Anti-CD4 induced transplantation tolerance in the rat: molecular and cellular mechanisms

by

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SUMMARY OF THESIS

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MONOCLONAL ANTIBODIES (mAb) directed against the CLUSTER OF DIFFERENTIATION (CD)4 molecule, expressed on the surface of T helper cells, are a potentially important tool for manipulating the *in vivo* immune response to a tissue or organ allograft. In rodent models of organ transplantation, administration of anti-CD4 mAb prolongs allograft survival and may even induce permanent transplant tolerance. However, clinical application of anti-CD4 mAb requires a better understanding of the molecular and cellular mechanisms by which anti-CD4 mAb promotes graft survival. Early mechanisms may include CD4 T cell depletion and impairment of residual CD4 T cell function by blocking interaction of CD4 with CLASS II MAJOR HISTOCOMPATIBILITY (MHC) antigen or disrupting T cell signalling pathways.

The experiments described in this thesis were undertaken to provide new insight into how one particular anti-CD4 mAb, (MEDICAL RESEARCH COUNCIL (MRC) OXFORD UNIVERSITY CELLULAR IMMUNOLOGY UNIT (OX)38), prolongs graft survival in a rat model of cardiac transplantation. Initial studies showed that OX38, a mouse IMMUNOGLOBULIN G (IgG)2a mAb, which binds to the membrane distal domain of rat CD4, when given as a brief treatment preoperatively, was able to prolong survival of fully allogeneic Lewis ($RT1^l$) heterotopic cardiac grafts in DA ($RT1^a$) recipients (MEDIAN GRAFT SURVIVAL TIME (MST) >100 days). Moreover, recipients bearing long-standing heart grafts developed specific tolerance to donor alloantigen since they accepted a second donor graft but rejected third party grafts.

Tolerance induction following OX38 mAb was associated with partial depletion (approximately 50%) of peripheral CD4 T cells. Initially, residual CD4 T cells were shown on the basis of phenotype, to consist of predominantly RECENT THYMIC EMIGRANT (RTE) ($CD4^{+ve}$ $CD45RC^{low}$ $Thy\text{-}1^{high}$), but over several weeks, peripheral CD4 T cell numbers recovered to near normal levels. Non-rejecting heart grafts in OX38 mAb treated recipients were heavily infiltrated with mononuclear cells, including numerous CD4 cells. Analysis of intragraft cytokine MESSENGER RIBONUCLEIC ACID (mRNA) transcripts by REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR) revealed the presence of both T HELPER 1 (Th1) and T HELPER 2 (Th2) cytokine mRNA in non-rejecting grafts with a pattern similar to that seen during unmodified rejection. Moreover, measurement of alloantibody isotypes in OX38 mAb treated animals did not support the view that anti-CD4 induced tolerance was attributable to a dominant Th2 cytokine response.

Residual CD4 T cells, following anti-CD4 mAb treatment, remained only transiently (2 days) coated with OX38 mAb. Encounter of CD4 T cells with alloantigen whilst they were still coated with mAb was not essential for tolerance induction. A small 'window of opportunity' was detected (up to 4 days following mAb treatment) during which cardiac transplantation failed to trigger allograft rejection. $CD4^{+ve}$ T cells obtained from OX38 mAb treated animals during this 'window of opportunity', showed altered tyrosine phosphorylation of a 36-38 KILO DALTON (kDa) protein on subsequent activation *in vitro* with immobilised anti-T CELL RECEPTOR (TCR) mAb when compared with anti-TCR activated, untreated CD4 T cells. The altered phosphorylation

pattern of *in vivo* OX38 mAb treated cells was similar to that seen in naïve CD4 T cells activated with anti-TCR after *in vitro* OX38 treatment. In addition, the pattern of phosphorylation observed in anti-CD4 treated cells was similar to that previously described in anergic CD4 T cells, suggesting that OX38 mAb treated cells may be functionally anergic.

An unexpected role for the thymus in OX38 mAb induced transplantation tolerance was identified. Anti-CD4 mAb treated recipients which had undergone thymectomy several weeks prior to transplantation, rapidly rejected their heart grafts, despite OX38 therapy. This observation suggested that CD4 T cell depletion alone was not, in itself, sufficient to prevent allograft rejection and that a product from the thymus was necessary. A role for RTE in anti-CD4 mAb induced transplantation tolerance was indirectly explored in experiments using immunopurified single positive ($CD4^{+ve}$ $\alpha\beta TCR^{+ve}$) thymocytes and LYMPH NODE CELLS (LNC). In reconstitution experiments using thymectomised anti-CD4 treated animals, adoptive transfer of purified single positive thymocytes on days 2 and 4 after heart grafting, restored the ability of OX38 mAb to prolong allograft survival in thymectomised animals. Furthermore, in anti-TCR proliferation assays, single positive thymocytes were less affected by *in vitro* OX38 mAb treatment than single positive LNC and were found to produce both Th1 and Th2 cytokine mRNA. A possible interpretation of these findings, is that in this experimental model, RTE may be analogous to naïve T HELPER 0 (Th0) T cells and may have an essential role in tolerance induction.

By adding to current understanding of the molecular and cellular mechanisms of anti-CD4 induced transplantation tolerance, the above observations may provide a more rational basis from which to further develop strategies for using anti-CD4 mAb in clinical transplantation.

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Background to Thesis

I. Introduction

Human organ transplantation has blossomed since the advent of immunosuppressive drug therapy in the 1960's. With the increased immunosuppressive efficacy of drugs such as Cyclosporin A, introduced in the late 1970's, graft survival figures have shown improvement in many clinical trials. However, current immunosuppressive regimes still remain immunolologically non-specific, are often toxic, and still do not assure 100% graft survival. Over the years, a number of isolated anecdotal case reports have highlighted patients enjoying long-term graft survival following transplantation, in the absence of continuing immunosuppressive drug therapy. The mechanism underlying this phenomena of graft tolerance is the focus of intense debate and current transplantation research. A better understanding of the cellular and humoral effector mechanisms involved in graft rejection and transplant tolerance should help in achieving the long-term goal of purposeful modulation of the immune response with therapies which have increased immunological specificity coupled with reduced toxicity and better graft survival.

The principle aim of the work described in this thesis was to study the mechanism of action of anti-CD4 mAb pre-treatment, in a rat transplantation model using fully mismatched cardiac allografts. The ability of an anti-CD4 mAb (OX38) to perturb CD4 T cell function, prevent graft rejection and promote transplant tolerance was investigated. The introduction chapter of the thesis concentrates on the molecular basis of CD4 T cell allorecognition, T cell activation, anti-CD4 treatment and tolerance induction. Other aspects of effector cell mechanisms in the context of transplantation, notably the role of lymphokines, intracellular signalling pathways and the role of naïve precursor cells in the induction of tolerance will be addressed in more detail in the later chapters.

II. Abbreviations used in Thesis

μg	Microgram(s)
μl	Microlitre(s)
μm	Micron(s)
βME	2-Mercaptoethanol
$[Ca^{2+}]_i$	Intracellular Calcium Ions
^0C	Degrees Centigrade
^{51}Cr	Chromium 51
Ag	Antigen
APC	Antigen Presenting Cell
ARAM	Antigen Recognition Activation Motif
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CDR	Complementarity Determining Region
cm	Centimetre
Con A	Concanavalin-A
CPM	Counts Per Minute
C-terminal	Carboxy-Terminal
C-terminus	Carboxy-Terminus
DAG	Diacylglycerol
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
DST	Donor Specific Blood Transfusion
DTT	Dithiothreitol
ER	Endoplasmic Reticulum

FasL	Fas Ligand
FCS	Foetal Calf Serum
FcεRI	Multi-Subunit High Affinity IgE Receptor
FcεRIγ	Gamma Chain of the Multi-Subunit High Affinity IgE Receptor
FITC	Fluorescein Isothiocyanate
g	Gram(s)
GDP	Guanosine Diphosphate
gp	Glycoprotein(s)
GRFs	Guanine Nucleotide Releasing Factors
GTP	Guanosine Triphosphate
GVH	Graft Versus Host Disease
H&E	Hematoxylin and Eosin
H_20_2	Hydrogen Peroxide
^{3}H-Thymidine	Tritiated Thymidine
hr	Hour(s)
HRP	Horse Radish Peroxidase
Ig	Immunoglobulin
IL-	Interleukin
IL-2Rα	Interleukin-2 Receptor Alpha
INF-γ	Interferon-Gamma
IP	Intraperitoneal
IP_3	Inositol 1,4,5-triphosphate
IU	International Units
IV	Intravenous
kDa	Kilo Dalton
kg	Kilogram(s)

KO	Knockout
LNC	Lymph Node Cell(s)
mAb	Monoclonal Antibody
MCF	Mean Channel Fluorescence
MHC	Major Histocompatibility
Min	Minute(s)
ml	Millilitre(s)
MLR	Mixed Lymphocyte Reaction
mm	Millimetre
MRC	Medical Research Council
mRNA	Messenger Ribonucleic Acid
MST	Median Graft Survival Time
NGS	Normal Goat Serum
NK	Natural Killer Cell
NMS	Normal Mouse Serum
N-terminal	Amino-Terminal
OD	Optical Density
OX	Oxford University Cellular Immunology Unit
$p50^{csk}$	50 Kilo Dalton Csk Protein Tyrosine Kinase
$p56^{lck}$	56 Kilo Dalton Lymphocyte Specific Tyrosine Kinase
$p59^{fyn}$	59 Kilo Dalton Fyn Protein Tyrosine Kinase
PBL	Peripheral Blood Lymphocyte(s)
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein Kinase C

PLCγ1	Phospholipase C Gamma 1 Isoform
PTK	Protein Tyrosine Kinase
PTPase	Protein Tyrosine Phosphatase
r.t.	Room Temperature
RaMo-antibody	Rabbit Anti-Mouse Antibody
RID	Radial Immune Diffusion
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RTE	Recent Thymic Emigrant
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SH2	Src Homology 2 Domain
SH3	Src Homology 3 Domain
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-Beta
Th0	T Helper 0 Subset
Th1	T Helper 1 Subset
Th2	T Helper 2 Subset
Thp	T Helper Common Precursor Subset
TNF-α	Tumour Necrosis Factor-Alpha
ZAP-70	70 Kilo Dalton Zeta-Chain Associated Protein

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IV. Declaration

I declare that all of the conceptual ideas and experimental design, *constituting the theoretical work described in this study*, were derived wholly by the author, BC Jaques and the supervisor, Professor JA Bradley.

I also declare that all of the experimental work, *constituting the results described in this thesis*, were carried out wholly by the author, BC Jaques, with exception of receiving technical assistance for:

- **RT-PCR assays** from Dr S Middleton.
- **Cytotoxic, alloantibody isotype and IL-2 bioassays** from Dr H Marshall.
- **Immunohistochemical analysis** from Dr EM Bolton and Mr JC Casey.
- **Anti-TCR activation and intracellular signalling experiments** from Mr D Kavanagh.
- **MLR assays** from Miss P Neison.

Some of the results described in Chapters 1 and 2 were presented orally at the *15th World Congress International Transplantation Society International Meeting: Kyoto, Japan (1994)* and at the *Autumn Meeting of the British Transplantation Society: London, UK (1994).*

While some results described in Chapters 1, 2 and 3 were presented orally at the *4th International Basic Science Symposium of the International Transplantation Society: Noordwijkerhout, The Netherlands (1995).*

1. Introduction

1.1 MAJOR HISTOCOMPATIBILITY ANTIGENS (MHC)

1.1.1 Introduction

Throughout recorded history, inquisitive 'physicians' have been documented as having attempted various kinds of tissue transplantation but, until the later half of this century, these attempts inevitably ended in graft failure. In the early 20th century, even after the technical difficulties of surgical anastomosis and infection control were, to a large extent, surmounted, solid organ transplantation still suffered the same outcome. However, it was only after the technical difficulties associated with transplantation were overcome that other mechanisms leading to allograft failure were recognised and then studied in more detail.

The suggestion that transplant rejection may be due to "inherited factors" was first documented by L Loeb [1,2] at the turn of the century following the work of the geneticists CC Little, EE Tyzzer and colleagues. They observed that tumours taken from a strain of Japanese waltzing mice were, when transplanted into other mice, accepted by mice of the same strain but rejected by F2 generations of waltzing mice crossed with other mouse strains. With considerable foresight, Tyzzer later pointed out in a review of the subject, [3] that the "foreignness" or "incompatibility" of the tumour in these transplantation experiments was dependent on "the presence of a large number of independently inherited factors". An understanding of the antigenic basis of tumour allograft rejection came over 20 years later following work by PA Gorer who demonstrated that tumour transplantation between mice strains was governed by antigens present on the donor's tissues which were absent from the recipient's. [4]. Gorer named this antigen, Antigen II, and showed that it was expressed on

blood cells and that rejection in allotransplantation experiments was secondary to the recipient animals making isoagglutinating antibody responses to Antigen II [5]. A decade later, GP Snell demonstrated that a complex of dominant genes existed which determined the transplantability of tumours and called these the "histocompatibility antigens" [6]. With this important discovery, the genetic locus in mice discovered by Gorer then became known as the H-2 complex, and further studies by these two men and their colleagues eventually set out the important framework on which the development of today's understanding of transplantation histocompatibility antigens has been built.

We now know that each species of animal possesses a MHC that encodes for both Class I (recognised by CD8 cytotoxic T cells) and Class II (recognised by CD4 T helper cells) antigens. The MHC is composed of a large number of highly polymorphic loci making the total number of inheritable antigenic combinations very large. In addition to alloantigens encoded by the MHC, each species also has a number of MHC independent alloantigenic loci which generally incite weaker alloreactive responses and have been named, collectively, the minor histocompatibility antigens. The major and minor histocompatibility antigens together are responsible for the cell-mediated and/or humoral response individuals within a species mount against grafts from other individuals of the same species and this immunological response constitutes allograft rejection.

MHC gene products are integral cell-surface proteins which are found in all higher vertebrates and are encoded by a similar genetic region in each species [7,8]. The main function of MHC gene products is to act as 'restriction' elements for the recognition of foreign antigens. Specifically, MHC molecules on the surface of ANTIGEN PRESENTING CELLS (APC) bind endogenously (Class I MHC) or exogenously (Class II MHC) derived peptide antigens, and present the peptides in the context of 'self MHC' to cells (T cells) in the immune system. It is

the ability of MHC to couple with peptide antigen which allows the host's immunological system almost limitless adaptation to a constantly changing environment occupied by mutating and novel pathogens [9], and gives the system specificity when recognising 'normal' self from 'altered' self following infection or oncogenic transformation.

1.1.2 Structure and function of MHC

The T cells responsible for initiating rejection of MHC incompatible grafts recognise and respond to intact allogeneic MHC molecules expressed on the surface of donor APC. This type of recognition is called direct allorecognition, and it is the high precursor frequency of alloreactive T cells for direct recognition which accounts for the exceptional high potency of the alloimmune response [10,11,12]. Recipient T cells may also recognise and respond to allogeneic MHC molecules which, like nominal protein antigens, have been enzymatically degraded, processed and then presented by host APCs in the peptide-binding groove of self MHC; this type of recognition is known as indirect allorecognition [13,14,15]. The extent to which T cells recognising alloantigens presented by the indirect pathway contribute to graft rejection is not clear but is likely to be influenced by several variables such as prior sensitisation of the recipient, the type of tissue transplanted, the nature of the MHC disparity, and the type of immunosuppressive agent used [16,17,18,19,20]. As already noted, in allotransplantation, the most important MHC gene products are Class I and Class II antigens.

Class I MHC molecules are expressed constitutively on the surface of most nucleated cells [21]. They are 45 kDa integral membrane GLYCOPROTEINS (gp) which comprise a short CARBOXY-TERMINUS (C-terminus) hydrophilic segment situated in the cytoplasm, a hydrophobic section anchoring the protein in the cell membrane, and 3 extra cellular domains,

(α1, α2, and α3). The α3 domain is relatively non-polymorphic and associates non-covalently with the non-glycosylated and non-polymorphic 12 kDa polypeptide β_2-microglobulin. This domain of Class I MHC also serves as the site of recognition by CD8 molecules [22,23]. The α1 and α2 hypervariable domains of the Class I molecule form two α-helices floored by a β-pleated sheet and this structure constitutes the peptide groove of the molecule. Uniformly folded non-polymorphic segments limit the peptide groove at either end, imposing physical restrictions on the size of the peptide which can be accommodated; Class I MHC bound peptides are between 8 and 10 amino acids in length. The α3 domain and β_2-microglobulin of Class I MHC both display a folding pattern typical of classical IMMUNOGLOBULIN (Ig) domains.

Class II MHC molecules have a much more restricted tissue distribution than Class I MHC. They are expressed constitutively on dendritic cells, B cells and activated macrophages and given the presence of appropriate cytokines, can be up-regulated and expressed on most other nucleated cell types. Class II MHC molecules are formed by the non-covalent association of a 33-34 kDa α-chain and a 28-29 kDa β-chain. Like Class I molecules, each component chain has a short hydrophilic cytoplasmic CARBOXY-TERMINAL (C-terminal) sequence, a hydrophobic membrane anchor, a β-sheet and α-helix domain and a classic Ig domain. The two sheet and helix portions of each chain align face to face to form the peptide binding groove. In contrast to Class I MHC, the peptide-binding groove is 'open ended' and can accommodate peptides from 13 to 25 amino acids in length. A non-polymorphic loop in the β chain Ig like domain (loop 3 region of β2) is the site of interaction with the CD4 molecule [24,25].

1.1.3 Peptide binding to MHC

The peptides found within the peptide-binding groove of Class I MHC molecules are usually endogenously derived from within the cell and result from proteolytic cleavage of self proteins or alternately from viral proteins. Conversely, MHC Class II peptides are derived mainly from the enzymatic breakdown of exogenous foreign protein and/or cell-surface proteins [26]. The fundamental difference between Class I and Class II MHC in peptide binding stems from the different route of intracellular trafficking for these proteins following their synthesis in the ENDOPLASMIC RETICULUM (ER) [27]. As newly synthesised Class I heavy chains are first folded and assembled, they associate non-covalently with β_2-microglobulin in the ER [26,28]. Concomitantly, cytosolic self proteins are digested by multi-subunit proteosomes [29] into 8-10 amino acid peptides and gain access to the ER by membrane bound transporters [30]. In the ER the binding of heavy chain, β_2-microglobulin and peptide stabilises the complex and with the help of an 88 kDa chaperone protein [31,32], the tri-molecular complex is transported to the cell-surface, via the Golgi apparatus, where glycosylation occurs. On the other hand, by temporally occupying the peptide groove of Class II MHC molecules [33], the invariant chain prevents Class II MHC from binding peptide in the ER. The invariant chain by its chaperone effect, also prevents glycosylation of Class II MHC molecules at the Golgi [34]. Following synthesis, Class II MHC molecules proceed directly from the ER to an acidic membrane bound compartment known as the endosome [35,36]. In the endosome, foreign proteins and the invariant chain are degraded by a variety of proteases which allows peptide fragments ranging from 13 to 25 amino acids in length to associate with Class II MHC [37]. The variation in length of Class II bound peptides suggests that the Class II molecule itself may play an active role in antigen selection by protecting bound peptide from complete degradation [38] or allowing exposed ends of

longer peptides to be truncated or trimmed [39]. Once peptides are bound to Class II, the resulting complex is stabilised and transported to the cell-surface.

1.2 THE CD4 MOLECULE

1.2.1 CD4 structure and function

The CD4 molecule is a 56 kDa transmembrane protein consisting of 4 Ig like extracellular domains, a transmembrane domain and an intracellular C-terminus cytoplasmic portion. The two AMINO-TERMINAL (N-terminal) domains of the CD4 molecule engage the β chain of Class II MHC by contact sites in the COMPLEMENTARITY DETERMINING REGIONS (CDR)1-like and CDR3-like loops of the first domain, and loop 6 of the second domain [40,41]. The CD4-Class II MHC β domain interaction may be species specific [42], but has not been shown to be affected by Class II polymorphisms. The hydrophilic cytoplasmic domain of CD4 associates non-covalently with the 56 KILO DALTON LYMPHOCYTE-SPECIFIC TYROSINE KINASE ($p56^{lck}$) via cysteine-dependent interactions [43,44,45,46,47] and probably confers the co-receptor function of CD4 during TCR activation (see below).

1.2.2 CD4 as a co-receptor

By physically associating with the TCR during its interaction with the Class II MHC-Peptide complex [48], CD4 acts as a co-receptor and leads to a 10 fold increase in TCR mediated lymphocyte activation [49,50,51]. The association of $p56^{lck}$ with the cytoplasmic tail of CD4

permits the initiation of tyrosine kinase signalling events following TCR-Peptide-Class II MHC-CD4 complex engagement and is likely to be responsible for the potentiation of T cell activation. The observation that some T cell clones are resistant to anti-CD4 antibody dependent inhibition [52,53,54] suggests that the dependency of T cells on CD4 co-receptor function may vary depending on the lineage or activation state of the T cell. In particular, primed T cells may be less susceptible to anti-CD4 treatment than naïve (unprimed) T cells. This *in vitro* observation is consistent with the *in vivo* finding that the induction of transplantation tolerance using anti-CD4 mAb is easier to accomplish in naïve animals as opposed to alloantigen primed recipients [55].

1.3 ANTIGEN RECOGNITION AND SIGNAL TRANSDUCTION

1.3.1 Structure and function of the T cell receptor (TCR)

The TCR is a member of the Ig superfamily and consists of a dimer of non-identical α and β or of γ and δ chains. Each α and β chain has a short cytoplasmic C-terminal segment, a hydrophobic membrane anchor, and a large extracellular domain which resembles the Ig light chain. Like the Ig variable region [56], the N-terminal portion of the α and β chains has also evolved hypervariability in loops 2, 3, and 6 forming CDR1, CDR2, and CDR3. The TCR α chain CDR1 and CDR2 engage the α helix of the α1 domain of Class I or Class II MHC [57], while the same CDRs on the TCR β chain engage the other α helix (either α2 domain of Class I or the β1 domain of Class II). This leaves the central region of the TCR αβ dimer, consisting of the two CDR3s, to bind peptide antigen presented in the groove of MHC

[58,59]. The structure of the TCR γδ receptor is probably analogous to that of the αβ TCR but this is yet to be confirmed.

Like the Immunoglobulin D receptor on the surface of B cells, the TCR confers T lymphocytes with the capacity for antigen specific recognition during an immune response. The TCR itself does not have intrinsic signal transduction capabilities and relies instead on its association with other cell-surface molecules during T cell triggering and generation of the intracellular signals needed for T cell activation. Recent evidence, utilising chimeric TCR receptors [60,61,62,63], and TCR mutants [64], has shown that at least 2 molecules which intimately associate with TCR, CD3 ε and ζ chain subunits, have signal transducing moieties. As discussed below, the two molecules responsible for transducing signalling events have highly conserved common peptide sequences within their cytoplasmic domains and these have been termed ANTIGEN RECOGNITION ACTIVATION MOTIFS (ARAMs).

1.3.2 CD3 complex

The invariant CD3 molecule comprises an γ, δ, and two ε subunits associated with a ζ chain existing as either a homo (ζζ), or heterodimer (ζη or ζγ); the homodimer form is the more commonly found configuration [65]. Like the αβ TCR complex, the invariant CD3 molecule is a transmembrane protein with an Ig like extracellular domain and a hydrophobic membrane anchor, but unlike the TCR, which has a short cytoplasmic domain, the CD3 γ, δ, and ε chains have large cytoplasmic domains. Each ζ chain protein has a small extracellular domain, a hydrophobic transmembrane anchor, and a large cytoplasmic domain. As alluded to above, a small percentage of TCRs have been shown to be comprised of the invariant molecule associated with a heterodimer of ζ coupled via an η chain which is a product of an alternated

splicing of the C-terminal end of the ζ chain [66,67,68] or complexed via the γ chain of the MULTI-SUBUNIT HIGH AFFINITY IGE RECEPTOR (FCεRIγ) [69]. The γ subunit of FCεRI has been shown to share sequence homology with both the cytoplasmic and transmembrane anchor region of the ζ chain [70]. Receptors containing either ζζ, ζη or ζγ can contribute to and alter TCR function [71,72].

As mentioned above, evidence suggesting the role for ζ and ε chain involvement in TCR mediated signal transduction arises mainly from studies of two separate murine T cell hybridoma variants which lack complete or functional ζ chains. The first variant, which lacks expression of ζ chain [73] was found, when reconstituted using full length ζ chain, to regain normal TCR expression and function. However, reconstitution using ζ chain with truncation of the cytoplasmic domain resulted in impaired antigen and superantigen induced INTERLEUKIN (IL)-2 production [74]. Further experiments using this hybridoma with chimeric receptors consisting of the extracellular domains of CD4, CD8 or INTERLEUKIN-2 RECEPTOR ALPHA CHAIN (IL-2Rα) fused to the cytoplasmic portion of ζ chain which could be expressed independently of the other TCR subunits, gave additional support for the role of ζ chain in TCR signalling. Antibody mediated cross-linking of CD8/ζ or IL-2Rα/ζ chimeras or gp120 cross-linking of CD4/ζ (as well as CD4/η and CD4/FCεRIγ) chimeras was sufficient to activate biochemical and cellular events indistinguishable from those mediated by the wild-type multi-subunit receptor complex itself [60,61,62].

The second murine T cell hybridoma studied in this context, was devoid of cytoplasmic ζ chain but was found to produce IL-2 in response to antigen or superantigen [75]. Experiments using this mutant, with a chimeric receptor consisting of the extracellular and transmembrane portions of the IL-2Rα chain and the cytoplasmic domain of the CD3ε

subunit (IL-2Rα/ε), showed that the CD3ε cytoplasmic tail was responsible for the signalling events and IL-2 production observed [63].

1.3.3 Antigen recognition activation motifs (ARAMs)

ARAMs are highly conserved protein sequences which are present in a variety of haematopoietic cell antigen receptors as well as viral plasma membrane proteins. They are thought to represent the structural basis for many forms of signal transduction. For example, the cytoplasmic domains of ζ chain, CD3ε, TCR η and FcεRIγ subunits share a common motif consisting of two ***N*-TYROSINE-X-X-LEUCINE-*C*** paired sequences separated by six to eight amino acids [76]. This ARAM sequence appears in triplicate in the ζ chain, as a doublet in TCR η, and as a single copy in CD3ε and FcεRIγ subunits. Chimeric receptors with cytoplasmic tails encompassing as few as 17 amino acids containing the isolated ARAM have been shown to be capable of transducing intracellular signals sufficient for the activation of the PROTEIN TYROSINE KINASE (PTK) pathway and subsequent TCR-mediated effector functions [77,64]. Alteration in either of the two tyrosine residues of the activation motif using the IL-2Rα/ε chimera results in a marked reduction of receptor function [63]. Loss of receptor function was also observed when the C-terminal leucine residue was deleted [64]. Thus, it would appear from these studies that the ARAM sequences encoded in the cytoplasmic tail of antigen receptor-associated signal transducing molecules such as the ζ chain and CD3ε subunit plays a critical role in coupling TCR ligation to the PTK pathway and to other effector molecules within the intracellular signalling cascade.

1.3.4 Protein tyrosine kinases (PTK) and phosphatases (PTPase) introduction

The events which follow TCR ligation with antigen and lead to cytokine production, gene upregulation and clonal proliferation have not been fully elucidated. However, some of the biochemical changes following TCR ligation with antigen, superantigen, or immobilised antibody, as well as the changes following CD3 ligation with immobilised antibody, which lead to IL-2 production have been the subject of recent studies. Biochemical and genetic evidence implicates at least 3 cytoplasmic PTKs in TCR signal transduction, namely Lck, Fyn, and 70 KILO DALTON ZETA CHAIN ASSOCIATED PROTEIN (ZAP-70). Additionally, the CD45 transmembrane PROTEIN TYROSINE PHOSPHATASE (PTPase) has been shown to be quintessential in this signalling cascade, suggesting perhaps that TCR activation represents an alteration in the dynamic equilibrium between phosphorylated and dephosphorylated substrates, as orchestrated by the PTKs and PTPases.

1.3.4.1 CD45 protein tyrosine phosphatase (PTPase)

The CD45 transmembrane protein (initially identified as the leukocyte common antigen) has been shown to have PTPase activity and is thought to be critically involved in regulating the activation of a number of haematopoietic cells through their antigen receptors. As its name implies, CD45 is abundantly expressed on the cell-surface of all leukocytes and exists in several different isoforms. All isoforms of CD45 have a membrane anchor and a large cytoplasmic domain which is highly conserved and contains a 300 amino acid tandem repeat which has considerable homology to the catalytic domain of the cytosolic phosphatase-1B [78,79]. Unlike the cytoplasmic tail, the extracellular domain exists in several structurally distinct isoforms [80]. This heterogeneity arises because of alternate splicing of the several

exons which encode the extracellular domain together with variable expression of the translated product. The different isoforms of CD45 have been categorised by their molecular weights and/or antigenic properties and may correspond to functionally distinct cell types [81,82].

The distinct isoforms of CD45 differentially associate with the TCR and its coreceptors, giving weight to the idea that the different isoforms may have discrete activation properties. The high molecular weight isoform of CD45 has been found to move within the cell membrane independently of the TCR complex on naïve human [83] and mouse [84] T cells, while experiments using cloned activated [84,85] or "memory like" [86] T cells expressing the low molecular weight isoform of CD45 have found that CD45 is associated with the CD4-TCR and/or the CD8-TCR complex. Interestingly, McKnight et. al. [82] have shown that rat T cell clones expressing high and low molecular weight CD45 isoforms secrete different cytokine profiles and they suggested that these isoforms may represent distinct T effector cell populations in the rat, analogous to the murine Th1 (which secrete mainly IL-2 and INTERFERON-GAMMA (INF-γ)) and Th2 (which secrete IL-4, IL-5, IL-6, and IL-10) cell types [87]. A problem with this interpretation is that the distribution of a particular CD45 isoform on the surface of the T cell does not necessarily equate to a maturational end-point of that particular cell. For example, naïve RTE $CD4^{+ve}$ T cells in the rat express the low molecular weight isoform of CD45 but are the precursors of both the high and low molecular weight isoforms found on more mature T cells [88]. Moreover, reconstitution of adult thymectomised bone marrow irradiated T cell depleted rats with high molecular weight CD45 $CD4^{+ve}$ T cells results in T cells which predominantly express the low molecular weight CD45 isoform. [89]. In addition, reconstitution of adult congenitally athymic nude rats with CD4 lymphocytes expressing one or other of the isoform of CD45 results, after CD4 T cell

repopulation, in lymphocyte populations, many of which express the reciprocal CD45 isoform to that of the transferred cells [90]. These results suggest that the two CD4 T cell subsets may have a precursor-product relationship depending on the state of antigen experience, activation, maturation or stage of cell division.

The earliest demonstration that CD45 might be important in TCR mediated signal transduction came from antibody cross linking studies. Interestingly, antibody cross linking of CD45 alone during TCR stimulation was found to result in either potentiation or inhibition of T cell activation, depending on the type of the anti-CD45 antibody used, or the nature of the T cell line examined [91,92,93,94,95]. However, when TCR and CD45 are co-aggregated by the use of cross-linking anti-TCR and anti-CD45 antibodies, inhibition of T cell activation is consistently observed [96]. This inhibition may occur because some forms of cross linking effectively inhibit the antigen receptor oligomerisation necessary for normal TCR function [97] but is more likely to occur as a result of antibody mediated alteration of the signalling activity of CD45 phosphatase.

Studies using T cell clones deficient in CD45 expression give support to the concept that CD45 is a positive regulator of TCR signal function since such clones display abrogation of TCR signal transduction. For example, a murine T cell clone lacking cell-surface expression of CD45 has been shown to lack the ability to proliferate or produce cytokines in response to antigen or anti-TCR antibodies [98]. Interestingly, this same T cell clone was still able to respond to exogenous IL-2, indicating that CD45 is required for TCR mediated cellular function but is not involved in the downstream signalling events mediated through the IL-2 receptor. Similarly, cytotoxic T cells which are deficient in CD45 expression are unable to lyse target cells and to produce cytokines [99].

Studies using Jurkat and HPB-ALL CD45 negative mutant cell lines have shown that the mutant cells do not undergo tyrosine phosphorylation and phosphatidylinositol hydrolysis, and do not increase their influx of intracellular Ca^{2+} following TCR ligation [100,101]. These deficient biochemical responses are restored following transfection of the mutant T cell lines with CD45 [100,102,103]. Because induction of tyrosine phosphorylation is one of the earliest events following TCR engagement [104,105], the lack of TCR mediated tyrosine phosphorylation in CD45 deficient cell lines suggests that during normal TCR induced signalling, CD45 is involved at a very proximal site in the signal transduction cascade.

One possible role of CD45 is to regulate the state of tyrosine phosphorylation of the tyrosine kinases $p56^{lck}$ and 59 KILO DALTON FYN PROTEIN TYROSINE KINASE ($p59^{fyn}$), both of which associate non-covalently with the TCR complex. Initial analysis of tyrosine phosphorylation patterns using CD45 deficient cell lines revealed a slight increase in the tyrosine phosphorylation of the lymphocyte specific protein tyrosine kinase $p56^{lck}$. Peptide mapping revealed that $p56^{lck}$ was hyper-phosphorylated on it's C-terminal $tyrosine^{505}$ residue, a known site of negative regulation for members of the Src family of PTKs [106,107,108,109]. Further experiments using CD45 deficient cell lines also showed Fyn to be hyper-phosphorylated on it's C-terminal $tyrosine^{528}$ residue, although to a lesser degree than Lck [108,109]. Additional credence for the idea that Lck and Fyn, but not Src are the specific *in-vivo* substrates of CD45, is provided by the observation that CD45 is unable to de-phosphorylate the C-terminal tyrosine residue of Src *in-vivo* [108]. Analysis of Lck [109] and Fyn [102,109] kinase activity from CD45 positive cells has revealed a two to three fold increase in their *in-vitro* enzymatic activity when compared to CD45 negative cells, although this was dependent on the substrate used and specific cell examined [102,106]. Finally, the

addition of purified CD45 to either Lck or Fyn *in-vitro* results in de-phosphorylation at the C-terminal tyrosine residues and a concomitant increase in kinase activity [110,111].

1.3.4.2 50 kilo dalton Csk protein tyrosine kinase ($p50^{csk}$)

The data outlined above suggest that the TCR signalling defect observed in CD45 deficient cell lines may be due to lack of dephosphorylation and activation of Lck and Fyn due to persistence of phosphorylation at the negative regulatory C-terminal tyrosine residue. A protein kinase capable of phosphorylating the C-terminal tyrosine residues of Src [112], Lck [113], and Fyn [114] has been identified as Csk PTK. This PTK is structurally similar to the Src family of PTK containing a SRC HOMOLOGY 2 (SH2), SRC HOMOLOGY 3 (SH3) and a kinase domain. However it lacks a myristylation site, an auto-phosphorylation site and a negative regulatory C-terminal tyrosine residue [115]. A negative regulatory role for Csk in TCR mediated signalling is suggested by experiments showing that a three fold increase in expression of Csk in CD45 positive cells resulted in a decrease in tyrosine phosphoprotein induction and IL-2 production [116]. Evidence that $p50^{csk}$ has a regulatory role on $p59^{fyn}$ enhanced TCR signalling comes from experiments using Jurkat cells expressing a constitutively active form of $p56^{lck}$, in which the regulatory tyrosine at position 505 was mutated to phenylalanine and thus could not be inhibited by phosphorylation. In these studies, over expression of $p50^{csk}$ leads to attenuation of TCR induced signalling by inactivation of $p59^{fyn}$ [117]. Thus, TCR mediated signalling appears to be balanced by events which allow the coupling of de-phosphorylation of upstream PTKs with their downstream substrates and uncoupling by phosphorylation of regulatory C terminal tyrosine on these PTKs by Csk like molecules.

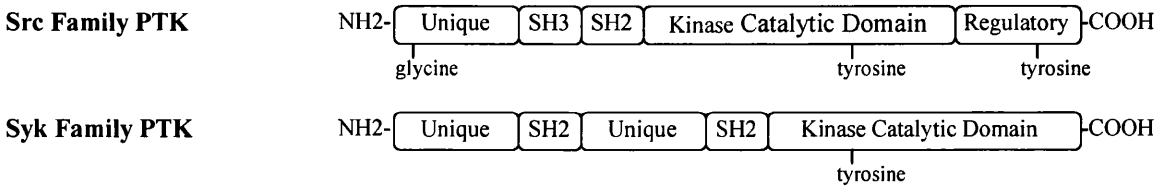
1.3.4.3 Src and Syk family of protein tyrosine kinases

Following TCR ligation with antigen or antibody, the PTK pathway is activated and results in a cascade of biochemical changes within the T cell, starting with tyrosine phosphorylation of a variety of cellular proteins [118,119]. One of the cellular proteins that undergoes rapid tyrosine phosphorylation is the γ1 ISOFORM OF PHOSPHOLIPASE C (PLCγ1) [120,121,122]. Tyrosine phosphorylation of PLCγ1 leads to an increase in its catalytic activity which in turn leads to hydrolysis of PHOSPHATIDYLINOSITOL 4,5-BIPHOSPHATE (PIP_2) to INOSITOL 1,4,5-TRIPHOSPHATE (IP_3) and DIACYLGLYCEROL (DAG) [104,123]. These bi-products act as second messengers within the cell and induce further changes such as the mobilisation of INTRACELLULAR CA^{2+} IONS ($[Ca^{2+}]_i$) and the activation of PROTEIN KINASE C (PKC) [124,125].

Three PTK enzymes $p56^{lck}$, $p59^{fyn}$, and ZAP-70, all situated in the cytoplasm, have been implicated in TCR mediated signalling. $P56^{lck}$ and $p59^{fyn}$ are both members of the Src family of protein kinases which share six common features. These are as follows, (1) a N-terminal myristylated glycine at residue 2 which permits membrane localisation, (2) a unique ~80 amino acid N-terminal region that may dictate specific associations of the kinase, (3) a ~60 amino acid SH3 domain involved in interacting with signalling molecules with prolene rich regions, (4) a ~100 amino acid SH2 domain that can specifically mediate the recruitment of tyrosine phosphoproteins, (5) a C-terminal catalytic domain, (6) and a C-terminal regulatory tyrosine residue.

