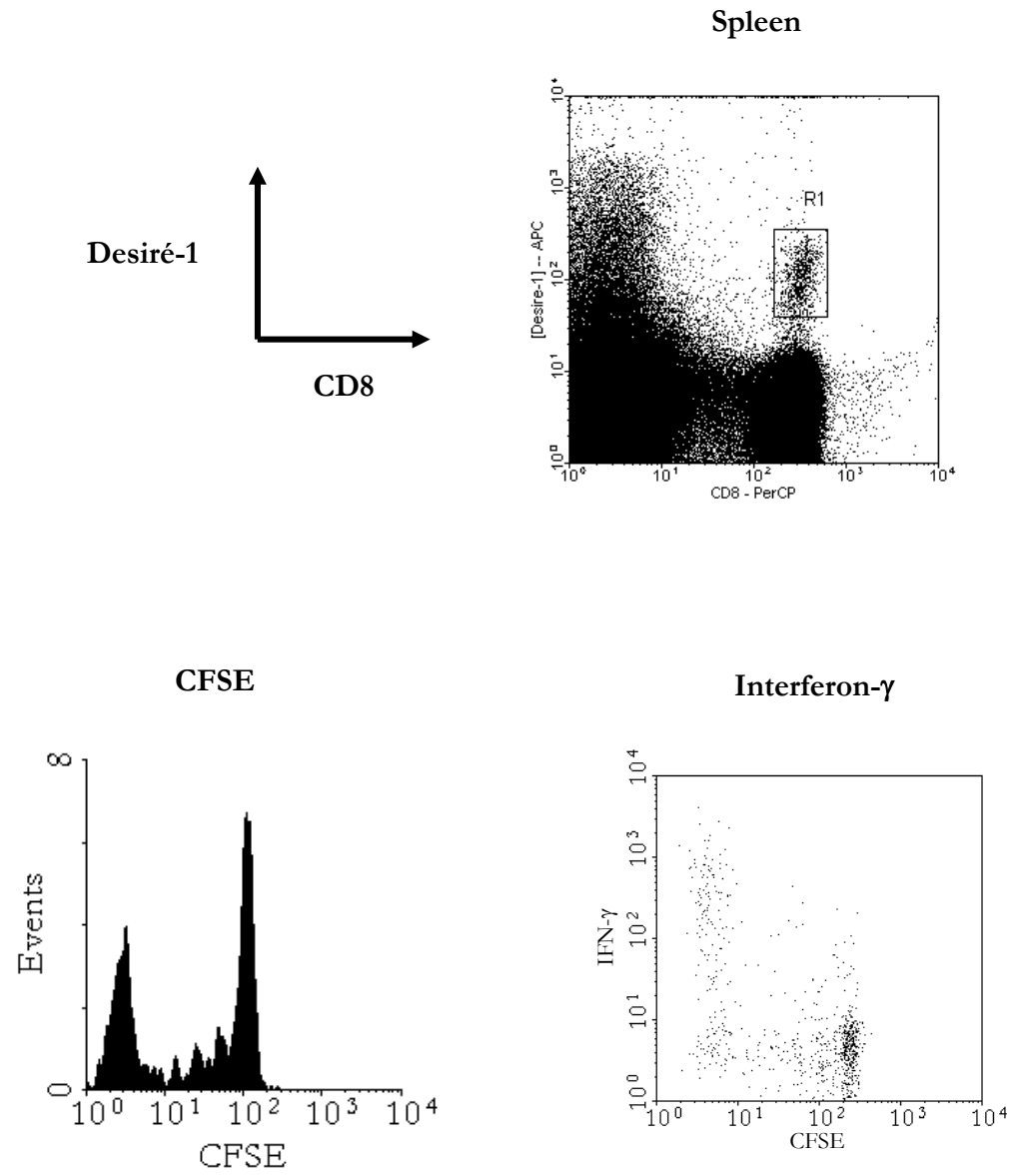


Figure 4.10 75 days following transplantation of H-$2K^{b+}$ (CBK x BALB/c F1) cardiac allografts (n=3) that were acutely rejected $CD8^+DES^+$ T cells were present in the spleen. CFSE analysis of gated $CD8^+DES^+$ T cells revealed a clear population of cells which had divided extensively that had not been present in the acute setting (Figure 4.4). Gated $CD8^+DES^+$ T cells in the spleen that were within the $CFSE^{low}$ peak produced interferon-γ, but not IL-2, IL-4 or IL-10.

Figure 4.11 Memory $CD8^+DES^+$ T Cells Possess a Distinct Surface Phenotype

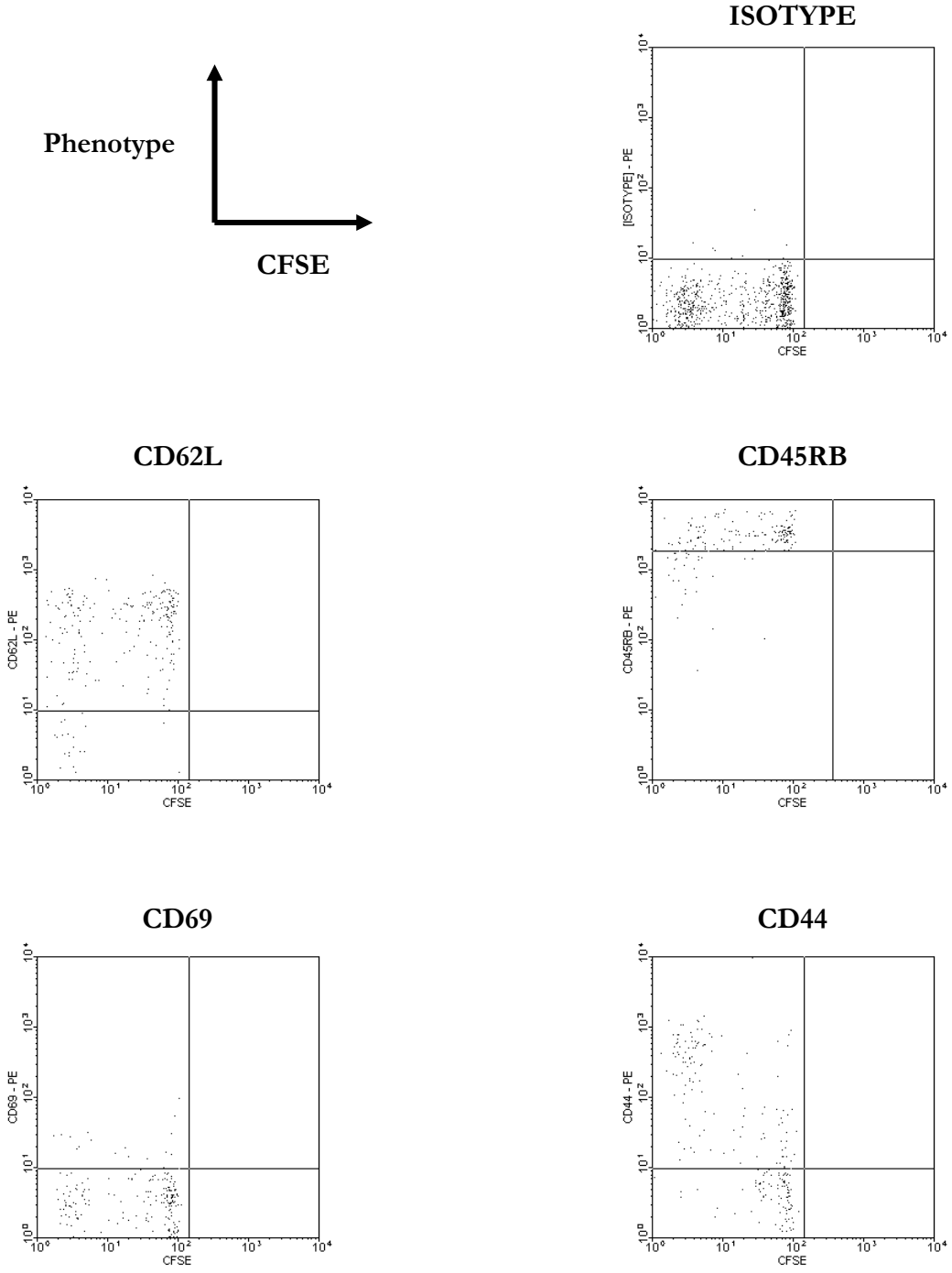


Figure 4.11 100 days following transplantation of H-$2K^{b+}$ (CBK x BALB/c F1) cardiac allografts (n=3) $CFSE^{low}CD8^+DES^+$ T cells possessed a memory $CD69^{low}CD44^{high}$ surface phenotype however CD45RB and CD62L expression remained heterogeneous. Representative FACS® plots are shown.

Chapter 5 - Induction of Unresponsiveness by Infusion of CBK Bone Marrow

5.1 Introduction

There is no doubt that successful tolerance inducing protocols would have a great impact on the practice of clinical transplantation and the treatment of end stage organ failure. However, despite over half a century of active interest in tolerance induction, reliable and safe strategies have not yet been brought to the clinic. Clinical reports continue to accumulate of patients with long-term functioning allografts even after cessation of immunosuppression indicating that tolerance to allografts is possible in human beings[163-165]. However, our understanding of the immune response to alloantigens is still relatively incomplete, preventing achievement of this goal as quickly as desired.

The powerful beneficial effect of allogeneic cells on the outcome of allotransplantation has been recognised for some time[159,162]. Pre-transplant blood transfusion has been shown to improve kidney allograft survival[309,310], but to date only one strategy has been demonstrated to reliably result in tolerance[311,312]. Patients requiring bone marrow transplantation prior to kidney transplantation and receiving them from the same living related donor have become tolerant, again showing that tolerance is a possibility.

Experimentally, our laboratory has been successful in producing donor specific unresponsiveness through the infusion of syngeneic CBK bone marrow expressing a

single donor MHC class I molecule, H-2K^{b}, prior to cardiac allograft transplantation. We have developed two protocols that lead to the indefinite survival (>100 days) of a fully allogeneic H-2K^{b+} (CBK x BALB/c F1) cardiac allograft following intravenous administration of H-2K^{b+} CBK bone marrow[215,216]. In the first, 50 x 10^{6} CBK bone marrow cells alone are infused 14 days prior to transplantation. In the second, 5 x 10^{6} CBK bone marrow cells are infused 27 days prior to transplantation under cover of depleting anti-CD4 monoclonal antibody YTA 3.1 (Figure 5.1). The cardiac allograft expressed H-2K^{b} as well as the full H-2^{d} haplotype to which the immune response has been suppressed. The spreading of unresponsiveness from H-2K^{b}, the "key" alloantigen, to H-2^{d} "bystander" alloantigens also expressed by the allograft, has been termed linked unresponsiveness, linked epitope suppression or infectious tolerance[214,219]. It requires that "bystander" and "key" alloantigens be expressed together by the same cardiac allograft[216]. When H-2K^{b} is absent, linked unresponsiveness is not operational, as is the case for (CBA/Ca x BALB/c F1) cardiac allografts that are acutely rejected despite CBK bone marrow infusion[214]. The mechanisms responsible for linked unresponsiveness remain somewhat obscure.

Bone marrow is not unique in its ability to induce linked unresponsiveness, the "key" antigen can be class I, class II or minor histocompatibility antigens, the "bystander" alloantigens can also be diverse, and the type of allograft can be skin or heart[217,218,220-223,313]. This diversity of protocols leading to linked suppression indicates that it is a robust mechanism for inducing unresponsiveness. It does not require a thymus[219] and is transferable to naïve animals[223,313], with the majority of data in the literature suggesting that CD4^{+} regulatory T cells mediate this.

Explanation of linked suppression requires the existence of an active mechanism of tolerance (e.g. regulation/suppression). For the better part of the past 30 years, evidence for a population of suppressor T cells has been lacking, disregarded or discredited. Gershon and Kondo in 1971, using a complicated experimental system observed that tolerance could be transferred from tolerant mice to naïve mice by lymphocytes[179]. With that, they introduced the phrase "infectious immunological tolerance" which hinted at the existence of "tolerant" lymphocytes able to regulate or suppress the activity of naïve lymphocytes as they respond to their nominal antigen. This provided some of the first support for the concept of suppressor T cells and the work of Gershon and Kondo, although questioned early on, identified a basic immunoregulatory mechanism necessary to explain linked suppression.

Qin et al. showed in an elegant experiment a clear example of regulatory T cells in vivo[180]. Thymectomised CBA/Ca mice transgenic for human CD2 were rendered tolerant to skin grafts with non-depleting CD4 and CD8 monoclonal antibodies. "Tolerant" T cells from these mice were infused into naïve CBA/Ca mice and were able to prevent rejection of skin grafts transplanted across the same minor histocompatibility mismatch (B10.BR ($H\text{-}2^k$) to CBA/Ca ($H\text{-}2^k$)). Infusion of tolerant mice with 50 x 10^6 non-tolerant splenocytes did not break tolerance to the skin grafts while it was shown, by depletion of tolerant T cells, that the naïve T cells in the adoptively transferred mice had the potential to reject the skin allografts. Further experiments showed that the ability to prevent rejection by the tolerant cells lay within the $CD4^+$ "tolerant" T cell population. However after 14 days of co-existence with "tolerant" cells, naïve T cells became themselves tolerant and also acquired the ability to suppress allograft rejection by other naïve populations.