In contrast, ZAP-70 belongs to the Syk family of PTKs which contain two tandemly arranged SH2 domains and a C-terminal kinase domain; Syk PTKs are not myristylated, do not contain SH3 domains and do not contain negative regulatory tyrosine residues at their C-terminal (See Figure 1.1 below).

Figure 1.1 Schematic diagram of the Src and Syk family of Protein Tyrosine Kinases.



1.3.4.4 P56[lck] protein tyrosine kinase

P56[lck] is a 56 kDa lymphoid specific PTK which non-covalently associates with the cytoplasmic domain of CD4 and CD8 molecules via cysteine-dependent interactions [43,44,45,46,47]. As mentioned earlier, the extracellular domains of CD4 and CD8 bind to the non-polymorphic region of Class II and Class I MHC respectively and act to stabilise the interaction between the T cell and the APC [126,127]. As well as serving as TCR coreceptors through their stabilising functions, CD4 and CD8 molecules, through their association with p56[lck], provide signal transducing co-receptor function with TCR [128]. Experiments using antibody to cross-link CD4 or CD8 to CD3 have found that such cross-linking results in enhanced TCR mediated signalling [129,130,131,132]. The significance of CD4 and p56[lck] in T cell induced signalling has been underscored by studies using a CD4 dependent antigen specific murine T cell line which lacks endogenous CD4. Transfection of this cell line with CD4 molecules containing mutant cytoplasmic domains, incapable of associating with p56[lck], results in a non-functional TCR, whereas transfection with normal CD4 molecules, capable of associating with p56[lck], restores normal TCR mediated PTK signalling [51]. Similar experiments using T cells expressing mutant CD8 molecules, incapable of associating with p56[lck], also results in a non-functional TCR, further highlighting the importance of p56[lck] in CD8 T cell signalling [133].

Experiments using genetic mutants of $p56^{lck}$ have helped to dissect further the role of $p56^{lck}$ in normal T cell signalling. If the mutant Jurkat leukaemic T cell line J CaM1.6, which lacks a functional $p56^{lck}$, is activated via the TCR, it fails to induce tyrosine phosphorylation of cellular proteins, to mobilise $[Ca^{2+}]_i$, or to express cell-surface activation molecules [134]. Reconstitution of the mutant line with wild type murine $p56^{lck}$ restores normal TCR mediated function. Similarly, another mutant, this time an IL-2 dependent cytotoxic T cell line which lacks $p56^{lck}$, is incapable of cytolysis until reconstituted with normal $p56^{lck}$ [135]. Interestingly, both of the above mutants have comparable levels of $p59^{fyn}$ activity, indicating that other members of the src family cannot compensate for isolated $p56^{lck}$ deficiencies and that $p56^{lck}$ must act proximally to $p59^{fyn}$ and other PTKs in the normal TCR signal transduction cascade.

1.3.4.5 FYN protein tyrosine kinase

Fyn is a 59 kDa PTK which is found predominantly in cells of neuronal and haematopoietic origin [136,137]. These two tissues display distinct isoforms of Fyn which differ as a result of alternate splicing of the several exons which encode the protein [138]. The association of Fyn with TCR ζ chain was first demonstrated in co-immunoprecipitation experiments [139]. The interaction of Fyn with the cytoplasmic domain of ζ chain appears to be mediated by the first 10 amino acids within the unique region of Fyn and at least two regions of the cytoplasmic domain of ζ each of which encompasses at least one ARAM sequence. The evidence for this comes from studies using ζ chain with only one ARAM sequence capable of interacting with Fyn, which revealed that the two molecules failed to associate [140]. Genetic evidence supporting the role of Fyn in TCR mediated signalling comes from a series of

transgenic experiments. Over-expression of wild type Fyn in the thymus of transgenic mice results in thymocytes that show increased levels of tyrosine phosphorylated proteins and are hyper-responsive with respect to TCR mediated PTK activity, when compared to thymocytes obtained from normal litter mates [141]. In contrast, expression of a mutant Fyn molecule with an inactive kinase catalytic site results in abrogation of TCR mediated proliferation and mobilisation of $[Ca^{2+}]_i$ as compared to normal cells. Mice that lack Fyn as a result of homologous recombination have grossly normal thymocyte subsets and normal TCR V_β repertoires. Interestingly, these Fyn deficient mice have marked signalling abnormalities in the single positive thymocyte population [142,143] but have essentially normal peripheral T cell signalling [143], suggesting that Fyn may play a role in the later stages of thymocyte development and may not be required for TCR mediated proliferation in peripheral T cells.

1.3.4.6 ZAP-70 protein tyrosine kinase

Zeta-chain associated protein (ZAP-70) is a member of the Syk family of PTKs [144]. ZAP-70 is a 70 kDa tyrosine phosphoprotein which, as its name implies, was initially found to be associated with the ζ chain of TCR following receptor stimulation [69]. More recently however, it has been shown to associate also with CD3ε subunit in stimulated cells [145,146]. The association of ZAP-70 with the ζ chain requires activation of the TCR and tyrosine phosphorylation of the CD3ζ chain and ZAP-70 by $p56^{lck}$. In experiments using a Jurkat cell line deficient in Lck, neither ZAP-70 nor ζ chain were found to phosphorylate or to associate together when the TCR was stimulated by immobilised cross-linking anti-TCR antibodies [147]. In studies using chimeric receptors expressing 2 forms of ζ chain, one expressed as part of the normal TCR and the other expressed with CD8, stimulation of TCR results in

association of ZAP-70 with TCR/ζ but not with CD8/ζ. Conversely, stimulation of CD8 results in association of ZAP-70 with CD8/ζ but not TCR/ζ [148]. The association of ZAP-70 with the ζ chain has been shown to be mediated through phosphorylation of ZAP-70 SH2 domains and ζ ARAM sequences [147]. Not surprisingly, the association of ZAP-70 with CD3ε is dependent on CD3ε tyrosine phosphorylation [146,149] and is probably mediated through the CD3ε ARAM sequence [150]. Exactly how ZAP-70 is involved in the TCR signalling cascade is not known. Studies using kinase inactive ZAP-70 constructs have reported normal phosphorylation of ZAP-70 and ζ chain, with normal association of the two molecules but with a marked decrease in cellular tyrosine phosphorylated proteins [147]. Thus, it appears that ZAP-70 specifically couples Lck mediated signalling to the TCR multi-subunit complex and is probably important in mediating signalling to downstream effector molecules. (See Figure 1.2 Below)

Figure 1.2 Schematic representation of the initiating events in TCR activation. Adapted from Chan et. al. *Annu Rev Immunol* 1994.12:555-92.

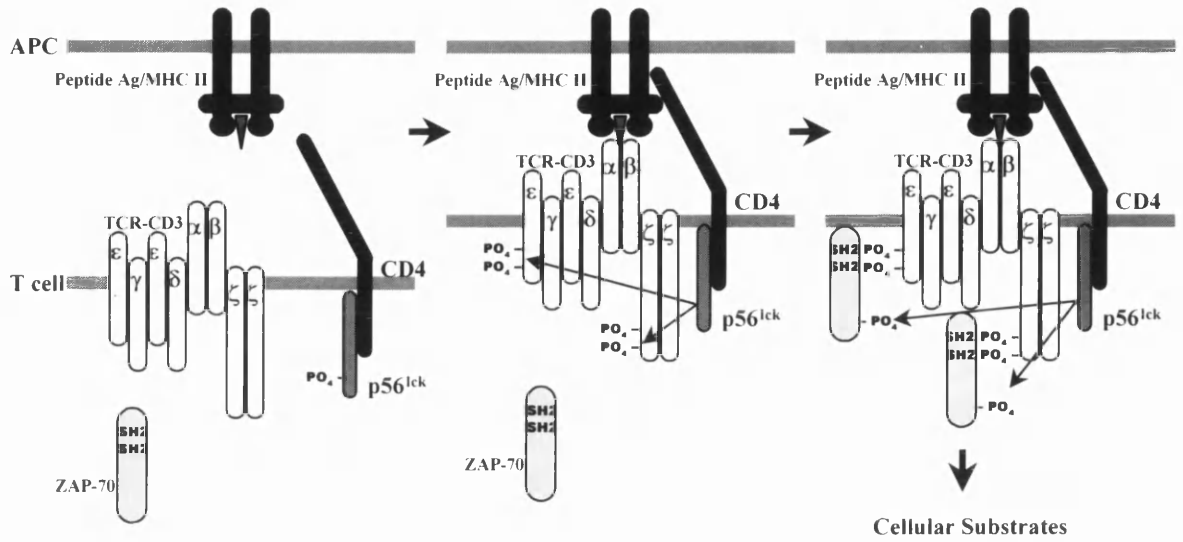


Figure 1.2 characterises the interaction of APC with CD4 T cells. Specifically, the presentation of peptide antigen (Ag) with MHC Class II to the αβTCR with subsequent activation of the intracellular signalling pathways and generation of active intracellular substrates is illustrated. The association of CD3 with TCR, the co-receptor function of the CD4 molecule, and the de-phosphorylation of $p56^{lck}$ and subsequent phosphorylation of tyrosine residues on the ε and ζ chains of CD3 and ZAP-70 are also depicted.

1.4 ANTI-CD4 TREATMENT AND TOLERANCE

1.4.1 Antibody induced tolerance introduction

Studies using monoclonal antibodies directed against T cell antigens have been shown to inhibit T cell proliferation *in vitro* [151,152,153,154,155] and to be capable of immunosuppression *in vivo* by inhibiting the development of autoimmune disease [156,157,158,159] and by increasing allograft survival [160,161,162,163] in animal models. In various rodent transplantation models using vascularised cardiac allografts, different systemic anti-CD4 mAb treatment protocols have been employed, some of which lead to profound depletion of CD4 T cells and others which block CD4 T cell function, partially or

completely spare CD4 T cell numbers. Studies using anti-CD4 in experimental transplant models have reported, that anti-CD4 treated animals not only enjoy increased survival of their cardiac allografts, compared to control animals, but additionally may develop allospecific tolerance when rechallenged with secondary grafts of the same strain as the original donor [160,162,164,165,166,167]. The mechanism of action of anti-CD4 mAb treatment in the development of transplantation tolerance is however, far from clear.

The first studies in which tolerance to a cardiac allograft was induced using anti-CD4 mAb, used anti-CD4 protocols in mice [165] or rats [162] which caused profound and long lasting depletion of CD4 T cell, thereby exposed the treated animals to long periods of non-specific immunosuppression. Although very useful in establishing models of tolerance, the almost-complete depletion of the $CD4^{+ve}$ T cell fraction, made it very difficult to determine how the other cell sub-populations and the re-emerging $CD4^{+ve}$ T cells each contributed to the transplant tolerance which ensued. More recent protocols of anti-CD4 treatment [164,166] have employed short courses of non-depleting or partially depleting anti-CD4 antibodies which allow for consistent monitoring of effector cell function without the need for experimental calculation and assumption due to the unnatural shifting of cell subsets as a result of depletion.

The principle mechanisms which have been proposed to explain peripheral T cell tolerance are clonal deletion [168,169,170], immune deviation or polarisation [171,172,173,174,175,176], T cell inhibition [177,178,179] and T cell anergy [180,181,182]. As indicated above, each individual mechanism probably represents a single facet of the multifaceted immune system, which when experimentally exploited, gives a false impression of uniqueness. It is the authors belief that transplantation tolerance using fully disparate MHC allografts is likely to be non-exclusive and will therefore incorporate some or all of the

above mechanisms, perhaps working together in an active or even synergistic fashion. A unifying explanation for transplantation tolerance must incorporate all of the pieces of the 'immunological jig-saw puzzle', explain the variation observed between experimental systems and hopefully predict the immune response when appropriately challenged. The potential mechanisms which are responsible for peripheral T cell tolerance are reviewed below.

1.4.2 Clonal deletion

Clonal depletion of potentially auto-reactive developing T cells within the thymus is the principal mechanism responsible in establishing central tolerance to potential auto-antigens. Clonal deletion of peripheral mature T cells, may in principle, also be partly responsible in the induction of tolerance following anti-CD4 mAb. In the context of anti-CD4 treatment and tolerance, it is believed that antibody treated cells which contact antigen, perhaps particularly those with the highest avidity for antigen [183], may receive inappropriate or inadequate intracellular signals which ultimately lead to programmed cell death or apoptosis. Additionally, one important element of T cell depletion by anti-CD4 antibodies appears to be secondary to cross-linking of the CD4 molecules on the cell-surface in the absence of TCR engagement and is associated with the up-regulation of Fas antigen [184,185]. The Fas antigen is usually only expressed on activated T cells and it is through the binding of Fas to its ligand (FasL) that immunoregulation through apoptosis is thought to occur. In this context, it is notable that mice which are deficient in either Fas or FasL expression tend to develop lymphoproliferative or autoimmune diseases [186].

Several models of peripheral tolerance secondary to clonal deletion have recently been described. Examples include the following. Mice given bacterial superantigens display an early and massive T cell proliferative response followed by clonal deletion through apoptosis of most, but not all, of their antigen reacting T cells. When the remaining T cell pool of such animals are subsequently re-challenged with superantigen, the animals display classical immunological tolerance [187]. Another elegant example of peripheral tolerance based on clonal elimination was provided by Scully et. al. [188] who transplanted the thymus from a B10.BR mouse under the kidney capsule of adult thymectomised CBA/Ca mice which had been depleted of $CD4^{+ve}$ and $CD8^{+ve}$ T cells using depleting anti-CD4 and CD8 antibodies. Once these animals had reconstituted their T cell pool in the presence of the new thymus, they were challenged with B10.BR skin grafts and were shown to be tolerant to them. However, the transplant tolerance observed could be broken if the transplanted mice were reconstituted with naïve CBA/Ca T cells. Interestingly, reconstitution of unmodified naïve CBA/Ca mice with tolerant T cells did not confer tolerance to the recipient mice. Likewise, reconstitution of CBA/Ca litter mates, which had been T cell depleted and thymectomised, with tolerant T cells did not restore graft rejection, so long as donor antigen was present in the system before cell transfer. This indicates that the missing cellular component in this experimental model was allospecific helper and/or effector cells. However, it is important to emphasise that clonal deletion of alloreactive T cells on its own is not the only mechanism, or indeed the major mechanism of tolerance induction, since, in many models of transplantation tolerance induced by monoclonal antibodies, restoration of allograft rejection is not achieved when naïve immunocompetent T cells are given to the tolerant recipients [179,181,189].

1.4.3 Immune deviation/polarisation

There is considerable evidence in the rat which suggests that the maintenance of peripheral tolerance to self antigens in normal animals is mediated by regulatory $CD4^{+ve}$ T cells, via the production of cytokines such as IL-4 and IL-10 [190]. Also, Hutchings et. al. [191] found an increase of $CD4^{+ve}$ T cells producing these cytokines around the islets of Langerhans of NOD mice made resistant to diabetes by the administration of non-depleting anti-CD4 antibodies. A similar regulatory mechanism for transplantation tolerance provides an attractive explanation for anti-CD4 induced allograft survival. As mentioned above, $CD4^{+ve}$ T cells are a heterogeneous population and can be divided into two functionally distinct subsets, Th1 and Th2, based, to a large extent, on their different cytokine repertoires. Th1 cells, through release of IL-2, TUMOUR NECROSIS FACTOR-ALPHA (TNF-α) and INF-γ, are responsible for mediating cellular effector responses such as cytotoxicity reactions, delayed type hypersensitivity, and B cell help for the production of complement-fixing antibodies. In contrast, Th2 cells produce IL-4, IL-5, IL-6 which provide B cell help for production of neutralising IgG1 or anti-parasitic IgE antibodies and IL-10 which down regulates Th1 cells. Th1 and Th2 effector cells arise from a T HELPER COMMON PRECURSOR CELL (Thp), and antigen inexperienced immature $CD4^{+ve}$ T cells are thought to correspond to Th0 clones which simultaneously secrete low levels of both Th1 and Th2 type cytokines. Because functionally distinct Th1 and Th2 subsets are mutually antagonistic and because each T cell subset has an autocrine response to its own pattern of cytokine, as an immune response begins to polarise, immature T cells are thought to be recruited towards the dominant response.

Normally a balance exists *in vivo* between Th1 and Th2 responses. Transplant rejection is generally associated with a strong Th1 immune response. Those advocating immune deviation as an explanation for transplantation tolerance, suggest that in the course of

tolerance induction, the antagonistic Th2 sub-population predominates and is responsible for inducing and/or maintaining the tolerant state. Several groups have shown preferential expression of IL-4 and/or IL-10 during the induction of tolerance to renal or cardiac allografts in rodents using tolerogenic strategies which include using low dose anti-CD4 antibody treatment [172,192]. Similarly, some investigators have been able to prevent Class II disparate skin graft rejection in mice by the transfer of thymocytes expressing Th2 cytokine repertoires [193]. However, it should be emphasised that there are a number of reports of tolerance induction occurring in the absence of an obvious Th2 cytokine response. For example, mice given rat anti-CD4 mAb to induce tolerance to skin allografts have also been shown to be tolerant of the rat anti-CD4 antibody itself; failure to generate a humoral response directed against the rat protein suggests that there is not necessarily a global bias towards a Th2 response in this model [189]. Also, in a similar skin allograft system but using both anti-CD4 and CD8 antibodies, mice which were made tolerant of K^b Class I MHC failed to make anti-K^b antibodies. However, such antibodies were found in high titres in untreated litter mates allowed to reject their grafts [194]. More recently, studies using IL-4 KNOCKOUT (KO) mice [195] or neutralising anti-IL-10 antibody [196], have shown that mice can be made tolerant to oral ovalbumin in the apparent absence of any IL-4 and/or any detectable Th2 cytokine response. From these studies, it is clear that although Th1 to Th2 immune deviation is an attractive explanation for peripheral tolerance in some experimental models, it cannot be invoked uniformly to account for anti-CD4 induced tolerance and therefore other potential mechanisms to explain tolerance must also be considered.

1.4.4 T cell inhibition, suppression and/or immunoregulation

T cell inhibition as a mechanism of immunological tolerance was a popular concept in the early 1970s and was originally called suppression. With the failure to clearly define a distinct population of 'suppressor' T cells, the concept fell out of favour. However, in the last few years the concept has been revived because several groups have been able to demonstrate, by adoptive transfer, the presence of cells which are capable of modulating the immune response *in vivo*.

One of the best examples of suppression using mAb therapy in a transplantation model is that reported by Qin et. al. In 1993 [179]. They used non-depleting anti-CD4 and anti-CD8 antibodies to induce tolerance to B10.BR multiple minor skin allografts in adult thymectomised CBA F1 hybrid recipients, obtained after crossing normal CBA mice with CBA mice expressing the human CD2 transgene ($hCD2^{+ve}$). After establishing tolerance, the authors tested for T cell suppression by transferring 5×10^7 normal spleen cells from naïve $hCD2^{-ve}$ CBA mice back into the tolerant graft recipients. They first showed that if they removed the tolerant cells from the recipients at the time of spleen cell transfer, by using anti-human CD2 antibody, then the skin grafts were be promptly rejected by the transferred immunocompetent cells. However, if they waited for at least two weeks after cell transfer before deleting the tolerant cells, then the original, and even a second B10.BR skin graft, was not rejected, despite the absence of any residual $hCD2^{+ve}$ tolerant cells. This elegant experiment demonstrates that the original population of lymphocytes in the tolerant animal were capable not only of inhibiting naïve T cells, but also that, in time, the naïve T cell population had itself become tolerant. In describing this phenomena, Waldmann's group coined the term "infectious tolerance", however, credit must go to Eardley and Gershon who first described infectious tolerance in 1975. Eardley and Gershon showed that tolerant cells

could make naïve cells tolerant when mixed together and called this concept "feedback suppression" [197]. Waldmann and collegues also showed, using their experimental model, that a T cell mixture taken from tolerant and naïve animals could be transferred back into new secondary recipients who would then also display tolerance to the original antigen; moreover, this process could be repeated several times over, transferring transplantation tolerance from one new naïve recipient to another. There are of course, still several important questions which remain unanswered in this experimental model. For example, is infectious tolerance one phenomena (i.e. inhibition/suppression) or does infectious tolerance also rely on multiple mechanisms? What type of cells are responsible for causing suppression? Do the "suppressor cells" actively produce cytokines which suppress other effector cells or are they inactive and unresponsive, and merely inhibit by interfering with a developing immune response by virtue of their numbers or their ability to consume important cytokines? To address these questions further, additional mechanisms must be considered such as T cell anergy.

1.4.5 Anergy

The term T cell anergy was coined by Schwartz and colleagues [198]and can be defined as the absence of a normal cellular response to antigen (proliferation and production of IL-2 and other cytokines) despite the continued presence of the cell capable of recognising the antigen (T helper/effector cell) and the antigen itself. Many explanations for T cell anergy have been proposed but it is now generally accepted that anergy in $CD4^{+ve}$ T cells is induced when these cells encounter antigen and fail to proliferate normally due to the absence of appropriate costimulation or cytokines. Anergy is more easily demonstrated within the Th1 cell subset

and it has been suggested that Th1 cells are more susceptible than Th2 cells to the induction of non-responsiveness. As discussed above, the absence of an appreciable Th2 response in many models of Th1 transplantation tolerance, suggests that Th2 cells can also be anergised, although direct testing for Th2 anergy in these models was not performed. Interestingly however, Yssel et. al. [199] showed that it is possible to induce anergy in human allergen specific Th2 cells by activating the cells in the absence of professional APC. The induction of anergy in this system was accompanied by phenotypic modulation of the cells and altered cytokine production upon activation.

As implied above, there has been a plethora of reports describing anergy involving Th1 responses which date back to the mid 1980s. Jenkins & Schwartz [198] showed that T cell clones when stimulated with antigenic peptides bound to Class II MHC on metabolically inactive or non-professional APC (lacking normal costimulatory accessory surface molecules) did not proliferate and became unresponsive when subsequently re-challenged with antigen presented by professional APC. They later showed [200] that murine Th1 clones stimulated with antigen presented by normal APC in the presence of anti-IL-2 and anti-IL-2 receptor (IL-2R) antibodies also became anergic. From these studies, they suggested that the lack of accessory molecules for costimulation is not the limiting step in the induction of anergy. Instead, they argued that lack of normal IL-2 production and cellular proliferation resulting from incomplete costimulatory activity was the critical event.

Quill et. al. [201] showed that MHC Class I restricted T-hybridoma cells could also be rendered anergic by using a system of antigen presentation in planar lipid membranes with or without Class I molecules. They showed that the T cells became unresponsive if antigen was presented by Class I without other accessory molecules, but remained responsive if antigen was presented in the lipid membranes directly without Class I. This finding suggests that

induction of anergy, through lack of accessory costimulation, requires the engagement of the T cell receptor in the context of normal self MHC-CD8/CD4 complex.

T cell anergy was implicated in transplantation tolerance by Alters et. al. [182]. This group transplanted mouse pancreatic islets of Langerhans from A/J(IE^{K+ve}) donors into streptozotocin induced diabetic C57B1/6 (IE^{K-ve}) recipients under the cover of depleting anti-CD4 antibody treatment. They found that the recipients became tolerant of their IE disparate grafts, as measured by normoglycaemia for greater than 200 days. Murine TCR V_β gene segments which encode reactivity with Class II MHC IE antigen are found in $V_\beta 5$, $V_\beta 11$ and $V_\beta 17$ subsets and animals which express IE antigen generate self tolerance by depleting the majority of their V_β 5, 11, and 17 T cells from the periphery [202,203,204,205]. Alters et. al. found no evidence of V_β 5, 11, or 17 depletion in their model of anti-CD4 induced transplantation tolerance. Furthermore, after isolating LNCs with specific V_β subsets from their tolerant mice, they showed that the $V_\beta 11^{+ve}$ subset proliferated poorly in response to immobilised anti-$V_\beta 11$ antibody, and that proliferation could be partially restored by adding exogenous recombinant IL-2. In a combination of mixing experiments using tolerant $CD4^{+ve}V_\beta 11^{+ve}$ or $CD8^{+ve}V_\beta 11^{+ve}$ cells, the authors could not suppress naïve C57B1/6 LNC proliferation in response to anti-$V_\beta 11$ antibody. These data suggest that in this particular model, anergy rather than clonal deletion or inhibition/suppression was responsible for tolerance induction. The presence of anergic cells in anti-CD4 mAb treated recipients bearing long-term surviving allografts has also been reported by other groups [181,189], providing further support for the hypothesis that anergy of peripheral CD4 T cells may be an important mechanism for maintaining the tolerant state.

1.4.6 Infectious anergy

Interestingly, in all the systems discussed above, tolerance once established appears to be very robust and to dominate any further tendency for the immune system to respond to a new challenge with the same antigen. In a recent review, Cobbold et. al. [206] have introduced a new term, "infectious anergy", which in part describes tolerance as being both inhibition and anergy working together. They have suggested [207,208] that anergic T cells might compete with immunocompetent T cells and, by depriving the immunocompetent cells of important paracrine proliferative cytokines, cause them to default to the anergic state. There is good *in vitro* data using mixing experiments with anergic and immunocompetent naïve cells which suggests that competition between these cells for the surface of APC can induce anergy in the immunocompetent fraction [209]. It seems likely that competition for the APC cell-surface molecules required for costimulation, particularly that of either B7-1 or B7-2 with CD28 [180] or the recently described costimulatory molecule for Th1 cells [210], is involved in the induction of infectious anergy. It is notable that blockade of CD28-B7 interaction after cardiac alloantigenic challenge, has been shown to induce anergy in the Th1 compartment but apparently spares the Th2 response [211]. There is a growing body of evidence which suggests that B7-2 and B7-1 may provide costimulation with some specificity for Th1 and Th2 cells respectively [180,212]. This might explain how, if competition for ligand is limiting, either Th1 or Th2, or both Th1 and Th2 responses could be inhibited, depending on expression and/or level of competition for each of the ligands involved.

Cobbold et. al. [206] have proposed a model which attempts to unify the different potential mechanisms underlying peripheral tolerance to alloantigen. They suggest that a cellular collaborative unit may involve competition between non-tolerant naïve Th0 like cells, anergic tolerant Th1 or Th2 $CD4^{+ve}$ T cells and non-tolerant $CD4^{+ve}$ and/or $CD8^{+ve}$ T cells for the

cell-surface of an APC. In this model, they propose that naïve cells encounter antigen and produce low levels of Th1 and Th2 cytokines. The small amount of IL-4 produced in turn stimulates the tolerant T cell pool to expand. During expansion, the tolerant $CD4^{+ve}$ T cells secrete INF-γ and TRANSFORMING GROWTH FACTOR-BETA (TGF-β) which further increases MHC expression and antigen presentation on the surface of the APC without increasing the APC's expression of costimulatory molecules. This implies that it becomes more likely that non-tolerant $CD4^{+ve}$ and/or $CD8^{+ve}$ T cells will contact antigen on the surface of the APC without receiving appropriate costimulation, which in-turn implies that they are driven towards the anergic state. A central element in this hypothetical model of infectious anergy, is the cohort of anergic T cells which are able to proliferate in response to low levels of IL-4 and are able to secrete INF-γ and TGF-β but otherwise have no direct effector cell function. The mechanism(s) involved in generating this initial T cell pool is not addressed in their model and could occur through any of the above mechanisms listed above.

1.5 AIMS AND OBJECTIVES OF THESIS

Using a full MHC mismatched rat heterotopic cardiac transplantation model, I hope to show that MRC OX38 anti-CD4 mAb treatment therapy can achieve long term tolerance in treated recipients. I hope to also show that this treatment therapy is associated with preferential depletion of Th1 like T effector cells and that transplantation tolerance can be achieved in mAb treated recipients without obvious bias to either a Th1 or Th2 polarised type response.

I hope to also show that anti-CD4 mAb treatment has an effect on the residual non-depleted T cell pool. To test if treated cells are functionally capable of initiating allograft rejection, anti-CD4 mAb treated animals will be challenged with heart grafts at a time following mAb therapy when CD4 T cells are no longer coated with antibody. Additionally, following OX38 mAb treatment, residual CD4 T cells will be analysed to determine whether they display abrogated intracellular signalling in response to TCR activation. Also, the pattern of signalling observed in anti-CD4 mAb treated cells will be compared to the signalling pattern described in anergic T cells.

I also hope to demonstrate that there is an additional requirement for transplantation tolerance induced by MRC OX38 mAb treatment in this experimental model. I hope to provide evidence that anti-CD4 induced transplantation tolerance can only be achieved is there are a sufficient number of RTE cells in anti-CD4 mAb treated animals. To provide a basis for the role of RTE in this experimental model, single positive $CD4^{+ve}$ thymocytes will be characterised *in vitro* using TCR activation assays and the mRNA cytokine repertoire of these cells will analysed by RT-PCR.

2. Materials and Methods

2.1 ANIMALS

All animals were purchased from Harlan UK.(Harlan UK Ltd. : Shaw's Farm, Blackthorn, Bicester, Oxfordshire, UK) and maintained in conventional facilities at the University of Glasgow Department of Surgery animal house. Mice used for antibody production were F1 (DBA/2 X BALB/c) males between 6 and 10 weeks old. Cardiac transplantation recipient rats were DA adult males which were between 8 and 12 weeks old at time of transplant. Animals used for thymectomy were DA adult males which were between 6 and 8 weeks at time of thymectomy. Organ and skin donor rats were all $RT1^l$ (Lewis) males which were between 8 and 14 weeks of age at time of sacrifice. Third party rats for skin and/or cardiac allografting were $RT1^c$ (PVG) adult males which were also between 8 and 14 weeks of age at time of sacrifice. The animals were fed using standard rodent animal feeds and were cared for and used in strict accordance of the Animal Scientific Procedures Act 1986.

2.2 ANTIBODIES USED

2.2.1 Hybridoma production

MRC OX38 [213], a mouse anti-rat IgG2a mAb, which is specific for the membrane distal domains of the rat CD4 molecule expressed on most rat T lymphocytes as well as some macrophages, was used to induce tolerance and for immunoprecipitation of CD4 bound

$p56^{lck}$. The hybridoma cell line was kindly provided by Dr. D. Mason (MRC Cellular Immunology Unit, Oxford, England).

R73 [214], a mouse IgG1 anti-rat mAb specific for the constant determinant of the rat αβTCR receptor was used for T cell staining and T cell activation experiments. The hybridoma cell line was kindly provided the European Collection of Animal Cell Cultures (ECACC) (Portondown, Wilkshire, UK).

The hybridomas for both OX38 and R73 were grown using standard tissue culture techniques (see below). When sufficient cell numbers were available, they were injected INTRAPERITONEALLY (IP) into (DBA/2 X BALB/c) F1 mice (2 x 10^6 cells/animal) (Harlan Olac) which had been primed 7 days earlier with 0.2mls of pristane injected IP. Seven to fourteen days after IP injection of hybridoma cells, 2-5mls of ascitic fluid was harvested from each mouse, pooled and allowed to clot. The clotted ascities was then centrifuged in a Minifuge GL Centrifuge (Heraeus-Christ Centrifuge : Osterode, Germany) at 200g for 10 MINUTES (Min) at room temperature (r.t.). The supernate was saved, further clarified by centrifuging at 1500g for 10 Min at r.t. in the same centrifuge and then filtering using millipore GS 0.22μm filters (Millipore S. A. : 67120 Molsheim, France) into sterile 50ml polypropylene tubes. Ascities samples were stored at -20^0C until further purification.

2.2.2 Other antibodies used

MRC OX8 [215], a mouse anti-rat IgG1 mAb which binds to rat CD8 expressed on rat T cytotoxic/suppressor lymphocytes as well as some rat NK cells and **MRC OX12** [216] a mouse IgG2a mAb which binds to rat Ig κ chains, expressed on B-lymphocytes, were used in

the purification of rat $CD4^{+ve}$ T lymphocytes and thymocytes. These mAb were kindly provided by Dr. D. Mason (MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford, England).

As well as R73 (previous page), **polyclonal RABBIT ANTI-MOUSE ANTIBODY (RaMo) IgG** (Serotec Ltd.) was used in preparation of anti-TCR coated plates and cytometer tubes (see below), but was also used in conjunction with OX38 in inhibition assays when it was employed as a cross-linking second antibody.

RC-20 (Affinity Research Products : Nottingham UK), a construct incorporating the hypervariable binding domain of an anti-phosphotyrosine antibody conjugated to HORSE RADISH PEROXIDASE (HRP), **polyclonal rabbit anti-human $p56^{lck}$** and **polyclonal rabbit anti-human ZAP-70** were both purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology : Santa Cruz, Ca), and **polyclonal goat anti-rabbit HRP** (DAKO A/S : Dakopatts Produktionsvet 42, P. O. Box 1359, DK-2600, Glostrup, Denmark) were all used in Western blotting.

MRC OX39 [217], is a mouse IgG1 mAb specific for the 50,000 kDa induceable IL-2Rα chain which is present on activated rat $CD4^{+ve}$ T cells, **MRC OX1** [218], a mouse IgG1 mAb which binds to the rat leukocyte common antigen, **MRC OX22** [219], mouse IgG1 mAb which binds to the high molecular weight isoform of the leukocyte common antigen (CD45RC antigen) and **MRC OX21** [220], a mouse IgG1 mAb specific for the human C3b inactivator (which was used as a control antibody) were kindly provided by Dr. D. Mason (MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford, England), **W3/25** [215], a mouse IgG1 mAb which also labels the membrane distal domain of rat CD4, was a kind gift from Dr. E. Bell (Dept of Immunology, Manchester School of Medicine,

Manchester, UK), **ED1** [221] (Serotec Ltd) a mouse IgG1 mAb which labels most rat tissue macrophages, monocytes and dendritic cells were used in immunohistochemical staining of cardiac tissue, PBL, LNC, thymocytes, and spleen cells and **MRC OX7** [88] a mouse IgG1 mAb which binds to the rat Thy 1.1 antigen expressed by thymocytes and by newly exported functionally naïve CD4 T cells was used in flow cytometric analysis.

Other antibodies used for fluorescent staining included PHYCOERYTHRIN (PE) conjugated OX8 and W3/25; FLUORESCEIN ISOTHIOCYANATE (FITC) conjugated R73, PE and FITC conjugated OX22, FITC conjugated mouse anti-rat IgM, IgG1, IgG2a, IgG2b, and IgG2c (All from Serotec Ltd.), as well as FITC CONJUGATED POLYCLONAL RABBIT ANTI-MOUSE ANTIBODY (RaMo-FITC) (both from DAKO A/S). A summary of the antibodies used in this thesis are provided in Table 2.1 below.

Table 2.1

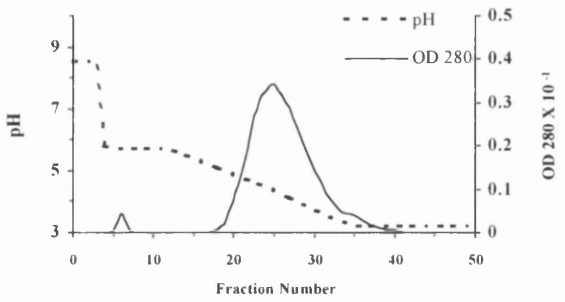
mAb	Species	Ig Class	Specificity	Reference
OX38	Mouse	IgG2a	Membrane distal domain rat CD4	[213]
R73	Mouse	IgG1	Rat αβTCR	[214]
OX8	Mouse	IgG1	Rat CD8	[215]
OX12	Mouse	IgG2a	Rat Ig κappa chain expressed on B cells	[216]
OX39	Mouse	IgG1	Inducible Rat IL-2 Receptor α chain	[217]
OX1	Mouse	IgG1	Rat leukocyte common antigen	[218]
OX22	Mouse	IgG1	Rat high molecular weight isoform of the leukocyte common antigen ($CD45RC^{+ve}$)	[219]
OX21	Mouse	IgG1	Human C3b inactivator	[220]
W3/25	Mouse	IgG1	Membrane distal domain rat CD4	[215]
ED1	Mouse	IgG1	Rat macrophages, monocytes and dendritic cells	[221]
OX7	Mouse	IgG1	Rat Thy 1.1 antigen	[88]

2.2.3 Prosep-A affinity purification of OX38 mAb from mouse ascitic fluid

Ascitic fluid was thawed, diluted 1:2 in 1.0M glycine, 0.3M NaCl pH 8.6 buffer, and applied to a Protein-A affinity column (PROSEP-A High Capacity Bioprocessing Ltd. : 1 Industrial Estate Consett Co, Durham, England) as recommended by the manufacturer. Briefly, aliquots of the diluted ascities were added to a 10ml PROSEP-A column which was equilibrated in 1.0M glycine, 0.15M NaCl pH 8.6 buffer and the bound protein fraction eluted using a pH 6 to 3 gradient of 0.1M citrate buffer. All effluents passed through a single wave length in-line

spectrophotometer (Single Path Monitor UV1, Pharmacia-LKB Biotechnology AB : Bjorkgatan, S-75182, Uppsala, Sweden) and peak fractions collected in a Frac-100 Pharmacia fraction collector. See Figure 2.1 below.

Figure 2.1 Affinity purification of OX38 ascities



This figure illustrates that as the PROSEP-A column elution buffer pH was reduced, protein (as determined by absorption of light at an OPTICAL DENSITY (OD) 280ηm wavelength) was eluted and collected by fraction from the column.

Pooled peak fractions were dialysed using Visking size 2-18/32" tubing (Medicell International Ltd. : 239 Liverpool Road, London) into Phosphate Buffered Saline pH 7.4 Dulbecco 'A', (PBS) and were stored at -20^0C until use.

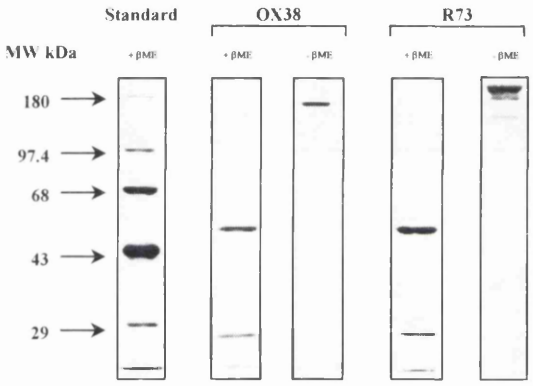
2.2.4 Radial immune diffusion (RID) determination of antibody concentration

The IgG2a concentration in each of the two pooled peaks obtained by PROSEP-A affinity purification (described above), were determined by Radial Immune Diffusion assay using a commercially available kit (Serotec IgG2a RID Kit, Serotec Ltd.).

2.3 SODIUM DODYCYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The starting ascities, unbound non-absorbed protein fraction from the PROSEP-A column, and pooled elution peaks from the same column, as well as TCR-activated CD4 T cell lysates or immunoprecipitates were analysed by SDS-PAGE on 10% gels as described by Laemmli, [222]. The Molecular Mass of protein bands were determined by running standard protein markers on each gel (Gibco BRL : Life Technologies Ltd, P. O. Box 35, Trident House, Renfrew Road, Paisley, UK). See Figure 2.2 below.

Figure 2.2 10% SDS-PAGE of affinity purified monoclonal antibodies



As demonstrated by 10% SDS-PAGE, this figure illustrates the purity of both OX38 and R73 mAb. The polyacrylamide gel was stained with 1% Coomassie blue dye for 30 Min and de-stained in 10% methanol/acetic acid overnight. The single lane on the left shows standard molecular weight protein markers, while the lanes on the right show 5μg protein samples of affinity purified OX38 and R73 run in the presence and absence of βME.

2.4 HETEROTOPIC CARDIAC TRANSPLANTATION

2.4.1 Donor animals

Rat heterotopic cardiac transplantation was performed as described by Ono and Lindsey [223]. Briefly, using sterile technique and halothane/oxygen inhalation anaesthesia, donor animals were heparinised using 100 INTERNATIONAL UNITS (IU) Heparin INTRAVENOUSLY (IV) (Leo Laboratories Ltd. : Princes Risbourgh, Bucks., England) and were exsanguinated. Thoracotomy was performed and the heart immediately chilled with topical 4^0C normal saline and ice. The right and left superior vena cavae, inferior vena cava, and pulmonary venous trunk were individually ligated using 6.0 silk ties and transected. All other venous and arterial branches, except the pulmonary artery and aorta, were ligated en-mass using a single 6.0 silk tie. The heart was excised and the donor organ immediately placed in cold (4^0C) sterile saline until required for transplantation. Cold ischaemic time (i.e. the time in cold sterile saline after removal of the heart from the donor until the anastomosis was started in the recipient) varied from 5 to 45 Min in total.

2.4.2 Recipient animals

Using sterile technique and halothane/oxygen inhalation anaesthesia, recipient animals had laparotomy performed on heated operating plates. The infra-renal abdominal aorta and vena cava were identified and isolated. Lumbar vessels were ligated using 8.0 silk ties. Two microsurgical vascular occlusion clamps were placed 1.5 cm apart across the aorta and vena cava together and arterotomy and venotomy performed. Using standard microsurgical techniques, the donor heart aorta and pulmonary artery were anastomosed, in an end to side fashion, onto the recipient aorta and vena cava respectively using a continuous 9.0 prolene

running sutures. Reperfusion of the donor organ occurred on average after a 15 to 25 Min warm anastomosis time (i.e. the time from removal of the donor organ from cold saline until reperfusion with recipient blood occurred) with the removal of the vascular occlusion clamps. The peritoneum-abdominal muscles, and skin were closed separately using a continuous 4.0 dexon running suture. Overall, donor organ retrieval and recipient transplantation took between 30 and 60 Min to perform. Graft function was checked daily by direct abdominal palpation of the donor organ.

2.5 ORTHOTOPIC RENAL TRANSPLANTATION

2.5.1 Donor animal

Briefly, using sterile technique and halothane/oxygen inhalation anaesthesia, donor animals were heparinised using 100 IU Heparin IV (Leo Laboratories Ltd. : Princes Risbourgh, Bucks., England). The left renal vein, artery and ureter were divided and the animal exsanguinated. The kidney was perfused with 5 mls of cold (4^0C) saline by direct cannulation of the renal artery using a 25 gauge needle and 5 ml syringe, and placed directly into the recipient for immediate transplantation.

2.5.2 Recipient animals

Using sterile technique and halothane/oxygen inhalation anaesthesia, after opening the abdomen using a midline incision, the native left renal vein and artery were isolated and

occluded using fine vascular clamps and then each was transected distal to the occlusion clamp. The left ureter was then divided near the lower pole of the native left kidney which was removed so that the donor kidney could be placed in an orthotopic position for transplantation. The donor renal artery and vein were anastomosed in an end to end fashion using the recipients renal artery and vein respectively using interrupted 10.0 prolene sutures. Reperfusion of the donor organ occurred on average after a 15 to 25 Min warm anastomosis time (i.e. the time from after perfusion of the donor kidney with cold saline until reperfusion with recipient blood occurred) with the removal of the vascular occlusion clamps. The donor ureter was then anastomosed to the recipients left ureteric stump using interrupted 10.0 prolene sutures. The peritoneum-abdominal muscles, and skin were closed separately using a continuous 4.0 dexon running suture. Overall, donor organ retrieval and recipient transplantation took between 30 and 60 Min to perform. Seven days after transplantation, using halothane/oxygen inhalation anaesthesia, through a separate posterior-retroperitoneal loin incision, the recipients right kidney was removed after mass ligation of the vascular and ureteric pedicle using a single 6.0 silk tie. The muscle and skin wound was closed en-mass using a 4.0 dexon running suture. The animals which were left to survive on the transplanted kidney alone, were assessed daily for clinical signs of uraemia and were bleed by tail tipping at regular intervals for serum creatinine and urea measurements.

2.6 SKIN GRAFTING

2.6.1 Donor animals

Donor animals were sacrificed so that skin from over the abdominal region which was shaved of fur and cleaned with 70% ethanol could be obtained for grafting. Once removed, skin samples were cut into 2 cm^2 patches and were used immediately for grafting.

2.6.2 Recipient animals

On the back between the fore legs (front shoulder region), recipient animals were shaved of fur and cleaned with 70% ethanol. Using sterile technique and halothane/oxygen inhalation anaesthesia, a 2 cm^2 patch of native skin was excised from the prepared area and was replaced with a similar sized portion of donor skin. The donor skin was sutured in place along the edges, using interrupted 4.0 dexon sutures. Animals were assessed daily for evidence of skin graft rejection, which was defined as the day that the skin graft sloughed off completely.

2.7 THYMECTOMY

Thymectomy of $RT1^a$ (DA) male rats (6 to 8 week old) was performed using sterile technique and halothane/oxygen inhalation anaesthesia. Briefly, with the rat prone and the neck hyperextended, a 10mm longitudinal skin incision was made over the sternal notch and proximal sternum. A 3-5 mm sternotomy was then performed by scissor dissection of the manubrium, taking care to avoid the external jugular and brachiocephalic veins. The

sternotomy was widened using blunt dissection until the thymus was easily visualised with the aid of an operating microscope (x 15 magnification). Sharp dissection of the connective tissue surrounding the thymic capsule was then performed until the thymus could be removed in one piece from the superior mediastinum and delivered through the skin wound. The sternotomy and skin wounds were then closed using continuous catgut suture and the rats given one to two breaths of positive pressure ventilation to expand their lungs by gently blowing through a 2.5 ml syringe barrel which was held over the rats nose and mouth.

2.8 TISSUE CULTURE

All tissue culture work, except centrifugation, was performed using standard tissue culture techniques in a Gelaire BSB 40 laminar flow hood (Flow Laboratories Ltd. : Woodcock Hill, Harefield Road, Rickmansworth, Herts., England). All centrifugation was performed at 4^0C for 8 Min at 200g in a Minifuge GL Centrifuge. Washes were performed by addition of 10mls medium to resuspended cell pellets, debris was allowed to settle, and suspended cells transferred to clean tubes with plastic Pasteur pipettes. Tissue culture medium, unless otherwise stated, was ROSWELL PARK MEMORIAL INSTITUTE (RPMI) 1640 with Penicillin (10 IU/ml), Streptomycin (10μg/ml), Glutamine (2mM) with or without 10% FOETAL CALF SERUM (FCS) and/or 2 x 10^{-5} M 2-Mercaptoethanol (Gibco BRL). Cell cultures were grown in 5% CO_2/air mix at 37^0C in a FLOW LABORATORY 210 incubator (Flow Laboratories Ltd.).

2.9 MIXED LYMPHOCYTE REACTION (MLR) ASSAY

2.9.1 Spleen cells (Stimulator and target cells)

Spleens were obtained, using sterile technique, from sacrificed Lewis rats and placed into 50ml petri dishes containing standard RPMI culture medium (RPMI 1640, with 10 IU/ml penicillin, 10μg/ml streptomycin, 2mM glutamine, Gibco BRL) with 10% FCS added. As described above, all work except centrifugation and cell irradiation was performed using standard tissue culture techniques. Splenocytes were obtained by gently crushing the splenic capsule with disposable sterile plastic forceps releasing the cells into the tissue culture medium. The cells were triturated free of clumps by using 5ml plastic Pasteur pipettes and were then transferred to 10ml conical centrifuge tubes for centrifugation at 200g for 8 Min at 4^0C. The supernate was discarded, the cell pellet resuspended in 10mls of medium, transferred to a fresh 10ml conical tube and the centrifugation repeated. Erythrocytes were lysed by resuspending the washed cell pellets in 5mls sterile water immediately followed by 5mls of sterile 0.3M NaCl. Cells were washed a further 3 times by centrifugation and resuspended in 5mls medium. The cells were then irradiated, (3000 Rads over 10 Min), washed twice by centrifugation and finally resuspended at the desired concentration in medium with 10% FCS and 2×10^{-5} M 2-Mercaptoethanol.

CONCANAVALIN-A (Con-A) (Sigma Chemicals Ltd.) transformed splenic blasts were prepared using standard tissue culture conditions after adding 50μg sterile Con-A to 2.5×10^7 Lewis splenocytes in 10mls RPMI with 10% FCS and incubating for 72 HOURS (hr).

2.9.2 Lymph node cells (LNC : Responder cells)

Cervical and mesenteric lymph nodes were obtained using sterile technique from sacrificed DA rats. Peri-lymphatic fat and connective tissue was dissected away from the lymph nodes which were then placed in 50ml petri dishes containing culture medium. Using standard tissue culture techniques, lymphocytes were obtained by teasing the lymph nodes through a sterile stainless steel tea strainer with the rubber end of a 2ml syringe plunger. The cells were washed twice by centrifugation (as described above) and were resuspended at the desired concentration in medium with 10% FCS and 2 x 10^{-5} M 2-Mercaptoethanol.

2.9.3 MLR assay

MLR assay were performed in U bottomed 96 well microtitre plates (Nunclon Microwell Plates, Nunclon Intermed : Denmark). 0.1ml of irradiated stimulator (1 x 10^6/ml) and responder (0.5 x 10^6/ml) cells were used per well. Responder and stimulator cells were incubated at 37^0C in 5% $C0_2$/air mix. On days 3, 4, and 5, culture wells were pulsed with 0.010ml (0.001 mci) of TRK 418 TRITIATED-THYMIDINE (^{3}H-Thymidine) (Amersham International Plc. : Lincoln Place, Green End, Aylesbury, Bucks., England). Eighteen hrs after pulsing, cells were harvested onto microtitration filter paper (ICN Flow Biomedicals Ltd. : Eagle House, Peregrine Business Park, Gomm Road, High Wycombe, Bucks., England) using a Microtiter Dynatech Automash II harvester (DYNEX Technologies : Billingshurst, West Sussex, England). Filter paper-cell circles were placed into 5ml polypropylene tubes and mixed with 3mls Ecoscint A scintillation fluid (National Diagnostics : 1013-1017 Kennedy Blvd., Manville, N. J., USA). ^{3}H-Thymidine uptake was determined by counting

samples on a 1209 Rank Beta Liquid Scintillation counter (LKB Wallac : Wallac OY, P. O. Box 10, 20101 Turku, Finland).

2.10 OTHER CELL PREPARATION / PURIFICATION

2.10.1 Thymocyte preparation

Thymus glands were obtained using sterile technique from sacrificed DA rats. Peri-thymic fat and connective tissue were dissected away from the glands which were placed in 50ml petri dishes containing standard culture medium. Using standard tissue culture techniques, thymocytes were obtained by teasing the thymic tissue through a sterile stainless steel tea strainer as described above. Cells were washed twice by centrifugation and resuspended at the desired concentration in Phosphate Buffered Saline (PBS).

2.10.2 Peripheral blood lymphocyte (PBL) enrichment

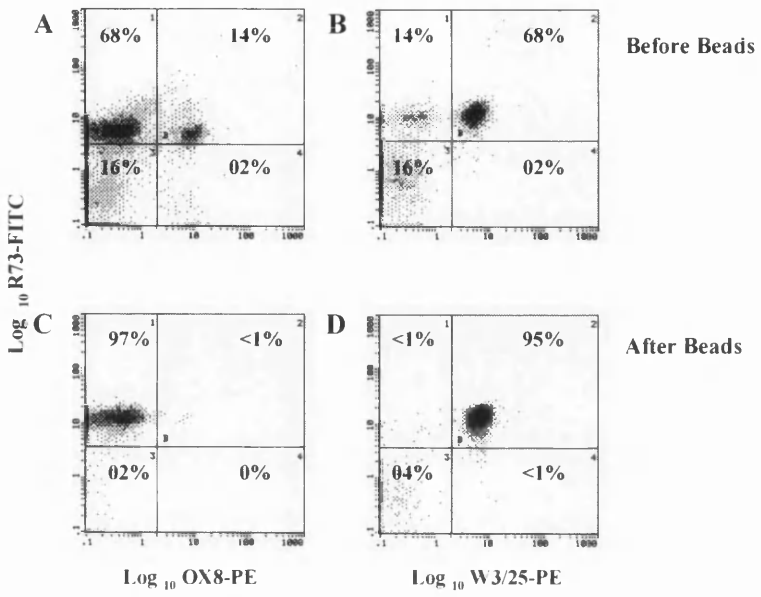
PBL were obtained using sterile technique by bleeding DA rats, by direct cardiac puncture, into syringes pre-loaded with 50 IU of heparin. The heparinised blood was then mixed with an equal volume of PBS. Five mls of diluted blood-PBS mix was layered on 5 mls of percol (ς = 1.088) and centrifuged at 400g for 20 Min at 22^0C. Following centrifugation, PBL were harvested from the interface and washed twice by centrifugation at 200g for 8 Min at 4^0C in PBS and were resuspended at the desired concentration in standard medium or PBS.

2.10.3 CD4 T cell and thymocyte purification

CD4 $^{+ve}$ T cells were obtained by negative selection using immunomagnetic cell separation. Purified LNC and Thymocytes, obtained as outlined above, were resuspended in PBS + 0.2% BOVINE SERUM ALBUMIN (BSA) (Ultra Pure, Sigma Chemical Co. : P. O. Box 14508, St. Louis, Mo., USA), and 50µl of OX8 and OX12 mAb were added per 10^9 cells. The cells were incubated for 30 Min at 4^0C and were then triple washed by centrifugation, as described above, and resuspended in 1ml of standard medium. The cells were counted using a standard haemocytometre, 1ml of goat anti-mouse IgG coated magnetic beads (Biomag, Advanced Magnetics Inc. : UK) was added per 10^8 cells. After incubation for 30 Min at 4^0C, the cells were pelleted, using a magnetic stand as recommended by the manufacturer. The supernate was added to a second portion of Bio Mag beads and the purification process repeated. CD4 enriched cells were then washed twice by centrifugation, counted and resuspended at the desired concentration in either medium or PBS. In subsequent discussion, CD4 enriched LNC are referred to as 'purified CD4 T cells' whereas CD4 enriched thymocytes are referred to as 'purified CD4 thymocytes'.

The purity of CD4 T cells obtained after negative selection immunomagnetic cell separation was routinely checked by double label flow cytometric analysis and a typical result is depicted in Figure 2.3 below. Here LNCs were stained directly using anti-αβTCR-FITC (y-axis) and either anti-CD8-PE (left panels, x-axis) or anti-CD4-PE (right panels, x-axis). Cells were stained both before (upper panels) and after (lower panels) negative selection of CD4 T cells. In all experiments to be described, the purity of CD4 preparations from LNC and thymocytes was always greater than 93 and 80% respectively.

Figure 2.3 Lymph node cell purity as determined by double label flow cytometry



Cells were resuspended at 1.4 x 10^6 / ml in PBS containing 0.2% BSA. The first 10,000 cells were analysed using an EPICS XL® analyser (Coulter : Luton, UK), see above for detailed protocol. (A) OX8-PE vs. R73-FITC before beads. (B) W3/25-PE vs. R73-FITC before beads. (C) OX8-PE vs. R73-FITC after beads. (D) W3/25-PE vs. R73-FITC after beads.

2.11 ANTI-TCR ACTIVATION EXPERIMENTS

2.11.1 Preparation of culture plates with αβTCR mAb for proliferation and cell signalling experiments

For proliferation and cell signalling studies, CD4 T cells were activated by cross-linking of αβTCR in culture plates containing immobilised R73 mAb. Using sterile tissue culture techniques, 0.2ml of polyclonal RaMo IgG (Serotec Ltd.) diluted to 10μg/ml in 0.1M $NaHCO_3$ pH 8.2 was added to each well of a U 96 Nunclon Microwell Plates (Nunclon

Intermed) and incubated for 2 hrs at 37^0C. The plates were triple washed with excess PBS, blotted dry, and 0.2ml of R73 mAb diluted to 5μg/ml in PBS added to each well. The plates were then incubated at 4^0C until ready to be used. Prior to use, the wells of the plate were triple washed with excess PBS and 'blocked' by addition to each well of 0.2ml of 1% NORMAL MOUSE SERUM (NMS) diluted in PBS and incubating for 1 hr at 4^0C. Finally the wells were triple washed with excess PBS and blotted dry ready for addition of CD4 T cells. Plates prepared in this way are subsequently referred to as 'standard TCR plates'.

2.11.2 Preparation of tubes with αβTCR mAb for use in Ca^{2+} signalling experiments

Polystyrene flow cytometer tubes (Coulter) were preincubated with 3mls of 50% ethanol for 1 hr at 22^0C. The ethanol was then removed and the tubes allowed to dry. 2ml of polyclonal RaMo IgG (Serotec Ltd.) diluted to 10μg/ml in 0.1M $NaHCO_3$ pH 8.2 was added to each tube and the tubes incubated for 2 hrs at 37^0C. After the tubes had been triple washed with excess PBS and blotted dry, 2mls of R73 mAb diluted to 5μg/ml in PBS was added to each tube and incubated at 4^0C until ready to be used. Prior to use, each tube was triple washed with excess PBS and 'blocked' by addition of 2ml of 1% NMS diluted in PBS and incubating for 1 hr at 4^0C. Finally, the tubes were triple washed with excess PBS and blotted dry ready for addition of CD4 T cells. Flow cytometer tubes prepared in this way are subsequently referred to as 'standard TCR coated cytometer tubes'.

2.11.3 TCR proliferation assays

Purified CD4 T cells (or thymocytes) were prepared at 1 x 10^6/ml in standard medium with 10% FCS and 2 x 10^{-5} M 2-Mercaptoethanol and 0.1 ml added to each well of standard TCR plates. For proliferation assays, the plates were incubated at 37^0C in 5% $C0_2$/air mix. On days 2, 3, and 4 of culture, the wells were pulsed with 0.010ml (0.001 mci) of TRK 418 ^{3}H-Thymidine (Amersham International Plc.). Eighteen hrs after pulsing, cells were harvested onto microtitration filter paper (ICN Flow Biomedicals Ltd.) using a Microtiter Dynatech Automash II harvester (DYNEX Technologies). Filter paper-cell circles were placed into 5ml polypropylene tubes and mixed with 3mls Ecoscint A scintillation fluid (National Diagnostics). ^{3}H-Thymidine uptake was determined by counting samples on a 1209 Rank Beta Liquid Scintillation counter (LKB Wallac).

2.11.4 TCR cell signalling experiments : Whole cell lysate preparation

Purified CD4 T cells were prepared at 1-2.5 x 10^7/ml in PBS and 0.1 ml of cells were added to each well of standard TCR plates. Plates were incubated for 0, 0.5, 2, 5, and 30 Min at 22^0C. At each time-point, T cell activation was arrested by the addition of 50µl of triple concentrated Laemmli SODIUM DODECYL SULFATE (SDS) Sample Buffer. The samples were then transferred to 0.5ml polypropylene microfuge tubes containing 15µl of 10mM Na Vanadate, 100mM Na Fluoride, 100mM Na Pyrophosphate and boiled for 5 Min. 15µl of each sample was then loaded on a 10% polyacrylamide gel as described above.

2.11.5 TCR cell signalling experiments : Immunoprecipitation

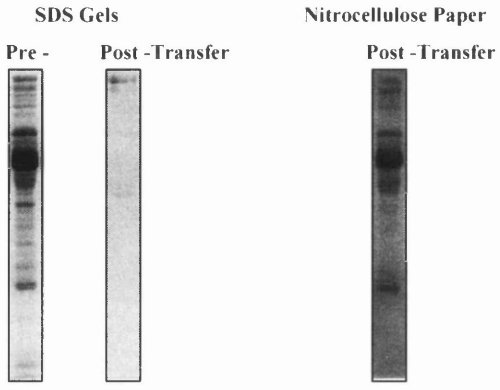
Purified CD4 T cells were prepared at 1-8 x 10^7/ml in PBS and 0.08ml of cells was added to 20µl of 10 times concentrated lysis buffer which contained 5% NP-40, 0.5M NaCl, 250mM Tris pH 8.3, 50mM Na pyrophosphate (Sigma Chemicals), 5mM Na ortho-Vanadate (Sigma Chemicals), 50mM Na Fluoride (Sigma Chemicals), 10µg/ml Leupeptin (Sigma Chemicals), 10µg/ml Aprotinin (Sigma Chemicals), 20µg/ml Pepstatin A (Sigma Chemicals), 0.8mM EDTA, and 10mM p-nitrophenylnitrophosphate (Sigma Chemicals). Ten µl of appropriate antibody (diluted to 1mg/ml in lysis buffer) was then added to each sample which was incubated for 30 Min at 4^0C. Following incubation, 10µl protein-A Sepharose (Sigma Chemicals), pre washed with PBS (at 20mg Protein-A/ml beads) was added and incubated for 10 Min at 4^0C. The samples were pelleted by centrifuging for 5 Min using a MSE Micro Centaur microfuge (Fischer Scientific UK : Bishop Meadow Road, Loughborough, UK), the supernate removed and the pellet triple washed by resuspending in 0.5ml of lysis buffer and recentrifuging as above. The washed pellet was then resuspended in 0.1ml of Laemmli SDS sample buffer and boiled for 5 Min. Fifteen µl of each supernate was then loaded on a 10% polyacrylamide gel as described above.

2.11.6 TCR cell signalling experiments : Anti-phosphotyrosine and anti-p56^{lck} signalling

Following activation of purified CD4 T cells using standard TCR plates, proteins lysates and immunoprecipitates were separated by 10% SDS-PAGE and transferred to nitrocellulose (Hybond, ECL : Amersham, Buckinghamshire, UK) using the Sartoblot (Sartorius Ltd. : Blenheim Road, Epson, Surrey, UK) semi-dry blotting method as described by the manufacturer. Blotting efficiency was tested by staining pre and post-transfer SDS gels as

well as post-transfer nitrocellulose paper with coomassie blue stain as shown in Figure 2.4 below.

Figure 2.4 Blotting efficiency as tested by Coomassie blue staining



- **4 x 10^5 whole cell lysates run per lane**
- **Nitrocellulose transfer performed by semi-dry western blotting at 4 mAmp/cm^2**
- **Gels and nitrocellulose stained with 1% Coomassie blue for 30 Min and de-stained in 10% methanol/acetic acid overnight**

The nitrocellulose transfers were then blocked for 1hr at r.t. in affinity wash buffer (0.1M Tris-Base, 0.1M NaCl, 0.1% Tween, pH 7.5) containing 5% BSA. To stain for phosphotyrosine containing proteins, a 1:2500 dilution of RC-20 (Affinity Research Products) in affinity wash buffer + 0.5%BSA was incubated at r.t. for 1hr or overnight at 4^0C. To stain for $p56^{lck}$, a two stage procedure was employed. (1) A 1:1000 dilution of anti- $p56^{lck}$ antibody (Santa Cruz Biotechnology : Santa Cruz, California, USA) (pre-absorbed with NMS) in affinity wash buffer + 0.5%BSA was incubated at r.t. for 1hr followed by washing twice with affinity wash buffer. (2) Addition of a 1:1700 dilution of a HRP coupled goat anti-rabbit antibody (DAKO A/S) (pre-absorbed with NMS) in affinity wash buffer + 0.5% BSA + 5% NORMAL GOAT SERUM (NGS) for 1hr at r.t.. After staining, blots were washed

three times in affinity wash buffer and the staining detected by chemoluminescence-autoradiography system as described by the manufacturer (Hybond, ECL) using X-OMAT O film (Eastman Kodak Co. : Rochester, NY)

2.11.7 TCR cell signalling experiments : Calcium signalling

One ml of purified CD4 T cells at 1 x 10^7/ml in standard RPMI, (RPMI 1640 with 10 IU/ml penicillin, 10μg/ml streptomycin, 2mM glutamine, Gibco BRL) containing 2.5mM of Ca^{2+} (signalling medium) was incubated with 5μM of Fluo-3 (Molecular Probes - Cambridge BioScience : Cambridge, UK) dissolved in 25% Pluronic F-127 (Molecular Probes - Cambridge BioScience) in Anhydrous DMSO (Sigma Chemicals) for 15 Min at 37^0C. The cells were then diluted 1:2 using signalling medium and were ready for activation experiments using standard TCR coated cytometer tubes as outlined above. All activation experiments were performed at r.t. using the EPICS XL®cytometer (Coulter) analysing between 600-1000 cells/second. Total Flou-3 florescence was determined by adding 2μM of ionomycin (Sigma Chemicals) to each sample.

2.12 IL-2 CYTOKINE ASSAY

Supernates from MLR were assayed for IL-2 by bioassay using the IL-2 dependent cell line CTLL (kindly provided by Dr. C. Lawrence University of Glasgow School of Veterinary Medicine, Glasgow, UK). Doubling dilutions of supernate were incubated with 5 x 10^3

CTLL in U 96 Nunclon Microwell Plates (Nunclon Intermed) for 24 hrs at 37^0C. Cells were then pulsed with 0.001 mci ^{3}H-Thymidine (Amersham International Plc) and Thymidine incorporation measured by counting samples on a 1209 Rank Beta Liquid Scintillation counter (LKB Wallac) as outlined above. An IL-2 standard curve was generated for each experiment by using recombinant human IL-2 (Galaxo : Geneva, Switzerland).

2.13 IMMUNOHISTOCHEMISTRY

2.13.1 Tissue preparation

Cardiac tissue was snap frozen in liquid nitrogen and cryostat sections of 5μm were cut at -20^0C onto gelatinised slides. A range of mAb were used to label tissue sections using an indirect immunoperoxidase technique [224]. Briefly, Cryostat sections were rehydrated in PBS then incubated with optimal dilutions of mouse mAb directed against rat leukocyte subpopulations for 30-60 Min at r.t.. After triple washing in PBS, a peroxidase-conjugated polyclonal RaMo-antibody (DAKO A/S) (which was pre-cleared using 10% Normal Rat Serum to prevent non-specific binding) was added at 1:40 dilution and incubated at r.t. for 30 Min. Sections were then triple washed in PBS and then incubated with substrate : 0.6mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemicals) in PBS with 0.01% HYDROGEN PEROXIDE (H_2O_2) for 5 Min at r.t. until the brown reaction product appeared. After further washing in PBS, sections were briefly counter stained in Harris's hematoxylin (BDH Ltd. - Merck Ltd. : Lutterworth, Leicester, UK), rinsed in H_20, dehydrated in ethanol, cleared in xylene, then mounted in DPX mountant (BDH Ltd. - Merck Ltd.). A portion of each heart

graft was also fixed in formalin, embedded in paraffin wax, sectioned and stained with HEMATOXYLIN AND EOSIN (H&E).

2.13.2 Morphometric analysis of cellular infiltrating cells

The mononuclear cell infiltrate of cardiac tissue was assessed by counting the number of positively stained cells per high powered field [400x magnification] using a microscope eyepiece graticule bearing a counting grid. Ten consecutive high power fields per section were counted and the results represented by the mean +/- STANDARD DEVIATION (sd) for each section.

2.14 FLUORESCENT ACTIVATED FLOW CYTOMETRY

Mesenteric and cervical LNC, PBL, Thymocytes or purified CD4 T cells or purified CD4 thymocytes were obtained as outlined above and were resuspended in PBS made with 0.2% BSA (Ultra Pure, Sigma Chemical Co.). 1 x 10^6 cells in 0.1ml were incubated for 1 hr at 4^0C with 0.01ml of purified antibody conjugate (Serotec Ltd) or OX38 diluted 1:10 with PBS with 0.2% BSA. Cells were then washed twice by centrifugation and the OX38 cell pellets resuspended in 0.020ml of RaMo IgG (diluted 1:20 in 10% Normal Rat Serum) conjugated with FITC (DAKO A/S) and incubated for 30 Min at 4^0C. The fluorochrome labelled cells were washed a further two times by centrifugation and were finally resuspended in 0.7mls of

PBS with 0.2% BSA. A total of 10,000 cells were then analysed on a EPICS XL® analyser (Coulter).

2.15 ANTIBODY MEDIATED CYTOTOXICITY ASSAYS

Serum taken from transplanted DA recipients was heat inactivated for 30 Min at 56^0C and tested for alloantigen-specific cytotoxicity using a standard ^{51}Cr-release assay. Briefly, 5Mbq ^{51}Cr (Amersham) was incubated for 90 Min at 37^0C with Lewis (Donor Strain) Con A-transformed splenic blast cells. After excess ^{51}Cr was removed from the blasts by centrifugation, 1 x 10^5 target cells in standard RPMI with 10mM Hepes and 5% FCS, were added to 50µl of serial diluted test sera in 96 well U-bottomed microtitre plates (Nunclon Intermed). The plates were incubated with the sera for 30 Min at 37^0C. Then, 100µl of guinea pig complement (Serotec Ltd) was added to each well and the plates incubated for a further 60 Min at 37^0C. Plates were then centrifuged for 5 Min at 200g at r.t., 100µl of supernate recovered and transferred to separate tubes for counting released ^{51}Cr on a LKB Compu-gamma counter (LKB Wallac). Specific ^{51}Cr release was calculated by the formula: Percent specific release = 100 x [(experimental release - spontaneous release) / (Maximum release - spontaneous release).

2.16 ALLOANTIBODY ISOTYPE DETERMINATION

Lewis LNC (1 x 10^6 cells in 0.1 ml PBS with 0.2% BSA) were incubated with tripling dilutions of heat inactivated serum obtained from test and control animals for 30 Min at 4^0C. Following incubation, the cells were washed twice by centrifugation in PBS with 0.2% BSA and cell pellets were resuspended using FITC-conjugated anti-isotype antibody (Serotec Ltd.) at the manufacture's recommended dilution. The target cells were incubated for a further 30 Min at 4^0C, washed twice by centrifugation, resuspended in 0.5mls PBS with 0.2% BSA and analysed on a EPICS XL® analyser (Coulter).

2.17 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

2.17.1 Ribonucleic Acid (RNA) isolation from cardiac samples

Three hundred to 400 mg of cardiac tissue was homogenised in 3ml of TRIzol (Gibco BRL) using a sterile Teflon pestle and glass homogenisation tubes for 1-2 Min at 4^0C. The homogenised sample was incubated a further 5 Min at 4^0C to permit the complete dissociation of nucleoprotein complexes. Two ml of the homogenate was added to 0.2ml of chloroform and after mixing by vortexing, the preparation was incubated at r.t. for 3 Min. The samples were then centrifuged at 12,000g for 15 Min at 4^0C using a SIGMA-2K15 centrifuge (SIGMA-Aldrich Co Ltd. : Fancy Road, Poole, Dorset, UK) to pellet the RIBONUCLEIC ACID (RNA). Each RNA pellet was then washed in 1ml of 75% Ethanol and re-centrifuged at 7,500g for 5 Min at 4^0C. Each pellet was resuspended in 100µl of DIETHYL

PYROCARBONATE (DEPC) - H_20 by repeated trituration of the sample using a micro-pipette and was incubated at 60^0C for 10 Min. The OD of each sample was determined at both 260 and 280ηm wavelengths using a Pye Unichem SP8-100 spectrophotometer (Philips Unichem : York Street, Cambridge, UK) to calculate, respectively, the quantity of RNA and the relative contamination with protein.

2.17.2 RNA isolation using DyNABEADS mRNA direct kit

Purified CD4 T cells or thymocytes (1-5 x 10^6), from TCR activation experiments were pelleted by spinning in the microfuge for 5 Min at r.t. and were either stored at -20^0C or used immediately for each experiment. 1ml of lysis/binding buffer (100mM Tris-HCl, pH 8.0, 500mM LiCl, 10mM EDTA, 1% LiDS, 5mM DITHIOTHREITOL (DTT)) was added to each pellet and lysis was performed by repeated trituration firstly through a micro pipette followed by a 21 gauge stainless steel needle. 0.25ml of Dynabeads® (DYNAL A.S. : Oslo, Norway) Oligo $(dT)_{25}$ were washed into 0.20mls of lysis/binding buffer by magnetic transfer and were added to the cell lysates and were annealed for 5 Min at r.t.. The magnetic beads were then washed twice using 0.5ml washing buffer (10mM Tris-HCl, pH 8.0, 0.15M LiCl, 1mM EDTA) with 0.1% LiDS added and triple washed in 0.5ml washing buffer alone by magnetic transfer. The mRNA was then displaced from the magnetic beads by adding 30μl DEPC-H_20 and incubating at 65^0C for 2 Min.

2.17.3 Complementary Deoxyribonucleic Acid (cDNA) synthesis

Twenty-four µl of RNA (representing 10µg of total RNA obtained from Cardiac tissue, or undiluted mRNA obtained from the Dynabead isolation method) was incubated with 2µl (1µg) oligo $(dT)_{12-18}$ primer (Boehringer-Mannheim UK : Bell Lane, Lewes, East Sussex) at 70^0C for 10 Min then cooled to 4^0C for 5 Min. 10µl of 5x reverse transcriptase buffer (Gibco BRL), 4µl (100mM) DTT, 2µl (10mM) dNTPs (Boehringer-Mannheim UK) was added and incubated at 37^0C for 2 Min. Finally 2µl (400U) of Superscript Rnase H Reverse Transcriptase (Gibco BRL) was added and COMPLEMENTARY DEOXYRIBONUCLEIC ACID (cDNA) synthesised using the Cetus 480 (Perkin-Elmer Ltd. : Maxwell Road, Beaconsfield, Bucks, UK) automated temperature cycling machine.

2.17.4 Reverse Transcriptase-Polymerase chain reaction (RT-PCR)

β-Actin, IL-2, IL-5, INF-γ, IL-10, IL-13, IL-2Rα and IL-2Rβ (+) and (-) specific primers were obtained from Cruachem (Cruachem Ltd. : Todd Campus, West of Scotland Science Park, Acre Road, Glasgow). All primers were used at a final concentration of 0.2µM except β-Actin which was used at 0.1µM. RT-PCR was performed using a Geneamp 9600 (Perkin-Elmer Ltd.) automated temperature cycling machine after mixing 0.1µl (0.5 units) Taq polymerase (Gibco BRL), 0.4µl (10mM) dNTPs (Boehringer-Mannheim UK), 0.75µl (50mM) Mg^{2+} (Gibco BRL) (except when IL-4 primers were used and 1.25 µl (50mM) Mg^{2+} was added instead), 2.5µl 10x PCR buffer (Gibco BRL), 5.0µl (+) and (-) primer mix, 5µl cDNA, and DEPC-H_20 to make 25µl reaction volume in Rnase free sterile PCR tubes (Perkin-Elmer Ltd.). Primer sequences used in RT-PCR are summarised in Table 2.2 below.

Table 2.2

Cytokine	Sequence	Reference
β actin sense (+)	5' ATGCCATCCTGCGTCTGGACCTGGC 3'	Nudel et. al. *Nucleic Acids Research* 1983.**11**:1759
β actin antisense (-)	5' AGCATTTCGGGTGCACGATGGAGGG 3'	
IL-2 sense (+)	5' CATGTACAGCATGCAGCTCGCATCC 3'	M^{c}Knight et. al. *Immunogenetics* 1989.**30**:145
IL-2 antisense (-)	5' CCACCACAGTTGCTGGCTCATCATC 3'	
INF-γ sense (+)	5' ATGAGTGCTACACGCCGCCTCTTGG 3'	Dijkema et. al. *EMBO Journal* 1985.**4**:761
INF-γ antisense (-)	5' GAGTTCATTGACAGCTTTGTGCTGG 3'	
IL-2Rα sense (+)	5' GTGGGGAGATAAGGTGGACGCAT 3'	Page and Dallman *Eur J Immunol* 1991.**21**:2133
IL-2Rα antisense (-)	5' GATCGAAAGGAGACAGGCACCC 3'	
IL-2Rβ sense (+)	5' TACTGGTCCTCGGCTGCTTCTTTG 3'	Page and Dallman *Eur J Immunol* 1991.**21**:2133
IL-2Rβ antisense (-)	5' GTGAAAGGCAGCAGAGGTGGGA 3'	
IL-4 sense (+)	5' TGATGGGTCTCAGCCCCCACCTTGC 3'	M^{c}Knight et. al. *Eur J Immunol* 1991.**21**:1187
IL-4 antisense (-)	5' CTTTCAGTGTTGTGAGCGTGGACTC 3'	
IL-10 sense (+)	5' GTGAAGACTTTCTTTCAAA 3'	Feng et. al. *Biochem Biophys Res Commun* 1993.**192**:452
IL-10 antisense (-)	5' TGATGAAGATGTCAAACTC 3'	
IL-13 sense (+)	5' CAGGGAGCTTATCGAGGAGC 3'	Lakkis and Cruet *Biochem Biophys Res Commun* 1993.**197**:612
IL-13 antisense (-)	5' AAGTTGCTTGGAGTAATTGAGC 3'	
IL-5 sense (+)	5' TTCTAACTCTCAGCTGTGTCTGGGC 3'	Uberla et. al. *Cytokine* 1991.**3**:72
IL-5 antisense (-)	5' AATGCCCACTCTGTACTCATCACGC 3'	

3. Effect of OX38 anti-CD4 monoclonal antibody on the CD4 T cell pool

3.1 INTRODUCTION

Following clinical organ transplantation, currently available anti-rejection treatment regimens are immunologically non-selective, need to be given long-term, and are associated with a variety of side effects and long-term hazards, such as increased susceptibility to infection and malignancy. $CD4^{+ve}$ T cells play a central role in initiating and amplifying the diverse effector mechanisms responsible for graft rejection. Adoptive transfer studies using congenitally athymic T cell deficient rats have clearly demonstrated that CD4 T cells alone are sufficient to initiate rejection of fully allogeneic grafts, whereas CD8 T cells are neither necessary, nor by themselves, sufficient to mediate rejection, even in high responder rat strain combinations [225,226,227,228,229,230]. In exceptional circumstances, notably mouse skin grafts bearing a mutant Class I MHC disparity, CD8 T cells alone appear able to mediate graft rejection autonomously, but in most other circumstances CD4 T cells play an essential role, [231]. The central role of CD4 T cells in graft rejection can be attributed to their ability to function effectively as T helper cells, releasing the necessary cytokines for orchestrating the various effector pathways, including cytotoxic T cell-mediated lysis [232,233], delayed type hypersensitivity responses [232,234] and B cell activation and humoral immunity [226,230]. The CD4 molecule is therefore an obvious target molecule for prevention of graft rejection and mAb therapies directed against CD4 T cells provide an attractive tool with which to manipulate the alloimmune response with the aim of promoting long-term graft survival.