The cellular and molecular mechanisms of cell-cell regulation remain the subject of debate however passive and active mechanisms have been proposed[314]. It has been suggested that tolerant T cells are able to regulate naïve T cells by cell-cell contact through the interaction of yet unidentified surface molecules[281] or by elaborating regulatory Th2 or Tc2 cytokines such as IL-4, IL-10, and TGF-β. Passively, tolerant T cells may out compete naïve T cells for ligands on the surface of activated antigen presenting cells while consuming important Th1 cytokines, such as IL-2, thereby depriving naïve T cells of ligands and T cell "help"[314]. What is clear though is that both "key" and "bystander" antigens must be expressed by the same antigen presenting cell in order for regulation to take place[315].

Our model offers the opportunity to focus on the role of $CD8^+$ T cells in linked suppression. A single mismatch for H-$2K^b$, an MHC class I molecule recognised by $CD8^+$ cytotoxic T cells, is the basis for the induction of linked suppression and is also required for acceptance of a fully allogeneic (CBK x BALB/c F1) cardiac allograft in our model. Focusing on $CD8^+$ T cells that recognise the "key" alloantigen via the direct pathway allowed us to assess the effect of tolerance induction on the $CD8^+$ T cell population. This would hopefully allow us to identify an active mechanism of tolerance induction operating through $CD8^+$ T cells.

It is with these ideas that we set forth, using the adoptive transfer model, to examine the role of $CD8^+$ T cells and determine if they could play a role in the establishment of linked unresponsiveness. We first investigated the effect of CBK bone marrow administration alone on the peripheral T cell repertoire. Next, we asked how this

would influence the response of $CD8^{+}DES^{+}$ T cells to the fully allogeneic H-$2K^{b+}$ cardiac allograft.

5.2 Results

For clarity, day 0 will refer to the day of transplantation. We assigned negative numbers for time points before and positive numbers for time points after transplantation irrespective of the protocol studied. For example, CBK bone marrow was infused on day –14 and analysis performed on days –9 and day +5 (Figure 5.1). Representative FACS® plots from each series of experiments have been chosen to illustrate key observations of $CD8^{+}DES^{+}$ T cells.

5.2.1 CBK Bone Marrow Infusion Prior to Transplantation Induced Operational Tolerance

5.2.1.1 Protocol 1

80% of CBA^{DES} mice infused with 50 x 10^{6} H-$2K^{b+}$ CBK bone marrow cells on day -14 accepted fully allogeneic H-$2K^{b+}$ (CBK x BALB/c F1) hearts for greater than 100 days (n=5). In contrast, H-$2K^{b-}$ (CBA/Ca x BALB/c F1) cardiac allografts were rejected by identically pretreated CBA^{DES} mice with a median survival time of 9.5 days (n=8) (Figure 5.2a).

5.2.1.2 Protocol 2

Five million CBK bone marrow cells infused into CBA^{DES} mice on day –27 along with two doses of YTA 3.1 (50μg) intravenously on days –27 & -28 also resulted in unresponsiveness to a fully allogeneic cardiac allograft. 100% of (CBK x BALB/c F1) hearts were accepted with a median survival time of greater than 100 days (n=7) while (CBA/Ca x BALB/c F1) hearts were rejected with a median survival time 9.5 days (n=8). Naïve CBA^{DES} (n=4) mice and CBA^{DES} mice treated with only YTA 3.1

(n=6) also rejected (CBK x BALB/c F1) hearts acutely with median survival times of 7.5 and 8.5 days, respectively (Figure 5.2b).

5.2.2 50 x 10^6 H-2K^{b+} CBK Bone Marrow Cells Induced Peripheral Clonal Deletion of CD8$^+$DES$^+$ T Cells

The effect of administering high dose CBK bone marrow intravenously was studied first. On day –14, 50 x 10^6 CBK (H-2K^{b+}) or NZW (H-2K^{b-}) bone marrow cells were administered intravenously to CBA^{DES} mice. Spleens were harvested on days –11, -9, -7, -5, -2, as well as +5, +30 and +100 days (n=3 for each group at each time point). The response of CD8$^+$DES$^+$ T cells was followed in the absence of transplantation (Figure 5.3).

CD8$^+$DES$^+$ T cells became fully activated in response to the infusion of CBK bone marrow cells. This was followed by massive clonal deletion in the longer term. Absolute counts of CD8$^+$DES$^+$ T cells revealed a 3 fold (307%) clonal expansion within three days (day –11) of CBK bone marrow infusion. This was followed by a reduction of mean absolute numbers of CD8$^+$DES$^+$ T cells to 54% of untreated CBA^{DES} controls by day +5, 39% by day +30 and 21% by day +100. Absolute counts of CD8$^+$DES$^+$ T cells in untreated CBA^{DES} mice (normalised to 100%) and CBA^{DES} mice infused with H-2K^{b-} NZW bone marrow cells did not significantly differ from each other (Figure 5.3).

5.2.3 Proliferation of $CD8^+DES^+$ T Cells Following CBK Bone Marrow Infusion

CFSE analysis convincingly confirmed that $CD8^+DES^+$ T cells underwent cell division in response to infusion of CBK bone marrow but showed no response to NZW bone marrow (Figure 5.4). At the peak of clonal expansion (day –11) following CBK bone marrow infusion, a mean of 99% of $CD8^+DES^+$ T cells had divided at least once.

5.2.4 Generation of Memory $CD8^+DES^+$ T Cells by Infusion of 50 x 10^6 CBK Bone Marrow Cells

In Figure 5.5, we show representative FACS® dot plots of gated $CD8^+DES^+$ T cells recovered from the spleen on day –2 following infusion of 50 x 10^6 CBK bone marrow (n=3) on day –14 (Figure 5.1) compared to control CBA^{DES} mice infused with NZW bone marrow (n=3). Plotting the cell surface phenotype (y axis) versus CFSE fluorescence (x axis) provided greater insight into the response $CD8^+$ T cells to H-$2K^{b+}$ bone marrow cells. This time point was indicative of the phenotypic state of the cells at the time when a cardiac allograft would normally be transplanted.

First, we observed that a spectrum of phenotypic states existed within the population of $CD8^+DES^+$ T cells at this time point. The phenotype of each individual cell was intimately related to it's division history. Those cells which had not divided possessed an essentially naïve phenotype ($CD62L^{high}CD69^{low}CD45RB^{high}CD44^{low}$). However, $CD8^+DES^+$ T cells which were within the CFSE fluorescence peaks with the lowest fluorescence intensity (i.e. >3 divisions) had a $CD69^{low}CD44^{high}$ memory phenotype. Once again, CD62L and CD45RB expression was heterogeneous.

Cytokine production by these cells was similarly influenced by division history (Figure 5.6). IL-2 and interferon-γ were produced by $CD8^+DES^+$ T cells that had undergone the most divisions in response to CBK bone marrow infusion. IL-4 and IL-10 were not produced at any time point between day –11 and day +100.

In summary, infusion of 50 x 10^6 CBK bone marrow cells resulted in $CD8^+DES^+$ T cells proliferating, developing a memory surface phenotype and Tc1 profile of cytokine production in the spleens of pretreated mice. Additionally, CD44 expression appeared to be the most reliable marker of memory in our experimental system, as previously observed (Figure 4.11). The fact that pretreatment with 50 x 10^6 CBK bone marrow cells resulted in operational tolerance suggested that H-$2K^b$ specific $CD8^+DES^+$ T cells were "tolerant" but functional studies were not performed to prove this.

5.2.5 Activated $CD8^+DES^+$ T Cells Infiltrate H-$2K^{b+}$ Cardiac Allografts Despite Pretreatment & Peripheral Clonal Deletion

In order to investigate the effect of CBK bone marrow infusion on the course of allograft rejection we prepared several groups of CBA^{DES} mice (n=3 per group) for analysis at days +5, +30, and +100 as follow:

(1) naïve CBA^{DES}

(2) 50 x 10^6 CBK BMC infused on day –14

(3) group 2 transplanted with H-$2K^{b-}$ (CBA/Ca x BALB/c F1) heart on day 0

(4) group 2 transplanted with H-$2K^{b+}$ (CBK x BALB/c F1) heart on day 0

(5) group 1 transplanted with H-$2K^{b+}$ (CBK x BALB/c F1) heart on day 0

$CD8^+DES^+$ T cells isolated from spleens and graft infiltrating cells were enumerated, and analysed for surface phenotype and the production of cytokines. Due to the small number of graft infiltrating cells, cells isolated from the same group of mice had to be pooled in order to perform a full phenotype analysis however samples for absolute counts were obtained prior to pooling. All animals studied in group 4 had strongly beating hearts at the time of each experiment, while all CBA^{DES} mice in groups 3 and 5 had rejected their hearts acutely (Figure 5.2a). We were only able to isolate graft infiltrating cells from rejecting and non-rejecting hearts at day +5 and from long-term surviving hearts in group 4.

Evident at all three time points (Figure 5.7) was the finding that infusion of 50 x 10^6 CBK bone marrow cells alone (group 2) was predominately responsible for the massive clonal deletion of $CD8^+DES^+$ T cells observed in the spleens of CBA^{DES} mice. Although when a H-$2K^{b+}$ cardiac allograft was transplanted on day 0, clonal deletion was more profound at all three time points (group 4). Despite pretreatment with 50 x 10^6 H-$2K^{b+}$ bone marrow cells and the peripheral clonal deletion that it caused, $CD8^+DES^+$ T cells still homed to H-$2K^{b+}$ cardiac allografts in similar numbers. There was a mean of 30,864 (±SD 13,555) graft infiltrating $CD8^+DES^+$ T cells in group 4 and a mean of 23,518 (±SD 7,829) graft infiltrating $CD8^+DES^+$ T cells in group 5.

As previously observed in chapter 4, $CD8^+DES^+$ T cells homed only to H-$2K^{b+}$ hearts and not to H-$2K^{b-}$ hearts. This was not changed by pretreatment since H-$2K^{b-}$ (CBA/Ca x BALB/c F1) hearts (group 3) were not infiltrated (n=3). However, we did observe that the mean absolute number of $CD8^+DES^+$ T cells present within H-

$2K^{b+}$ cardiac allografts transplanted into CBA^{DES} mice pretreated with 50 x 10^6 CBK bone marrow declined over time from 30,864 (±SD 13,555) $CD8^+DES^+$ T cells at day +5 to 5,177 (±SD 2,481) $CD8^+DES^+$ T cells at day +30 to undetectable at day 100.

In Figure 5.8, we examined the surface phenotype of $CD8^+DES^+$ T cells at day +5 within the H-$2K^{b+}$ allografts of mice in group 4 (n=3) and group 5 (n=3). Groups 4 and 5 were destined to accept and reject their H-$2K^{b+}$ heart grafts respectively, giving us the opportunity to compare the $CD8^+DES^+$ T cell response to cardiac allografts under tolerising versus rejecting conditions. Graft infiltrating cells from three CBA^{DES} mice in each group were pooled for analysis due to the limited number of infiltrating cells after determining the absolute counts individually. The spleens of all CBA^{DES} mice were analysed separately.

$CD8^+DES^+$ T cells present in the spleens of naïve CBA^{DES} mice (group 1) remained undivided and retained a naïve phenotype at all time points examined. $CD8^+DES^+$ T cells present in the spleens of CBA^{DES} mice in groups 2, 3 and 4 resembled those previously observed on day –2, prior to transplantation ($CFSE^{low}CD69^{low}CD44^{high}$ memory cells, Figure 5.5 & Figure 5.8), while $CD8^+DES^+$ T cells present in the spleens of mice in group 5 lacked a discrete population of $CD8^+DES^+$ T cells with a memory phenotype.

The surface phenotype of pooled graft infiltrating $CD8^+DES^+$ T cells was analysed for group 4 and group 5. We found that $CD8^+DES^+$ T cells infiltrating H-$2K^{b+}$ cardiac allografts in both groups generally possessed an activated/effector $CD62L^{low}CD69^{high}CD45RB^{low}CD44^{high}$ phenotype (Figure 5.8). However, in group 4

$CD8^+DES^+$ T cells infiltrating transplanted hearts were less heterogeneous. A mean of 97% (n=3) of these were $CFSE^{low}$ (>3 divisions) with a strongly activated $CD62L^{low}CD69^{high}CD45RB^{low}CD44^{high}$ phenotype. In contrast, a mean of 24% (n=3) of $CD8^+DES^+$ T cells infiltrating hearts in group 5 had not divided at all (0 divisions). One possible interpretation of these data is that in pretreated CBA^{DES} mice (group 4) $CD8^+DES^+$ T cells which had been exposed to CBK bone marrow but that had not been driven to extensive division were now defective in their ability to respond fully on rechallenge with a $H\text{-}2K^{b+}$ allograft.

CFSE vs. phenotype plots of $CD8^+DES^+$ graft infiltrating T cells suggested an ongoing response in group 5 (Figure 5.8). Interestingly naïve $CD8^+DES^+$ T cells in untreated CBA^{DES} mice (group 5) appeared to enter the graft with an activated non-memory $CD62L^{low}CD69^{high}CD45RB^{high}CD44^{low}$ surface phenotype. However, with proliferation these cells also progressed to acquire a $CD45RB^{low}CD44^{high}$ surface phenotype. In an untreated host (group 5), the modulation of these "memory" surface markers within the allograft would suggest that partial transformation of $CD8^+DES^+$ T cells from activated/effector T cells to memory T cells occurred within the allograft. However, true memory cells are $CD69^{low}$. CD69 expression is potentially down-regulated with exit from the allograft resulting in true $CD69^{low}CD44^{high}$ $CD8^+DES^+$ memory T cells present in the periphery as was seen in figure 4.11. The loss of CD69 expression with exit from the graft was not confirmed or contradicted by these data.

Pooled graft infiltrating cells from group 4 and group 5 were also analysed for cytokine production (Figure 5.9). As in figure 5.8, $CD8^+DES^+$ T cells from group 4

appeared less heterogeneous and produced only interferon-γ similar to those in group 5.

5.2.6 5 x 10^6 CBK Bone Marrow in Combination with YTA 3.1 Impairs Infiltration of H-2K^{b+} Cardiac Allografts by CD8$^+$DES$^+$ T Cells

As both high and low dose protocols lead to unresponsiveness to the same fully allogeneic H-2K^{b+} (CBK x BALB/c F1) cardiac allograft we briefly analysed mice pretreated using the second protocol (Figure 5.1) on day +5. Five experimental and control groups were prepared (n=3 CBA^{DES} mice/group):

(1) naïve CBA^{DES}

(2) group 1 treated with 50μg YTA 3.1 intravenously on days –28 and –27

(3) group 2 infused with 5 x 10^6 CBK bone marrow cells on day –27

(4) group 3 transplanted with an H-2K^{b-} (CBA/Ca x BALB/c F1) cardiac allograft on day 0

(5) group 3 transplanted with an H-2K^{b+} (CBK x BALB/c F1) cardiac allograft on day 0.

First, we observed that no clonal deletion had taken place in the periphery following pretreatment with low dose CBK bone marrow and anti-CD4 (Figure 5.10). No CFSE^{low}CD69^{low}CD44^{high} memory population was detectable in the spleens of pretreated CBA^{DES} mice following pretreatment with H-2K^{b+} bone marrow and anti-CD-4 (data not shown). The lower dose of CBK bone marrow with anti-CD4 mAb was not as immunogenic for donor specific T cells, perhaps due to the lack of effective CD4$^+$ T cell help. CD8$^+$DES$^+$ T cells only infiltrated (CBK x BALB/c F1)

allografts following pretreatment. However, the most striking observation was that infiltration of the graft by $CD8^+DES^+$ T cells was reduced following pretreatment with 5 x 10^6 CBK bone marrow cells in combination with YTA 3.1 (Figure 5.11). This made meaningful analysis of graft infiltrating $CD8^+DES^+$ T cells difficult, however the small number of $CD8^+DES^+$ T cells that successfully infiltrated H-$2K^{b+}$ allografts possessed a $CD62L^{low}CD69^{high}CD45RB^{low}CD44^{high}$ surface phenotype (data not shown). Due to limited numbers, graft infiltrating cells were pooled for surface phenotype analysis. There were too few $CD8^+DES^+$ T cells for analysis of cytokine production. These observations suggested that $CD8^+DES^+$ T cells remaining in the periphery were impaired in their ability to infiltrate H-$2K^{b+}$ cardiac allografts by pretreatment with low dose CBK bone marrow in combination with depleting anti-CD4.

5.2.7 Adoptively Transferred $CD8^+DES^+$ T Cells Do Not Infiltrate an Established H-$2K^{b+}$ Cardiac Allograft in a Tolerant Host

In situations of tolerance and long-term unresponsiveness, the allograft persists long after the immediate consequences of the alloantigen pretreatment have resolved. However, naïve T cells continue to be produced in euthymic recipients, and established allografts could be vulnerable to attack by newly generated allograft specific T cells. Here we explored how an established allograft interacts with naïve $CD8^+DES^+$ T cells that enter the peripheral lymphoid pool in a resting state.

We prepared CBA/Ca mice pretreated with YTA 3.1 and 5 x 10^6 CBK bone marrow cells which were then transplanted with (CBK x BALB/c F1) hearts (n=7). All 7 hearts were beating at day +196. 2 x 10^6 naïve $CD8^+DES^+$ T cells were adoptively

transferred into these mice at this time point. Spleens and cardiac allografts were harvested for analysis, five days later on day +201. Allografts were still beating strongly at this time.

There was no detectable response against H-2K^{b} by the naïve CD8^{+}DES^{+} T cells infused (data not shown). No cell division (CFSE) was detected in the periphery. CD8^{+}DES^{+} T cells did not infiltrate (CBK x BALB/c F1) allografts, or produce cytokines (IL-2, interferon-γ, IL-4 or IL-10) and possessed an entirely naïve surface phenotype 5 days after infusion. These data were taken to indicate that H-2K^{b} specific cells infused were either "ignorant" of the allograft or were themselves being suppressed by a regulatory population of cells. This serves as a possible model for the maintenance of allograft tolerance in the face of a functioning thymus.

5.3 Discussion

In this chapter, we investigated the induction of unresponsiveness to fully allogeneic H-2K^{b+} cardiac allografts following infusion of syngeneic H-2K^{b+} bone marrow (CBK). Two protocols that lead to acceptance of a (CBK x BALB/c F1) allograft were studied (Figure 5.1). We confirmed that CBA^{DES} mice would also accept (CBK x BALB/c F1) cardiac allografts following pretreatment with CBK bone marrow cells in both protocols (Figure 5.2a & Figure 5.2b). The objective of this chapter was to investigate the effect of these tolerance protocols on H-2K^{b} specific CD8^{+} T cells and possibly identify a mechanism of tolerance operating through alloreactive cytotoxic T cells.

We showed that infusion of high dose CBK bone marrow cells caused a significant peripheral clonal deletion of H-2K^{b} reactive CD8^{+}DES^{+} T cells (Figure 5.3) and that this was associated with the indefinite survival of a fully allogeneic cardiac allograft (Figure 5.2a). Infusion of a lower dose of bone marrow in combination with depleting anti-CD4 monoclonal antibody treatment also induced indefinite allograft survival (Figure 5.2b), but clonal deletion of CD8^{+}DES^{+} T cells was not observed (Figure 5.10). These data are consistent with previous observations of the effect of bone marrow dosage in other experimental settings[198,313].

A full time course analysis of the effect of infusion of a high dose of CBK bone marrow on CD8^{+}DES^{+} T cells showed that infusion of 50 x 10^{6} CBK bone marrow cells resulted initially in massive clonal expansion (approx. 307%) of donor specific CD8^{+}DES^{+} T cells (Figure 5.3). CD8^{+}DES^{+} T cells became fully activated within three days expressing CD69 and dividing up to 6 times. However, by the time of

transplantation (Day 0), the size of the population of $CD8^+DES^+$ T cells had essentially returned to control levels (Figure 5.3) and possessed a $CD69^{low}CD44^{high}$ memory surface phenotype (Figure 5.5). In the absence of a $H\text{-}2K^{b+}$ heart allograft clonal deletion continued to day +100 (Figure 5.7, group 2). The fact that bone marrow cells are non-professional antigen presenting cells may play a role. It would have been interesting to determine for how long and in which tissues $H\text{-}2K^{b+}$ bone marrow cells persisted after infusion. These experiments are currently in progress in the laboratory.

As bone marrow infusion leads to linked unresponsiveness and acceptance of a fully allogeneic cardiac allograft, we were interested to know if $CD8^+DES^+$ T cells had developed a putative regulatory phenotype as determined by cytokine production. At day –2, a full analysis of $CD8^+DES^+$ T cells present within the spleens of pretreated CBA^{DES} mice was performed (Figure 5.5 & Figure 5.6). We found that the cells possessed an antigen experienced memory phenotype. IL-2 and interferon-γ production by these cells indicated a Tc1 phenotype. At no time point were we able to demonstrate production of Tc2 cytokines (IL-4 or IL-10) by $CD8^+DES^+$ T cells. Production of IL-4 or IL-10 by the residual population might have been suggestive of the involvement of regulatory T cells, although we did not explore whether the potentially immunosuppressive cytokine TGF-β was produced by these cells.

Although, we were not able to show that $CD8^+DES^+$ T cells were producing regulatory cytokines on day -2, a small residual population of donor specific cells persisted in the longer term. The full activation of $CD8^+DES^+$ T cells, clonal expansion followed by peripheral deletion is a pattern that has been observed by other investigators[169,170,204]. In response to antigens constitutively expressed by

the host[169], to overwhelming LCMV viral infection[204], or to superantigen exposure[170] mature $CD8^+$ antigen specific cells were found to behave in a similar way to our findings, but a residual population of anergic antigen specific cells persisted in the long-term[169,204]. The obvious question is whether a population of anergic $CD8^+DES^+$ T cells is also generated in response to high dose CBK bone marrow infusion. What further evidence do we have suggesting that $CD8^+DES^+$ T cells were anergic? Indirectly, we can say that survival of a fully allogeneic H-$2K^{b+}$ allograft following pretreatment indicates that residual $CD8^+DES^+$ T cells were at least tolerant and possibly anergic (Figure 5.2a). Alternatively, because of clonal deletion, the number of residual $CD8^+DES^+$ T cells was too low to mount an effective rejection response. One possible approach to test this would have been to harvest $CD8^+DES^+$ T cells from pretreated CBA^{DES} mice, re-label them with CFSE, adoptively transfer them into CBA/Ca mice and re-challenge them with H-$2K^b$ in some form (bone marrow, skin or heart). If these re-adoptively transferred $CD8^+DES^+$ T cells failed to respond to this challenge by proliferation or cytokine production, this would provide further evidence in support of anergy.

While we investigated the mechanisms of the maintenance phase of tolerance, we demonstrated a potential manifestation of regulation or ignorance. We found that when naïve $CD8^+DES^+$ T cells were adoptively transferred into CBA/Ca mice that had accepted H-$2K^{b+}$ (CBK x BALB/c F1) cardiac allografts they remained in a naïve, undivided state and did not infiltrate the established H-$2K^{b+}$ cardiac allografts (Section 5.2.7). This serves as a model for the behaviour of newly generated T cells released from the thymus long after pretreatment and transplantation have occurred. However, this also provides support for the idea that unresponsive, anergic or "tolerant" cells persisting in tolerant hosts may be able to regulate or suppress the

activation of naïve graft specific T cells. A regulatory population may have been established in the presence of an accepted allograft or during the pretreatment phase.

A second possibility explaining our observation is that other factors that normally promote T cell activation were absent in mice with long-term surviving grafts. (1) donor antigen presenting cells from cardiac allografts were lacking and (2) inflammation due to surgical trauma was long resolved. These two points could have resulted in an unawareness of the allograft by T cells, in other words "ignorance" as described by Ohashi et al.[178]. It is clear that in hosts tolerant of allografts the activation of naïve graft specific T cells is limited either by regulatory T cells or a non-immunogenic allograft.

The residual population of potentially anergic cells following bone marrow pretreatment may allow an active tolerance mechanism mediated by graft specific H-$2K^b$ specific $CD8^+$ T cells to be postulated. "Linked suppression" has been closely investigated in vitro by several investigators[281,314-317]. The data obtained suggest that it can be mediated by anergic T cells and that antigen presenting cells involved in linked suppression must bear both alloantigens. Anergy is an attractive possibility since it could explain the lack of response to both "key" and "bystander" alloantigens. Anergic H-$2K^b$ specific $CD8^+DES^+$ T cells could via an allograft expressing both "key" and "bystander" alloantigens suppress the response or activation of T cells specific for the entire H-2^d haplotype in our model. This would provide a mechanism for the suppression of T cell responses against "bystander" alloantigens. In effect, H-$2K^b$ specific cells would not respond themselves in addition to meditating the suppression of "bystander" specific T cell responses. This would offer an elegant and unifying explanation.