The use of anti-CD4 mAb to perturb the function of CD4 T cells has been shown to be an effective strategy for prolonging the survival of skin and vascularised organ allografts in various rodent models of transplantation [160,161,162,163]. Moreover, in some animal

models, anti-CD4 therapy has been shown to induce a state of permanent and specific tolerance, such that treated recipients accept further grafts of the original donor strain but promptly reject those from unrelated strains [160,162,164,165,166,167]. Early studies employing anti-CD4 mAb used antibody treatment protocols which resulted in profound and prolonged depletion of CD4 T cells. More recent studies however, have focused on the use of partially depleting or non-depleting anti-CD4 regimes and have shown that complete elimination of CD4 T cells is not an essential prerequisite for prolongation of allograft survival. Since non-depleting or partially depleting protocols are likely to be associated with a considerably shorter period of non-specific immunosuppression than depleting protocols, they are likely to be of greater clinical value.

The central aim of this thesis was to investigate the possible mechanisms involved in the induction and maintenance of transplantation tolerance resulting from administration of a partially depleting pre-operative course of the mouse mAb MRC OX38 [213] which is directed against the rat CD4 molecule. Previous studies using MRC OX38 have shown that transplantation tolerance, which is dependent on the presence in the recipient of an intact thymus gland, can be induced in ACI ($RT1^a$) rats receiving Lewis heart allografts [160]. For the present studies, the same low responder strain combination was employed in a heterotopic cardiac allograft model. In the experiments described in this chapter, the depletion of CD4 T cells and their subsets was determined at various time-points following antibody treatment and cardiac transplantation and the ability of the residual T cells to proliferate and produce IL-2, when re-challenged *in vitro* with alloantigen, was analysed. To seek evidence for polarisation of the alloimmune response to either a Th1 or Th2 response, the cytokine mRNA profile within rejecting and tolerant heart allografts was assessed using RT-PCR. Furthermore, to provide additional information as to whether a dominant Th1 or Th2 response

was evident in the tolerant recipients, the isotype of the alloantibody response was determined.

3.2 RESULTS

3.2.1 Protocol for all in vivo pre-treatment of animals using MRC OX38 mAb

For all of the studies in this thesis, unless otherwise stated, the anti-CD4 mAb OX38 was administered IP at a dose of 10 mg/kg on day minus 3, and 2 mg/kg on days minus 2, minus 1, and 0 (relative to the day of transplant). This treatment protocol of OX38 mAb was based on that found by Shizuru et. al. [162] to promote tolerance of Lewis ($RT1^l$) hearts in ACI ($RT1^a$) rats. For convenience, this protocol of OX38 mAb treatment is subsequently referred to as the 'standard protocol'. For all of the studies described in this thesis, OX38 mAb was affinity purified. In addition, unless otherwise stated, all studies were performed in adult male DA rats.

3.2.1.1 Pre-treatment of DA recipients with MRC OX38 mAb results in long-term acceptance of Lewis cardiac allografts

An initial study was performed to confirm that the standard treatment protocol of OX38 was able to prolong the survival of Lewis heart grafts in fully allogeneic DA recipients. The results are shown in Table 3.1. The control group received MRC OX21 mAb [220] instead of OX38. Although OX21 is an IgG1 mAb whereas OX38 is an IgG2a subclass mAb, it was

readily available in the laboratory and it was decided that it would be an acceptable control antibody.

Table 3.1

Group	Monoclonal Antibody Treatment (mAb)*	n	Graft Survival (days)	MST (days)
1	OX21 (Control Antibody)	7	8,9,10 x 2,11,14 x 2	10
2	OX38	7	8,>100 x 6	>100

***mAb was administered by IP injection at a dose of 10mg/kg (day –3), and 2mg/kg (days –2, –1, 0). DA recipient animals received a Lewis cardiac allograft on day 0. OX21 (Control Antibody) is a mouse mAb which is directed against human C3b inactivator. Graft rejection was defined as complete cessation of ventricular contraction.**

As expected, all animals receiving the control mAb, OX21, rejected their cardiac allografts rapidly with a MST of 10 days. In contrast, although one animal given anti-CD4 mAb OX38 rejected its heart graft promptly at 8 days, the other 6 animals treated with OX38 showed long-term allograft survival and had readily palpable beating allografts for greater than 100 days following transplant (the duration of the experiment).

3.2.1.2 Following transplantation using OX38 pre-treatment, DA animals display allospecific, but not tissue specific tolerance

To test if DA animals with long-term Lewis heart allografts after pre-transplant OX38 treatment were tolerant to donor alloantigen, four animals with functioning primary cardiac allografts at greater than 100 days were challenged with both a PVG ($RT1^c$, third party) and a Lewis ($RT1^l$, allospecific) skin allograft. In addition, the four animals received a Lewis orthotopic renal allograft and underwent contralateral nephrectomy 7 days later, leaving their

survival dependent on the function of the transplanted kidney. The transplanted animals were examined daily and the end point for rejection of the individual skin grafts was taken as the day on which complete graft destruction had occurred. Table 3.2 summarises the graft survival data from this experiment. It can be seen that all recipients rejected third party PVG skin grafts whereas Lewis skin and kidney allografts were accepted indefinitely (>100days). However, it is notable that the rejection times of the PVG skin grafts (MST 26 days) are longer than might have been expected in a normal DA rat.

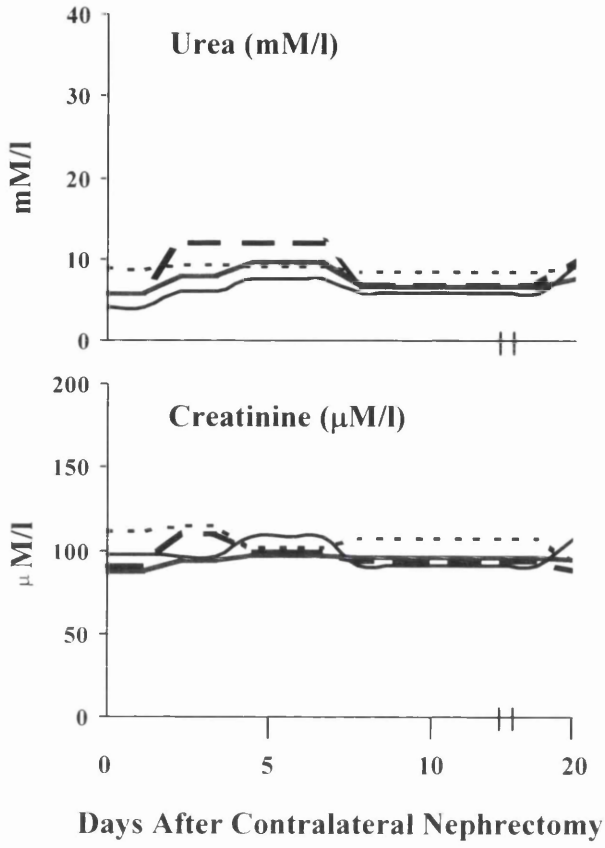
Table 3.2

Group	Recipient*	Strain and type of 2nd donor allograft	n	Graft Survival (days)	MST (days)
1	DA*	PVG Skin (3rd party)	4	21,26 x 3	26
2	DA*	Lewis Skin (Donor Specific)	4	>100 x 4	>100
3	DA*	Lewis Kidney (Donor Specific)	4	>100 x 4	>100

***All recipients were OX38 treated DA rats bearing a long-standing (>100 days) primary Lewis cardiac allograft.**

Sequential serum samples were also obtained from the four transplant recipients following renal allograft transplantation to determine urea and creatinine levels as an index of kidney allograft function. The results are shown in Figure 3.1.

Figure 3.1 Measurement of renal function of transplanted RT1^{l} (Lewis) kidneys



Results for 4 individual rats plotted separately

Urea plotted as mM/l and Creatinine plotted as μm/l

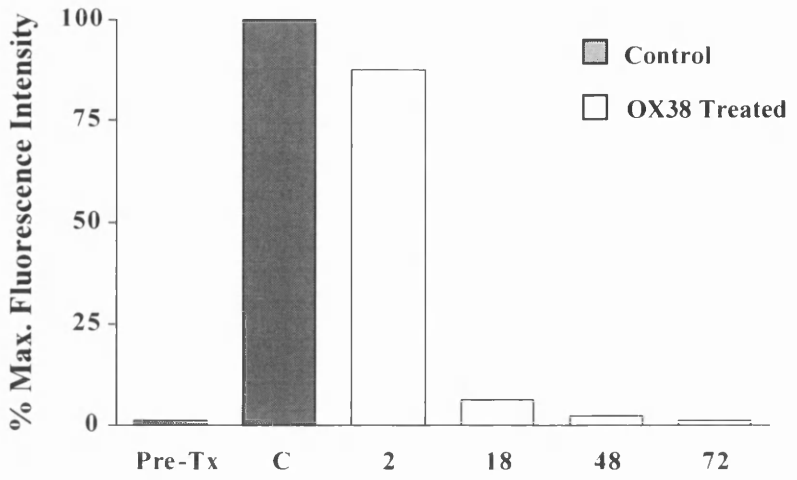
It can be seen from Figure 3.1 that, following kidney transplantation, recipients with a long standing heart allograft maintained normal renal function, indicating that the kidney grafts did not suffer from significant graft rejection. From previous studies in the laboratory it was known that unmodified allograft recipients which reject renal allografts usually die of gross renal failure within 2 days of contralateral nephrectomy and develop serum urea levels of greater than 50 mMol/L and creatinine levels of greater than 1000 μM/L (Data not shown; Personal communication Mr. J Tweedle Dept of Surgery, Western Infirmary, Glasgow).

Taken together, these data indicate that animals with long-term cardiac allograft survival following pre-operative OX38 mAb, display donor specific but not tissue specific tolerance.

3.2.2 Following treatment with OX38, the mAb, is quickly cleared from the serum

The observation that pre-transplant OX38 mAb treatment led to long-term impairment of allograft rejection, raises the question as to how long the administered mAb persists in treated animals. To determine the extent to which administered OX38 mAb persisted in the serum of treated rats, animals given the standard protocol of OX38 were bled serially from the tail vein to obtain serum samples at 2, 18, 48, and 72 hrs after the last injection of anti-CD4 antibody. The serum samples were then used to stain normal LNCs *in vitro* in order to determine whether they contained residual OX38 mAb. Briefly, the serum samples were heat inactivated and then incubated neat with DA LNCs for 30 Min at 4 ^{0}C. The target cells were washed three times by centrifugation and were then incubated with RaMo-FITC and analysed by flow cytometry using an EPICS XL® fluorescent cell sorter (Coulter, Luton, UK), to detect bound OX38 mAb. As a negative control, serum from normal DA rats was used and as a positive control, a saturating dose of OX38 mAb was added to the serum from normal DA rats. The results of this experiment are shown in Figure 3.2. It can be seen that there is no evidence of residual OX38 mAb in serum samples taken at 72 hrs and that even by 18 hrs, only low amounts of residual OX38 mAb were present which were insufficient to saturate CD4 binding sites on the target cells. Thus, OX38 antibody is rapidly cleared from the serum of treated animals.

Figure 3.2 Ability of serum obtained from OX38 treated animals to label normal CD4 T cells *in vitro*



DA LNC target cells were stained with serum which was obtained from animals prior to OX38 treatment (Pre-Tx) and at times indicated, following the last dose of the standard *in vivo* anti-CD4 treatment protocol.

Control (C) is LNC staining using pre-treatment serum to which a saturating dose of OX38 had been added *in vitro* .

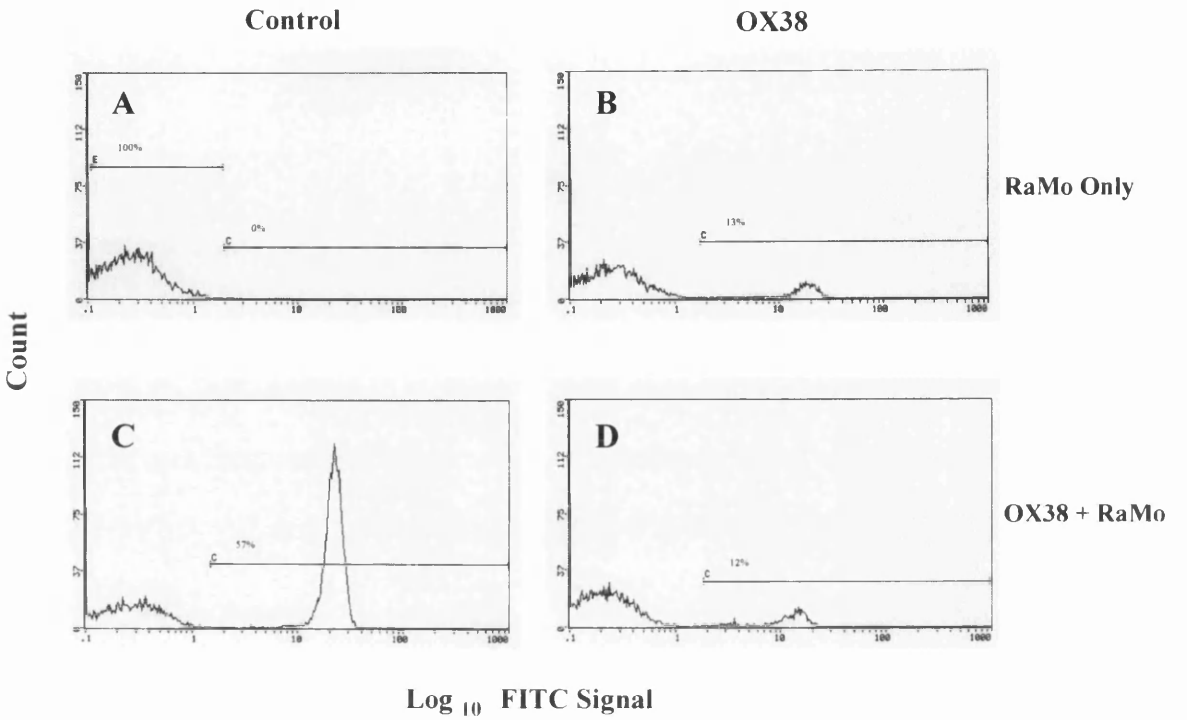
3.2.3 In-vivo treatment with OX38 causes modest levels of modulation of CD4 molecules and the mAb is no longer detected on the surface of PBL or LNC by 72 hrs after treatment

Having shown that OX38 mAb persists for a relatively short time in the serum of treated animals, studies were then performed to determine how long OX38 persists on the surface of CD4 T cells in the peripheral blood and lymph nodes of anti-CD4 treated animals. In addition, evidence for modulation of CD4 molecules following OX38 treatment was sought. Determination of cell-surface CD4 expression was measured after sacrificing treated and/or

transplanted animals (a minimum of four animals in each group) at various time-points and analysing fluorochrome labelled cells. Each antibody employed in the analysis was used at a saturating concentration pre-determined using PBL and LNCs taken from control untreated animals (data not shown).

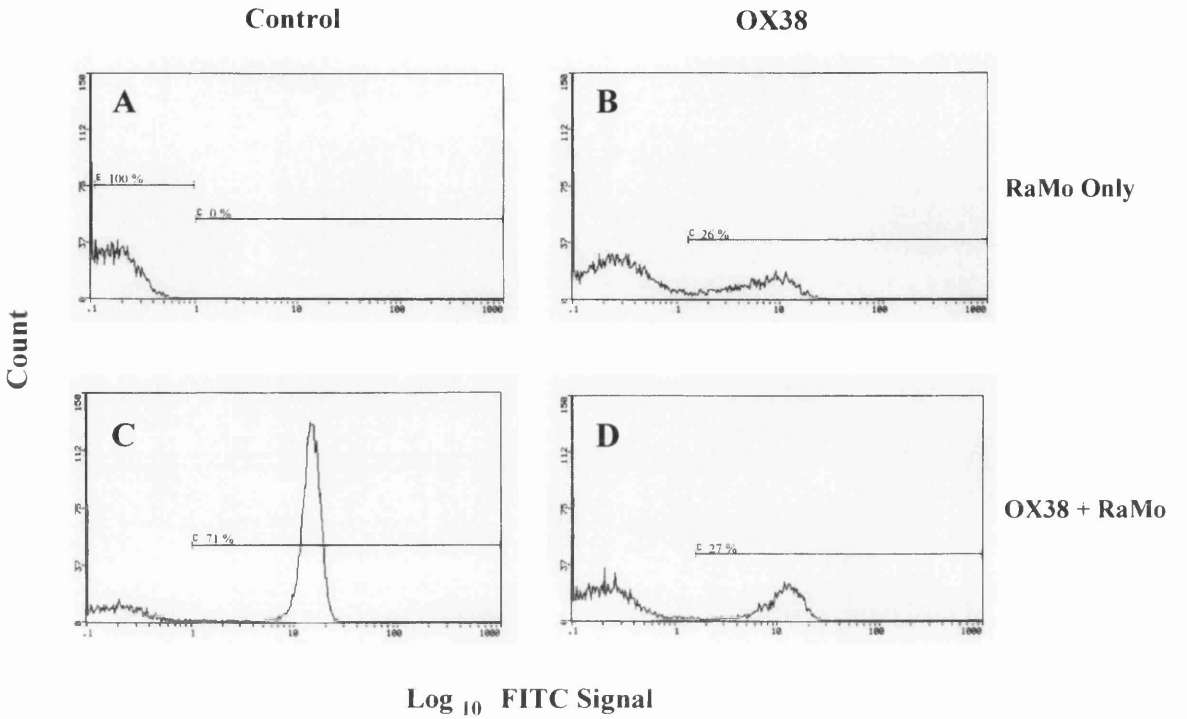
PBL and LNC were obtained from animals at 2, 48, 72 and 96 hrs after the last injection of OX38 mAb. They were then stained *in vitro* with RaMo-FITC to determine residual OX38 labelling. In addition, a saturating amount of OX38 mAb was added to the cells *in vitro* and they were then stained with RaMo-FITC to provide a measurement of total OX38 binding sites. Figures 3.3 and 3.4 show representative results of single label flow-cytometry analysis from this experiment. The figures show labelling of cells from a control untreated animal (left panels) and labelling of cells obtained 2 hrs following the last injection of mAb using the standard protocol of OX38 pre-treatment (right panels). Residual (upper panels), and total OX38 labelling (lower panels) of PBL and LNC are shown. The horizontal gating in this analysis shows the relative % of cells in each area of the profile. The y-axis shows the cell count and the x-axis shows the Log_{10} of the FITC signal and is indicative of the intensity of the signal. The results illustrated are typical of the results obtained from several other experiments.

Figure 3.3 Residual and total binding of OX38 to PBL following *in vivo* anti-CD4 treatment



Typical single colour fluorescence analysis. Cell number (Count) is depicted on y-axis and the Log_{10} of the FITC fluorescence is depicted on the x-axis. Left panels (A) and (C) are PBL cells from a control naïve untreated animal whereas the right panels (B) and (D) are PBL cells from a Day 0 (2 hr after last treatment) OX38 mAb treated animal. Upper panels (A) and (B) show the profiles using FITC-conjugated rabbit-anti-mouse antibody alone and the lower panels (C) and (D) depict the cell staining when a saturating amount of OX38 mAb was added exogenously to the cells prior to FITC-RaMo.

Figure 3.4 Residual and total binding of OX38 to LNC following *in vivo* anti-CD4 treatment

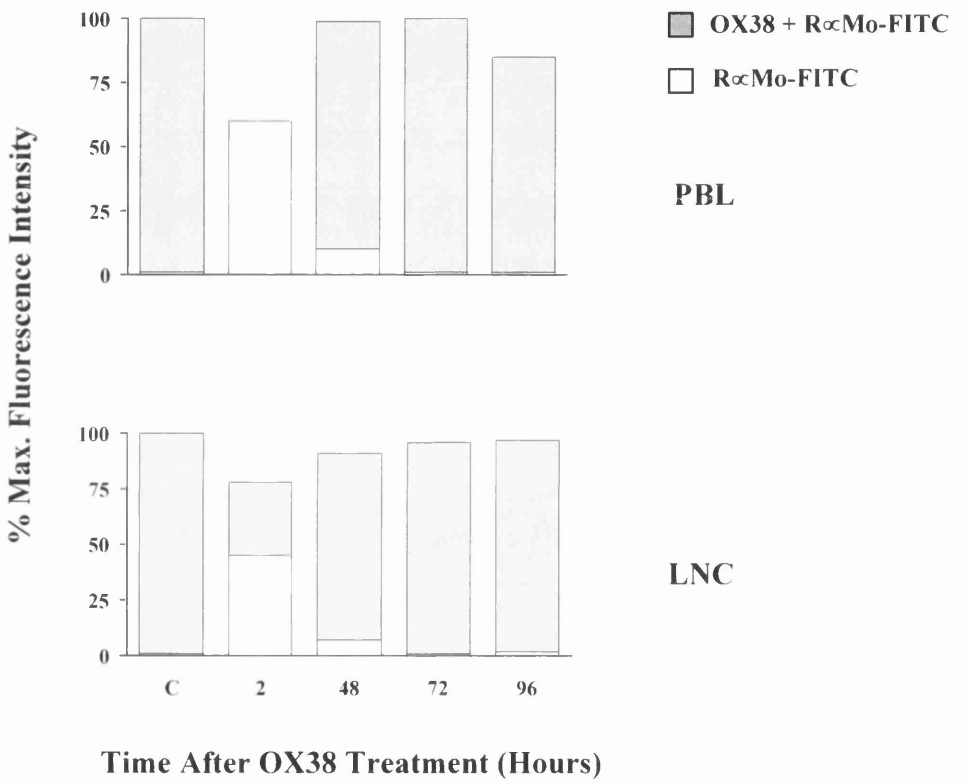


Typical single colour fluorescence analysis. Cell number (Count) is depicted on y-axis and the Log_{10} of the FITC fluorescence is depicted on the x-axis. Left panels (A) and (C) are LNC from a control naïve untreated animal whereas the right panels (B) and (D) are LNC from a Day 0 (2 hr after last treatment) OX38 mAb treated animal. Upper panels (A) and (B) show the profiles using FITC-conjugated rabbit-anti-mouse antibody alone and the lower panels (C) and (D) depict the cell staining when a saturating amount of OX38 mAb was added exogenously to the cells prior to FITC-RaMo.

Figure 3.5 shows the *in vivo* labelling and CD4 molecule modulation following the treatment of DA recipients with the standard *in vivo* protocol of OX38 mAb treatment. In this figure, the results of several flow cytometric analysis are combined and expressed as a bar chart. 100% maximum fluorescent intensity refers to the value obtained when normal LNC or PBL are labelled with a saturating amount of OX38 mAb followed by RaMo-FITC. Values below the 100% level, indicate an increasing degree of CD4 molecule modulation from the

lymphocyte cell-surface. The unfilled portion of each bar in this figure, represents the residual *in vivo* labelling with OX38, whereas the filled portion represents additional CD4 molecules which are labelled when exogenous OX38 mAb is added *in vitro* to the cell population (i.e. total CD4 labelling).

Figure 3.5 ***In vivo*** **labelling and modulation of PBL and LNC CD4 molecules following OX38 treatment**



Results are expressed as the mean number (from a minimum of 4 animals in each group) of the % maximum fluorescence intensity of PBL and LNC following staining with RaMo-FITC (residual *in vivo* OX38 labelling) or OX38 then RaMo-FITC (total CD4 binding sites). Open columns represent *in vivo* labelling and filled columns represent total CD4 binding. A drop down from %100 Max Fluorescence Intensity represents the degree of modulation of CD4 molecules. Control untreated naïve animal cells are represented by (C) whereas the OX38 treated animal cells at each point are represented by the time in hrs after last injection of mAb. Upper panel are from PBL and lower panel are LNCs.

Overall, the data from this analysis show that the OX38 treatment protocol used in these studies results in partial modulation of CD4 molecules at 2 hrs post treatment. The CD4 modulation observed was slightly more pronounced in PBL than in LNC. However, it is evident that significant CD4 modulation was no longer detected at 48 hrs after *in vivo* treatment with OX38 mAb. The data also show that 2 hrs after the last dose of OX38 mAb, CD4 molecules on lymphocytes from PBL were saturated with the OX38 mAb but only 60% of CD4 T cells from LNC had bound OX38 still detectable on their cell-surface. The loss of anti-CD4 mAb from the surface of both PBL and LNC lymphocytes by 72 hrs after the last *in vivo* treatment with OX38 mAb is consistent with the lack of residual OX38 mAb in the serum of treated animals at this time-point.

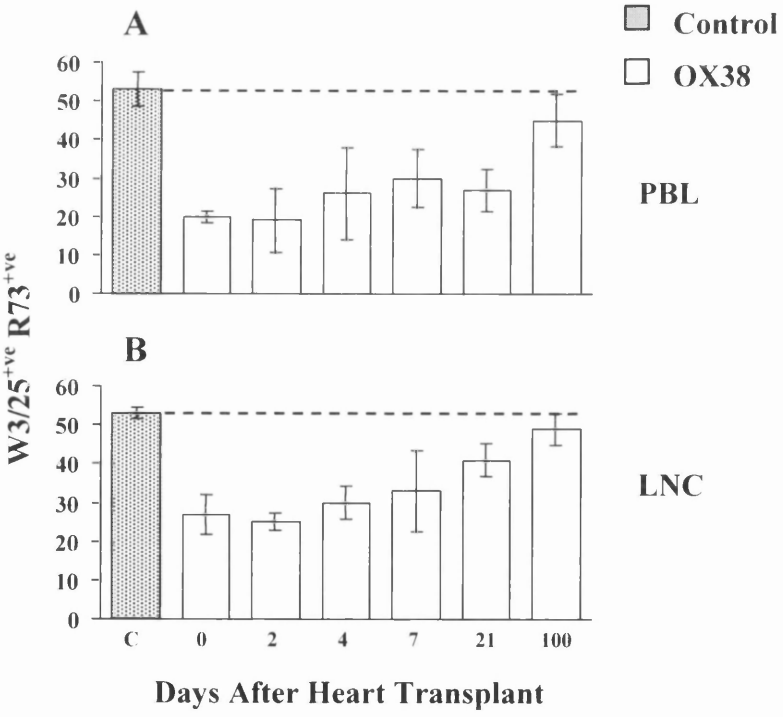
3.2.4 OX38 mAb treatment partially depletes both PBL and LNC CD4 T cells

The extent to which OX38 mAb treatment depleted $\alpha\beta TCR^{+ve}$ $CD4^{+ve}$ lymphocytes was determined by double label Flow Cytometric analysis using directly conjugated fluorochrome labelled antibodies against the CD4 and αβTCR molecules. In each experiment, several internal controls, including labelling for other cell-surface antigens in combination with either anti-αβTCR or anti-CD4, were included and cells from normal animals which had not received OX38 mAb were also analysed for comparison. In each analysis, therefore, the absolute number of double positive ($CD4^{+ve}$ $\alpha\beta TCR^{+ve}$) cells could be compared to the number of $CD4^{+ve}$ or $\alpha\beta TCR^{+ve}$ cells determined indirectly.

To determine the effect of OX38 treatment on depletion of CD4 T cells, animals were sacrificed on days 0, 2, 4, 7, 21 and 100 following OX38 mAb treatment and/or transplantation. Both PBL and LNC were labelled with R73-FITC and OX38 + RaMo-PE

(day 0 animals) or W3/25-PE (days 2, 4, 7, 21, 100 animals). The mAb W3/25 labels the rat CD4 molecule and competes for the same binding site as OX38 mAb [215]. Figure 3.6 summarises the results of this analysis. Each column represents the mean ± SD for a minimum of 4 animals. The results show that on the day of transplant (day 0), the $CD4^{+ve}$ $\alpha\beta TCR^{+ve}$ cell fraction had fallen from 53% to 20% in PBL and from 52% to 27% in LNC. By day 4 after transplant, there appeared to be some recovery of CD4 T cell numbers in both peripheral blood and lymph nodes. Interestingly, CD4 T cell numbers never recovered completely, but by day 100, had returned to near pre-operative levels.

Figure 3.6 Residual CD4 T cells in PBL and LNC following OX38 treatment and cardiac allograft transplant



PBL and LNC were double labelled to detect CD4 and αβTCR cells (minimum of 4 animals in each group). Results are expressed as the % mean cell number ± SD. Control untreated naïve animals cells are represented by filled columns (C) whereas the OX38 treated animals are represented by open columns. Upper panel (A) are PBL and lower panel (B) are LNCs.

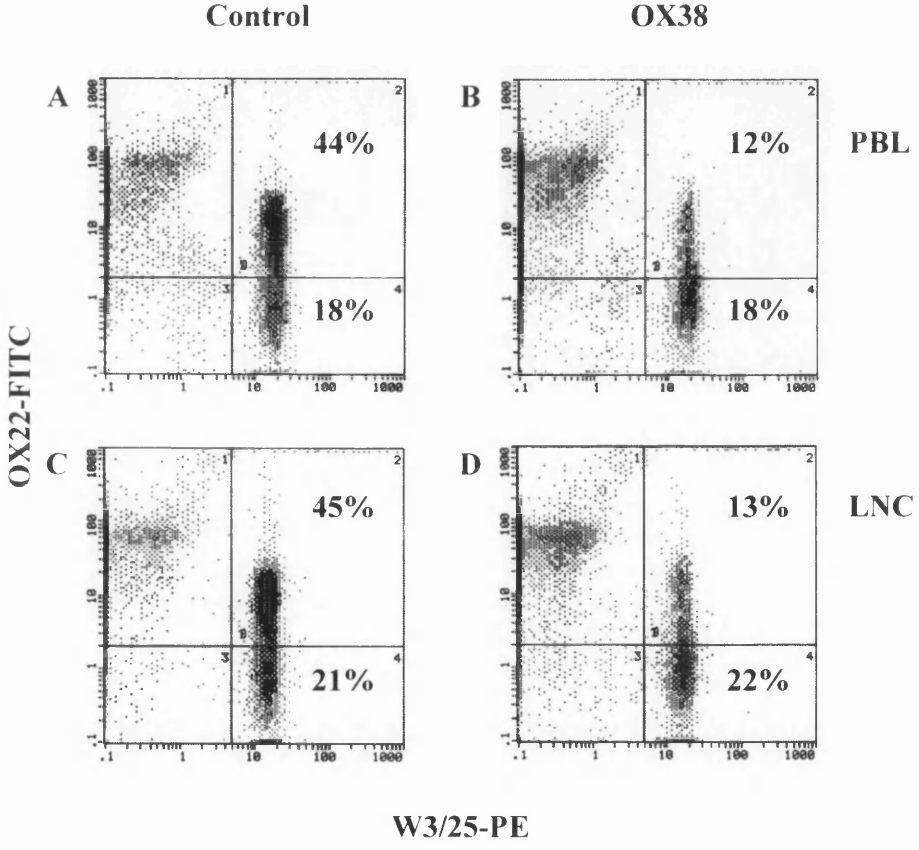
3.2.5 The depletion of CD4 T cells following OX38 mAb treatment is more pronounced in the CD45RC^{+ve} (MRC OX22^{high}) subset than in the CD45RC^{-ve} (MRC OX22^{low}) subset

Rat CD4 T cells can be subdivided according to their expression of the high molecular isoform of the leukocyte common antigen, CD45RC, by their reactivity with the mAb MRC OX22 [219]. Although the CD45 molecule is found on the cell-surface of all leukocytes, the OX22 determinant is restricted to rat B cells, non-activated CD8 T cells, NATURAL KILLER (NK) cells, some bone marrow cells, 2% of thymocytes and two-thirds of CD4 T cells [235]. Rat leukocytes expressing the high molecular weight isoform of CD45 (CD45RC^{+ve}) labelled with OX22 are typically described as OX22^{+ve}, OX22^{high} or OX22^{bright}, whereas cells expressing the low molecular weight isoforms are typically described as OX22^{-ve}, OX22^{low} or OX22^{dull}. As a result of the profile continuum on flow-cytometric analysis due to differential expression of CD45RC antigen and binding of OX22 mAb, the distinction between the CD4 T cell subsets is probably best described by the terms high and low. Consequently, CD4 T cells labelled with OX22 in the studies described in this thesis are referred to using the nomenclature of OX22^{high} and OX22^{low}. The observation that functional heterogeneity exists between the OX22^{high} and OX22^{low} CD4 T cell subsets, namely the ability of the OX22^{high} CD4 T cell subset to induce GRAFT VERSUS HOST DISEASE (GVH) [219] and restore allograft rejection and normal T cell effector mechanisms in CD4 T cell deficient nude rats [236], lead me to explore the possibility that OX38 mAb treatment may preferentially affect one, or the other of the CD4 T cell subsets.

To analyse the effect of OX38 mAb treatment on the two CD4 T cell subsets, peripheral blood and LNCs from treated animals were labelled using OX22 mAb. To exclude the possibility of analysis bias, the flow cytometric gates were standardised for each experiment and were identical for each cell sample. Figure 3.7 show typical two colour flow cytometric

profiles obtained after labelling PBL (upper panels) and LNC (lower panels) of control (left panels) and OX38 treated animals (right panels) with the OX22 mAb. As an internal control, each experiment included labelling the cells for CD4 and αβTCR. The number of double positive cells ($CD4^{+ve}$ $\alpha\beta TCR^{+ve}$) was compared with the number of $CD4^{+ve}$ $OX22^{high + low}$ cells and in each experiment, the percentage of cells labelled was found to be very similar, indicating that flow cytometric gating and cell staining was uniform for each experiment.

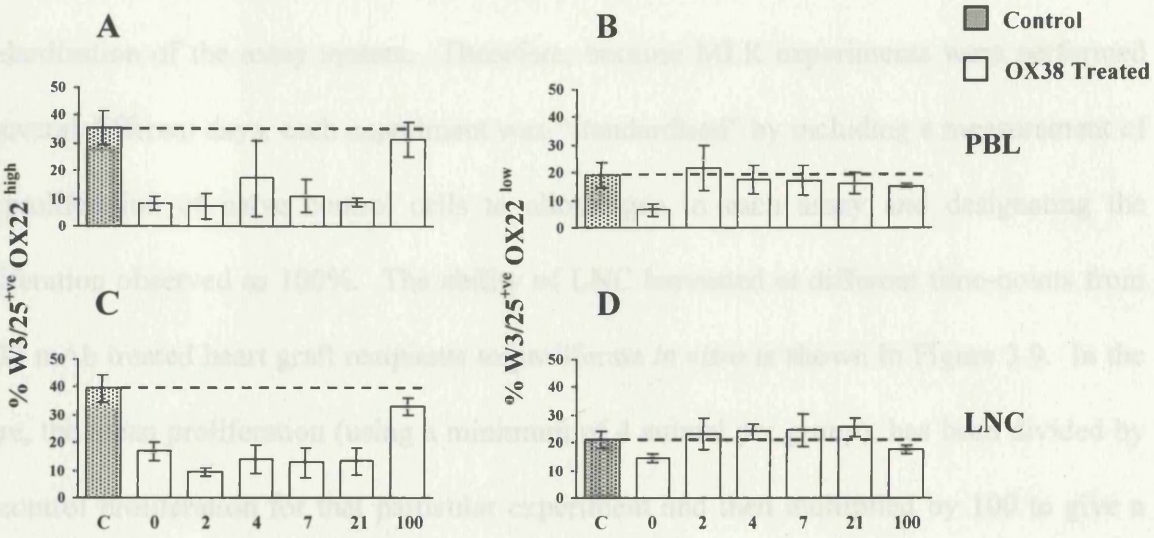
Figure 3.7 Labelling for CD45RC antigen and for CD4 using 2 colour Flow Cytometric analysis



Typical two colour fluorescent analysis using FITC-conjugated anti-CD45RC y-axis (Log_{10} FITC signal) and PE-conjugated anti-CD4 x-axis (Log_{10} PE signal). Left panels (A) and (C) represent cells from a control naïve untreated animal whereas the right panels (B) and (D) are from a Day 4 OX38 treated and transplanted animal. Upper panels (A) and (B) shows the staining for PBL and the lower panels (C) and (D) depict the staining for LNC.