It would have been interesting to isolate $CD8^+DES^+$ T cells from CBA^{DES} mice following pretreatment in order to test for anergy by performing cytotoxicity, cytokine and mixed lymphocyte culture assays in vitro. However, this would have required the development of a technique to purify trace numbers of $CD8^+DES^+$ T cells from CBA^{DES} mice. High speed FACS® sorting would have offered one possibility, but the absolute number of $CD8^+DES^+$ T cells remaining in the long-term surviving mice was relatively small (<300,000 cells/CBA^{DES} spleen). Moreover, FACS® sorting of these cells would have required the use of anti-CD8 and Désiré-1 monoclonal antibodies which may have interfered with the antigen recognition mechanisms of $CD8^+DES^+$ T cells at a molecular level, confounding further analysis.

At day +5 $CD8^+DES^+$ T cells in CBA^{DES} mice infused with 50 x 10^6 CBK bone marrow cells on day –14 infiltrated (CBK x BALB/c F1) cardiac allografts in roughly equal numbers to $CD8^+DES^+$ T cells in naïve. At this time point $CD8^+DES^+$ T cells in the pretreated mice differed very little from those in naïve mice in that they also possessed a fully activated surface phenotype and produced interferon-γ. However, although the general phenotype of the cells was similar in both groups, cells infiltrating hearts in pretreated CBA^{DES} mice were less heterogeneous. Interestingly, the number of $CD8^+DES^+$ T cells in mice with surviving allografts declined with time after transplantation such that by day +100, they were entirely absent.

Two striking differences between the two tolerance induction protocols were observed. Interestingly, infusion of 5 x 10^6 CBK bone marrow cells on day –27 under cover of a depleting anti-CD4 monoclonal antibody did not result in clonal deletion and despite this, infiltration of the H-$2K^{b+}$ allografts by $CD8^+DES^+$ T cells

was significantly reduced (Figure 5.11). Clearly, there was a deficit in the ability of $CD8^+DES^+$ T cells to enter cardiac allografts after bone marrow pretreatment in combination with anti-CD4. Besides the dose and conditioning in this protocol, we must consider that there is a difference of timing between pretreatment and transplantation (28 days). In this protocol, pretreated $CD8^+DES^+$ T cells had an additional 14 days to "mature" possibly explaining why $CD8^+DES^+$ T cells had lost, for the most part, their ability to infiltrate the cardiac allograft.

In a preliminary experiment we attempted to extend our analysis in both the low dose and high dose CBK bone marrow protocols. With the availability of $CD8^+DES^+$ and $CD8^+1B2^+$ T cells, we were able to follow immune responses to H-$2K^b$ and H-$2L^d$ independently of one another. $CD8^+1B2^+$ T cells offer an opportunity to visualise T cells specific for a "bystander" MHC class I antigen H-$2L^d$ that in theory would not be mounting an effective immune response against a "bystander" when present with cells recognising the "key" alloantigen. Hence, we could potentially gain insight into the immune response against "key" and "bystander" alloantigens within the same host – both MHC class I molecules in our experimental system. We were interested to see if $CD8^+1B2^+$ T cells behaved differently to $CD8^+DES^+$ T cells when H-$2K^b$ was expressed by the cardiac allograft. We generated $CBA^{DES/2C}$ mice seeded with the two populations of $CD8^+TG^+$ T cells. Experiments utilising both protocols with $CBA^{DES/2C}$ mice were performed. These mice were transplanted with H-$2K^{b+}$ (CBK x BALB/c F1) or H-$2K^{b-}$ (CBA/Ca x BALB/c F1) cardiac allografts. Analysis of $CBA^{DES/2C}$ mice was performed on day +5. Unfortunately, both experiments failed due to technical limitations and complexity. In the first experiment we were unable to enrich adequate numbers of $CD8^+1B2^+$ T cells for the large experiment and in the second $CD8^+1B2^+$ T cells in

untreated/untransplanted $CBA^{DES/2C}$ mice were found to be differentiated to $CD44^{high}$ interferon-γ producing cells consistent with a memory phenotype. Analysis of $CD8^+DES^+$ T cells in these mice confirmed the results described in chapter 4 & chapter 5. This component of the study requires further experiments that will be carried out by other members of the group in the future.

Both a strength and a weakness of the experimental system developed for this thesis is that only direct allorecognition of class I alloantigens can be studied. An adoptive transfer system utilising an indirect $CD4^+$ TCR transgenic specific for processed H-$2K^b$ peptides presented by H-$2IA^k$ or H-$2IE^k$ would be complimentary to the model we have developed. Such a system would allow us to study this MHC class I mismatched system via indirect recognition, possibly identifying active mechanisms of tolerance induction within the $CD4^+$ T cell subset.

In conclusion, we have observed that the small residual population of $CD8^+DES^+$ T cells that persist following high dose CBK bone marrow infusion possess a memory phenotype, but elaborate Tc1 inflammatory cytokines and not regulatory Tc2 cytokines such as IL-4 or IL-10. Despite homing to H-$2K^{b+}$ allografts by day +5, they evacuated or were deleted within the allograft over time and allowed the allograft to survive indefinitely. Some of these residual (potentially anergic) cells may offer a route to an active mechanism of tolerance (suppression) as has been suggested by the in vitro work of several investigators[281,314,315,317]. Presumably, a similar mechanism could also play a role in the low dose/anti-CD4 protocol. We cannot show conclusively that a regulatory population exists among the residual donor reactive T cells, however it is fair to argue that they may contribute to linked suppression via an anergy mediated mechanism.

Figure 5.1 Analysis of $CD8^+DES^+$ T Cells Following Alloantigen Pretreatment (Experimental Protocols)

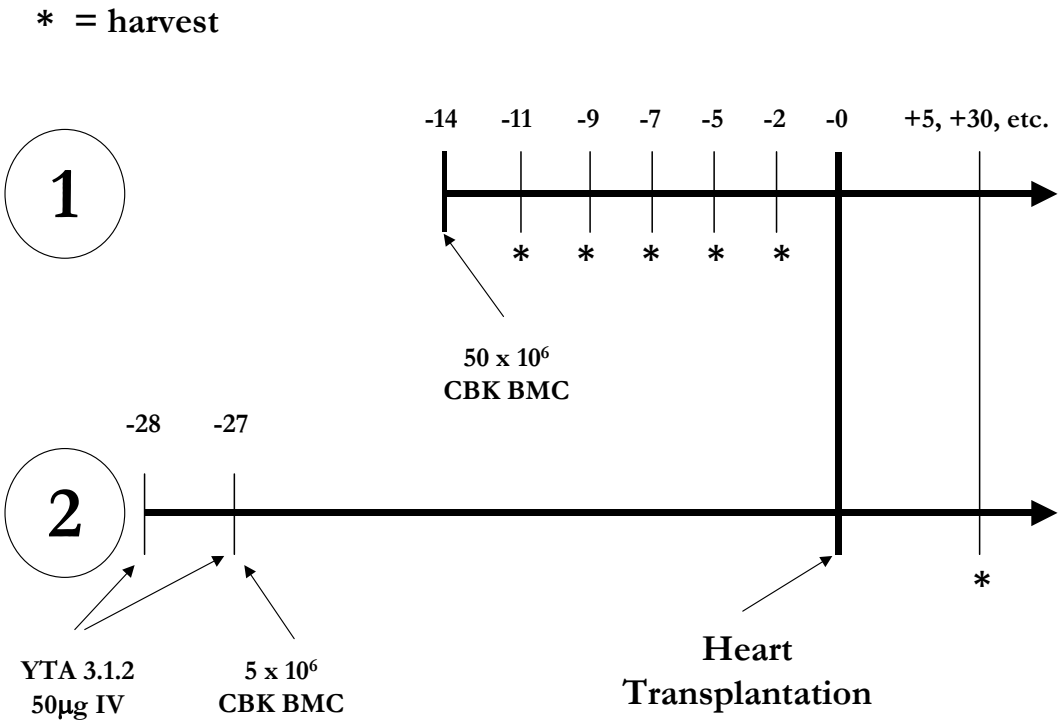
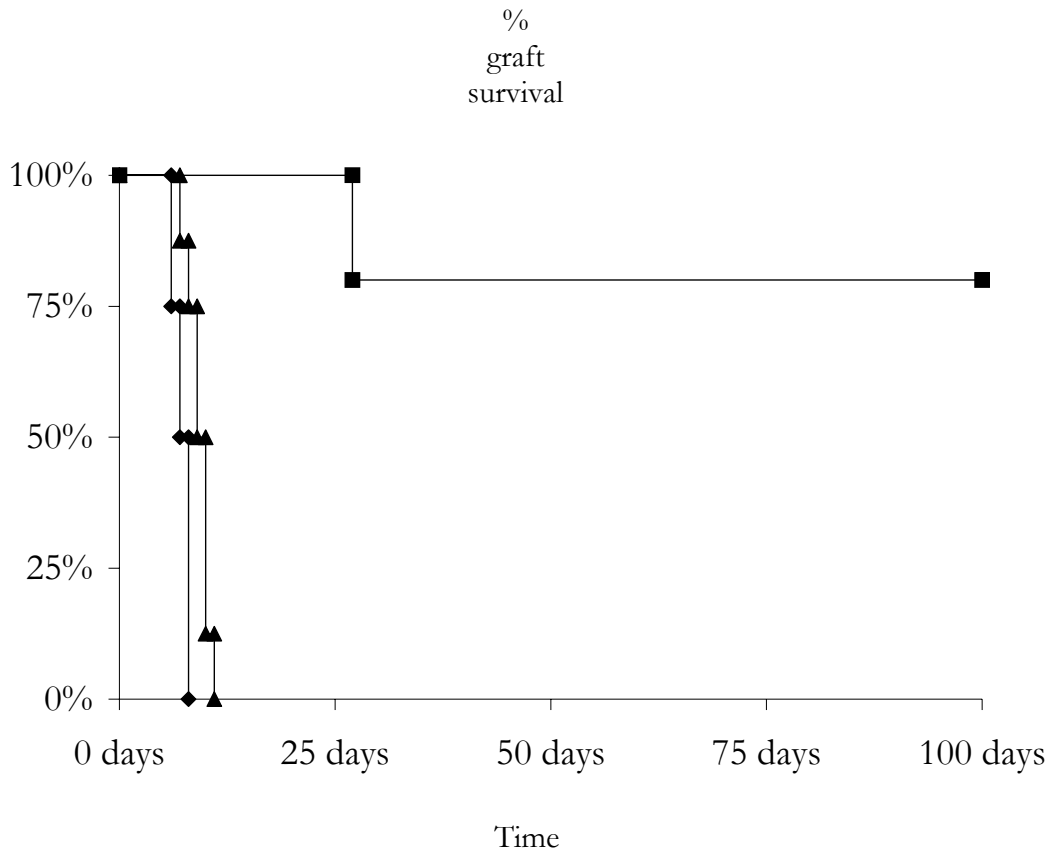


Figure 5.1 The intravenous infusion of unfractionated CBK ($H\text{-}2^k$ + $H\text{-}2K^b$) bone marrow cells into CBA/Ca ($H\text{-}2^k$) mice induced unresponsiveness to fully allogeneic $H\text{-}2K^{b+}$ (CBK x BALB/c F1) cardiac allografts. These cardiac allografts survived for greater than 100 days. Two protocols leading to unresponsiveness were studied. In protocol 1, 50 x 10^6 CBK bone marrow cells were infused on day –14. In protocol 2, 5 x 10^6 CBK bone marrow cells were infused under cover of two doses of depleting anti-CD4 monoclonal antibody (YTA 3.1) on days –28 & -27. CBA^{DES} mice were pretreated by these two protocols and adoptively transferred $CD8^+DES^+$ T cells were analysed at the indicated harvest days.

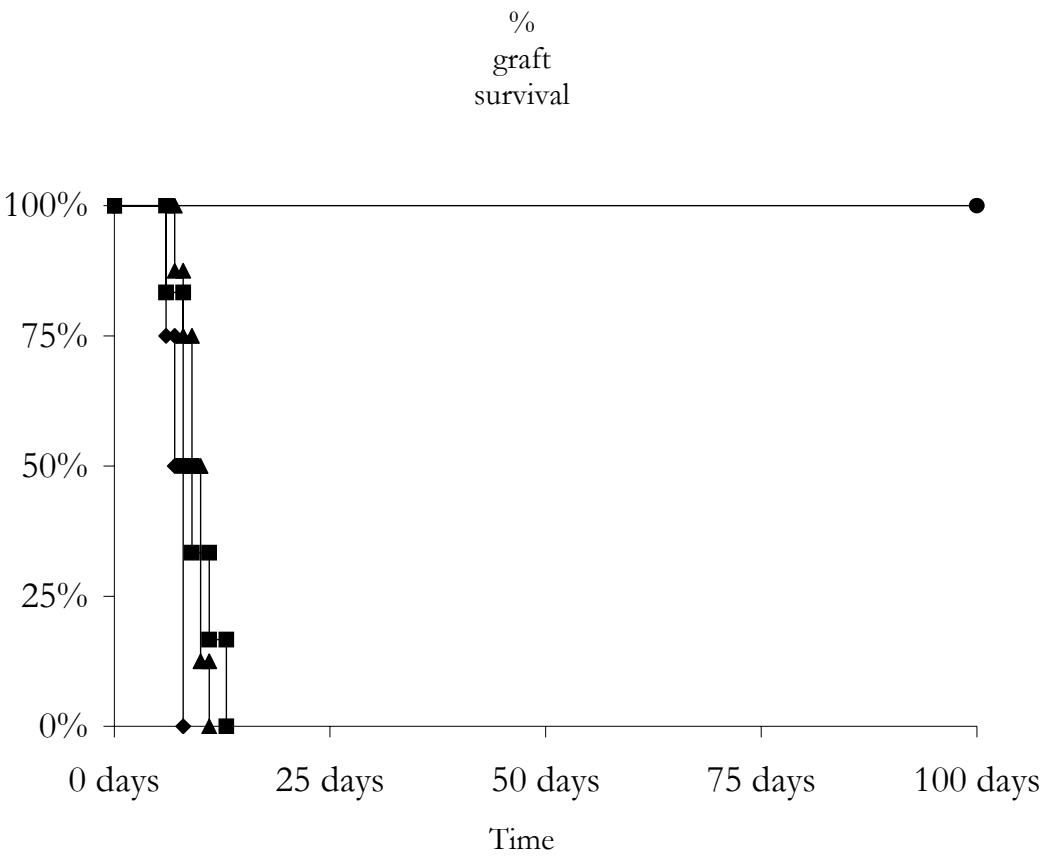
Figure 5.2a Operational Tolerance Following Infusion of 50 x 10^6 H-2K^{b+} CBK Bone Marrow Cells



Label	50 x 10^6 CBK BMC	Donor	(n=)	(mst=)
◆	Ø	(CBK x BALB/c F1)	6	8 days
■	✓	(CBK x BALB/c F1)	5	>100 days
▲	✓	(CBA x BALB/c F1)	8	9.5 days

Figure 5.2a High dose CBK bone marrow infusion into CBA^{DES} mice 14 prior to transplantation induced unresponsiveness to fully allogeneic H-2K^{b+} (CBK x BALB/c F1) hearts (protocol 1). This unresponsiveness was dependent on the expression of H-2K^b by the donor.

Figure 5.2b Operational Tolerance Following Infusion of 5 x 10^6 H-2K^{b+} CBK Bone Marrow Cells in Combination With Anti-CD4 mAb



Label	50μg x 2 YTA 3.1	5 x 10^6 CBK BMC	Donor	(n=)	(mst=)
◆	Ø	Ø	(CBK x BALB/c F1)	4	7.5 days
■	✓	Ø	(CBK x BALB/c F1)	6	8.5 days
▲	✓	✓	(CBA x BALB/c F1)	8	9.5 days
●	✓	✓	(CBK x BALB/c F1)	7	>100

Figure 5.2b Low dose CBK bone marrow infusion into CBA^{DES} mice 27 days prior to transplantation under cover of depleteing anti-CD4 monoclonal antibody (YTA 3.1) also induced unresponsiveness to fully allogeneic H-2K^{b+} (CBK x BALB/c F1) hearts (protocol 2). This unresponsiveness was also dependent on the expression of H-2K^b by the donor.

Figure 5.3 Effect of Pretreatment with 50 x 10^6 H-2K^{b+} Bone Marrow Cells on CD8$^+$DES$^+$ T Cells

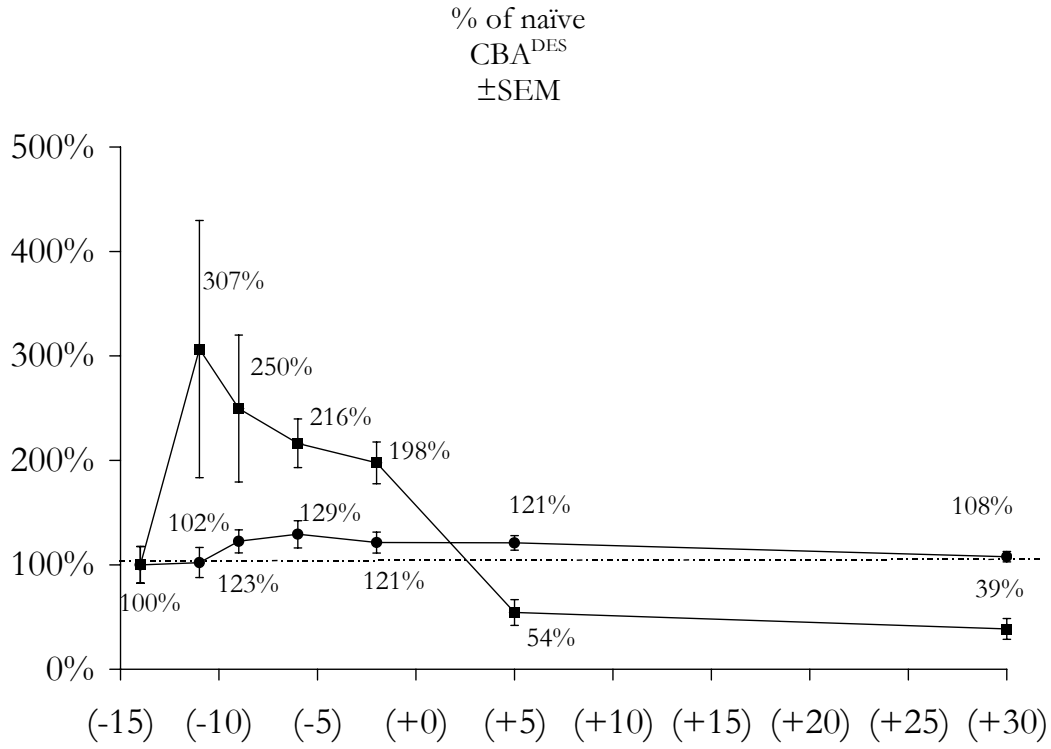


Figure 5.3 The effect of pretreatment (protocol 1) on adoptively transferred CD8$^+$DES$^+$ T cells was studied by infusing CBA^{DES} mice with H-2K^{b+} CBK or third party H-2K^{b-} NZW bone marrow cells on day –14 of protocol 1. The absolute number of CD8$^+$DES$^+$ T cells ±SEM present in the spleens of CBA^{DES} mice relative to the absolute number of CD8$^+$DES$^+$ T cells in the spleens of control naïve CBA^{DES} mice (100%) is shown. 50 x 10^6 CBK bone marrow cells induced clonal expansion followed by clonal deletion of CD8$^+$DES$^+$ T cells (n=3 at each time point analysed). These CBA^{DES} mice were not transplanted.

Figure 5.4 Proliferative Response of Alloantigen Specific T Cells Induced by Infusion of 50 x 10^6 H-$2K^{b+}$ CBK Bone Marrow Cells

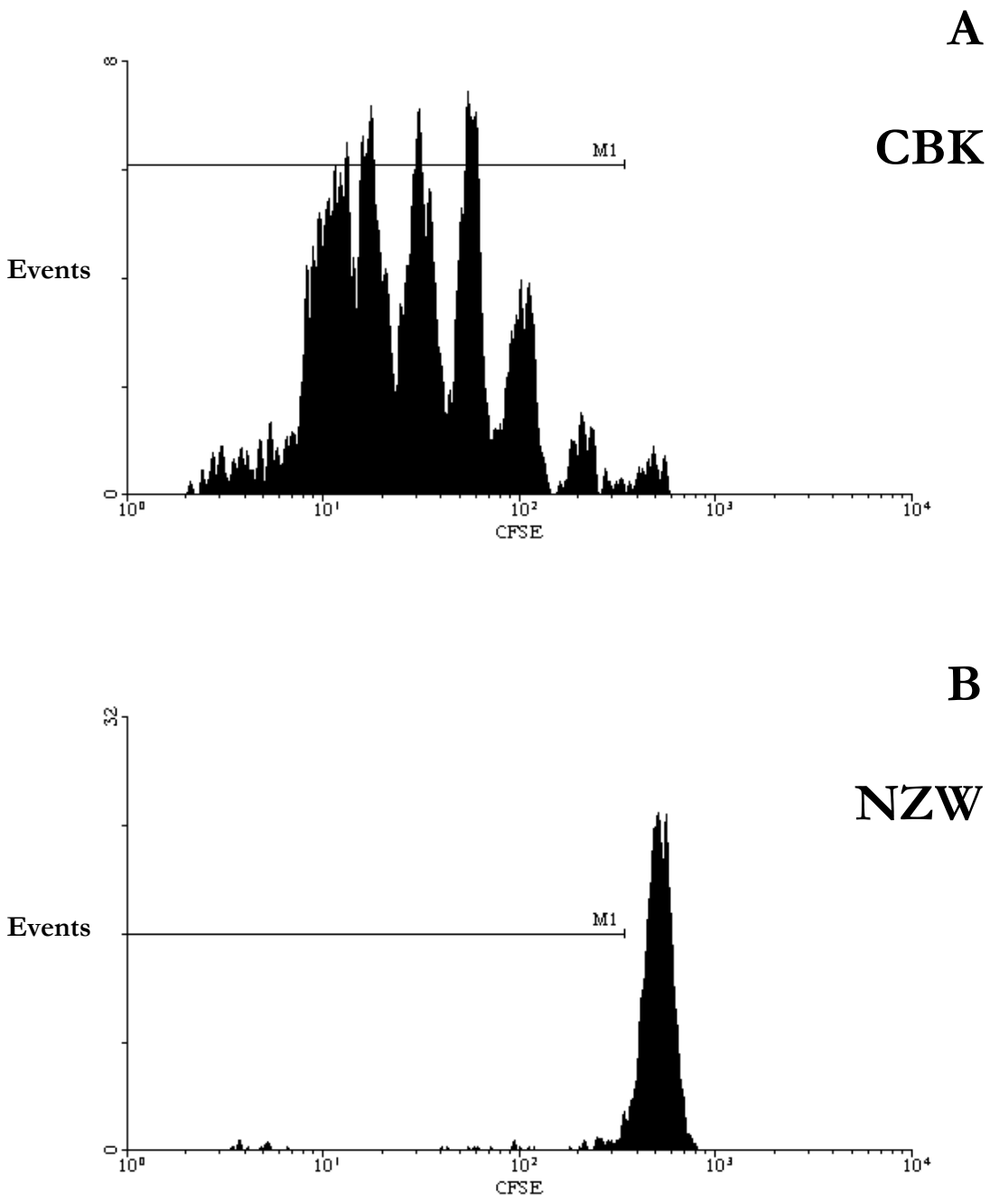


Figure 5.4 Representative CFSE histograms of gated $CD8^+DES^+$ T cells are shown (n=3 per group). CBA^{DES} mice were infused with 50 x 10^6 H-$2K^{b+}$ CBK or control H-$2K^{b-}$ NZW bone marrow cells per protocol 1. $CD8^+DES^+$ T cells present in spleen were analysed three days (day –11) following bone marrow infusion by FACS® to determine division history. At the peak of the proliferative response, H-$2K^{b+}$ CBK bone marrow cells clearly induced a vigourous proliferative response among adoptively transferred $CD8^+DES^+$ T cells while H-$2K^{b-}$ NZW bone marrow cells had not.