Figure 3.8 shows the results of several different flow cytometric analysis experiments expressed as a bar chart. This figure shows the percentage of $CD4^{+ve}$ $OX22^{high}$ (left panels) and $CD4^{+ve}$ $OX22^{low}$ (right panels) obtained after labelling PBL (upper panels) and LNC (lower panels) from control and OX38 treated animals on days 0, 2, 4, 7, 21, and 100 after OX38 treatment and/or cardiac allograft transplant. It can bee seen that the depletion of CD4 lymphocytes following OX38 treatment was associated with preferential loss of the $OX22^{high}$ fraction of $CD4^{+ve}$ T cells. There was almost complete sparing of the $OX22^{low}$ fraction. This observation can be interpreted as either preferential depletion of the $OX22^{high}$ subset by OX38 mAb, or CD4 depletion associated with isotype switching to the low molecular weight form of the residual CD4 T cells.

Figure 3.8 Preferential depletion of the CD45RC^{+ve} CD4 T cell subset following OX38 treatment and cardiac allograft transplant



Results are expressed as the % mean cell number ± SD of PBL and LNC staining double positive for CD4 and CD45RC$^{(+ve\ and\ -ve)}$ from a minimum of 4 animals in each group. Control untreated naïve animals are represented by filled columns (C) whereas the OX38 treated animals are represented by open columns. Panels (A) and (C) represent the proportion of cells labelling positive for CD4 and CD45RC^{+ve}. Panels (B) and (D) represent the proportion of cells which labelled CD4 positive but were CD45RC^{-ve}. Upper panels (A) and (B) are from PBL and lower panels (C) and (D) are LNCs.

3.2.6 Ability of LNC from OX38 mAb treated graft recipients to proliferate and produce IL-2 in the MLR

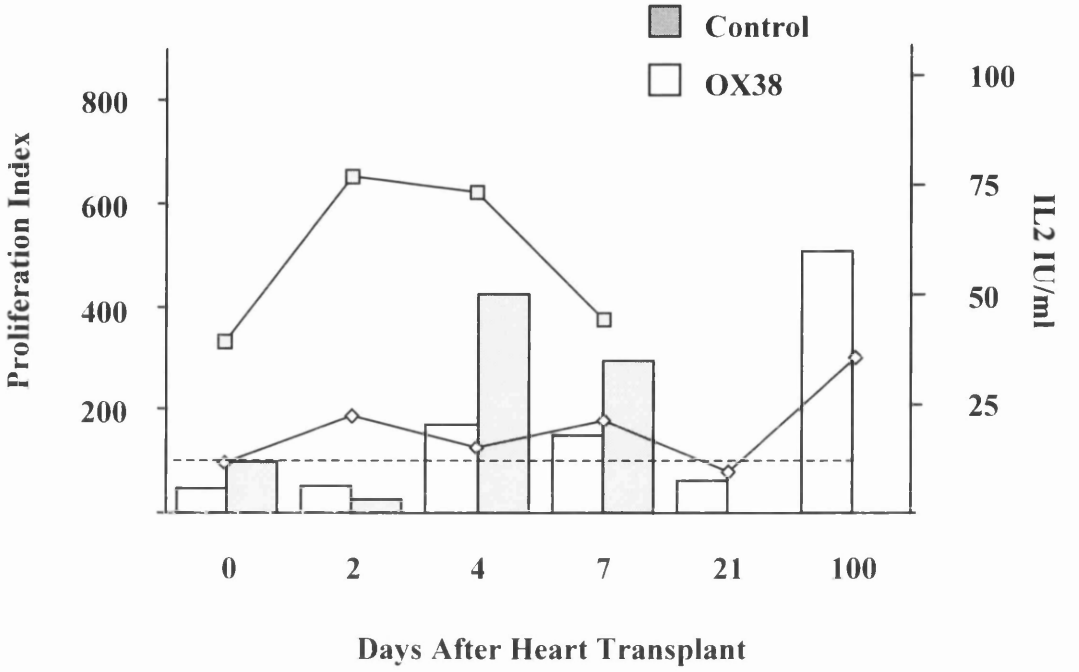
Although OX38 mAb treatment made DA recipients tolerant to a Lewis cardiac allograft, it was apparent from the preceding studies that significant numbers of CD4 T cells remained after OX38 treatment, and that T cell depletion was no longer apparent by day 100 after transplant. It was therefore of interest to determine the ability of LNC from such OX38 treated animals to proliferate and produce IL-2 when restimulated *in vitro* with irradiated

donor strain (i.e. Lewis) stimulator cells in a one way MLR. A feature of MLR which can make assays performed on separate days difficult to compare, is that there can be wide experiment to experiment variation in the levels of proliferation observed, despite careful standardisation of the assay system. Therefore, because MLR experiments were performed on several different days, each experiment was "standardised" by including a measurement of the proliferation of naïve control cells to alloantigen in each assay and designating the proliferation observed as 100%. The ability of LNC harvested at different time-points from OX38 mAb treated heart graft recipients to proliferate *in vitro* is shown in Figure 3.9. In the figure, the mean proliferation (using a minimum of 4 animal per group), has been divided by the control proliferation for that particular experiment and then multiplied by 100 to give a value relative to the 100% control value. Supernates from proliferating cells were obtained on the day of maximum proliferation and were tested for IL-2 activity using an IL-2 dependent cell line as described in the Materials and Methods (Section 2.12). To test the ability of cells taken from OX38 treated DA animals to proliferate *in vitro* in response to alloantigen, LNCs were obtained from animals at 2, 4, 7, 21 and 100 days post transplant and were challenged *in vitro* using Lewis irradiated spleen cells. Untreated DA animals bearing a rejecting Lewis heart graft were used as a comparison at 2, 4, and 7 days following transplantation. Day 0, OX38 treated and naïve untreated untransplanted animal cells were also used and have been plotted in the figure. The bar graph in Figure 3.9 shows the peak proliferation (day 3 MLR) results, while the line plot shows IL-2 production measured from the supernates of the proliferating cells from each sample.

As shown in Figure 3.9, the proliferation of cells from OX38 treated animals at day 0 is only 50% that of the naïve cells. This reduction in proliferation is in keeping with the observation that OX38 can inhibit LNC proliferation in MLR when added exogenously *in vitro* since at

day 0, the CD4 T cells obtained from anti-CD4 treated animals are still coated with mAb. At days 4, 7 and 100, OX38 treated animals show allospecific sensitisation and proliferate more than cells obtained from normal animals. Interestingly, the sensitisation which occurred in the OX38 treated animals was not as marked as that observed in untreated animals which rejected their allografts where proliferation was two fold greater than that observed in OX38 treated heart graft recipients.

Figure 3.9 Mixed lymphocyte proliferation and IL-2 production of LNC from control rejecting and OX38 treated animals



LNC from unmodified DA animals with rejecting Lewis heart grafts are represented by filled boxes and columns (Control) whereas responses from LNC taken from the OX38 treated animals at each point are represented by open diamonds and columns (OX38). Results are expressed as mean number from 4 animals at each of the time-points, of Day 3 LNC proliferation and IL-2 production from an MLR using DA responder cells cultured with irradiated Lewis stimulator cells. Cell cultures were pulsed with ^{3}H-Thymidine, harvested 18 hrs later onto microtitration filter paper, and analysed using a Beta Liquid Scintillation counter. Supernates from proliferating cells were used in an IL-2 bioassay as described in the Materials and Methods Section 2.12. The proliferative response of untreated naïve control animal cells (Day 0) is taken as a proliferation index of 100%. Proliferation results are expressed as a Bar chart (Left y-axis) whereas the IL-2 results are expressed as a Line chart (Right y-axis).

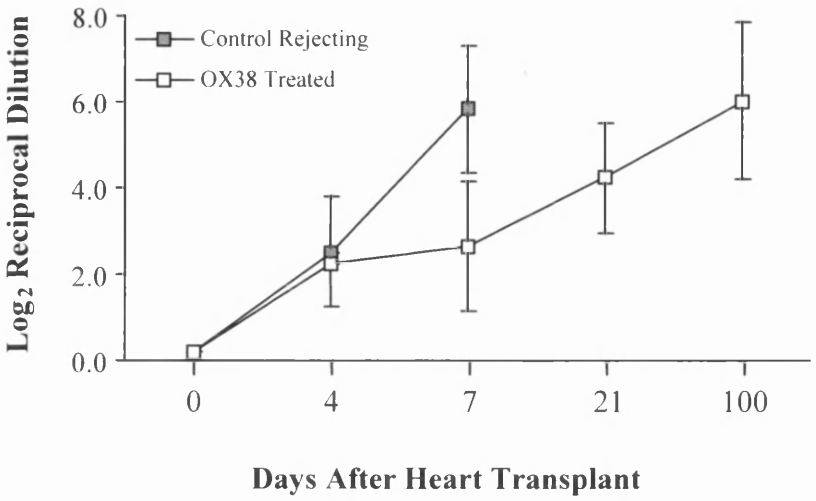
When analysing the IL-2 data, it is interesting to note that even when LNC from OX38 treated animals (on days 4, 7, and 100) proliferated in excess to LNC from normal animals (day 0) and control rejecting (day 2) cells, the IL-2 production from the OX38 treated animals was

less than that seen in cells from normal animals and never reached 50% of the peak IL-2 observed in the control rejecting cells on day 2.

3.2.7 OX38 mAb treated heart graft recipients show a delayed alloantibody response

After observing that LNC from OX38 mAb treated heart graft recipients displayed differences in IL-2 production in the one way MLR when compared with untreated control LNC, further evidence for abrogated effector function in anti-CD4 treated recipients was sought. In particular, the ability of OX38 treated and unmodified heart graft recipients to mount a cytotoxic alloantibody response was determined as another measure of CD4 T helper cell function. Serum samples were obtained from graft recipients on days 0, 4, 7, 21 (OX38 treated) and 100 (OX38 treated) and tested for the presence of cytotoxic antibody against donor strain (Lewis) target cells in a standard complement dependent cytotoxicity assay using guinea pig complement as a source of complement. The results are depicted in Figure 3.10 which shows the cytotoxic antibody titre, expressed as Log_2 of the reciprocal dilution of serum which gave ≥50% specific release of ^{51}Cr from the target cells.

Figure 3.10 Cytotoxic alloantibody response of control rejecting and OX38 mAb treated tolerant animals



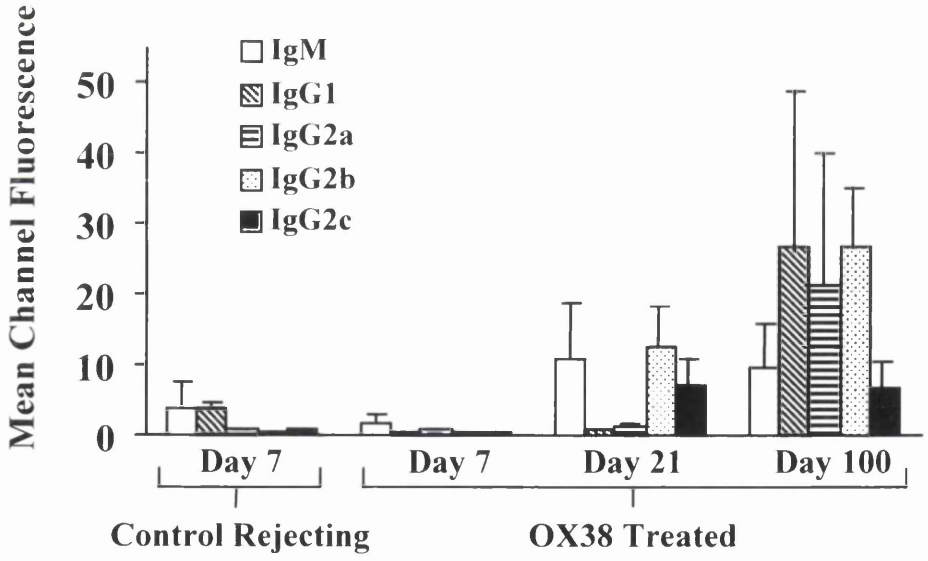
Serum samples from control rejecting (filled boxes) and OX38 treated (open boxes) DA recipients of a Lewis heart allograft were assessed for cytotoxic alloantibody levels against ^{51}Cr labelled Lewis Con-A blasts. Results shown are mean number ± SD of 4 animals per group. The y-axis represents Log_2 of the reciprocal dilution of serum and the x-axis is the day following heart transplantation on which serum was collected.

As expected, cytotoxic antibody levels were initially low in both groups of animals. They rose to a maximum titre of 1:64 at day 7 in unmodified recipients bearing rejecting heart allografts. The OX38 mAb treated graft recipients showed a delayed cytotoxic alloantibody response, but by day 100 the levels of cytotoxic alloantibody observed were comparable to those observed at day 7 in the unmodified graft recipients.

To determine the antibody isotype of alloantibody in serum taken from DA recipients of Lewis cardiac allografts, target LNC from normal Lewis rats were incubated with serum samples obtained from unmodified and from OX38 mAb treated DA rats bearing Lewis cardiac allografts. The target cells were then stained with fluorescent conjugated mouse anti-

IgM, IgG1, IgG2a, IgG2b, or IgG2c antibodies. The MEAN CHANNEL FLUORESCENCE (MCF) was then determined after analysing the fluorochrome labelled target cells. The results of this antibody isotype analysis are shown in Figure 3.11. It can be seen that at day 7 all isotypes of alloantibody were respectively low in both groups of animals, despite the previously noted cytotoxic alloantibody found at day 7 in unmodified recipients bearing a rejecting heart allograft. By day 21, the OX38 treated group showed a rise in the IgM, IgG2b and IgG2c alloantibody levels and by day 100, all alloantibody isotypes were increased. In retrospect, it would have been interesting to determine both cytotoxic alloantibody and alloantibody isotypes in unmodified graft recipients at days 21 and 100, but unfortunately these animals were sacrificed after they had rejected their grafts.

Figure 3.11 Class and subclass of alloantibody response in unmodified and OX38 mAb treated DA recipients of a Lewis cardiac allograft

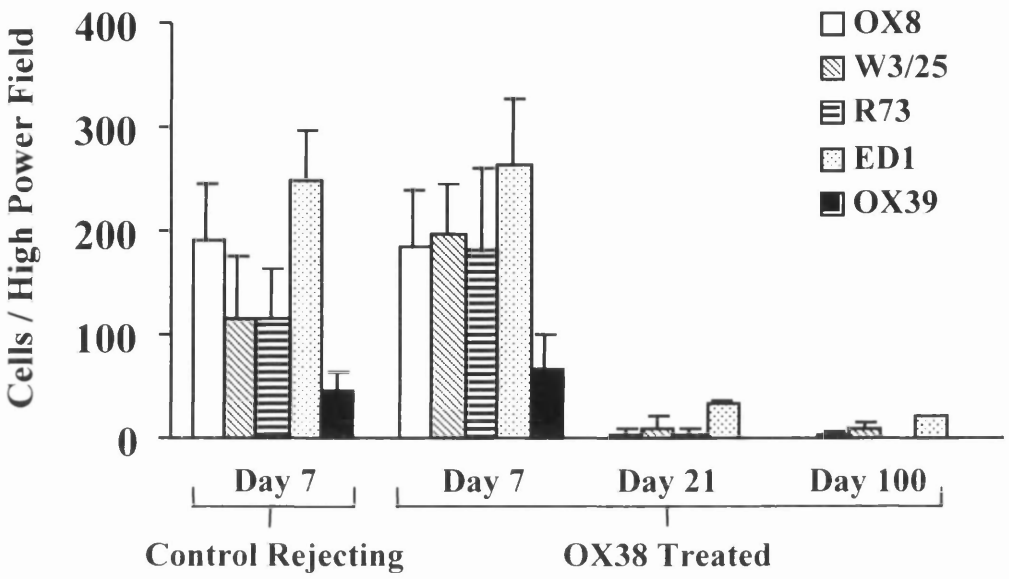


DA recipients of a Lewis cardiac allograft were assessed for alloantibody isotypes against Lewis LNC. Serum samples from control rejecting Day 7 animals are represented in the first group, whereas the OX38 treated Days 7, 21, and 100 animals are represented in the second group. The results are the mean + SD of the mean channel fluorescence. (Minimum of 4 animals in each group at each time-point).

3.2.8 Non-rejecting cardiac allografts in OX38 mAb treated recipients are heavily infiltrated by mononuclear cells

As the humoral effector response appeared intact in OX38 mAb treated DA recipients bearing Lewis cardiac allografts, evidence for impaired cellular infiltration of heart allografts was sought. The cellular infiltrates in rejecting cardiac allografts from unmodified recipients and from non-rejecting heart allografts in OX38 treated recipients were assessed by immuno-histochemistry to determine the magnitude and phenotype of the graft infiltrate. Cryostat sections of heart grafts were stained with a panel of monoclonal antibodies and a quantitative assessment of the infiltrate made. Figure 3.12 shows the cellular infiltrate of heart grafts from both unmodified and anti-CD4 treated animals at day 7, and of grafts from anti-CD4 treated animals at days 21 and 100 after transplantation. Both rejecting and non-rejecting cardiac allografts were, by day 7, heavily infiltrated by mononuclear cells. In all heart grafts there were large numbers of $CD8^{+ve}$ (OX8), $CD4^{+ve}$ (W3/25) and $\alpha\beta TCR^{+ve}$ (R73) cells, as well as heavy infiltrates of macrophages (ED1) and IL-2 receptor-α (OX39) positive cells. The infiltrate in non-rejecting heart grafts from OX38 treated recipients declined by day 21 and at day 100 and only occasional mononuclear cells were present. These were predominately $ED1^{+ve}$ macrophages. This data shows that tolerance induction using anti-CD4 mAb does not prevent the early infiltration of cardiac allografts by mononuclear cells, even those which are CD4 positive. However, with time, the mononuclear cell infiltrate in tolerant allografts declines.

Figure 3.12 Infiltration of Lewis cardiac allografts in control rejecting and OX38 mAb treated DA recipient rats



Lewis cardiac allografts obtained from DA recipients were assessed for mononuclear cell infiltration by immunoperoxidase staining. Cardiac allograft samples from control rejecting Day 7 animals are represented in the first group, whereas the OX38 treated Days 7, 21, and 100 animal allograft samples are represented in the second group. The results are expressed as cells per high power field. Heart sections from 4 animals in each group were examined and results are depicted as mean + SD.

3.2.9 Rejecting cardiac allografts from unmodified recipients and tolerant allografts from OX38 mAb treated recipients show similar mRNA cytokine transcripts

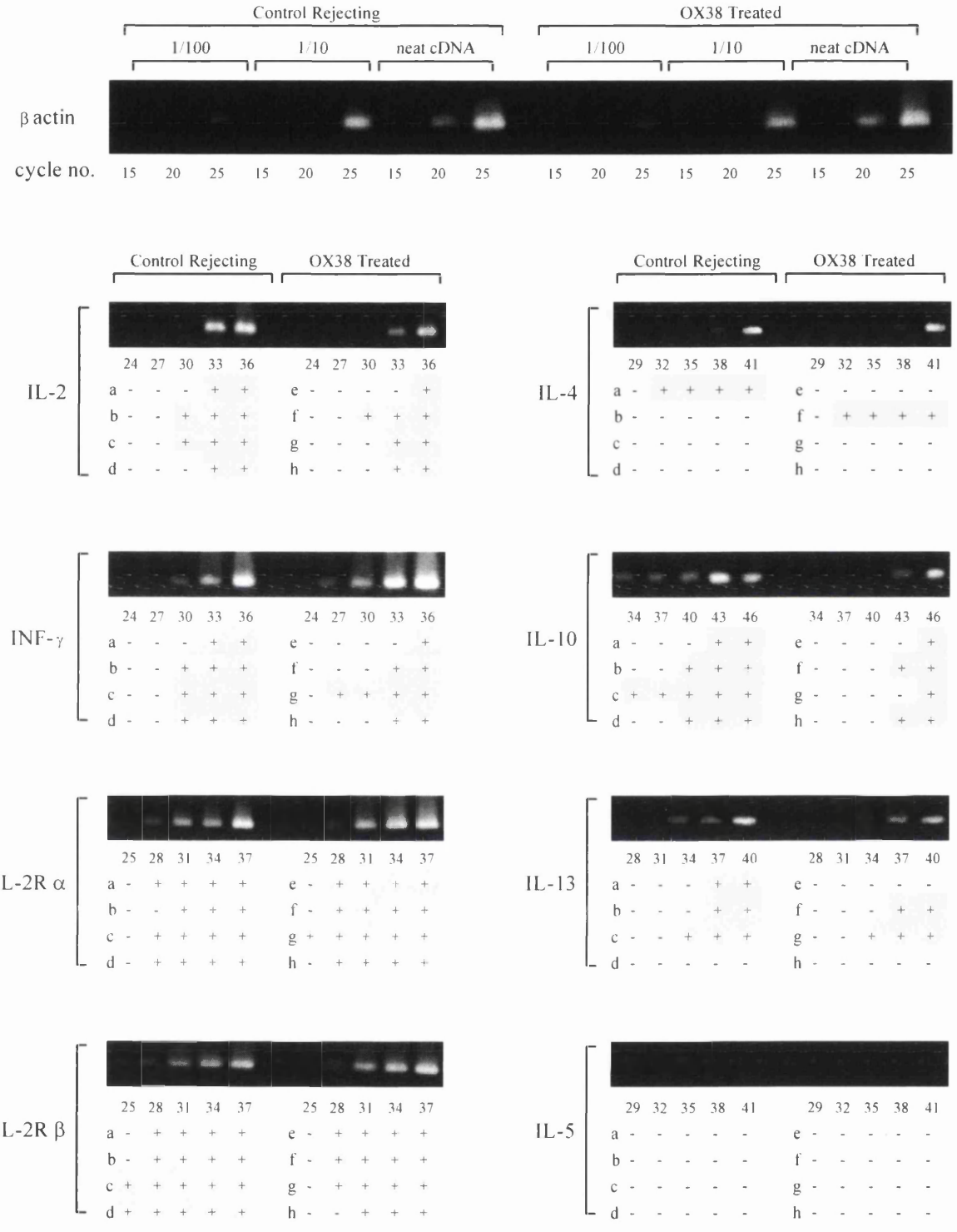
As both rejecting and tolerant cardiac allografts were initially heavily infiltrated with mononuclear cells, it was therefore of interest to assess whether there were differences in the cytokines present in rejecting and non-rejecting cardiac allografts. To determine the cytokine activity associated with the graft infiltrates, cytokine mRNA transcripts were assessed semi-quantitatively in rejecting and non-rejecting hearts using the polymerase chain reaction (RT-PCR). Primers specific for IL-2, INF-γ, IL-2Rα, IL-2Rβ, IL-4, IL-10, IL-13, and IL-5 were

used (see Materials and Methods Section 2.17 for details). The results obtained for heart grafts from both control rejecting and anti-CD4 treated animals on days 2, 4, and 7 after transplantation are displayed in Figures 3.13, 3.14, and 3.15 respectively. The results obtained for heart grafts from anti-CD4 treated animals at day 21 and day 100 after transplantation are shown in Figure 3.16. For each of the time-points after transplantation, 4 individual cardiac allografts from each experimental group were examined and these are labelled (a) to (h) in each of the figures. RT-PCR reactions were stopped at five different cycle numbers and the RT-PCR product was analysed after electrophoresis using 1% agarose gels which were stained with Ethidium Bromide. The presence of visible PCR product after ultra-violet illumination is indicated by a "+" for each sample. To ensure that an equal amount of starting material was used for each comparison, the cDNA from each heart graft was analysed at 3 different dilution's, (1/100, 1/10, and neat) and material at each dilution was analysed at 3 different cycle numbers, using the housekeeping gene primer, β-actin. A representative Polaroid photographic image of the gels from each experiment and for each cytokine have been included in the figures and in each case these were obtained from the cardiac allograft which had the most intense staining; it should be pointed out that the presence of visible PCR product by naked eye examination of the ultra violet illuminated gel (on which the scoring of RT-PCR product was based) did not necessarily equate to visible reproduction of the PCR product on the photographic image.

The cytokine profiles of rejecting heart allografts obtained 2 days after transplant from unmodified recipients and non-rejecting day 2 heart grafts from OX38 mAb treated recipients are compared in Figures 3.13. It can be seen that there were similar amounts of cytokine message in heart grafts from the two experimental groups for all primers used, with exception of those for IL-10. Message for IL-10 was present in reduced amount in the heart grafts from

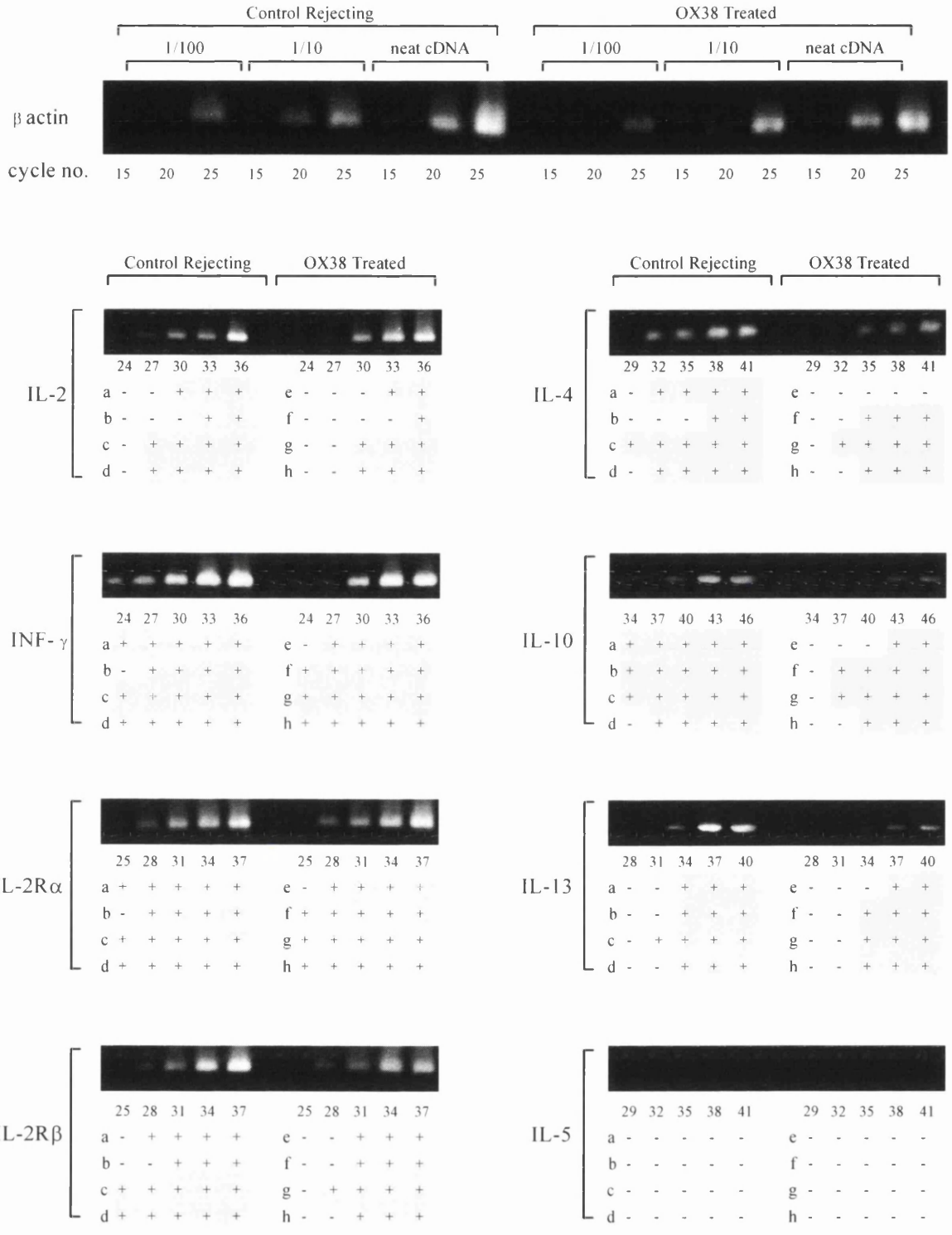
OX38 treated recipients than grafts from unmodified recipients. Figures 3.14 and 3.15, compare day 4 and day 7 rejecting and OX38 treated non-rejecting cardiac allografts respectively and demonstrate that there was no major difference in the level of cytokine message between the two experimental groups. The cytokine profiles from day 21 and day 100 cardiac allografts in the OX38 treated recipients are depicted in Figure 3.16. This figure shows that day 21 and day 100 cytokine profiles are similar to the day 4 and day 7 graft profiles, with the exception that increased levels of IL-13 were detected in both the day 21 and 100 hearts grafts. Additionally, very little cytokine message for IL-10 was detected in the day 100 heart allografts obtained from OX38 mAb treated recipients.

Figure 3.13 Cytokine PCR from Day 2 rejecting and OX38 treated cardiac allografts



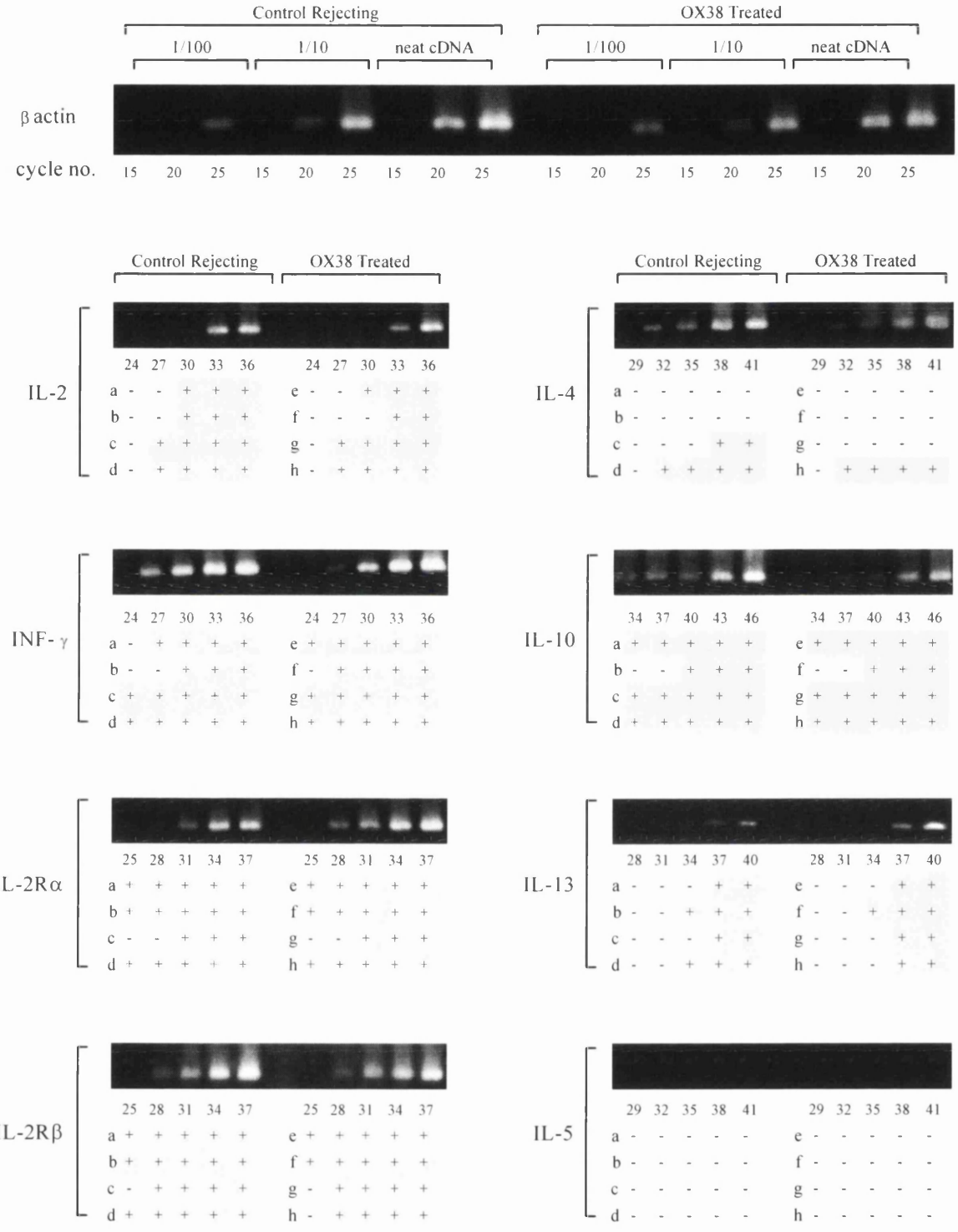
Day 2 PCR Summary. Individual animals represented by letters (a)-(d) (Control Rejecting) or (e)-(h) (OX38 Treated). A "+" with shading represents visible product whereas "-" represents absence of visible product. A typical staining pattern for one control animal and one OX38 treated animal are depicted for each cytokine and the individual cycle numbers are shown underneath. To ensure that an equal amount of starting material was used in each analysis, every sample was checked using 3 dilutions of cDNA for the house keeping gene β-actin, a typical gel depicting this analysis is shown at the top of the figure.

Figure 3.14 Cytokine PCR from Day 4 rejecting and OX38 treated cardiac allografts



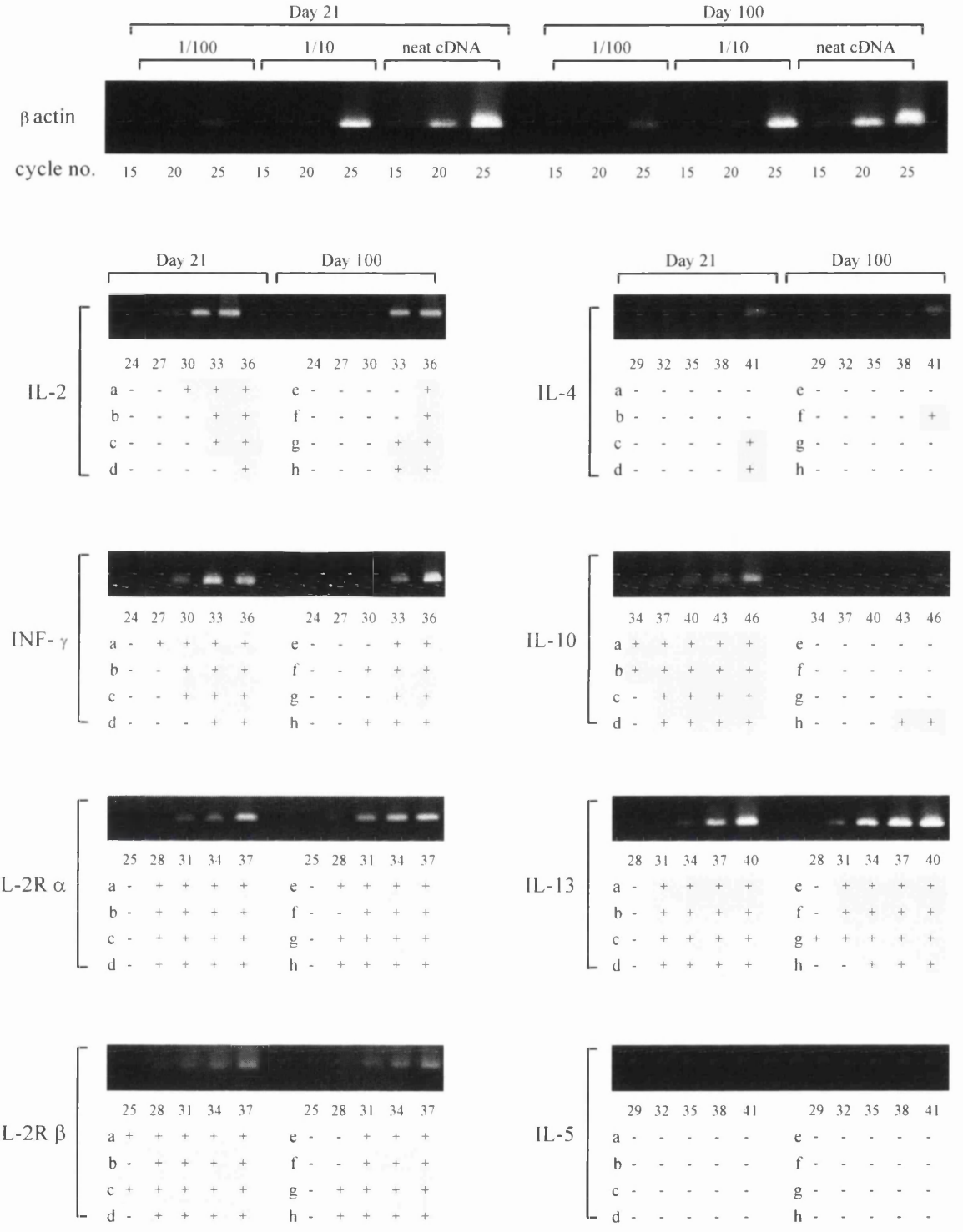
Day 4 PCR Summary. Individual animals represented by letters (a)-(d) (Control Rejecting) or (e)-(h) (OX38 Treated). A "+" with shading represents visible product whereas "-" represents absence of visible product. A typical staining pattern for one control animal and one OX38 treated animal are depicted for each cytokine and the individual cycle numbers are shown underneath. To ensure that an equal amount of starting material was used in each analysis, every sample was checked using 3 dilutions of cDNA for the house keeping gene β-actin, a typical gel depicting this analysis is shown at the top of the figure.

Figure 3.15 Cytokine PCR from Day 7 rejecting and OX38 treated cardiac allografts



Day 7 PCR Summary. Individual animals represented by letters (a)-(d) (Control Rejecting) or (e)-(h) (OX38 Treated). A "+" with shading represents visible product whereas "-" represents absence of visible product. A typical staining pattern for one control animal and one OX38 treated animal are depicted for each cytokine and the individual cycle numbers are shown underneath. To ensure that an equal amount of starting material was used in each analysis, every sample was checked using 3 dilutions of cDNA for the house keeping gene β-actin, a typical gel depicting this analysis is shown at the top of the figure.

Figure 3.16 Cytokine PCR from Days 21 and 100 OX38 treated cardiac allografts



Day 21 & 100 PCR Summary. Individual animals represented by letters (a)-(d) (Day 21) or (e)-(h) (Day 100) OX38 treated tolerant animals. A "+" with shading represents visible product whereas "-" represents absence of visible product. A typical staining pattern for one control animal and one OX38 treated animal are depicted for each cytokine and the individual cycle numbers are shown underneath. To ensure that an equal amount of starting material was used in each analysis, every sample was checked using 3 dilutions of cDNA for the house keeping gene β-actin, a typical gel depicting this analysis is shown at the top of the figure.