Figure 5.5 Generation of "Memory" $CD8^+DES^+$ T Cells Following Infusion of 50 x 10^6 H-$2K^{b+}$ CBK Barrow Cells

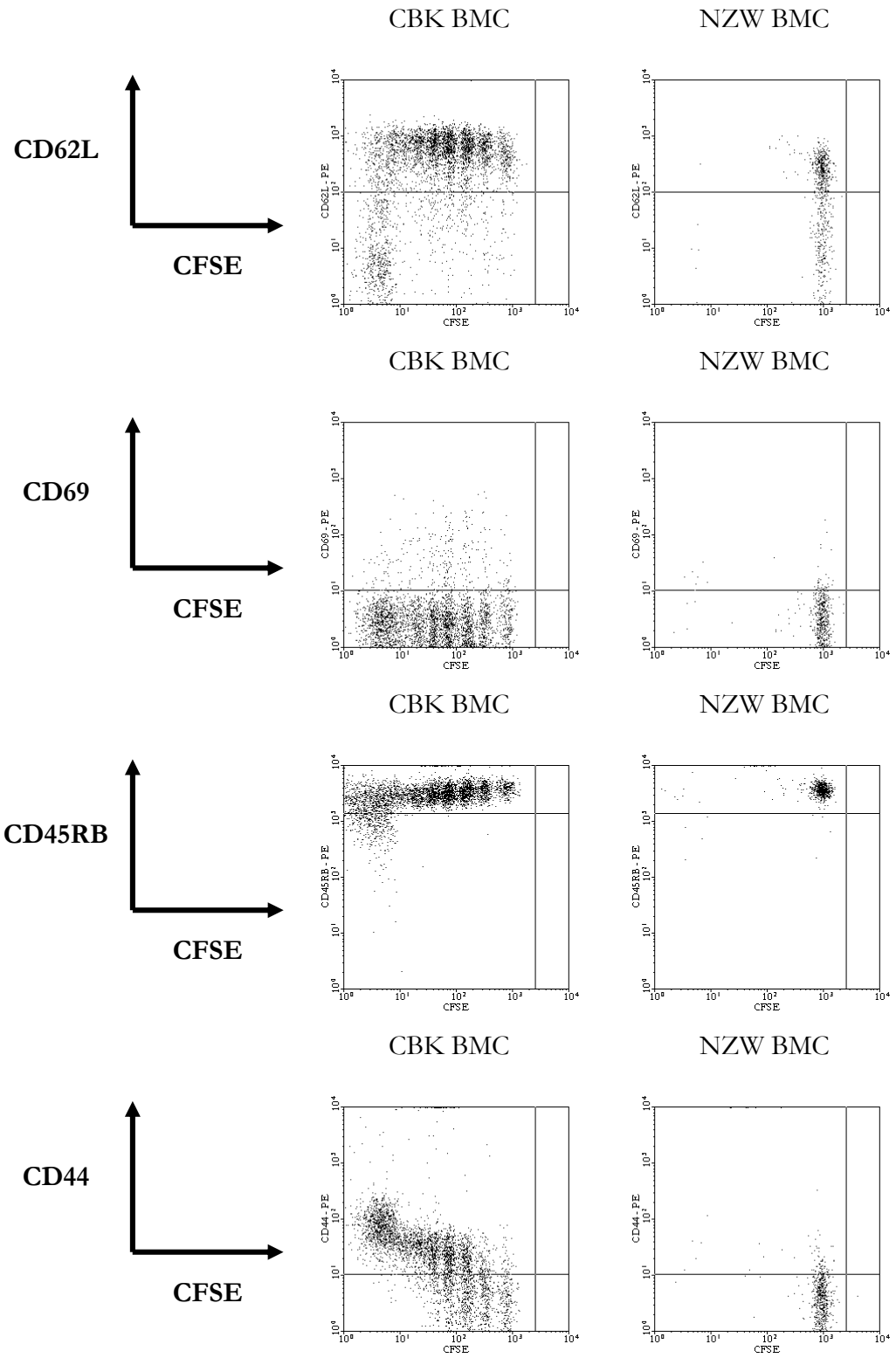


Figure 5.5 Representative plots of gated $CD8^+DES^+$ T cells present in the spleens of CBA^{DES} mice following infusion of H-$2K^{b+}$ CBK or H-$2K^{b-}$ NZW bone marrow cells on day –2 of protocol 1 are shown (n=3 per group). "Memory" $CD8^+DES^+$ T cells were present in the spleens by the time of transplantation. They were predominately $CFSE^{low}CD69^{low}CD44^{high}$.

Figure 5.6 CBK Bone Marrow Infusion Induces $CD8^+DES^+$ T Cells with a Tc1 Phenotype

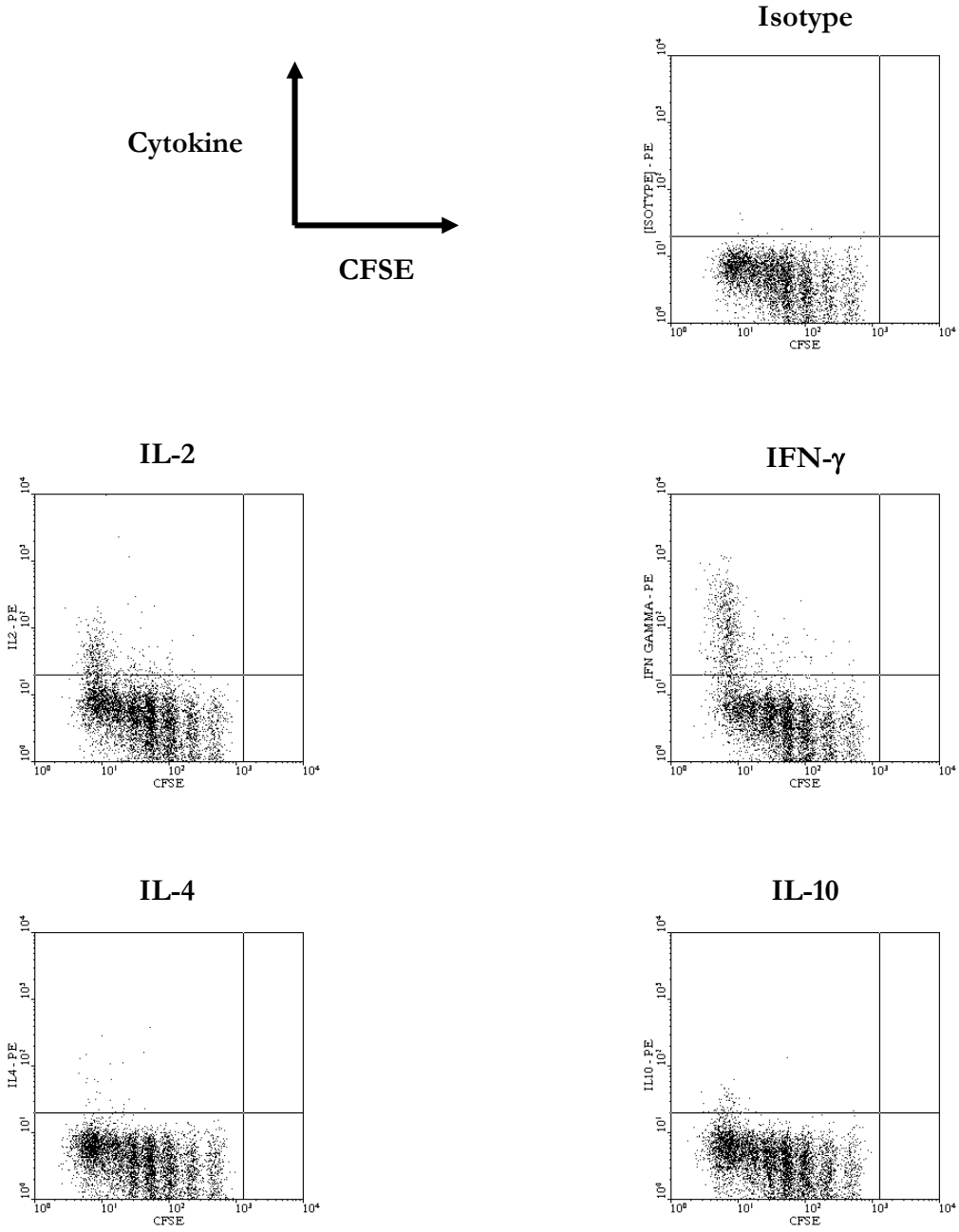
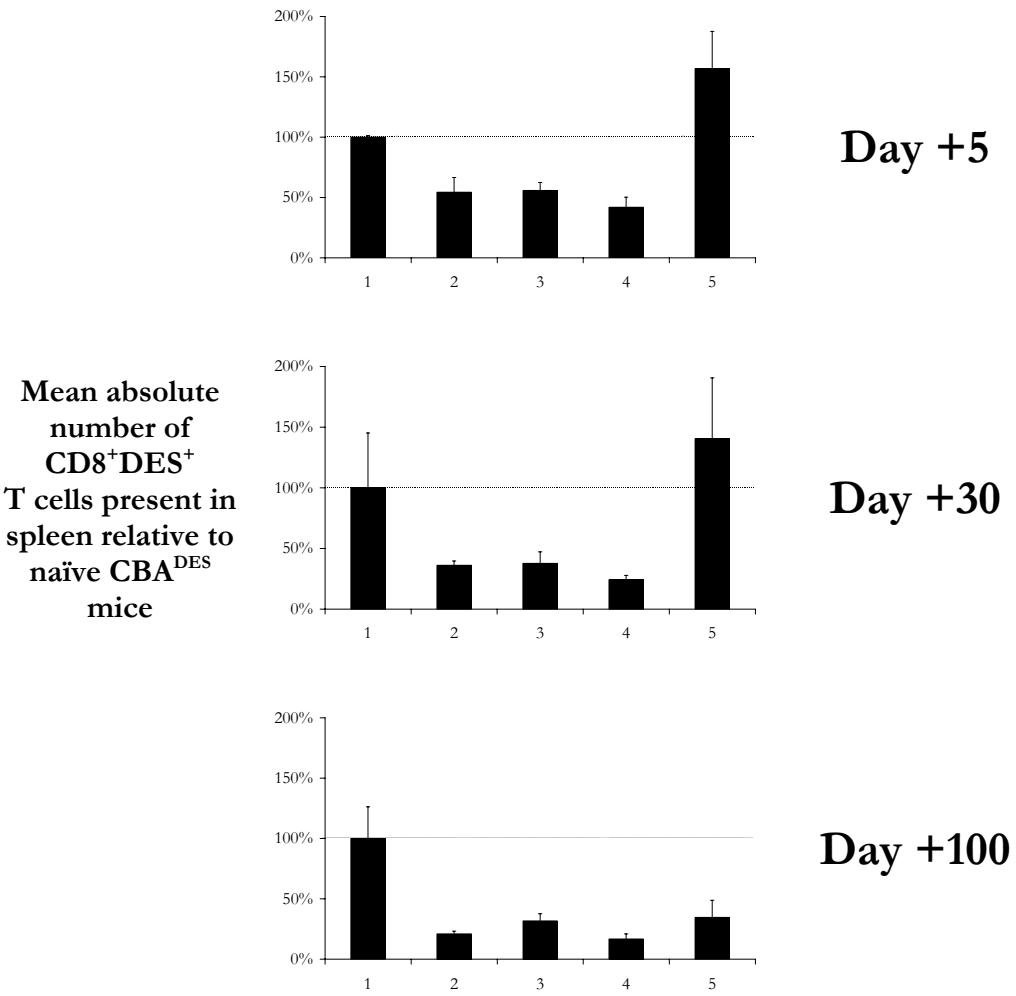


Figure 5.6 Representative plots of gated $CD8^+DES^+$ T cells present in spleens of CBA^{DES} mice following infusion of 50 x 10^6 CBK bone marrow cells on day –2 of protocol 1 are shown (n=3). The production of IL-2 and interferon-γ by adoptively transferred $CD8^+DES^+$ T cells was most evident among cells which had undergone the greatest number of divisions. Gated cells in control CBA^{DES} mice infused with NZW bone marrow did not proliferate or produce Tc1 or Tc2 cytokines.

Figure 5.7 Clonal Deletion Results from Infusion of 50 x 10^6 CBK Bone Marrow Cells



Group	50 x 10^6 CBK BMC day -14	Donor heart transplanted day 0
1	Ø	Ø
2	✓	Ø
3	✓	(CBA/Ca x BALB/c F1)
4	✓	(CBK x BALB/c F1)
5	Ø	(CBK x BALB/c F1)

Figure 5.7 Following pretreatment (protocol 1) of CBA^{DES} mice and transplantation of either H-$2K^{b+}$ or H-$2K^{b-}$ cardiac allografts on day 0, $CD8^+DES^+$ T cells present in the spleens of CBA^{DES} mice were enumerated on the indicated days; +5, +30, +100. All groups were normalised relative to naïve untransplanted CBA^{DES} mice. The mean relative absolute number +SEM of $CD8^+DES^+$ T cells present in spleens of group 1 through group 5 are shown (n=3 per group). Peripheral clonal deletion is a dominant feature induced by infusion of 50 x 10^6 CBK bone marrow cells.