3.3 DISCUSSION

The initial results described in this chapter confirmed the observation [160] that a brief period of treatment with the anti-CD4 mAb, MRC OX38, at the time of heart transplantation, leads to donor specific transplant tolerance in the fully allogeneic Lewis ($RT1^l$) to DA ($RT1^a$) rat strain combination. In order to gain insight into the possible mechanisms whereby anti-CD4 mAb could promote graft survival and tolerance induction in this experimental model, a detailed phenotypic analysis of the lymphocyte populations of the antibody treated transplanted animals was made, followed by an analysis of the cellular and humoral responses of the graft recipients to donor strain alloantigens.

The results of the flow cytometric analysis to determine the duration of OX38 binding to residual CD4 T cells and the assessment of CD4 modulation were of particular interest. Following OX38 treatment, residual CD4 T cells in the blood and lymph nodes remained only transiently coated with anti-CD4 mAb and the residual CD4 cells showed no detectable *in vivo* labelling 72 hrs after transplant. The short period of residual CD4 T cell coating with mAb contrasts sharply with similar studies in the mouse [167] in which the antibody persisted for several weeks. The OX38 mAb pre-treatment protocol resulted, initially, in partial modulation of CD4 molecules from residual cells in both blood and LNC. However the CD4 modulation observed was short lived and expression of surface CD4 molecules recovered by 48 hrs in both compartments. Additionally, the anti-CD4 treatment protocol saturated all available CD4 molecules on the surface of PBL, but not LNC. These results imply that modulation of CD4 from the T cell-surface is unlikely to play a major role in the induction of tolerance observed in this experimental model. This finding contrasts with those from models of anti-CD4 induced transplantation tolerance in which either non-depleting or depleting anti-CD4 protocols were found to cause profound and long lasting modulation of

CD4 molecules from the T cell-surface [167,237]. The CD4 modulation observed in these studies, may, however, not be a critical event in the perturbation of T cell function, but, rather, may be one step in a cascade which involves other molecular mechanisms. These may include interruption of downstream intracellular signalling events within the T cell resulting in T cell inhibition and subsequent tolerance induction.

The number of T cells required to initiate and effect allograft rejection is relatively small, for example transfer of 5 x 10^6 purified T cells is sufficient to cause rejection of fully allogeneic kidney allografts in the rat [225]. Consequently, T cell depletion is not likely to be the principal mechanism involved in OX38 mAb induced transplantation tolerance. Analysis of CD4 T cell numbers in the peripheral blood and lymph nodes of OX38 treated animals revealed that the mAb treatment produced approximately 50-60% CD4 T cell depletion around the time of heart transplantation. CD4 T cell numbers gradually recovered thereafter and were near normal by 100 days. Additional results, to be described in Chapter 5, provide further support for the view that CD4 T cell depletion is not the sole reason for induction of tolerance after OX38 mAb. These results show that OX38 mAb treated animals which have been thymectomised as adults before anti-CD4 treatment, reject their heart allografts promptly with kinetics similar to unmodified control animals.

Interestingly, when the phenotype of the residual CD4 T cells in OX38 treated animals was examined, there was apparent sparing of the $CD4^{+ve}$ $OX22^{low}$ fraction, suggesting preferential depletion of the $CD4^{+ve}$ $OX22^{high}$ T cell subset in these animals. It has been suggested that the rat $OX22^{high}$ CD4 T cell subset may be equivalent to murine Th1 cells because of the ability of these cells to initiate GVH [219], provide B cell help in antibody production [89,238] and because of the Th1 like cytokine profile of $OX22^{high}$ cells in response to mitogenic and allogeneic stimulation *in vitro* [238]. Additionally, Bell and colleagues have

shown that reconstitution of congenitally athymic $RT1^c$ (PVG) nude rats with $OX22^{high}$ CD4 T cells, obtained from euthymic naïve $RT1^c$ (PVG) donors, restores normal peripheral T cell function to the nude recipients which develop normal GVH responses, allograft rejection and thymus-dependent antibody production [236]. In contrast, reconstitution of nude rats with $OX22^{low}$ T cells does not restore peripheral T effector cell function.

The apparent preferential depletion of the $CD4^{+ve}$ $OX22^{high}$ T cell subset in the present experimental model, is consistent with the recent observations of Stumbles and Mason [239]. They found that activation of rat CD4 T cells *in vitro,* in the presence of a non-depleting anti-CD4 antibody (W3/25), led to a preferential Th2 type cytokine response by the CD4 T cells. Stumbles and Mason suggested that this was due to preferential inhibition of the Th1 CD4 T cells by the anti-CD4 mAb. This observation is also consistent with the results of Goedert and colleagues [240], who showed that anti-CD4 mAb selectively inhibited the differentiation *in vitro* of Th1 like, but not Th2 like, $CD4^{+ve}$ T cells.

Another explanation for the present observation is that after OX38 mAb treatment, CD45 isotype switching from the $CD4^{+ve}$ $OX22^{high}$ to the $CD4^{+ve}$ $OX22^{low}$ isoform may have occurred. However, as will be seen later, analysis of the $CD4^{+ve}$ $OX22^{low}$ fraction, (discussed fully in Chapter 5), revealed that this particular subset was made up predominantly of Thy-1^{+ve} T cells. The Thy-1 antigen in the rat is expressed by immature thymocytes and RTE cells and it is unlikely, therefore, that isotypic switching may have occurred. Why preferential depletion of the $CD4^{+ve}$ $OX22^{high}$ T cell subset should occur after OX38 mAb treatment is not known. One possibility is that this subset of CD4 T cells is more likely to apoptose, either as a result of cross linking with bivalent anti-CD4 mAb or after contacting alloantigen whilst coated with anti-CD4 mAb. Functional differences between $CD4^{+ve}$ $OX22^{high}$ and $CD4^{+ve}$ $OX22^{low}$ cells regarding cytoplasmic expression of protein tyrosine phosphatase (PTPase) and

their intracellular signalling capabilities have been described [241]. Differential depletion may be a result of the differential intracellular signalling capabilities of these cells, because alternate intracellular signalling pathways are utilised following CD4 ligation with mAb. If so, it is reasonable to speculate that this may lead to differential up-regulation of CD95 (Fas) and hence apoptosis in the $CD4^{+ve}$ $OX22^{high}$ T cell subset, but not in the $CD4^{+ve}$ $OX22^{low}$ T cells.

Addition of anti-CD4 mAb to rat T cells *in vitro* prevents them from proliferating to allogeneic stimulators and impairs their ability to produce IL-2 [239]. It was of interest, therefore, to examine whether LNC from tolerant anti-CD4 treated recipients were able to proliferate and produce IL-2 *in vitro* in a one way MLR. As described, CD4 T cells from anti-CD4 treated and transplanted animals showed allospecific sensitisation and proliferated to a greater extent than LNC obtained from normal DA rats. However, when the supernatants from the proliferating LNC were tested for IL-2 activity, using an IL-2 dependent cell line, OX38 mAb treated animals showed less IL-2 activity, compared to samples obtained from naïve LNC. It should be noted, however, that interpreting measurements of IL-2 production in the MLR is potentially problematic. For example, low levels of IL-2 in the supernates may be attributable to reduced production of IL-2 by the responding T cell population, but could also arise if the responding T cell population consumes the IL-2 after it has been released. Another point to note is that in the MLR experiments, an equal number of responder LNC was used for each well. Therefore, when cells were obtained from OX38 treated animals, at the early time-points, the fraction of $CD4^{+ve}$ T cells in each MLR well was proportionally less than the number of $CD4^{+ve}$ T cells obtained from rejecting control animals. Nevertheless, IL-2 production *in vitro* was still reduced when cells were obtained from tolerant OX38 treated animals 100 days after transplantation where the proportion of $CD4^{+ve}$ cells in LNC was

similar to that found in control animals. Despite reduced IL-2 production, T cells obtained from OX38 mAb treated tolerant animals responded vigorously in MLR to donor alloantigen. This T cell proliferation could, in principle, have been due to a T cell growth factor other than IL-2. Unfortunately, after testing for IL-2, there was not sufficient supernate to allow testing for other pro-proliferative cytokines, such as IL-4.

Initial assessment of mononuclear cell infiltration of the Lewis cardiac allografts obtained from unmodified and from anti-CD4 treated DA recipients, showed that grafts from both experimental groups were heavily infiltrated with $CD8^{+ve}$, $CD4^{+ve}$, $\alpha\beta TCR^{+ve}$ and $IL\text{-}2R\alpha^{+ve}$ cells and cells of the macrophage lineage with no apparent difference between the experimental groups. To determine whether there was evidence for a shift from one type of T helper response to another, cardiac allografts were also assessed for the presence of Th1 and Th2 cytokine message. Intragraft mRNA transcripts for IL-2, INF-γ, IL-2Rα, IL-2Rβ and IL-4 were readily detectable by RT-PCR to a similar extent in both tolerant and rejecting heart grafts. The amount of IL-10 message was greater in rejecting heart grafts at day 2 and was almost undetectable in tolerant grafts at day 100. At days 2,4 and 7 following transplantation, IL-13 levels were detectable to a similar extent in both rejecting and non-rejecting grafts. The preliminary cytokine mRNA data, therefore, provided no support for the suggestion that transplant tolerance in this particular experimental model was associated with a Th1 to Th2 cytokine shift. A degree of caution is, however, required in interpretation of the cytokine mRNA data in terms of the Th1/Th2 paradigm. The presence of mRNA for IL-4, IL-10 and IL-13 in rejecting heart allografts does not necessarily equate with the presence of Th2 $CD4^{+ve}$ T cells, as non-lymphoid cells may, under certain circumstances, also produce IL-10 [242], and cells of the mast cell and basophil lineage may produce IL-4 [243]. Interestingly, message for the Th2 cytokine IL-13 was present to a similar extent in both tolerant and

rejecting animal grafts early on, and appeared to be increased in heart grafts obtained from tolerant animals at days 21 and 100. The gene for rat IL-13 has only recently been cloned [244] and while human IL-13 shares many of the properties of IL-4, including B cell stimulation and Ig isotype switching [245], it is not yet clear to what extent rat B cells respond to IL-13.

Split tolerance (i.e. the ability of cells from tolerant animals to respond to donor alloantigen *in vitro*) is well recognised in a variety of experimental models where tolerance is induced by various means such as DONOR SPECIFIC BLOOD TRANSFUSION (DST) [246], anti-CD4 treatment [162], neonatal inoculation of antigen [247,248] and Cyclosporin A treatment [249]. A recent report in which non-depleting anti-CD4 treatment was used to prolong survival of rat heterotopic cardiac allografts, noted that cells obtained during the induction phase of tolerance showed normal proliferation to donor antigen in a one way MLR but cells obtained from the long-term tolerant animals showed diminished proliferation *in vitro* [250]. In this experimental system the authors suggested that lack of T cell proliferation in the established tolerant state was due to inhibition of CD4 T cell help by the anti-CD4 mAb but they failed to explain why this phenomenon took several months to develop. The authors suggested that the emergence of stable tolerance was associated with a dominant polarised Th2 state and inferred that reduced T cell help was due to diminished IL-2 production.

Dallman et al [251] originally showed that tolerance induction, using a model of DST in rats, was associated with reduced IL-2 mRNA transcripts in the grafts of tolerant animals and that transplant tolerance in this experimental model could be overcome by the administration of exogenous IL-2 at the time of tolerance induction. On the basis of the Th1/Th2 paradigm, it seemed reasonable to assume that if tolerance was associated with a diminished Th1 response, then Th2 cytokines might be enhanced and contribute to tolerance, and several

groups involved in transplantation research rushed to find the evidence for Th2 dominance using a plethora of experimental models. Decreased Th1 associated cytokines, with sparing of Th2 cytokines, has now been observed in several other transplantation models where organ graft survival was observed, including induction therapies using CTLA4-Ig [211] and non-depleting anti-CD4 monoclonal antibodies [252,253]. Although there is a suspicion that immune deviation to a Th2 pattern of response in a model of Th1 mediated graft rejection may be permissive for long-term engraftment, a Th2 type of alloimmune response may, paradoxically, support the development of chronic rejection in the longer term [211]. This suggestion is supported by some studies which have utilised immune modulating therapies aimed at polarising the alloimmune response towards a Th2 response. While this strategy has resulted in prevention of Th1 mediated early rejection, grafts were lost in the longer term [254,255,256]. This data suggests that simply inhibiting a Th1 response and enhancing a Th2 response, is unlikely to be the panacea for preventing allograft rejection. Additional evidence also suggests that a polarised Th2 response does not necessarily confer transplant tolerance in some models of transplantation. Interestingly, liver transplantation in the same low responder rat strain combination which was utilised for the work in this thesis, (Lewis to DA), results in spontaneous tolerance of some allografts, whereas transplantation in the reciprocal high responder strain combination, (DA to Lewis), results in prompt rejection. When graft infiltrating cells were analysed by semi-quantitative RT-PCR following liver transplantation, the immune response in both strain combinations was found to be remarkably similar, the only difference being reduced mRNA expression of IL-4 in cells from the low responder tolerant group [257]. Moreover, using IL-2 KO mice in a pancreatic islet transplantation model, Steiger et. al. [258] were able to demonstrate that IL-2 was not required for allograft rejection and that the presence of IL-4 and IL-10 expression in the absence of IL-2 did not result in transplant tolerance - all transplants were rejected, albeit in a slower fashion than

grafts in control animals. A potential problem with this and other cytokine KO mice is, as the authors themselves point out, that KO mice are not 'normal' animals with a single cytokine deficiency. One can imagine that as the immune system in KO animals develops, cells not dependent on the missing cytokine for growth or differentiation are positively selected for, and that other cytokines whose function overlaps with the missing cytokine, may also play an increased role within what is undoubtedly a highly redundant biological system.

In the present studies, the assessment of the alloantibody response in unmodified and anti-CD4 treated animals gave further insight into the nature of the T helper response. In the rat, on the basis of the selective effects of IL-12 on IgG isotype alloantibody responses, alloantibodies of the IgG2b and IgG2c subclass appear to be Th1 mediated, and IgG1 subclass antibodies are of Th2 T helper dependent isotypes [259]. The experiments in this chapter revealed a weak cytotoxic alloantibody response which was present in both rejecting and anti-CD4 treated tolerant animals. However, in the anti-CD4 treated recipients, the cytotoxic antibody response was slower to develop. The alloantibody isotype data confirms that early antibody responses in this model are relatively weak with only modest levels of alloantibody detected early on. Alloantibody levels were substantially higher in the long-term tolerant recipients but there was no evidence for dominance of any particular antibody isotype and hence no support, for the suggestion that tolerance in this particular experimental model is associated with a Th1 to Th2 cytokine shift.

In the context of transplantation rejection and tolerance, the Th1/Th2 paradigm cannot be invoked universally to explain whether graft rejection or tolerance will result. A large number of studies have been published with contrasting results, some supporting and some negating the hypothesis. The apparent discrepancy between the results using different experimental systems, is perhaps, not surprising if one considers that the immune system in

mammals has evolved by developing strategies to match the variation imposed by the pathogens it faces. Some experimental models may only exploit one facet of the immune response and with such a constraint the response may be skewed in one way or the other. For example, if an antigen which only generates a Th1 or Th2 response is chosen to challenge the immune response and one manipulates the response and induces tolerance, it may not be surprising to find an intact opposing Th2 or Th1 compartment (which could be interpreted as the important mechanism in tolerance induction). In allograft transplantation, it seems clear that immune responses dominated by both cell-mediated or humoral effector mechanisms may be detrimental to organ graft survival and depending on the species used, the strain combination tested, the influence of the immunosuppressive protocol employed and the time frame examined, domination of either cell-mediated or humoral effector mechanisms may be apparent. It remains to be determined whether manipulating the cytokine repertoire from one T helper response to the other will result in long-term allograft survival and tolerance induction.

If polarisation from one type of T helper response to another is not the explanation for transplantation tolerance then what other mechanism may be responsible? Cobbold et. al. [206] and Strom et. al. [260] who recently reviewed the mechanisms of peripheral tolerance and the Th1/Th2 paradigm respectively, have implied in their reviews, that the local autocrine effect of cytokines on T cell growth and differentiation in the milieu of an immune response modified by some form of immunosuppression (e.g. altering cytokine production, reducing critical T cell mass, blocking necessary T cell-costimulation and perturbing T cell signalling) may have a certain hierarchy and if appropriately manipulated initially away from the dominant T helper response responsible for rejection, (i.e. away from a Th1 response if Th1 mechanisms are principally responsible for rejection, or away from a Th2 response if Th2

mechanisms are dominant) then tolerance may be induced. This understanding allows the important point that once "non-rejection" occurs, perhaps with other constraints being met and maintained, then tolerance can be generated without the need for polarisation to the alternate type of T helper response.

3.4 KEY POINTS

- **Pre-operative anti-CD4 (OX38) treatment results in allospecific (but not tissue specific) tolerance in the Lewis to DA model of heterotopic cardiac transplantation.**

- **Following treatment, anti-CD4 mAb is cleared rapidly from the lymphocyte cell-surface of PBL and LNC and is not detectable in the serum at 72 hrs.**

- **The anti-CD4 treatment protocol employed, results in partial depletion of CD4 T cells with preferential sparing of the CD45RC^{-ve} T cell subset.**

- **Lymph node cells taken from tolerant animals display 'split tolerance' when re-stimulated *in vitro* in standard one way MLR (i.e. show allospecific sensitisation) but produce reduced amounts of IL-2 compared to normal LNC.**

- **Alloantibody responses and cellular infiltration in cardiac allografts are similar in tolerant and unmodified heart graft recipients.**

- **RT-PCR analysis of cardiac allografts obtained from tolerant and unmodified graft recipients at days 2, 4, 7, 21 and 100 provided no evidence for a Th1 to Th2 cytokine shift with tolerance.**

4. Effect of OX38 monoclonal antibody on intracellular signalling events

4.1 INTRODUCTION

Ligation of the αβT cell receptor with peptide antigen presented in the grove of MHC Class II triggers an intracellular signalling response, the nature of which is common to that which is seen in other cells of haemopoietic origin following their activation. The signalling response involves the activation of PTKs and a cascade of tyrosine phosphorylation of intracellular proteins, including components of the antigen receptor itself. During T cell – APC interaction, the membrane glycoprotein CD4 coaggregates with TCR - CD3 - MHC Class II complex [48] and enhances antigen mediated activation of Class II MHC restricted T cells [49,50,51]. This positive effect of CD4 in the T cell activation process has been attributed to its non-covalent association [43,44] with the PTK $p56^{lck}$, which becomes activated upon CD4 aggregation [128]. As discussed earlier, activation of $p56^{lck}$ probably involves dephosphorylation at the negative regulatory C-terminal $tyrosine^{505}$ site by CD45 phosphatase and auto phosphorylation at the positive regulatory $tyrosine^{394}$ site. Once activated, $p56^{lck}$ potentiates signal transduction by phosphorylating and activating downstream substrates including PTKs such as ZAP-70.

An additional means by which CD4 associated $p56^{lck}$ may enhance downstream tyrosine protein phosphorylation is through a mechanism unrelated to its tyrosine kinase activity, but relying instead on its SH2 sequence [261]. Xu and Littman observed that the SH2 domain of $p56^{lck}$ is able to recruit other phosphotyrosine containing signalling molecules or, alternatively, protect tyrosine phosphorylated substrate sequences from the action of phosphatases, thus complexing and stabilising tyrosine phosphorylated CD3 ζ chains, ZAP-70, and possibly other molecules, with CD4 and TCR.

In the recent years significant progress has been made in identifying the T cell proteins which serve as targets for $p56^{lck}$ during TCR induced T cell activation. As discussed earlier, several components of the CD3 complex, including the ε and ζ chains [76,146,149,262], phospholipase-Cγ1 (PLCγ1) [120,121], and ZAP-70 [144,145,148] undergo rapid tyrosine phosphorylation during T cell activation and may recruit and/or phosphorylate other down stream effector molecules in the signalling cascade. For example, following activation by phosphorylation, activated PLCγ1 mediates, in turn, the hydrolysis of PIP_2 to IP_3 and DAG [263,264]. The latter molecules are ultimately responsible for increasing the concentration of cytoplasmic calcium and activating PKC [265,266]. Other proteins which undergo phosphorylation during T cell activation are Vav, a 95 kDa polypeptide which is thought to possess guanine nucleotide exchange activity towards low molecular weight G-proteins, [267,268,269], Shc, a 46-52 kDa protein which once phosphorylated may interact with ζ chain [270] and become involved in recruitment of Grb-mSos complex to the plasma membrane [271], and a 36-38 kDa protein [272] which may also complex with Grb2. Through their action on the GUANOSINE TRIPHOSPHATE (GTP) binding protein, $p21^{ras}$, these proteins may have an important regulatory function in TCR mediated activation and proliferation although this remains to be fully clarified. Ras proteins cycle between an inactive, GUANOSINE DIPHOSPHATE (GDP) bound state and an active GTP bound form [273,274,275]. Hydrolysis of bound GTP is accelerated by GTPASE ACTIVATING PROTEINS (GAPs) which inactivate ras [276] whereas GUANINE NUCLEOTIDE RELEASING FACTORS (GRFs) exchange GDP for GTP which activate ras [277]. An elegant series of transfection experiments using mutant and wild type $p21^{ras}$ protein genes has underscored the importance of $p21^{ras}$ in expansion of effector T cells. In these studies, the expression of a dominant negative $p21^{ras}$ protein resulted in suppression of IL-2 gene induction, whereas expression of

active $p21^{ras}$ resulted in activation of transcriptional factors, such as AP-1, and increased IL-2 production [278,279]. Although the mechanism and role of $p21^{ras}$ in T cells activation has yet to be fully elucidated, activation of $p21^{ras}$ in T cells occurs in both a PKC dependent [280] and a PKC independent [281] manner. The PKC independent pathway is the one which appears to involve the 36-38 kDa $p21^{ras}$ binding protein [272].

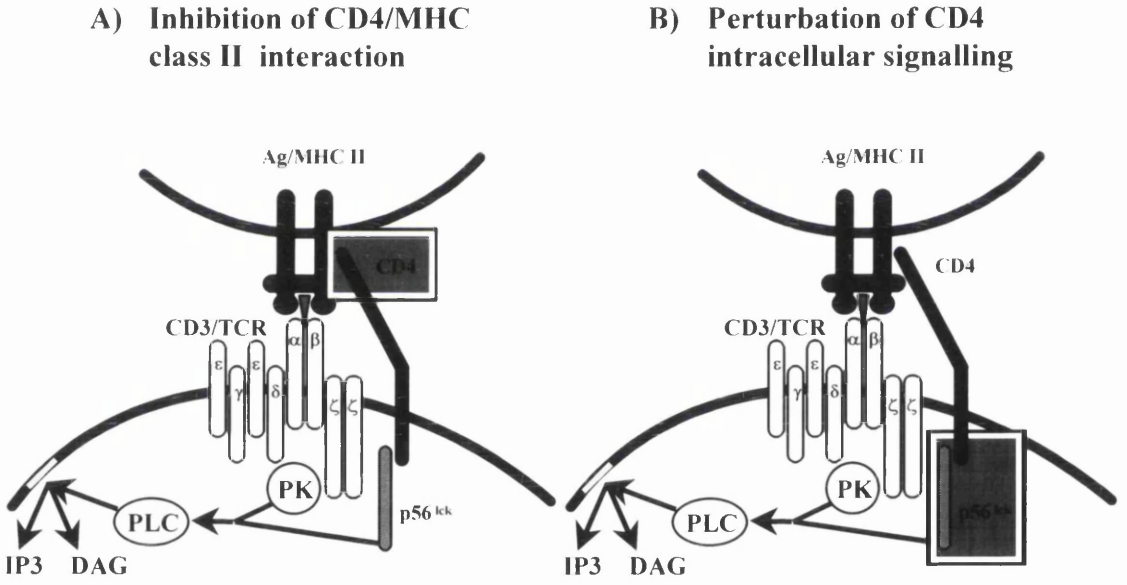
In several studies using a diverse range of T cells, including immortalised human T cell clones [282], normal human PBL's [283] and murine LNC's [284] treatment with anti-CD4 mAb has been shown to alter the intracellular signalling cascade which is orchestrated through CD4 and $p56^{lck}$. From these studies, it seemed reasonable to hypothesise that anti-CD4 (OX38 mAb) pre-treatment in the rat experimental model which forms the basis of this thesis, may also lead to differences in T cell function as a result of altered intracellular signalling. This possibility was therefore investigated using an *in vitro* activation system employing immobilised anti-αβTCR mAb to activate rat CD4 T cells. Anti-TCR induced activation was chosen instead of activation in response to alloantigen because it avoids the possibility of introducing additional phosphorylated proteins into the system by the use of allogeneic stimulator cells. In the studies performed, attention was focused particularly on the 36-38 kDa protein because lack of tyrosine phosphorylation of this protein has been shown to correlate with the development of T cell anergy [285,286]. Consequently, the SDS-PAGE system used in the experiments described was optimised using 10% gels to maximise separation of proteins in the 20 to 70 kDa range. As a result, it was accepted that changes in tyrosine phosphorylation of other activation proteins may have been over looked under experimental conditions used.

4.2 RESULTS

4.2.1 Effect of anti-CD4 treatment on the normal T cell intracellular signalling cascade

In principle, anti-CD4 mAb treatment may abrogate T cell activation either by sterically interfering with the interaction of CD4 on the T cell and MHC Class II on the APC, and/or by perturbing the normal T cell signalling cascade. The two mechanisms, which are not mutually exclusive, are depicted schematically in Figure 4.1 below.

Figure 4.1 Possible mechanisms whereby anti-CD4 mAb may interfere with T cell activation



The figure illustrates two mechanisms whereby anti-CD4 mAb may interfere with T cell activation

A). Anti-CD4 mAb may by binding to the CD4 molecule, form a physical barrier which interferes with the normal interaction of the CD4 molecule with the non-polymorphic region of the MHC Class II molecule;

B). Anti-CD4 mAb may perturbate the normal intracellular signalling mechanisms invoked by CD4 through its interaction with $p56^{lck}$ or other cytoplasmic kinases.

4.2.2 Delaying cardiac transplantation until after anti-CD4 mAb has been cleared from treated animals still results in prolonged allograft survival

To investigate the possibility that anti-CD4 mAb may perturb T cell signalling and to exclude $CD4^{+ve}$ $\alpha\beta TCR^{+ve}$ T cell depletion as an exclusive mechanism for tolerance induction in this experimental model, advantage was taken of the finding, discussed earlier in Chapter 3, that in OX38 mAb treated animals, the anti-CD4 mAb is rapidly cleared from the blood of treated animals and remains only transiently detectable on the lymphocyte surface. In contrast, following OX38 mAb, the CD4 T cell depletion observed was relatively slow to recover and the level of CD4 depletion observed was similar at days 0, 4 and 7 following *in vivo* OX38 treatment. Based on these observations, it was decided to delay heart transplantation from day 0 until either day 4 or day 7 following the last dose of OX38 mAb. Table 4.1 shows the heart graft survival data for the different experimental groups.

Table 4.1

Group	Day of Transplant (relative to mAb)	mAb*	n	Graft Survival (days)	MST (days)
1	0	OX21	7	8,9,10 x 2,11,14 x 2	10
2	0	OX38	7	8,>100 x 6	>100
3	+4	OX38	7	8,14,>100 x 5	>100
4	+7	OX38	6	9,12 x 2,18,>100 x 2	15

***mAb was administered by IP injection at a dose of 10mg/kg (day –3), and 2mg/kg (days –2, –1, 0). DA recipient animals received a Lewis cardiac allograft on days 0, plus 4 and plus 7 relative to last treatment.**

Experimental Groups 1 and 2 received anti-CD4 mAb (OX38) according to the standard protocol (10mg/kg on days -3, 2 mg/kg on days -2, -1, and 0 the day of transplant). Group 1 received a control mAb OX21 and this group had a MST of 10 days. Group 2 received OX38

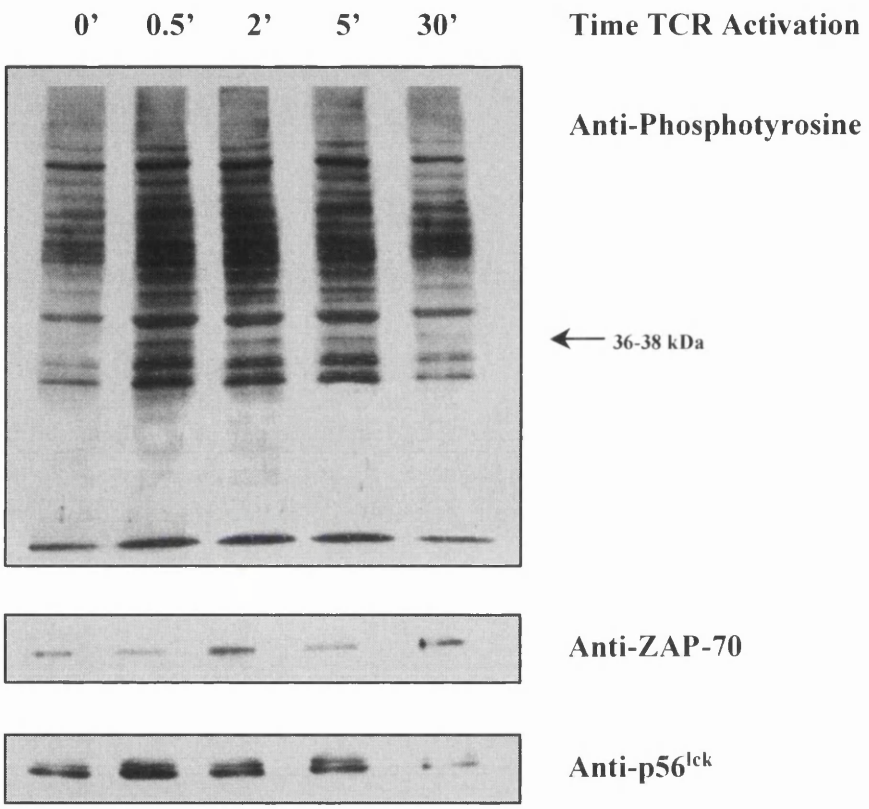
mAb and, as already noted in Chapter 3, had a MST of greater than 100 days. Groups 3 and 4 also received OX38 mAb and were then transplanted with cardiac allografts either 4 or 7 days after the last dose of anti-CD4. The MST in Group 3 was >100 days and the MST in Group 4 was 15 days. Since the level of CD4 T cell depletion after anti-CD4 mAb was similar on days 0, 4, and 7 after treatment, but residual labelling of CD4 T cells was no longer detectable at day 4, these data are consistent with the idea that anti-CD4 mAb treatment may impart some degree of 'negative' signalling to the T cell. If so, this process is partially reversed by seven days after the last dose of anti-CD4. This observation suggests a 'window of opportunity' exists (up to day 4), in which to seek evidence for functional and biochemical differences in anti-CD4 treated cells at a time when they are no longer coated with anti-CD4 mAb but before they have regained normal *in vivo* function.

4.2.3 TCR activation is associated with changes in intracellular tyrosine phosphorylation

The CD4 molecule is intimately associated with $p56^{lck}$ tyrosine kinase and it was therefore of interest to look for evidence of tyrosine phosphorylation following ligation of the αβTCR using immobilised anti-αβTCR mAb. It can be seen from the upper panel of Figure 4.2, which shows tyrosine phosphorylation patterns, that the most striking observation was the phosphorylation of a 36-38 kDa protein which was most apparent after activation of CD4 T cells for a period of 30 seconds. This protein subsequently became partially dephosphorylated at the 30 Min time-point. In order to ensure that the differences observed in tyrosine phosphorylation, in this and subsequent experiments, were not due to differences in the amount of cell lysate loaded on to the sample lanes, the same nitrocellulose blots were stripped and re-stained using anti-ZAP 70 and/or anti-$p56^{lck}$ antibodies. The results of

staining this western blot with anti-ZAP 70 and anti-p56^{lck} antibodies is displayed in the lower two panels of Figure 4.2. It can be seen in the bottom two panels of this figure, that the same magnitude of staining for p56^{lck} and ZAP-70 was observed for all sample, with exception of the 30 Min time-point sample, which stained less heavily with both antibodies.

Figure 4.2 TCR activation dependent tyrosine phosphorylation of CD4 T cells



Immunopurified CD4^{+ve} T cells were activated *in vitro* using immobilised anti-αβTCR mAb. Assays were quenched by the addition of SDS sample buffer after 0, 0.5, 2, 5 and 30 Min of activation. Cell lysates were then analysed by 10% SDS-PAGE and proteins transferred to nitrocellulose paper by western blotting. Specific staining of proteins was performed using antibody mediated chemoluminescence and autoradiography. The top panel shows the anti-phosphotyrosine staining. The bottom two panels represent the anti-ZAP-70 and anti-p56^{lck} staining of the same western blot. The time of activation is indicated on the top of the figure for each lane in Min.

4.2.4 Addition of OX38 mAb to CD4 T cells alters the tyrosine phosphorylation pattern of cytoplasmic proteins

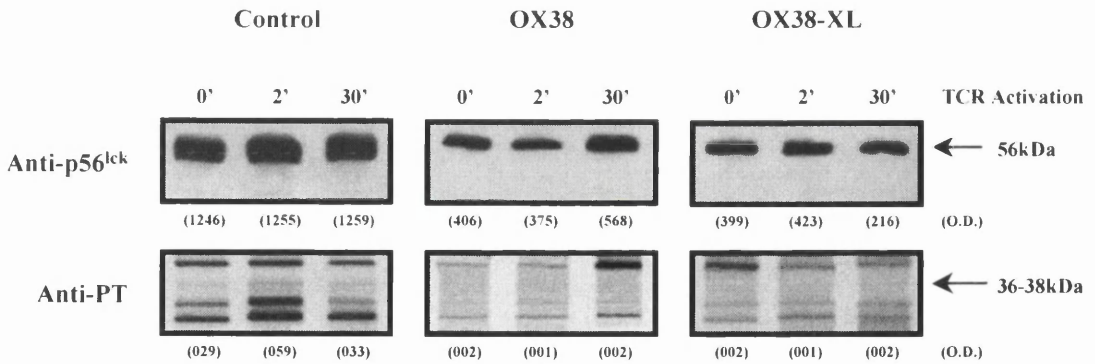
To determine the effect of *in vitro* OX38 mAb treatment on immunopurified $CD4^{+ve}$ T cells using the anti-αβTCR activation system described above, purified CD4 T cells were activated after pre-treatment with either PBS (Control), OX38 mAb, or OX38 which was then cross-linked by the addition of RaMo-antibody. It has been observed in previous studies that when using anti-CD4 antibodies to inhibit CD4 T cell activation, valence of the anti-CD4 antibody is critical and that polyvalent, but not monovalent antibody, inhibited normal intracellular signalling pathways [287] and also that IgM was more effective than IgG in inhibiting TCR induced activation [154] (and reviewed by Janeway [81]). It was therefore, reasonable to assume that cross-linking OX38 mAb with RaMo-antibody would ensure polyvalent attachment and clustering of CD4 molecules on the lymphocyte cell-surface and may lead to alteration in T cell activation induced intracellular signalling.

A representative autoradiograph from one experiment is shown in Figure 4.3. The autoradiographs obtained in this, and similar experiments, were also analysed using a BioRad Gel Doc 1000 soft laser densitometer and image analysis software system. The OD of the 36-38 kDa band and $p56^{lck}$ is displayed within brackets for each sample below the lanes of Figure 4.3. It can be seen from Figure 4.3 (lower panel), that anti-phosphotyrosine antibody chemoluminescence staining revealed a two fold or greater increase in the phosphorylation of the 36-38 kDa protein upon activation of control CD4 T cells at the 2 Min time-point, as detected by OD. It was notable, however, that there was no appreciable change in the phosphorylation of the 36-38 kDa protein upon T cell activation in the presence of either OX38 mAb or OX38 mAb cross-linked by RaMo-antibody. The top panels of Figure 4.3 show the staining of anti-$p56^{lck}$ in this experiment using the same western blot. Although the

density of the $p56^{lck}$ bands are three times higher in the control CD4 T cells when compared to the OX38 mAb treated samples, the important observation is that the relative amount of $p56^{lck}$ within each experimental group is similar. This indicates that any changes observed in the phosphorylation of the 36-38 kDa band within a group was not an artefact caused by unequal loading of lysate in that sample.

Overall, the data from these experiments suggests that *in vitro* pre-treatment of $CD4^{+ve}$ T cells with the anti-CD4 mAb OX38 leads to an impairment of the normal intracellular signalling cascade which occurs following TCR activation. This is evident as a reduction in the phosphorylation of a 36-38 kDa protein in OX38 mAb treated cells when compared to untreated control CD4 T cells.

Figure 4.3 Inhibition of TCR activation-dependent tyrosine phosphorylation in CD4 T cells by *in vitro* OX38 mAb treatment

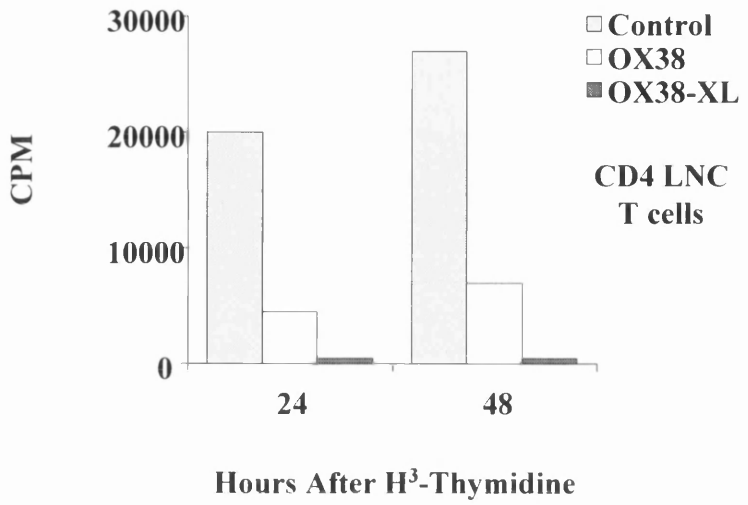


Immunopurified $CD4^{+ve}$ T cells pre-treated with PBS (Control, left panels) OX38 (middle panels), or OX38 which was cross-linked using Rabbit anti-Mouse antibody (right panels), were activated using immobilised anti-αβTCR mAb. Assays were quenched by the addition of SDS sample buffer after 0, 2 and 30 Min of T cell activation. Cell lysates were analysed by 10% SDS-PAGE and proteins transferred to nitrocellulose paper by western blotting. Specific staining of proteins was performed using antibody mediated chemoluminescence and autoradiography. The lower 3 panels depict the typical tyrosine phosphorylation patterns observed using anti-phosphotyrosine antibody staining. The upper 3 panels represent staining of the same gel using anti-$p56^{lck}$ antibody. The optical density (O.D.) of the 36-38 kDa protein band and $p56^{lck}$ are shown below each lane for each gel.