Figure 5.8 Surface Phenotype of $CD8^+DES^+$ T Cells in CBA^{DES} Mice Pretreated with 50 x 10^6 CBK Bone Marrow Cells Vs. Naive CBA^{DES} Mice

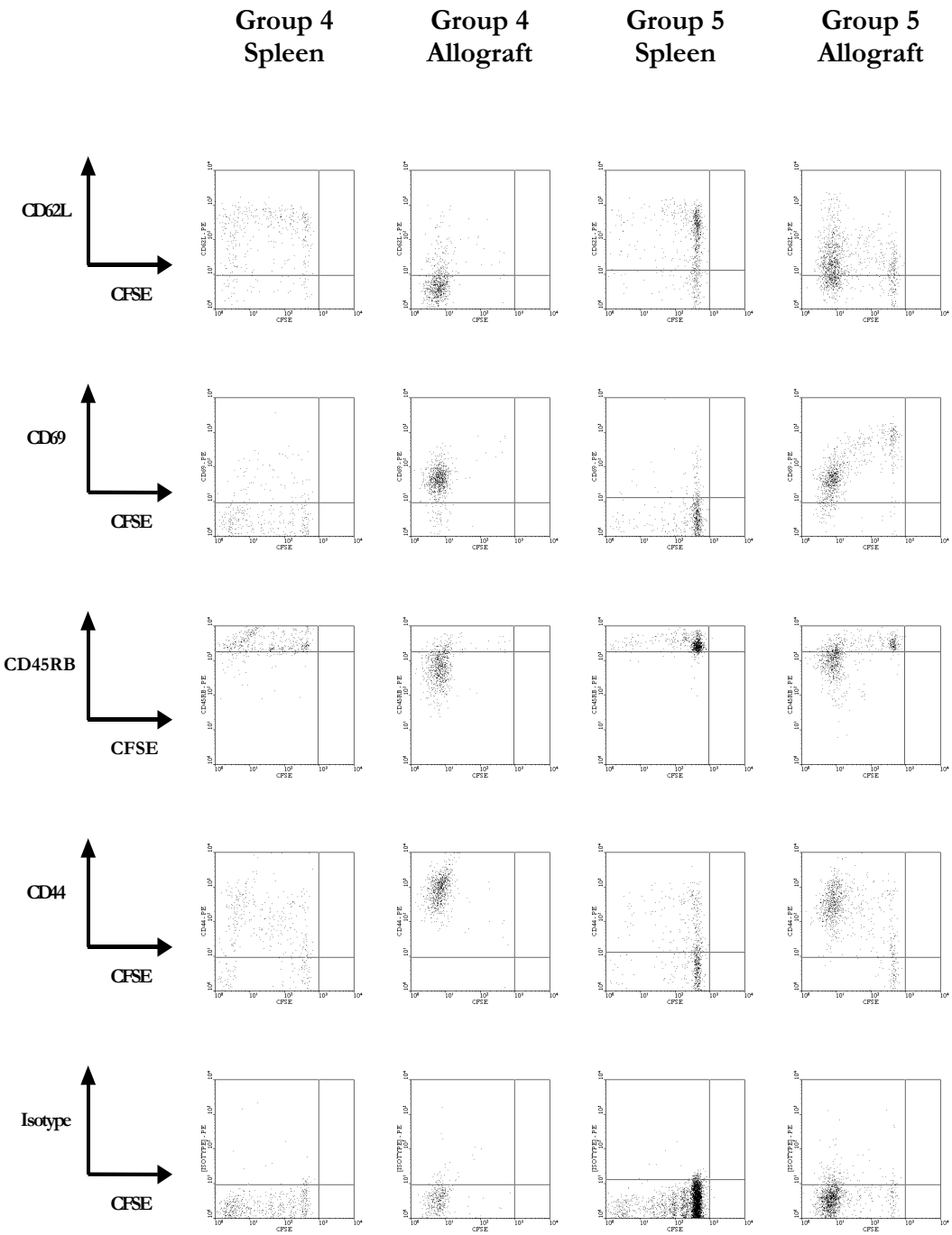


Figure 5.8 Representative FACS® profiles of gated $CD8^+DES^+$ T cells present in spleens and (CBK x BALB/c F1) H-$2K^{b+}$ allografts of CBA^{DES} mice in group 4 and group 5 on day +5 are shown (n=3 per group). Due to limited numbers, graft infiltrating cells were pooled in order to perform the analysis. We compared the phenotype of $CD8^+DES^+$ T cells under conditions leading to allograft survival (group 4) and allograft rejection (group 5). Although $CD8^+DES^+$ T cells in group 4 underwent a greater number of division, in both group 4 and group 5 adoptively transferred cells infiltrating the allograft possessed an activated surface phenotype.

Figure 5.9 Cytokine Production by Graft Infiltrating $CD8^+DES^+$ T Cells in Mice Pretreated with 50 x 10^6 H-$2K^{b+}$ CBK Bone Marrow Cells

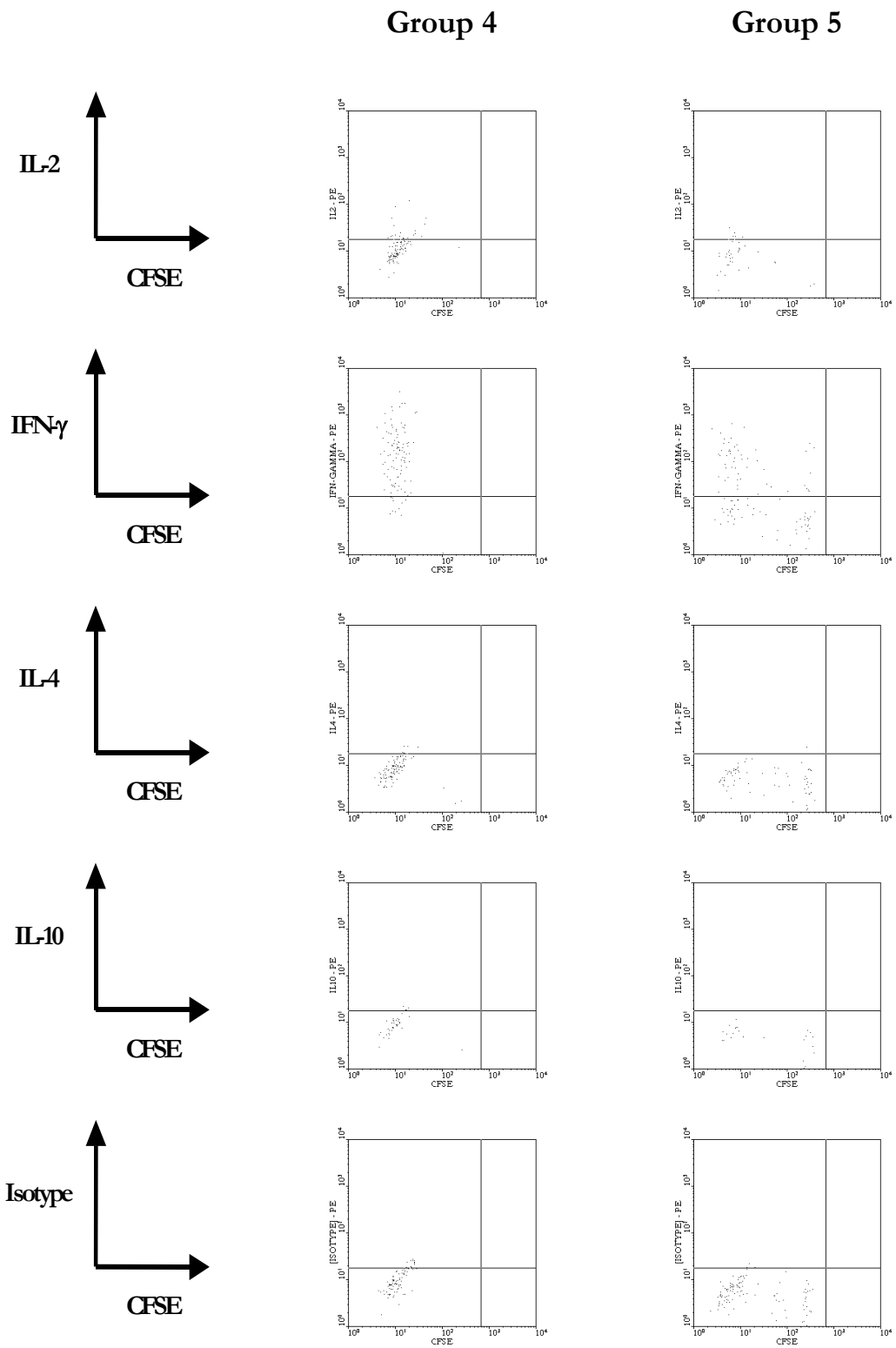
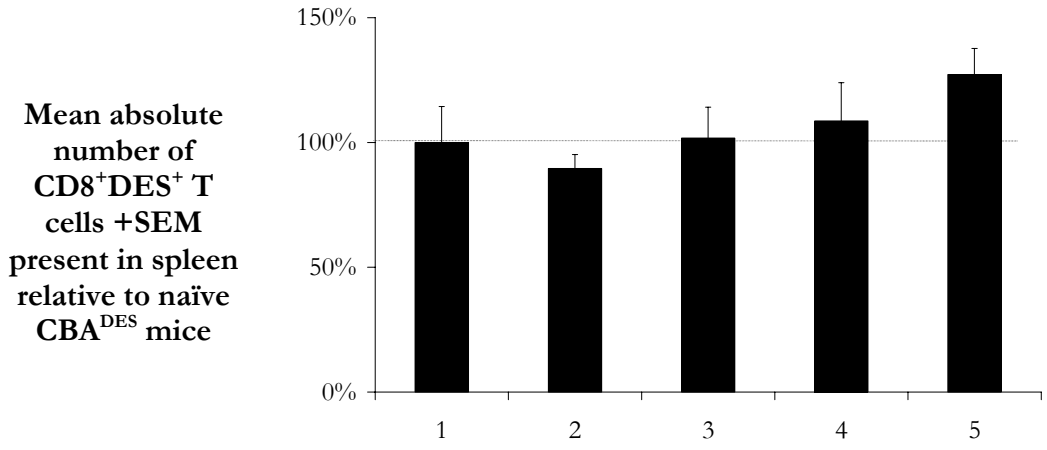


Figure 5.9 FACS® profiles of gated adoptively transferred $CD8^+DES^+$ T cells present in H-$2K^{b+}$ allografts (CBK x BALB/c F1) on day +5 in group 4 (protocol 1) and group 5 (untreated) are shown. Pooled graft infiltrating cells from each group (n=3 per group) were analysed. $CD8^+DES^+$ T cells present in allografts of both groups produced interferon-γ.

Figure 5.10 Infusion of 5 x 10^6 CBK Bone Marrow Cells with Depleting Anti-CD4 (YTA 3.1) did not Induce Peripheral Clonal Deletion of $CD8^+DES^+$ T Cells In Spleen at Day +5



Group	50μg x 2 YTA 3.1 day –28/-27	5 x 10^6 CBK BMC day -27	Donor heart tranplanat day 0
1	Ø	Ø	Ø
2	✓	Ø	Ø
3	Ø	✓	Ø
4	✓	✓	(CBA/Ca x BALB/c F1)
5	✓	✓	(CBK x BALB/c F1)

Figure 5.10 The mean relative absolute number of $CD8^+DES^+$ T cells +SEM present in the spleens of CBA^{DES} mice (n=3 per group) at day +5 following pretreatment with protocol 2 is shown for groups 1 through 5. Low dose CBK bone marrow infusion under cover of depleting anti-CD4 monoclonal antibody did not induce peripheral clonal deletion in contrast to protocol 1.

Figure 5.11 Pretreatment with 5 x 10^6 H-2K^{b+} CBK Bone Maarrow Cells in Combination with Anti-CD4 Impairs CD8$^+$DES$^+$ T Cell Infiltration of H-2K^{b+} Cardiac Allografts

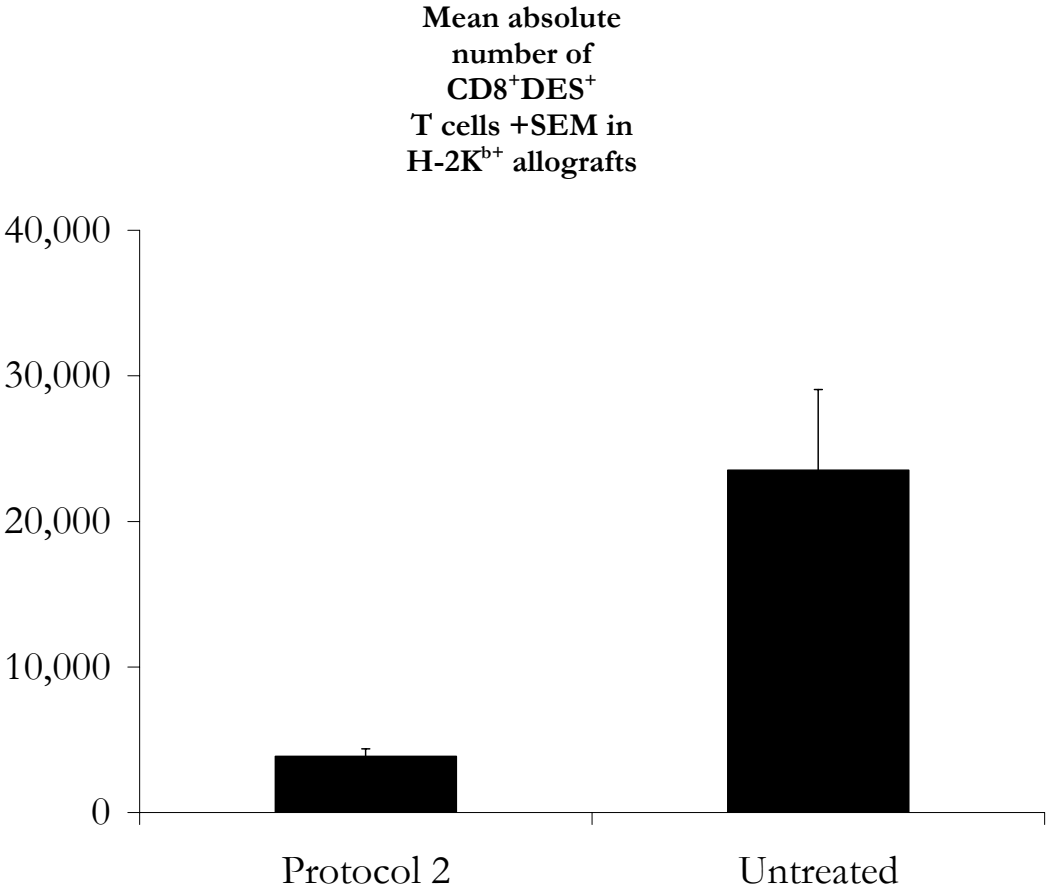


Figure 5.11 CD8$^+$DES$^+$ T cells present in H-2K^{b+} allografts five days after transplantation into CBA^{DES} mice were enumerated. CDB^{DES} recipients pretreated with 5 x 10^6 CBK bone marrow cells and YTA 3.1 were compared to untreated CBA^{DES} mice. The mean absolute number of CD8$^+$DES$^+$ T cells +SEM infiltrating H-2K^{b+} (CBK x BALB/c F1) allografts is shown. Pretreatment significantly impaired infiltration of H-2K^{b+} cardiac allografts by day +5.

Chapter 6 - Discussion

6.1 Introduction

Although clinical transplantation is now considered an important treatment for end organ failure, our knowledge of basic immune responses to alloantigens remains incomplete. Over the past fifty years, investigators have elucidated the critical role of T cells in allograft rejection, but effective strategies to specifically prevent, interrupt and monitor transplant rejection are still lacking. The achievement of donor specific unresponsiveness or tolerance in the clinic remains the central objective for transplantation immunologists and has driven the work presented in this thesis. Although advances in surgical techniques, organ procurement, HLA matching and immunosuppressive drugs have permitted the everyday treatment of end-organ failure with transplantation, the recipient must maintain life long non-specific immunosuppression with the risks of opportunistic infection and malignancy forever present. The induction of donor specific unresponsiveness would minimise or eliminate the need for such powerful, but clearly harmful drugs.

We hope that by investigating the mechanisms of allograft rejection it may be possible to develop strategies and drugs able to more effectively prevent or arrest rejection once it has begun. Similarly, careful identification and dissection of the mechanisms that play a role in successful experimental tolerance induction protocols are of utmost importance as we attempt to design protocols that can work in the clinic. We have chosen to examine how the infusion of syngeneic bone marrow cells expressing the single allogeneic MHC class I molecule, H-2K^{b}, results in the indefinite survival of a fully allogeneic H-2K^{b+} cardiac allograft. The ability to regulate the immune response against "bystander" alloantigens expressed by an allograft has been termed linked unresponsiveness. Exploitation of this ability of T

cells to regulate the response of naïve T cells to previously unencountered alloantigens via linked unresponsiveness has wide implications and is a promising strategy for the design of tolerance induction protocols for the clinic.

Therefore, we initially aimed to visualise T cells in vivo as they responded to alloantigens expressed by a vascularised heterotopic cardiac allograft under conditions leading to rejection. Subsequently we explored the events that occur to H-2K^{b} specific CD8^{+} T cells during alloantigen pretreatment and then how these pretreated cytotoxic T cells interacted with fully allogeneic H-2K^{b+} cardiac allografts in an attempt to identify the mechanisms of tolerance operating in these protocols.

6.2 Summary of Results

6.2.1 Chapter 3 - Establishment of the Adoptive Transfer Model

It has until recently not been possible to visualise T cells during an immune response against an organ graft in vivo. We have developed an experimental adoptive transfer system that enabled us to do this. CD8^{+} transgenic T cells specific for MHC class I alloantigens were transferred into syngeneic CBA/Ca hosts and studied by flow cytometry. In vitro, CD8^{+} transgenic T cells were specific for their nominal alloantigens and were non-reactive against third party MHC class I and class II alloantigens. In vivo, adoptively transferred CD8^{+}TG^{+} T cells were detectable by flow cytometry using anti-clonotypic monoclonal antibodies and remained in a resting state over the long-term. They homed to the secondary, but not the primary lymphoid tissues of naïve host. Adoptively transferred CD8^{+}TG^{+} T cells were functional in their new host as demonstrated by proliferation and clonal expansion.

The adoptive transfer system was sound and permitted the in vivo visualisation of T cells during an alloimmune response. In theory, a trace number of adoptively

transferred alloreactive transgenic $CD8^+$ T cells were returned to a normal milieu, where they competed with a diverse T cell repertoire, and where $CD4^+$ T cell help was abundant. In CBA^{TG}, we propose that the adoptively transferred cells did not alter or determine the immune response against alloantigens, but rather reflected the activity of naturally occurring alloreactive T cells when challenged with allogeneic cells or grafts.

6.2.2 Chapter 4 - In Vivo Generation of Effector and Memory $CD8^+DES^+$ T Cells

CBA^{DES} mice were used to investigate the role of alloreactive $CD8^+$ T cells during the acute rejection of fully allogeneic H-$2K^{b+}$ cardiac allografts. CBA^{DES} mice rejected C57BL/10 and (CBK x BALB/c F1) cardiac allografts with a similar tempo to naïve CBA/Ca mice. We determined the kinetics of $CD8^+$ T cell infiltration of H-$2K^{b+}$ cardiac allografts in CBA/Ca mice and chose to study $CD8^+DES^+$ T cells five days after transplantation of cardiac allografts as this was during the development of the immune response but prior to tissue necrosis.

H-$2K^{b+}$ cardiac allografts induced proliferation of $CD8^+DES^+$ T cells present in the secondary lymphoid organs. Roughly 50% of $CD8^+DES^+$ T cells present in the spleens of transplanted CBA^{DES} mice had divided at least once and produced mainly interferon-γ, but had not modulated surface activation markers. We found that $CD8^+DES^+$ T cells only infiltrated H-$2K^{b+}$ cardiac allografts. Several different strains of fully allogeneic H-$2K^{b-}$ cardiac allografts failed to induce either proliferation of H-$2K^b$ specific T cells in the periphery or infiltration into the transplanted hearts.

Most interestingly, the environment within a H-$2K^{b+}$ cardiac allograft appeared to be unique in that this was the only site where $CD8^+DES^+$ T cells possessing an activated

surface phenotype were found. These cells had been driven to divide many more times than those in the peripheral lymphoid tissues, produced interferon-γ and were $CD62L^{low}CD69^{high}CD45RB^{low}CD44^{high}$. In the longer term after H-$2K^{b+}$ cardiac allografts had been rejected, $CD8^{+}DES^{+}$ T cells possessing a $CD69^{low}CD44^{high}$ memory surface phenotype were found in the spleen. These putative memory T cells had divided extensively ($CFSE^{low}$) and produced interferon-γ.

MHC class I alloantigens expressed by the cardiac allograft or passenger leukocytes contained within it were able to specifically induce the activation and maturation of alloreactive $CD8^{+}$ T cells. These cells acquired the ability to infiltrate H-$2K^{b+}$ cardiac allografts. The graft appeared to be an important site for the maturation of alloreactive T cells since only cells within the allograft had extensively divided, and expressed an activated surface phenotype. There was a clear difference between cells in the allograft and those that continued to circulate in the periphery in the midst of a rejection response.

6.2.3 Chapter 5 - Induction of Unresponsiveness by Infusion of CBK Bone Marrow

In order to study alloreactive $CD8^{+}$ T cells under conditions resulting in the development of operational tolerance, we took advantage of a tolerance induction protocol previously established in our laboratory; where by survival of a fully mismatched H-$2K^{b+}$ cardiac allograft required only the administration of syngeneic CBK bone marrow prior to transplantation. As a single MHC class I antigen H-$2K^{b}$ was used for pretreatment and had to be expressed by the allograft, this protocol offered the opportunity to specifically investigate the role of alloreactive $CD8^{+}$ T

cells in the tolerance induction process with the adoptive transfer model that we had developed.

Intravenous administration of 50 x 10^6 CBK bone marrow cells (Protocol 1) lead to the indefinite survival of (CBK x BALB/c F1) cardiac allografts in CBA^{DES} recipients. We found that adoptively transferred $CD8^+DES^+$ T cells underwent massive clonal deletion as a result of infusion of a high dose of bone marrow and that this was associated with the indefinite survival of (CBK x BALB/c F1) allografts. Infiltration of (CBK x BALB/c F1) allografts by $CD8^+DES^+$ T cells after pretreatment was not reduced in the acute phase. However, with the passage of time $CD8^+DES^+$ T cells either migrated out of the allograft or were deleted within the allograft. Interestingly, graft infiltrating $CD8^+DES^+$ T cells from pretreated and naive CBA^{DES} mice did not differ from one another by surface phenotype or cytokine production in the early post-transplant course.

Intravenous infusion of 5 x 10^6 CBK bone marrow cells under cover of depleting anti-CD4 mAb also lead to indefinite survival of (CBK x BALB/c F1) cardiac allografts in CBA^{DES} recipients. However, this protocol did not cause clonal deletion of $CD8^+DES^+$ T cells in contrast to the data obtained when 50 x 10^6 CBK bone marrow cells were infused alone. Protocol 2 did however interfere with the infiltration of (CBK x BALB/c F1) cardiac allografts when compared to naïve CBA^{DES} mice rejecting (CBK x BALB/c F1) cardiac allografts.

Finally, we observed that resting $CD8^+DES^+$ T cells, when adoptively transferred into pretreated CBA/Ca mice which had subsequently accepted (CBK x BALB/c F1) cardiac allografts for greater than 100 days did not proliferate produce cytokines

or modulate surface markers. Surprisingly, these resting cells did not infiltrate established H-2K^{b+} (CBK x BALB/c F1) allografts.

The aim of this chapter was to understand and determine the fate of CD8^{+}DES^{+} T cells following pretreatment with CBK bone marrow. Secondly, we attempted to determine if CD8^{+}DES^{+} T cells persisting could be assigned a regulatory phenotype. We were not able to demonstrate immune deviation of CD8^{+}DES^{+} T cells from Tc1 to Tc2 cytokine phenotype. Clonal deletion was evident in protocol 1 while significantly reduced infiltration of H-2K^{b+} cardiac allografts occurred following pretreatment with protocol 2. However, since cardiac allografts were accepted indefinitely, we believe that the residual H-2K^{b} specific CD8^{+}DES^{+} T cells following pretreatment were likely tolerant and possibly anergic. We conclude that anergic alloreactive CD8^{+} T cells may play a role in the mediation of suppression of naïve responses against bystander alloantigens through linked suppression.

6.3 Discussion

The studies described in this thesis are largely observational. They do confirm and extend much of what has been learned by previous investigators using in vitro and often complicated experimental systems that have been difficult to interpret. By visualising the responses of alloreactive $CD8^+$ T cells in vivo, we have shown that $CD8^+$ T cells participate actively in rejection and possibly in the establishment of allograft tolerance. Under all circumstances in our studies, $CD8^+$ T cells developed a Tc1 profile of cytokine production suggesting that production of Tc2 cytokines was not a requirement for the indefinite survival of cardiac allografts in this experimental model. Furthermore, the environment within rejected and accepted cardiac allografts was found to be unique and distinct from peripheral lymphoid tissues. Alloreactive $CD8^+$ T cells were driven to proliferate within the allograft and possessed an activated phenotype solely within the graft. This implied that the allograft itself provides a strong stimulus to alloreactive T cells. Passenger leukocytes that migrate from the allograft to recipient secondary lymphoid tissues may be directly responsible for the peripheral proliferation demonstrated in the spleen, but this is clearly not as strong as the stimulus provided by the allograft itself. One further implication of these observations is that, at least for $CD8^+$ T cells, invasive biopsy of transplanted organs will be required for the foreseeable future in order to asses the immune status within the allograft since the state of cytotoxic T cells inside and outside the allograft did not correlate.

Despite the majority of the literature suggesting that $CD8^+$ T cells play a redundant role in allograft rejection, we clearly demonstrated an active role in rejection and we proposed a possible role for the $CD8^+$ T cell population in linked unresponsiveness

in our experimental system. These observations encourage us to continue to study $CD8^+$ T cells as targets for achieving tolerance. Only by increasing our understanding of immune responses to organ allografts might we succeed in controlling allograft rejection.

We were among the first to be able to visualise alloreactive T cells in response to an organ graft. We hope that the work presented in this thesis will contribute to improved treatment of end organ failure through the achievement of tolerance and better monitoring of the immunological state of the allograft. However, the potential benefits of such experimental systems is not limited to transplantation and should be useful in answering other basic immunological questions that affect the lives of patients afflicted with auto-immune diseases, or simply to further our understanding of basic immunologic mechanisms.

….. on ne voit bien qu'avec le couer. L'essentiel est invisible pour les yeux.

Antoine de Saint-Exupéry

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