4.2.5 Anti-CD4 treatment of CD4 T cells is associated with inhibition of TCR mediated activation in vitro

To determine whether the alteration in intracellular signalling brought about by anti-CD4 mAb treatment correlated with any functional differences in anti-CD4 treated cells, the proliferative response of CD4 T cells to immobilised R73 antibody was assessed by ^{3}H-Thymidine incorporation at 24 and 48 hrs after T cell activation. Briefly, in the experiment shown, CD4 T cells were treated, prior to activation, with PBS (Control), OX38 mAb or OX38 mAb which was cross linked by RaMo-antibody. The CD4 T cells were added to culture wells in which anti-αβTCR mAb (R73) had been bound to the plate. Cells were then pulsed with ^{3}H-Thymidine, harvested onto microtitration filter paper and ^{3}H-Thymidine uptake determined by counting samples on a beta liquid scintillation counter. The results of this experiment are shown in Figure 4.4. It can be seen that anti-CD4 mAb treatment reduced the ability of CD4 T cells to proliferate in response to TCR cross-linking and that inhibition was more pronounced when the anti-CD4 mAb was cross-linked on the T cell-surface by RaMo-antibody.

Figure 4.4 Ability of OX38 to inhibit CD4 LNC activation by immobilised anti-TCR antibodies



Immunopurified $CD4^{+ve}$ T cells were treated *in vitro* with PBS (Control, lightly filled columns), OX38 mAb (OX38, open columns), and OX38 mAb which was then cross-linked using Rabbit anti-Mouse antibody (OX38-XL, darker filled columns) and were activated using immobilised anti-αβTCR mAb. Cell cultures were pulsed with ^{3}H-Thymidine after 24 and 48 hrs, and were harvested 18 hrs later onto microtitration filter paper. Thymidine uptake was analysed using a Beta Liquid Scintillation counter. The y-axis represents the COUNT PER MINUTE (CPM) of ^{3}H-Thymidine, and the x-axis shows the time in hrs of cell culture.

4.2.6 CD4 T cells obtained from animals treated with OX38 mAb in vivo display signalling defects identical to those observed in CD4 T cells treated in vitro with OX38

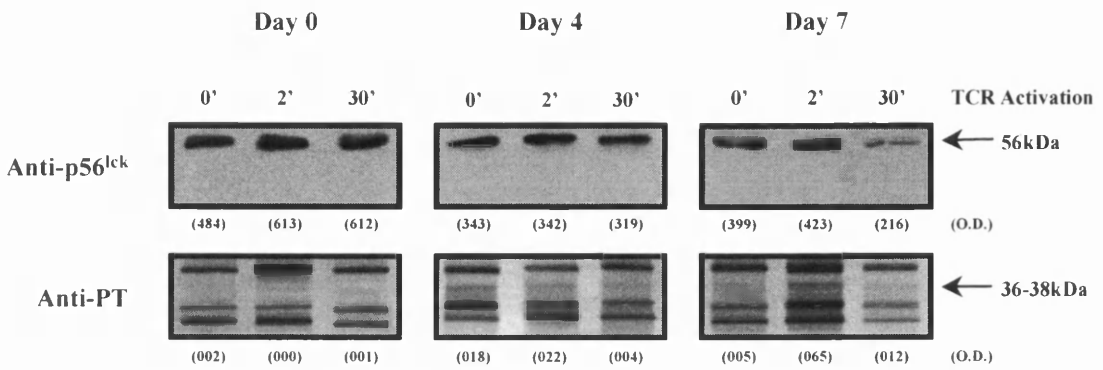
Following the observation that *in vitro* OX38 mAb treatment of CD4 T cells leads to altered intracellular signalling in these cells, studies were performed on CD4 T cells treated with OX38 mAb using the standard *in vivo* treatment protocol. Specifically, these studies were designed to determine whether the perturbation of CD4 T cell function responsible for the increased cardiac allograft survival in recipients given a heart allograft 4 days after the last

dose of OX38 mAb, was associated with alteration in the normal biochemical signalling cascade. CD4 T cells were obtained from animals at various time-points following the standard *in vivo* OX38 treatment protocol and then tested *in vitro* using the same immobilised anti-αβTCR activation system described above. Figure 4.5 shows the results of one such experiment and the results obtained in this experiment were typical of replicate experiments performed using the same conditions.

The format of Figure 4.5, is identically to that used previously for Figure 4.3, but the CD4 T cells used for this study were obtained from animals treated with OX38 mAb *in vivo* and were harvested after 0, 4 and 7 days following the last dose of anti-CD4 mAb.

Interestingly, the phosphorylation of the 36-38 kDa protein observed after activation of normal CD4 T cells was not observed in CD4 T cells obtained from the animals on day 0 or day 4 after OX38 mAb treatment. However, a marked increase in phosphorylation of this protein was apparent after 2 Min of T cell activation in the CD4 T cells obtained from animals 7 days after OX38 mAb treatment (OD 005 compared to 065 at 0 and 2 Min TCR activation respectively). It is notable that the difference observed in protein phosphorylation could not be accounted for by differences in the quantity of lysate loaded onto the gel. The top panel of Figure 4.5 confirms that within each group the OD of the $p56^{lck}$ staining is similar for the different lysate samples loaded on the gel.

Figure 4.5 Inhibition of TCR activation-dependent tyrosine phosphorylation of CD4 T cells by *in vivo* OX38 mAb treatment



Immunopurified $CD4^{+ve}$ T cells were obtained from animals treated with the standard *in vivo* OX38 mAb protocol. CD4 T cells were obtained on Day 0 (left panels), Day 4 (middle panels) and Day 7 (right panels) after OX38 mAb treatment and were activated using immobilised anti-αβTCR mAb. Assays were quenched by the addition of SDS sample buffer after 0, 2 and 30 Min of activation. Cell lysates were analysed by 10% SDS-PAGE and proteins transferred to nitrocellulose paper by western blotting. Specific staining of proteins was performed using antibody mediated chemoluminescence and autoradiography. The lower 3 panels show the typical tyrosine phosphorylation patterns observed using anti-phosphotyrosine antibody staining. The upper 3 panels represent staining of the same gel using anti-$p56^{lck}$ antibody. The optical density (O.D.) of the 36-38 kDa protein band and $p56^{lck}$ are shown below each lane.

The data from this experiment suggest that the alteration in intracellular signalling observed after *in vitro* pre-treatment of $CD4^{+ve}$ T cells with the mAb OX38 is also observed in CD4 T cells from animals treated with OX38 *in vivo*. However, the impairment in CD4 T cell signalling after *in vivo* OX38 mAb appears to be transient and/or reversible, as CD4 T cells obtained from animals 7 days following the last dose of OX38 mAb have signalling patterns identical to those seen in normal CD4 T cells. As noted already, the signalling defect observed in the CD4 T cell obtained from *in vivo* OX38 mAb treated recipients correlates with a functional impairment in the animals since those transplanted with an allogeneic

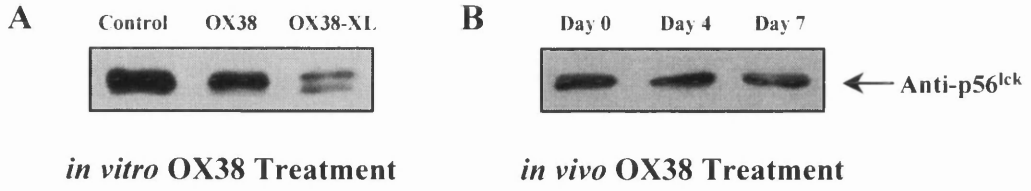
cardiac allograft on day 4 after OX38 mAb treatment do not reject their heart grafts, whereas those transplanted on day 7 following OX38 are able to mediate heart allograft rejection.

4.2.7 Analysis of CD4 immunoprecipitates for evidence of dissociation of $p56^{lck}$

A possible mechanism for altered intracellular signalling in OX38 mAb treated CD4 T cells is the dissociation of $p56^{lck}$ from the cytoplasmic tail of the CD4 molecule following anti-CD4 mAb binding. To determine whether evidence for this could be found in the present experimental system, *in vitro* OX38 treated CD4 T cells and CD4 T cells from OX38 treated animals were lysed in non-ionic buffer and the CD4 immunoprecipitated using anti-CD4 and Staph Protein A. Immunoprecipitates were then analysed by SDS-PAGE -nitrocellulose western blotting and were stained directly using anti-$p56^{lck}$. The results of this experiment are depicted in Figure 4.6. The left panel (A) of Figure 4.6, shows the staining pattern of CD4 immunoprecipitated $p56^{lck}$ which was observed in cell lysates from CD4 T cells after *in vitro* pre-treatment with PBS, OX38 and OX38 cross-linked with RaMo-antibody. It can be seen that OX38 treatment results in only partial dissociation of $p56^{lck}$ from the CD4 molecule and this dissociation is most marked when OX38 mAb is cross-linked by RaMo-antibody.

When CD4 T cells were obtained from animals on days 0, 4 and 7 following the last dose of *in vivo* anti-CD4 treatment, using the standard OX38 treatment protocol, and analysed in the same fashion as the *in vitro* OX38 treated cells, no change in the staining pattern of CD4 immunoprecipitated $p56^{lck}$ was observed. The results from this experiment are displayed in the right panel (B) of Figure 4.6. This latter observation argues against the idea that the altered intracellular signalling which occurs after anti-CD4 treatment can be attributed wholly to dissociation of $p56^{lck}$ from the CD4 molecule.

Figure 4.6 CD4 associated p56^{lck} following *in vitro* and *in vivo* OX38 pre-treatment



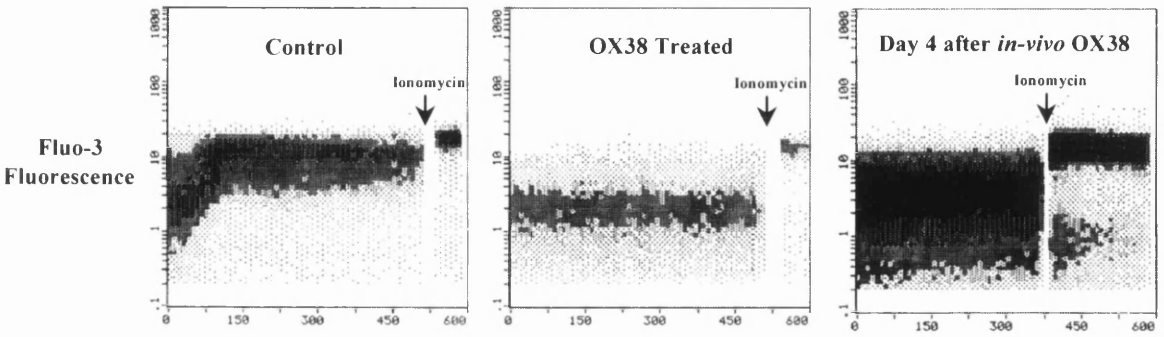
Immunopurified CD4^{+ve} T cells were obtained from naïve untreated animals and pre-treated *in vitro* with PBS (Control, panel A left), OX38 (OX38 Treated, panel A centre) and OX38 which was cross-linked using Rabbit anti-Mouse antibody (OX38-XL, panel A right) or were obtained on day 0 (Day 0 panel B left), on day 4 (Day 4 panel B centre) and day 7 (Day 7 panel B right) from animals treated with *in vivo* OX38 mAb (Standard Protocol). CD4 T cells were lysed using non-ionic buffer and immunoprecipitated using anti-CD4 antibody and staph protein A sepharose. Immunoprecipitated pellets were solubilised in SDS sample buffer and analysed by 10% SDS-PAGE. Proteins were transferred to nitrocellulose paper by western blotting and specific staining performed using anti-p56^{lck} antibody mediated chemoluminescence and autoradiography.

4.2.8 Effect of OX38 on intracellular Ca^{2+} influx following anti-TCR induced activation

In view of the observed differences in the phosphotyrosine labelling pattern of CD4 T cells after *in vitro* and *in vivo* OX38 mAb treatment, it was decided to determine if the same OX38 mAb therapy also abrogated downstream signalling events, such as influx of intracellular calcium, which normally follow ligation of the αβTCR. After TCR activation, the level of intracellular Ca^{2+} increases, due to the activation of Phospholipase C which cleaves PIP_2 to produce IP_3. The latter molecule mediates Ca^{2+} release from intracellular Ca^{2+} vesicles and initiates an influx of Ca^{2+} from the extracellular milieu. This rapid and sustained increase in intracellular Ca^{2+} is then thought to influence Ca^{2+} / calmodulin -dependent events which are essential for the production of key cytokines such as IL-2 [288,289,290,291].

To test the integrity of the intracellular signalling pathway distal to the PTKs, changes in intracellular Ca^{2+} were measured indirectly by pre-loading immuno-magnetically enriched $CD4^{+ve}$ T cells with the calcium dependent dye Fluo-3. Fluo-3 requires active cell metabolism for loading and therefore only labels living cells. CD4 T cells were obtained from naïve animals and pre-treated *in vitro* with PBS (Control) or OX38 mAb. Alternatively, CD4 T cells were obtained from animals 4 days after the last dose of OX38 mAb using the standard *in vivo* treatment protocol. All CD4 T cells were activated *in vitro* using immobilised R73 mAb and continuous assessment of cell fluorescence was performed using the flow cytometer. The results of such an experiment are depicted in Figure 4.7. It can be seen that there is an increase in Fluo-3 fluorescence with time after TCR activation in Control CD4 T cells. However, the increase in fluorescence is abrogated in CD4 T cells after *in vitro* OX38 mAb treatment and in CD4 T cells obtained on Day 4 after *in vivo* OX38 mAb treatment. After addition of the calcium ionophore, ionomycin, to each of the different cell populations, maximum Fluo-3 fluorescence was observed and was equivalent in each of the samples. The latter finding confirms that loading of the intracellular dye was comparable in the different experimental groups and that lack of fluorescence after activation in the OX38 treated T cells was not due, therefore, to absence of Fluo-3 dye. It can be seen from Figure 4.7 that the width of the fluorescent band is slightly broader in the analysis of CD4 T cells from anti-CD4 treated animals. This is probably attributable to a slightly higher cell number in the sample of this particular experiment.

Figure 4.7 Inhibition of αβTCR-activated calcium-dependent fluorescence by *in vitro* and *in vivo* OX38 mAb pre-treatment



CD4 T Cells were obtained from naïve animals and pre-treated *in vitro* with PBS (Control, left panel), or OX38 (OX38 Treated, centre panel) or were obtained from animals 4 days after using the standard *in vivo* OX38 treatment protocol (Day 4 after *in vivo* OX38, right panel). CD4 T cells were activated using immobilised R73 mAb while assessing the change in fluorescence of the calcium dependent dye Fluo-3, using an EPICS XL®Flow cytometer (Coulter, Luton, UK). The y-axis represents the Log_{10} of Fluo-3 fluorescence and the x-axis represents the time in seconds after initiation of TCR activation.

4.3 DISCUSSION

CD4 T cell inhibition by anti-CD4 mAb therapy may be mediated by at least three non-exclusive mechanisms. Firstly, by binding to the CD4 molecule, the mAb may cause steric inhibition of the interaction of the CD4 molecule with the non-polymorphic region of the β chain of MHC II (Loop 3 β2). Secondly, after binding to the CD4 molecule, the mAb may sequester or 'cap' cell-surface CD4 molecules to a particular region on the cell-surface so that association of CD4 with MHC Class II - TCR - CD3 is not able to readily occur. Third, by

binding to CD4 molecule, the anti-CD4 mAb may *'stimulate' or 'inhibit' the normal intracellular signalling mechanism in such a way that once the CD4 - TCR - MHC Class II complex is formed, the CD4 T cells are not able to undergo normal activation. This latter process has been collectively called "negative signalling". Previous studies have shown that OX38 mAb can inhibit CD4 T cell function both *in vivo* and *in vitro* but have not sought evidence for negative intracellular signalling.

The results reported in this chapter demonstrate that anti-CD4 mAb inhibits normal CD4 T cell function and is associated with an alteration in tyrosine phosphorylation pattern which follows αβTCR activation. The perturbation of CD4 T cell function caused by CD4 ligation with OX38 mAb, outlasted the physical interaction between antibody and ligand, suggesting that the antibody pre-treatment did result in some form of "negative signalling" within the CD4 T cell itself. This observation has not previously been reported. Negatively signalled CD4 T cells were, when challenged *in vivo* with alloantigen in the form of a full MHC mismatched vascularised cardiac graft, unable to mediate allograft rejection, and when challenged *in vitro*, using immobilised anti-αβTCR antibody, failed to phosphorylate the 36-38 kDa protein and failed to mobilise intracellular Ca^{2+} stores. The alteration in intracellular signalling could not be attributed to dissociation of $p56^{lck}$ from CD4, as no appreciable differences were detected between normal CD4 T cells and anti-CD4 treated CD4 T cells in this experimental system.

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* Stimulation of $p56^{lck}$ when it is juxtaposed to inappropriate substrate could result in the generation of negative signals.

Following TCR activation, tyrosine phosphorylation of cytoplasmic T cell proteins is a necessary prerequisite for induction of IL-2 synthesis [212,292]. Using an activation system employing immobilised cross-linked anti-αβTCR mAb, an increase in the tyrosine phosphorylation of a 36-38 kDa protein band over time was observed. Additionally, in cells which had been treated with OX38 mAb, *in vitro* or *in vivo*, phosphorylation of this protein was inhibited in this experimental system. Other published studies using Th1 T cell clones [285] or naïve LNC [286] have also shown TCR activation-dependent phosphorylation of a 38 and a 75 kDa T cell protein. Interestingly, in these studies when the Th1 clones were rendered anergic *in vitro* or when the normal T cells were made unresponsive to superantigens using an *in vivo* anergy protocol, there was a reduction in the phosphorylation of these two proteins following T cell activation. In addition, these studies found that the alteration in protein phosphorylation was associated with a concomitant defect in both IL-2 production and utilisation, suggesting that the two proteins may, in part, be involved in IL-2 production and/or up-regulation. The defect in tyrosine phosphorylation observed in the present studies correlated with a functional defect in CD4 T cells, as demonstrated by decreased T cell proliferation *in vitro* and by lack of cardiac allograft rejection *in vivo* (when transplantation was delayed to day 4 following last OX38 mAb treatment). Furthermore, the anti-CD4 treated T cells displayed additional functional defects consistent with abnormalities in IL-2 production. In preliminary experiments, when *in vitro* or *in vivo* anti-CD4 treated T cells were analysed for TCR activation-dependent calcium mobilisation, the anti-CD4 treated T cells did not, in contrast to normal cells, release intracellular calcium freely into the cytoplasm.

Active $p21^{ras}$ has been shown to be important in the induction of the IL-2 gene in T cells [278,279]. Moreover, as mentioned earlier, the PKC independent $p21^{ras}$ activating pathway

appears to involve the participation of a 36-38 kDa protein which phosphorylates following TCR activation [272] but which fails to phosphorylate in anergic T cells. It is reasonable to suggest that the 36-38 kDa protein which has been the focus of attention in this chapter, may be a $p21^{ras}$ activating protein or an early down stream substrate of active $p21^{ras}$ which is necessary for normal IL-2 production. Further characterisation of the 36-38 kDa protein is necessary to elucidate the relationship between this protein and $p21^{ras}$. To provide evidence for association of the 36-38 kDa protein with $p21^{ras}$, immunoprecipitation-immunochemoluminescence experiments using antibodies raised against these proteins would be helpful. Additionally, once the 36-38 kDa protein has been further characterised, examination of tyrosine phosphorylation and IL-2 production using CD4 T cells from 36-38 kDa KO animals would provide further insight into the role and relationship between the 36-38 kDa protein with $p21^{ras}$ and with IL-2 production.

One possible mechanism by which anti-CD4 mAb therapy may result in altered intracellular signalling events is by modulation of $p56^{lck}$ from the cytoplasmic tail of the CD4 molecule. Anti-CD4 mAb treatment has been shown to lead to a reduction in the CD4 bound $p56^{lck}$ fraction in *Herpes saimiri* virus immortalised human T cell clones and to cause a concomitant reduction in total $p56^{lck}$ kinase activity [282]. In another study using murine lymph node T cells, dissociation of CD4 from $p56^{lck}$ after anti-CD4 mAb pre-treatment was also noted [284]. However the latter study, when comparing anti-CD4 treated and untreated control T cells following anti-$\alpha\beta$TCR stimulation, failed to demonstrate any detectable differences in the early tyrosine phosphorylation pattern or Ca^{2+} mobilisation. A ten fold inhibition of DEOXYRIBONUCLEIC ACID (DNA) synthesis in the anti-CD4 treated group was, however noted. Both of the above studies suggested that the alteration in T cell activation observed after anti-CD4 mAb, was a consequence of dissociation of $p56^{lck}$ from the CD4 molecule,

thus removing effective kinase activity from the TCR and other downstream targets. However, not all studies using protocols which demonstrate altered intracellular signalling have been able to show dissociation of $p56^{lck}$ from CD4. For example, a study which examined the effect of humanised mouse anti-CD4 antibody on resting and activated human CD4 T cells, found qualitative differences between the mAb treated and untreated T cells and showed partial modulation of the CD4 molecule associated with reduced tyrosine phosphorylation of cytoplasmic proteins following anti-CD4 antibody pre-treatment. However, when remaining CD4 molecules were isolated by immunoprecipitation, this study found no measurable dissociation of $p56^{lck}$ from CD4 [283]. Interestingly, treatment of CD4 T cells with phorbol esters and anti-CD3 antibodies [293] and ligation of CD2 in Jurkat cells [294] have been shown to provoke dissociation of $p56^{lck}$ from CD4. Treatment with phorbol esters, mitogenic lectins, and stimulation via the antigen receptor complex also causes marked phosphorylation of $p56^{lck}$ at N-terminal serine residues and this is associated with reduced autophosphorylation and $p56^{lck}$ activity [295]. It is also associated with retardation in the electrophoretic mobility in SDS-PAGE, increasing the apparent molecular weight of the molecule to 59-60 kDa and is likely to change the interactions of $p56^{lck}$ with a subset of its regulators, including CD4 itself [296,297]. Both of the studies discussed above, showing dissociation of $p56^{lck}$ from CD4 following anti-CD4 antibody treatment, found that the $p56^{lck}$ which was lost from the pelleted immunoprecipitate was the heavier of a protein doublet fraction and the possibility remains that the altered signal transduction and observed dissociation may have been due to serine phosphorylation provoked by the anti-CD4 pre-treatment or some other factor unique to the individual experimental design in these studies. In the experiments described in this chapter, immunoprecipitation using <u>non-activated</u> immunopurified $CD4^{+ve}$ LNCs treated with anti-CD4, administered either *in vivo* or *in vitro*, failed to demonstrate appreciable dissociation of $p56^{lck}$ from CD4. A prerequisite for $p56^{lck}$

dissociation from CD4 may be modulation of CD4 with subsequent degradation of one or both components in the lysosomal compartment of the cell [298]. As OX38 mAb causes little in the way of CD4 modulation, perhaps this pathway of $p56^{lck}$ – CD4 dissociation is not utilised in this experimental model.

Exactly how OX38 pre-treatment leads to altered tyrosine phosphorylation of cytoplasmic proteins in the studies described remains unsolved. The anti-CD4 antibody used in the experiments described were all bivalent. One can envisage that potential sequestering of $p56^{lck}$ via CD4, away from the natural downstream targets could be responsible for the observed results. Further experiments utilising monovalent Fab fragments (which do not lead to aggregation of CD4) may help to elucidate this point. Another possible mechanism for the observed alteration in intracellular signalling could be that, as a result of OX38 binding to CD4, that dephosphorylation or autophosphorylation of $p56^{lck}$ may occur. There were no demonstrable changes in tyrosine phosphorylation as a result of CD4 antibody binding observed in the present experimental, but further studies looking specifically phosphorylation and dephosphorylation at other amino acid residues such as serine moieties would be helpful.

4.4 KEY POINTS

- **The pre-operative anti-CD4 treatment protocol employed in this study results in a 'window of opportunity' for tolerance induction which is not dependent on the physical presence of residual antibody: Allograft survival occurs when animals are transplanted early after antibody has been cleared from the cell-surface and serum.**

- **Activation of CD4 T cells using immobilised anti-TCR antibody results in tyrosine phosphorylation of a 36-38 kDa protein band which is inhibited by *in vitro* OX38 treatment.**

- **Cells taken from animals treated with anti-CD4 *in vivo* during the "window of opportunity" for tolerance induction, also display defective TCR activation induced tyrosine phosphorylation of the 36-38 kDa protein band.**

- **Neither *in vitro* or *in vivo* anti-CD4 treatment results in significant dissociation of $p56^{lck}$ from CD4 molecules.**

- **Both *in vitro* and *in vivo* anti-CD4 treatment affects normal intracellular calcium release from CD4 T cells.**

5. The role of the thymus in anti-CD4 monoclonal antibody induced tolerance

5.1 INTRODUCTION

The role of the thymus in transplantation rejection is well recognised as congenitally athymic T cell deficient animals readily accept a variety of allografts, with no evidence of rejection. The importance of the thymus in transplantation tolerance is less well understood. Recently, there has been much interest in the concept of inducing transplantation tolerance by injection of donor alloantigens directly into the thymus gland. A number of experimental studies using adult rats [299,300,301,302,303] have shown convincingly that intrathymic injection of donor antigen, in most cases together with some form of systemic immunosuppression, can induce tolerance to an organ allograft, underscoring the potential importance of the thymus gland as a site for tolerance induction. It should be emphasised that it is far form clear, however, how introduction of alloantigen directly into the thymic microenvironment in such studies promotes the development of transplant tolerance.

Interestingly, the role of the thymus in transplantation tolerance extends beyond the direct inoculation of antigen into the thymic gland itself and the thymus gland has been found to be important in several models employing different strategies to induce transplantation tolerance in both rodents and larger animals. Of particular relevance to the studies described in this thesis, Herbert and Roser [160], using OX38 mAb pre-treatment in ACI ($RT1^a$) recipient rats receiving Lewis ($RT1^l$) cardiac allografts found that thymectomy of the recipient animal, impaired the induction of tolerance in this experimental model. David Sachs and his collegues in Boston U.S.A. [304] using inbred miniature swine in which transplant tolerance was induced by treatment of recipient animals with a 12 day course of Cyclosporin A, showed

that thymectomy prior to transplantation (performed 21 and 42 days before transplant) abrogated tolerance induction to Class I MHC disparate renal allografts. Interestingly, if thymectomy was performed following renal transplantation it did not affect induction or maintenance of tolerance in this model.

The thymus is able, in principle, to directly affect the balance between allograft rejection and tolerance, by exporting new T cells to the periphery or by re-education of mature T cells which circulate within the medulla of the gland. It is likely that the local thymic environment which newly exported or re-educated T cells encounter strongly influences the subsequent response of these T cells to alloantigen. In a number of elegant experiments using euthymic PVG ($RT1^c$) and congenitally athymic T cell deficient PVG ($RT1^c$) nude rats, Bell and his group in Manchester U.K. have studied in detail the maturation of re-circulating single positive ($CD4^{+ve}$ $CD8^{-ve}$ $\alpha\beta TCR^{+ve}$) thymocytes, naïve and memory like T cells. Interestingly, they have shown that RTE ($CD4^{+ve}$ $CD8^{-ve}$ $\alpha\beta TCR^{+ve}$ $CD45RC^{-ve}$ $Thy\text{-}1^{+ve}$) when injected into euthymic animals, rapidly lose their Thy-1 antigen and express the $CD45RC^{+ve}$ high molecular weight isoform by day 7 [305]. Furthermore, presumably after antigen contact, by day 14 up to 30% of the transferred cells were found to have reverted back to the $CD45RC^{-ve}$ low molecular weight isoform, showing that expression of the CD45RC isoform *in vivo* in normal animals is dynamic and correlates to the isotype switching of these cells observed *in vitro* [306]. Additionally, when athymic PVG nude animals are reconstituted with $CD45RC^{+ve}$ single positive CD4 T cells taken from naïve PVG animals, they show complete restoration of peripheral T cell function with normal GVH responses, allograft rejection and thymus-dependent antibody responses [236]. Conversely, adoptive transfer of the $CD45RC^{-ve}$ $Thy\text{-}1^{-ve}$ subset does not confer normal T cell responses in nude recipients. However, if donor PVG ($RT1^c$) animals are given a DA blood transfusion prior to

cell harvesting, it is the CD45RC^{-ve} Thy-1^{-ve} and not CD45RC^{+ve} subset which is responsible for initiating DA allograft rejection in the PVG nude rat. Interestingly, however, if PVG nude rats are given a DA allogeneic blood transfusion prior to transplantation and cell transfer, then the CD45RC^{-ve} subset is prevented from regaining an alloaggressive capacity in these cell transfer experiments. Taken together, this data is consistent with the idea that isotypic switching of the leukocyte common CD45RC antigen (probably in both RTE and peripheral single positive CD4 T cells) reflects a major functional re-programming of CD4 T cells and significantly affects the ability of the T cell to respond to antigen.

In the work presented in this section of the thesis, the role of the thymus gland in anti-CD4 mAb induced tolerance induction was further investigated and some of the possible mechanisms whereby the thymus may contribute to tolerance were explored. Adult thymectomy was performed prior to transplanting DA recipients with Lewis cardiac allografts, and the effect of thymectomy on anti-CD4 induced tolerance was examined. The ability of prior thymectomy to abrogate the capacity of T cells from tolerant animals to adoptively transfer tolerance into secondary recipient animals with unmodified primary Lewis cardiac allografts was also explored. After examining the residual CD4 T cell subset population following anti-CD4 mAb treatment, as discussed earlier in Chapter 3, the role of RTE cells was assessed. RTE were found to be preferentially spared from the depleting effects of OX38 mAb treatment. A possible role for RTE in linking the induction and maintenance of tolerance in this experimental model was, therefore, sought by a series of adoptive transfer experiments in which thymectomised animals were reconstituted with single positive (CD4^{+ve} $\alpha\beta$TCR^{+ve}) thymocytes. Finally, because of the observation that RTE were preferentially spared following anti-CD4 mAb treatment in this model, and because studies using murine thymocytes has found that such cells secrete a mixed pattern of cytokines when

stimulated *in vitro* [307], the ability of OX38 mAb to inhibit the proliferation and alter the cytokine mRNA profile of single positive $CD4^{+ve}$ $\alpha\beta TCR^{+ve}$ thymocytes, following *in vitro* activation using immobilised anti-αβTCR mAb was examined.

5.2 RESULTS

5.2.1 Adult thymectomy prevents the ability of anti-CD4 mAb to induce tolerance to cardiac allografts

The effect of adult thymectomy on the ability of OX38 mAb to induce tolerance to Lewis cardiac allografts in DA rats was examined by thymectomising DA animals and then transplanting them with a Lewis heart more than 40 days later, under the cover of anti-CD4 (OX38) therapy. As expected from the studies of Herbert and Roser [160], adult thymectomy prevented the induction of transplant tolerance in this experimental model. All, except one of eight thymectomised animals, rejected their heart allografts and the kinetics of rejection were similar to those observed for control untreated animals. Table 5.1 below summarises the graft survival data of these experiments.

Table 5.1

Group	Pre-Transplant Treatment	mAb*	n	Graft Survival (days)	MST (days)
1	None	OX21	7	8,9,10 x 2,11,14 x 2	10
2	None	OX38	7	8,>100 x 6	>100
3	Thymectomy > -40 days (before Transplant)	OX38	8	7 x 2,8,11 x 3,23,>100	11

***mAb was administered by IP injection at a dose of 10mg/kg (day –3), and 2mg/kg (days –2, –1, 0). DA recipient animals received a Lewis cardiac allograft on day 0.**

The data for first two experimental groups in Table 5.1, showing the heart allograft survival times of euthymic animals treated with either OX38 or control OX21 mAb, have been described earlier and have only been included here for comparison. The MST in group 3 (Thymectomised animals) receiving OX38 mAb was only 11 days. The early graft rejection observed in this experimental group implies that either the thymus gland itself, or a recently emerged product of the thymus have an important role in the induction phase of anti-CD4 mAb induced tolerance in this particular model.

5.2.2 Adult thymectomy also abrogates the ability to transfer tolerance using splenocytes from tolerant donor animals

Recent studies have demonstrated that in some rodent transplantation models, transplant tolerance can be adoptively transferred from long-standing tolerant animals into new recipients bearing primary allografts [179]. Typically, such studies transfer spleen cell from the tolerant animal into naïve syngeneic secondary recipients at the time of transplantation. To determine whether T cell suppression could be observed in the present model of anti-CD4

induced tolerance, 2 x 10^8 splenocytes from DA animals bearing long-term Lewis cardiac allografts after OX38 treatment, were adoptively transferred into secondary unmodified DA recipients. The secondary animals were then challenged with a Lewis heart allograft the day following lymphocyte transfer. Table 5.2 (Group 1) shows the graft survival data following such an experiment. Four of six euthymic recipient animals receiving syngeneic splenocytes from long-term tolerant donors showed long-term graft survival and the MST for the group was greater than 100 days. Cells from tolerant OX38 mAb treated animals which had only had their heart allografts *in situ* for 21 days were, however, unable to induce tolerance when transferred to secondary recipients, as shown in Table 5.2 (Group 2). All 6 animals receiving spleen cells from OX38 mAb treated donors 21 days after heart transplant rejected their grafts with a MST of only 10 days. Thus, the ability of spleen cells from tolerant animals to transfer tolerance to secondary syngeneic recipients is critically dependent on the length of time the tolerant animal has had its heart graft *in situ*. One interpretation of this result is that the cardiac allograft itself promotes the emergence of a regulatory T cell population and the development of such regulatory T cells therefore increases with time.

Since the thymus appears important in the induction phase of OX38 mAb induced transplant tolerance, it was of interest to determine whether the presence of the thymus gland facilitated the ability of adoptively transferred "tolerant lymphocytes" to induce tolerance in the secondary recipients. To address this question, recipient DA animals were thymectomised more than 40 days prior to their use as secondary recipients of adoptively transferred T cells. Splenocytes were then obtained from long-term (greater than 100 days) OX38 treated DA animals and transferred into the thymectomised secondary recipients. The secondary DA recipients were then challenged, the following day, with a Lewis cardiac allograft. Intriguingly, adult thymectomy was found to abrogate the ability to transfer tolerance in this

model. Table 5.2 (Group 3) shows the graft survival data for the thymectomised recipients receiving cells from tolerant donors and it can be seen that they rejected their cardiac allografts with a MST of only 9.5 days.

Table 5.2.

Group	Thymectomy	Splenocytes Transferred*	n	Graft Survival (days)	MST (days)
1	None	2 x 10^8 Cells (Day >100)	6	11,13,>100 x 4	>100
2	None	2 x 10^8 Cells (Day 21)	6	10 x 5,11	10
3	> -40 days	2 x 10^8 Cells (Day >100)	6	8,9 x 2,10,11,12	9.5

***Splenocytes were obtained from OX38 treated DA rats bearing a long-standing (>100 or 21 days) Lewis cardiac allograft. Secondary syngeneic recipient animals were transplanted with Lewis cardiac allografts the day after adoptive transfer of spleen cells.**

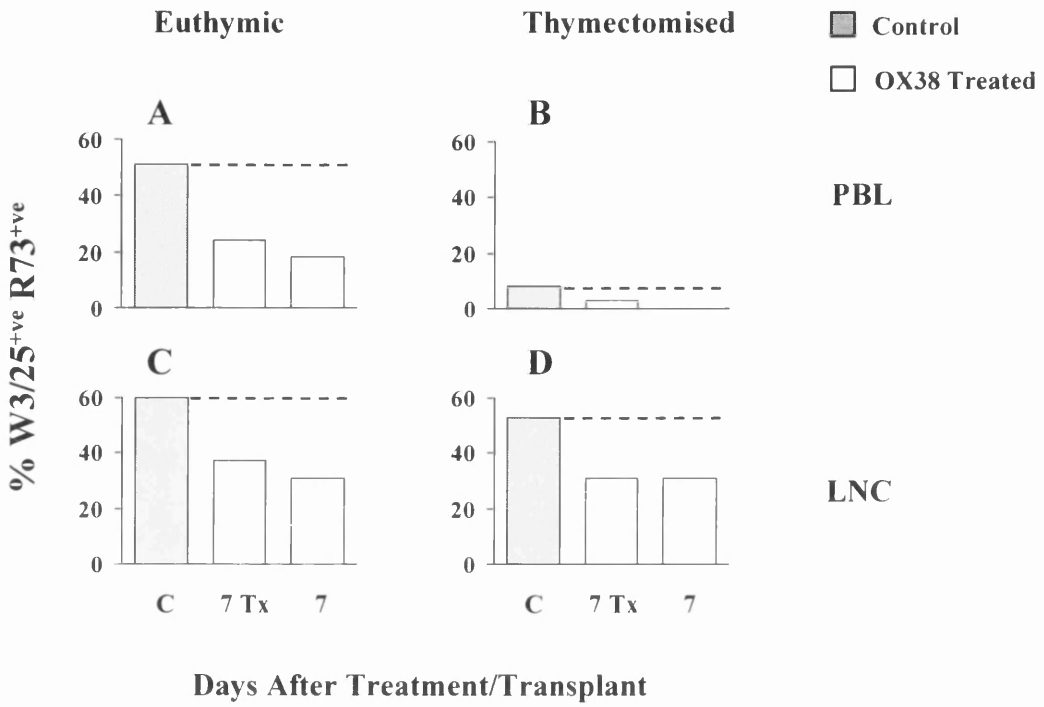
The above data demonstrates that the thymus gland plays a role in the induction phase of OX38 mAb induced transplant tolerance, and in addition, that the thymus gland (or a product thereof) facilitates the ability of tolerant lymphocytes to induce tolerance when adoptively transferred into syngeneic recipients of an organ allograft.

5.2.3 Effect of OX38 mAb on T cell depletion in thymectomised recipients

In view of the observation that thymectomy abrogated OX38 mAb induced transplant tolerance, it was of interest to determine the effect of OX38 mAb treatment on the peripheral T cell pool of adult thymectomised DA rats. Euthymic and adult thymectomised DA rats were treated with OX38 mAb, using the standard *in vivo* protocol, and sacrificed following treatment and/or transplant. As a first step to investigate the possible mechanism underlying the abrogation of tolerance induction by thymectomy, residual LNC and PBL following OX38

treatment were analysed by flow cytometry. PBL and LNC from euthymic and thymectomised untreated controls, and animals sacrificed 7 days following the standard *in vivo* OX38 treatment protocol were examined. To assess the role of exposure to alloantigen in these groups of animals, LNC and PBL were also obtained from OX38 treated euthymic and thymectomised animals which had been transplanted with a Lewis cardiac allograft 7 days earlier. As a first step, the cells which stained double positive for CD4 and αβTCR were analysed and the results are depicted in Figure 5.1. It can be seen that thymectomy alone reduced the percentage of $CD4^{+ve}$ T cells in both PBL and LNC from 53% and 60% respectively in euthymic control animals to 8% and 53% respectively in thymectomised animals. Interestingly, the presence of a heart allograft did not affect the relative depletion of CD4 T cells observed in either euthymic or thymectomised animals 7 days following OX38 treatment.

Figure 5.1 Depletion of CD4 T cells following OX38 treatment and/or transplant in euthymic and thymectomised animals



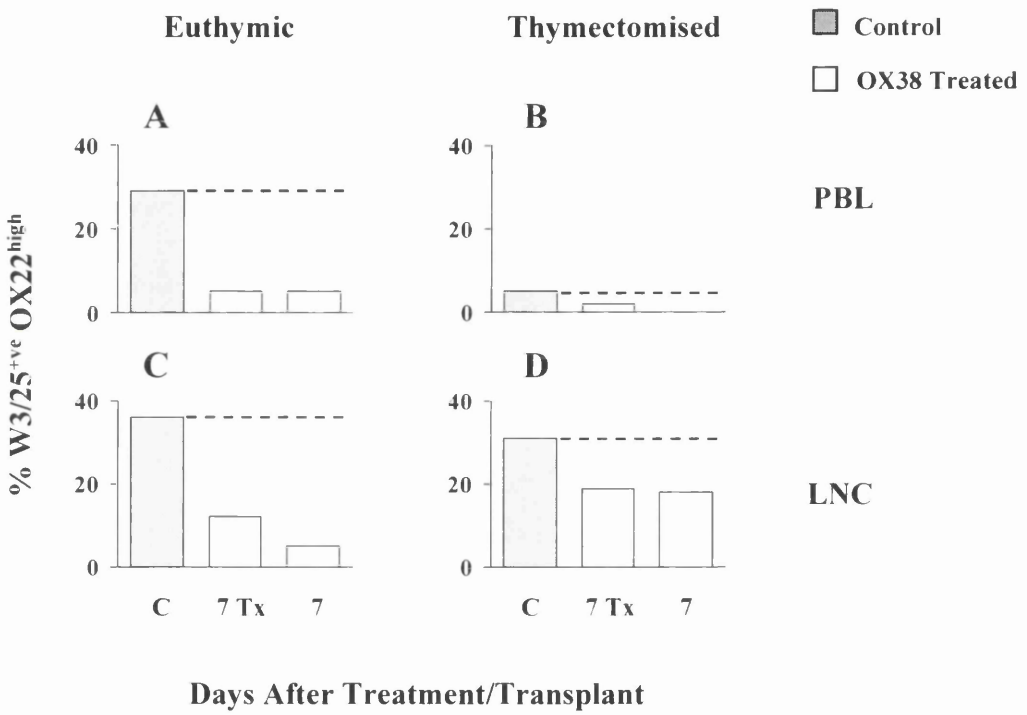
Results are expressed as the % mean number of PBL and LNC staining double positive for CD4 ($W3/25^{+ve}$) and αβTCR ($R73^{+ve}$) from a minimum of 2 rats in each group. Control untreated animals are represented by filled columns (C) whereas the Day 7 OX38 treated and transplanted (7Tx) and Day 7 OX38 treated (7) animals are represented by open columns. Left panels (A) and (C) show results in euthymic animals whereas the right panels (B) and (D) show results in thymectomised animals.

5.2.3.1 The relative depletion of $CD45RC^{+ve}$ ($MRC\ OX22^{high}$) CD4 T cell subset, is similar in euthymic and thymectomised animals following OX38 treatment

To further analyse the effect of thymectomy on animals treated with OX38 mAb, the CD4 T cell subsets were analysed by flow cytometry using MRC OX22 to stain the high molecular weight isoform of the leukocyte common antigen (CD45RC). To allow a direct comparison, both euthymic and thymectomised animals were used in each of the analysis performed.

Figure 5.2 shows that untreated thymectomised animals have substantially lower levels of W3/25^{+ve} OX22^{high} T cells in PBL than euthymic control animals. The figure also shows that the reduction in the W3/25^{+ve} OX22^{high} T cell fraction in the PBL and LNC following pre-treatment of euthymic animals with OX38 mAb is mirrored by a similar reduction of this cell population in thymectomised animals.

Figure 5.2 Depletion of the CD45RC^{+ve} CD4 T cell subset following OX38 treatment and/or transplant in euthymic and thymectomised animals

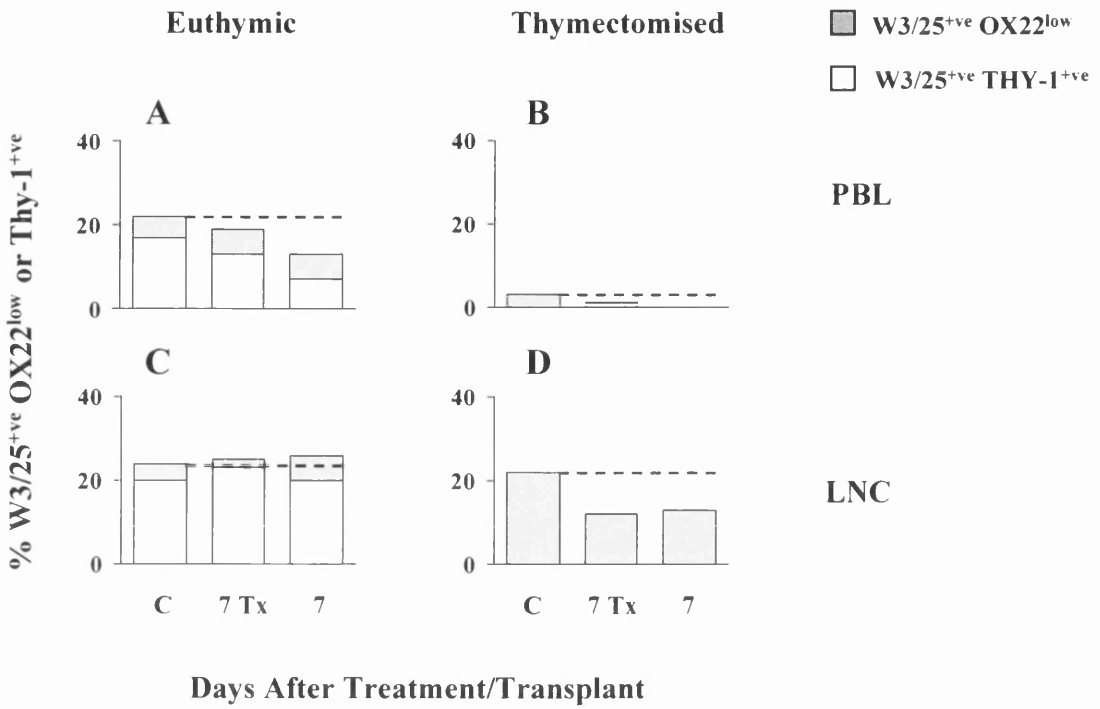


Results are expressed as the % mean number of PBL and LNC staining double positive for CD4 (W3/25^{+ve}) and CD45RC^{+ve} (OX22^{high}) from a minimum of 2 rats in each group. Control untreated animals are represented by filled columns (C) whereas the Day 7 OX38 treated and transplanted (7Tx) and Day 7 OX38 treated (7) animals are represented by open columns. Left panels (A) and (C) show results in euthymic animals whereas the right panels (B) and (D) show results in adult thymectomised rats. Upper panels (A) and (B) are from PBL and lower panels (C) and (D) are LNCs.

5.2.3.2 There is no sparing of the $CD4^{+ve}$ $OX22^{low}$ T cell fraction following anti-CD4 mAb treatment in thymectomised animals

Next, the reciprocal CD4 T cell subset, namely CD4 cells displaying the low molecular weight isoform of the leukocyte common antigen, $OX22^{low}$, were analysed by flow cytometry and the results are depicted in Figure 5.3. This figure shows that untreated thymectomised animals have far fewer $CD4^{+ve}$ $OX22^{low}$ T cells in their PBL than do euthymic animals (3% vs. 21% respectively). The data presented in this figure also confirms the previously noted observation (Chapter 3 Figure 3.8) that OX38 treatment does not appear to cause significant depletion of the $CD4^{+ve}$ $OX22^{low}$ T cell subset in euthymic animals. In contrast to euthymic animals, however, there was no sparing of the $CD4^{+ve}$ $OX22^{low}$ T cells subset in thymectomised animals. In both PBL or LNC of thymectomised animals, there was greater than 50% depletion of $CD4^{+ve}$ $OX22^{low}$ T cells following OX38 treatment. To confirm that complete thymectomy had been undertaken, and to ascertain the proportion of RTE cells in this experimental model, cells were also stained with the antibody to Thy-1 antigen [88], a transient marker of naïve RTE [305] and of thymocytes in the rat. Interestingly, when the $CD4^{+ve}$ $OX22^{low}$ T cells were counter stained with mAb to Thy-1, it could be seen that the majority of such cells in euthymic animals were Thy-1 positive (i.e. residual cells were mostly RTE). These cells could not, therefore, have been $OX22^{high}$ cells which had reverted back to the $OX22^{low}$ phenotype. Cells obtained from the thymectomised animals showed no staining with anti-Thy-1 mAb, thereby confirming the completeness of thymectomy in these animals.

Figure 5.3 Depletion of the CD45RC^{-ve} CD4 T cell subset following OX38 mAb treatment and/or transplant in euthymic and thymectomised animals



Results are expressed as the % mean number of PBL and LNC staining double positive for either CD4 (W3/25^{+ve}) and CD45RC^{-ve} (OX22^{low}), or CD4 (W3/25^{+ve}) and Thy-1 (Thy-1^{+ve}) from a minimum of 2 rats in each group. The closed columns represent the percentage of W3/25^{+ve} OX22^{low} cells whereas the open columns represent the proportion of these which are also Thy-1^{+ve}. Untreated control animals are represented as (C) whereas the Day 7 OX38 treated and transplanted and Day 7 OX38 treated only animals are represented as (7Tx) and (7) respectively. Left panels (A) and (C) show results for euthymic animals whereas the right panels (B) and (D) show results for adult thymectomised rats. Upper panels (A) and (B) are PBL and lower panels (C) and (D) are LNCs.

5.2.4 The timing of thymectomy influences tolerance induction using OX38 mAb

Because it takes several days for RTE to become functionally mature and lose their expression of the Thy-1 antigen [305], the timing of thymectomy relative to heart transplantation was altered to test, indirectly, whether residual RTE were able, in the absence

of the thymus gland itself, to facilitate induction of transplant tolerance with OX38 mAb. Thymectomy was performed on days minus 3 and plus 7 relative to cardiac transplantation. All animals received OX38 mAb according to the standard *in vivo* treatment protocol. The results of these experiments are displayed in Table 5.3.

Table 5.3.

Group	Timing of Thymectomy (relative to Tx)	mAb*	n	Graft Survival (days)	MST (days)
1	None	OX38	7	8,>100 x 6	>100
2	> -40 days	OX38	8	7 x 2,8,11 x 3,23,>100	11
3	day -3	OX38	6	28,>100 x 5	>100
4	day +7	OX38	6	>100 x 6	>100

***mAb was administered by IP injection at 10mg/kg (day –3), and 2mg/kg (days –2, –1, 0). DA recipient animals received a Lewis cardiac allograft on day 0.**

For comparison, groups 1 and 2 which have already been described earlier (Table 5.1) have been included in the Table 5.3. It can be seen from the table that delaying thymectomy to the peri-operative period, (day minus 3 and day plus 7 relative to transplant), confers protection against the allograft rejection observed when anti-CD4 mAb was given to animals which had been thymectomised >40 days before transplantation. These results suggest a role for naïve, RTE in anti-CD4 induced tolerance induction and indicate that the presence of an intact thymus gland is not, therefore, and essential requirement for OX38 mAb induced transplant tolerance.

5.2.5 Purification of CD4 single positive thymocytes using negative immuno-magnetic beading was monitored by flow cytometry

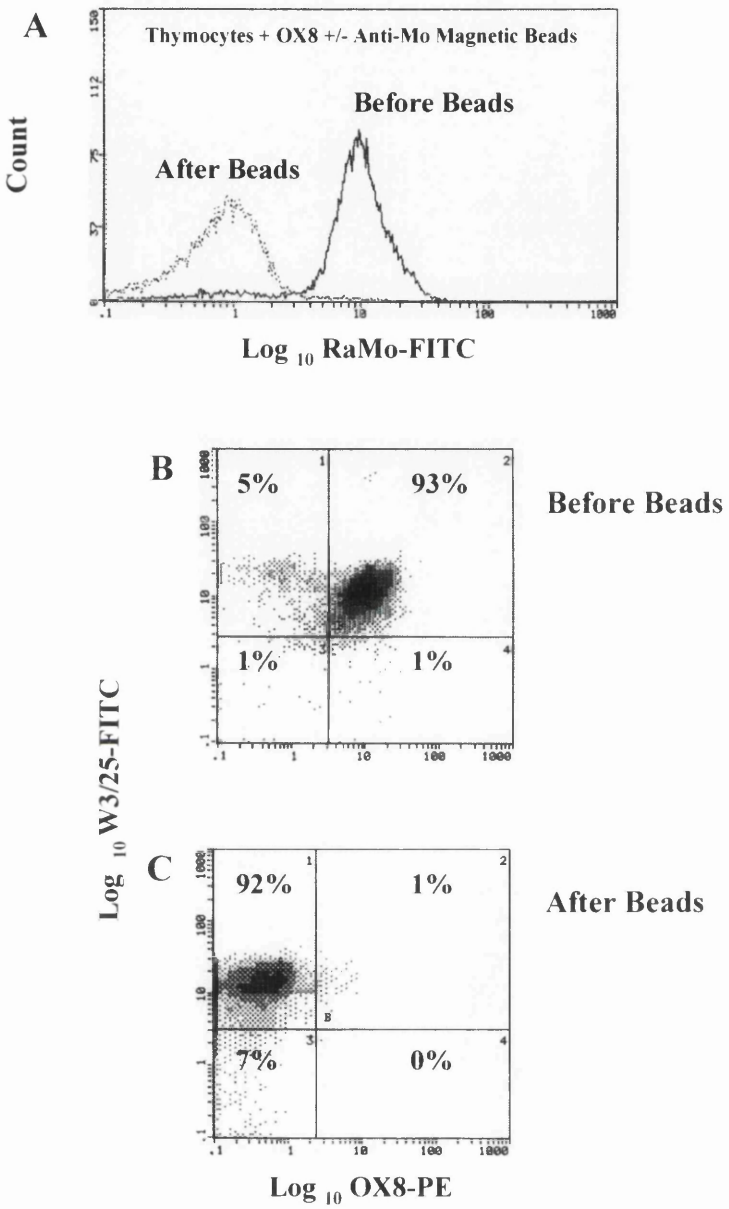
Following the demonstration that peri-operative thymectomy in anti-CD4 treated animals did not abrogate tolerance induction, and in view of the large numbers of naïve thymocytes found in the peripheral blood and lymph nodes of OX38 treated rats, it was decided to determine the role of single positive $\alpha\beta TCR^{+ve}$ $CD4^{+ve}$ thymocytes in tolerance induction, using a series of reconstitution experiments and *in vitro* cell assays.

For these experiments, whole thymus glands were obtained from 6-8 week old adult DA donors and thymocytes prepared as described in the Materials and Methods Section 2.10.1. Briefly, single positive $\alpha\beta TCR^{+ve}$ $CD4^{+ve}$ thymocytes were fractionated by negative selection using anti-CD8 (OX8) mAb and magnetic conjugated Goat-anti-Mouse beads. The purity of the fractionated thymocytes was assessed by flow cytometry and the results are depicted in Figures 5.4 and 5.5.

The top panel of Figure 5.4 shows the Log_{10} signal for Rabbit-anti-Mouse-FITC (RaMo-FITC) verses cell Count, after the addition of OX8, before and after cell separation using magnetic beads. The lower two panels of Figure 5.4 show typical double label flow cytometric analysis using anti-CD4 (W3/25-FITC) and anti-CD8 (OX8-PE) antibodies, before and after the cell separation. It can be seen that 93% of unseparated rat thymocytes stain double positive for CD4 and CD8 (Figure 5.4 panel B). After negative selection, 92% of the separated cells express CD4 and only 1% are CD8 positive (Figure 5.4 panel C). It can also be seen from Figure 5.4 (panel A), that following cell separation, no cells stained positive with RaMo-antibody. This suggests that most OX8 positive cells have been removed during the cell separation process.

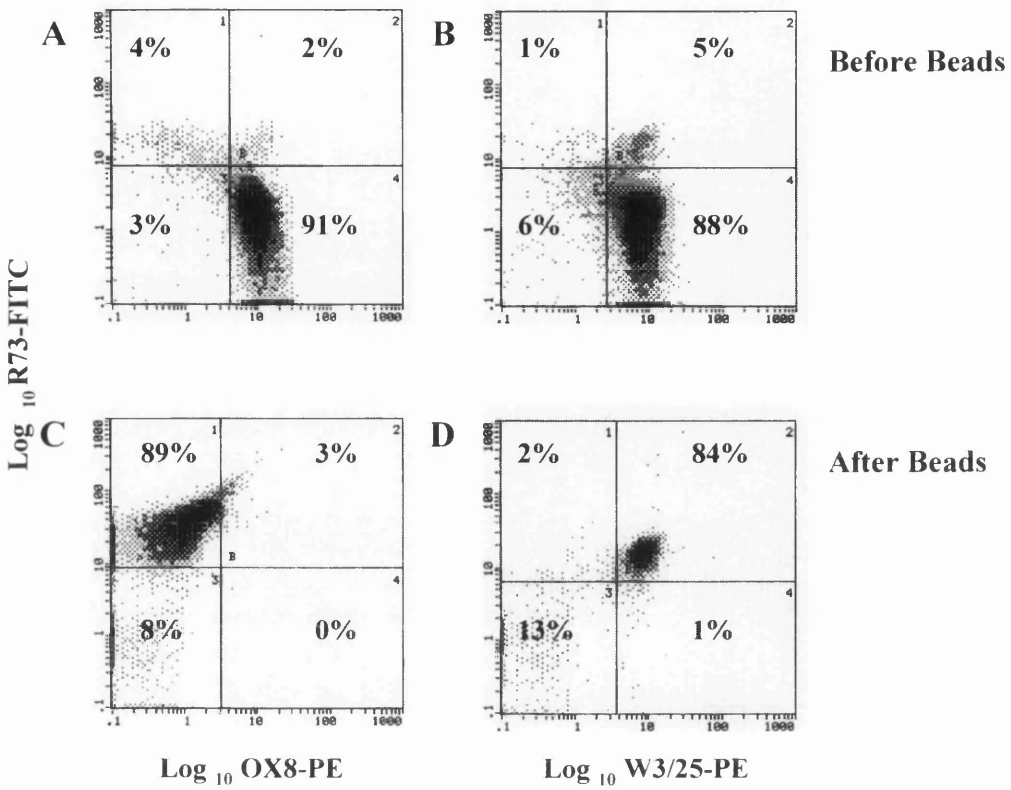
Figure 5.5, the format of which is similar to Figure 5.4, shows typical double label flow cytometric profiles of thymocytes before and after immunomagnetic cell separation, except that antibodies directed against the rat αβTCR (R73) and CD4 (W3/25) and CD8 (OX8) molecules were used to stain the separated cells. These profiles confirm that before enrichment, the majority of thymocytes were $\alpha\beta TCR^{-ve}$ $CD4^{+ve}$ $CD8^{+ve}$ (double positive-TCR negative) and following enrichment were 84% single positive $\alpha\beta TCR^{+ve}$ $CD4^{+ve}$. The remaining cell fractions comprised 2-3% single positive $\alpha\beta TCR^{+ve}$ $CD8^{+ve}$ and 8-13% $W3/25^{-ve}$ $R73^{-ve}$ (double negative) cells. These double negative cells were not characterised and may represent thymic epithelial and or stromal cells.

Figure 5.4 Single and two colour flow cytometric analysis of thymocytes before and after OX8 mAb depletion



Upper panel (A) shows the single colour fluorescence of thymocytes using FITC-conjugated rabbit-anti-mouse after OX8 treatment before and after magnetic cell separation. Single colour fluorescent analysis results expressed as count on the y-axis and Log_{10} of the FITC signal on the x-axis. Middle panel (B) and lower panel (C) shows two colour fluorescent analysis of thymocytes using FITC-W3/25 and PE-OX8 before and after magnetic cell separation respectively. Two colour fluorescent analysis results expressed as Log_{10} of the FITC signal on the y-axis and Log_{10} of the PE signal on the x-axis.

Figure 5.5 Flow cytometry of thymocytes before and after cell separation using negative selection immuno-magnetic beading



Two colour fluorescent analysis with results expressed as Log_{10} of the FITC signal on the y-axis and Log_{10} of the PE signal on the x-axis. Left panels (A) and (C) represent analysis of thymocytes after staining using FITC-conjugated anti-TCR (y-axis) and PE-conjugated anti-CD8 (x-axis). Right panels (B) and (D) depict fluorescent analysis using FITC-conjugated anti-TCR (y-axis) and PE-conjugated anti-CD4 (x-axis). Upper panels (A) and (B) shows results when staining before magnetic cell separation whereas the lower panels (C) and (D) show the staining after thymocyte separation.

5.2.5.1 Reconstitution of adult thymectomised OX38 treated animals with CD4 single positive thymocytes results in prolongation of cardiac allograft survival

Single positive CD4 thymocytes purified as described above, were utilised for adoptive transfer studies to reconstitute thymectomised anti-CD4 treated DA recipients receiving a

Lewis cardiac allograft. The CD4 thymocytes were also used in anti-αβTCR activation assays *in vitro*, to determine their cytokine repertoire after activation. Table 5.4 shows the results obtained after reconstitution of adult thymectomised (>40 days before transplant) anti-CD4 treated rats which were given 2-3 x 10^7 single positive $\alpha\beta TCR^{+ve}$ $CD4^{+ve}$ enriched thymocytes on day 2 and day 4 following heart transplantation. The CD4 reconstituted thymectomised animals (Group 3), showed prolongation of cardiac allografts survival, when compared to the thymectomised but non-reconstituted anti–CD4 treated animals (Group 2). The MST of these two groups was 81 and 11 days respectively.

Table 5.4.

Group	Thymectomy	SP Thymocytes	mAb*	n	Graft Survival (days)	MST (days)
1	None	None	OX38	7	8,>100 x 6	>100
2	> -40 days	None	OX38	8	7 x 2,8,11 x 3,23,>100	11
3	> -40 days	2-3 x 10^7	OX38	6	18,62 x 2,>100 x 3	81

***mAb was administered by IP injection at a dose of 10mg/kg (day –3), and 2mg/kg (days –2, –1, 0). DA recipient animals received a Lewis cardiac allograft on day 0. Single positive thymocytes were given by IV injection on days plus 2 and plus 4 relative to transplant.**

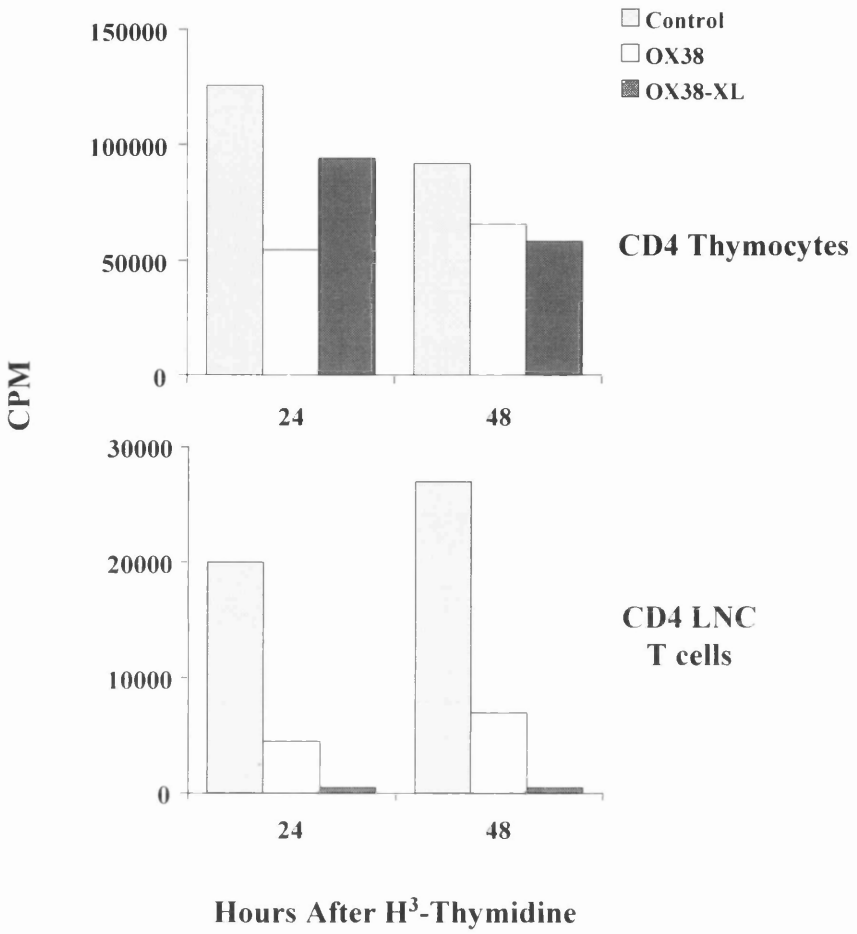
5.2.5.2 Thymocytes appear resistant to inhibition by OX38 treatment in vitro and display a Th0 like cytokine message pattern when stimulated using immobilised anti-TCR mAb

The above data suggests that the absence, in thymectomised animals, of RTE may abrogate the ability of OX38 anti-CD4 mAb to promote transplantation tolerance. The evidence for this comes from the observation that in euthymic animals given OX38 treatment, these cells appear to be preferentially spared in contrast to mature $CD4^{+ve}$ T cells, and from reconstitution experiments where, in thymectomised recipients, adoptively transferred CD4

single positive thymocytes confer tolerance. To investigate the effect of OX38 on CD4 thymocyte function, the ability of the mAb to inhibit proliferation of $CD4^{+ve}$ $\alpha\beta TCR^{+ve}$ enriched thymocytes *in vitro* in response to activation using immobilised anti-TCR was assessed. The cytokine repertoire of the thymocytes in response to TCR stimulation in the presence and absence of OX38 mAb was also determined.

Figure 5.6 (upper panel) shows the incorporation of ^{3}H-Thymidine by CD4 thymocytes after 24 and 48 hrs of culture in the presence of bound anti-αβTCR mAb. For comparison, the proliferation of CD4 LNC (lower panel), which has been described earlier (Chapter 4 Figure 4.4) is also shown. In the experiment illustrated, CD4 thymocytes and LNC were treated, prior to activation with PBS (Control), OX38 mAb or OX38 mAb which was cross linked by RaMo-antibody. The cells were added to culture wells in which anti-αβTCR mAb (R73) had been bound. Cells were then pulsed with ^{3}H-Thymidine, harvested onto microtitration filter paper and ^{3}H-Thymidine uptake determined by counting samples on a beta liquid scintillation counter. It can be seen from Figure 5.6 that single positive LNCs are far more susceptible to inhibition by OX38 mAb than are single positive CD4 thymocytes. Moreover, this difference between CD4 thymocytes and LNC is even more pronounced if the OX38 mAb was cross-linked using RaMo-antibody.

Figure 5.6 Inhibition of anti-TCR induced proliferation of single positive $CD4^{+ve}$ TCR^{+ve} thymocytes and LNC by OX38 mAb

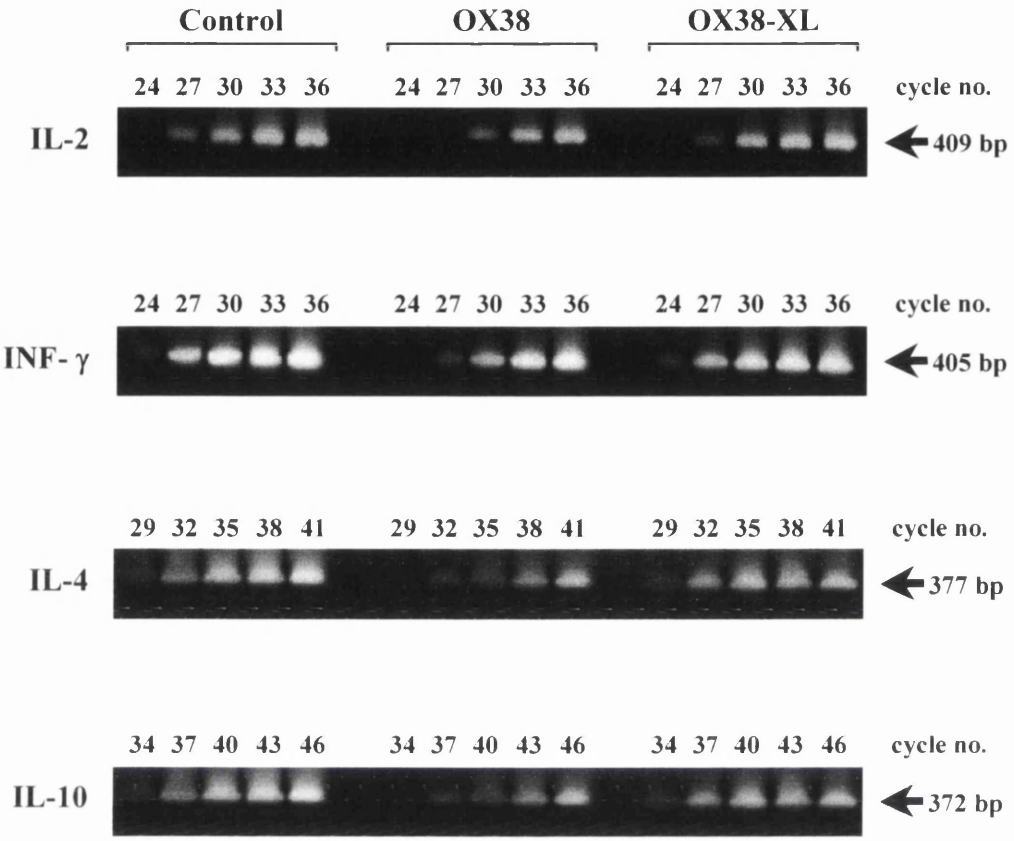


Immunopurified single positive $CD4^{+ve}$ $\alpha\beta TCR^{+ve}$ thymocytes (upper panel) or $CD4^{+ve}$ T cells (lower panel) were treated *in vitro* using PBS (Control, lightly filled columns), OX38 (OX38, open columns), and OX38 which was cross-linked using Rabbit anti-Mouse antibody (OX38-XL, darker filled columns) and were activated using immobilised anti-αβTCR mAb. Cell cultures were pulsed with ^{3}H-Thymidine at 24 and 48 hrs, harvested 18 hrs later onto microtitration filter paper, and analysed using a Beta Liquid Scintillation counter. The y-axis represents the cpm whereas the x-axis shows the time in hours when the cells were pulsed with ^{3}H-Thymidine.

Following TCR activation, thymocyte cell pellets were analysed for cytokine message by RT-PCR. Briefly, tissue culture plates were centrifuged at 200 x g for 8 Min and supernatants

removed so that mRNA could be harvested directly from the cell pellets. Each cell pellet was lysed as described in the Materials and Methods (Section 2.17.2) and mRNA extracted using Dynabead Oligo $(dT)_{25}$ (DYNAL A.S. Oslo, Norway) direct mRNA kit. Following cDNA transcription of mRNA, RT-PCR was performed using primers specific for rat IL-2, INF-γ, IL-4 and IL-10 cytokines. Figure 5.7 shows Polaroid photographic images obtained after stopping each RT-PCR reaction at five different cycle numbers and analysing the RT-PCR products by electrophoresis on 1% agarose gels and staining with Ethidium Bromide. The RT-PCR results show that anti-TCR activated thymocytes express high levels of both Th1 (IL-2 and INF-γ) and Th2 (IL-4 and IL-10) cytokines and that pre-treatment with OX38 did not preferentially skew this pattern towards either a dominant Th1 or Th2 response, irrespective of whether the OX38 antibody was cross-linked or not. The results also show that, in keeping with the modest inhibiting effect of OX38 on thymocyte proliferation, there was a small reduction in the cytokine message.

Figure 5.7 Effect of OX38 mAb pre-treatment on thymocyte cytokine message as determined by RT-PCR



Immunopurified single positive $CD4^{+ve}$ $\alpha\beta TCR^{+ve}$ thymocytes pre treated with PBS (Control), OX38 (OX38) and OX38 which was cross-linked using Rabbit anti-Mouse antibody (OX38-XL), were activated using immobilised anti-αβTCR antibody. RNA was extracted from the activated cell pellets and RT-PCR performed using IL-2, INF-γ, IL-4, and IL-10 specific primers. The cycle number for each cytokine are shown on the top of each figure and the size in base pairs (bp) shown on the right.

5.3 DISCUSSION

The immuno-regulatory mechanisms involved in the development of transplantation tolerance can be divided into two phases, namely the induction phase and the maintenance phase of tolerance. During the induction phase, the tolerant state appears somewhat precarious and

depending on the model employed, can be 'broken' by, for example, the administration of exogenous cytokines [182,251,308], by depleting $CD4^{+ve}$ [250,308] T cells or by temporarily removing alloantigen from the host animal [308]. Conversely, the maintenance phase of tolerance appears to be far more robust and is usually resistant to attempts at reversal by exogenous administration of IL-2 or even injection of alloreactive lymphocytes. It is only during the maintenance phase of transplant tolerance that cells from tolerant animals are able, on adoptive transfer, to confer tolerance to new unmodified recipients [250,308,309], ("Infectious Tolerance").

In the model of anti-CD4 induced tolerance utilised in this thesis, the induction phase of transplant tolerance was shown to be thymus dependent; thymectomised animals rejected their allografts with a MST of 11 days. This finding was of interest because it underscored two important issues concerning the mechanism responsible for tolerance in this experimental model. First; because CD4 T cell depletion following OX38 treatment was no greater in euthymic than in thymectomised animals, (which promptly reject their cardiac allografts in the presence of OX38 mAb treatment), it demonstrates that CD4 T cell depletion is unlikely to be responsible for tolerance in this model although, of course, CD4 T cell depletion may play an important contributory role. Second; because thymectomised, anti-CD4 treated animals reject their allografts with kinetics similar to unmodified animals, it indicates that residual T cells following OX38 treatment are sufficient in number and function to initiate graft rejection. The results described in this chapter of the thesis also suggest that induction of transplant tolerance by OX38 mAb requires additional events over and above 'negative signalling' of CD4 T cells. If 'negative signalling' occurs, it is clearly reversible.

Following partial depletion of peripheral CD4 T cells with OX38 mAb, CD4 T cell numbers gradually recover and by day 100 following transplantation, T cell numbers have returned to

near normal. Similarly, in T cell reconstitution experiments using congenitally athymic animals, adoptive transfer of mature thoracic duct isolated CD4 and CD8 T cells leads, over time, to the expansion of the transferred cells to levels comparable to those found in euthymic animals [310]. This underscores the point that the peripheral T cell pool is under strict homeostatic regulation, although the mechanisms responsible are not clear.

Following OX38 treatment, CD4 depletion was far more marked in thymectomised than in euthymic animals. It is possible that the pressure to re-expand the peripheral CD4 T cell pool following OX38 induced depletion, imposed on thymectomised anti-CD4 treated animals, is sufficient to overcome the 'inhibitory' effect of anti-CD4 treatment on residual T cells. Such a mechanism is unlikely, however, if one takes into consideration the survival data obtained when comparing OX38 treated animals which were thymectomised on days >minus 40, minus 3 or plus 7 relative to transplantation. Altering the timing of thymectomy resulted in allograft survival in the two groups of animals undergoing delayed thymectomy groups (days minus 3 and plus 7 relative to transplant), and these animals are presumably comparable to the early thymectomised (day >minus 40 relative to transplant) anti-CD4 treated animals in terms of the pressure on the residual peripheral CD4 T cell pool to proliferate. Although T cell depletion was not formally measured and compared in these 3 groups of transplanted recipients, it is unlikely that the CD4 depletion observed in the group which had thymectomy >40 days before transplant would be significantly different to the other 2 experimental groups, with the exception that Thy-1^{+ve} RTE would be absent at the time of transplant in the former group.

The thymus has a key role in producing all the αβT cell lineages. During maturation these cells develop in a stepwise fashion and are not complete until the single positive $CD4^{+ve}$ or $CD8^{+ve}$ stages are reached [311]. The thymus is also critical in the selection of T cells prior to

exportation to the periphery in that developing thymocytes with TCR which recognise self antigens are deleted. It is easy to imagine that following transplantation, foreign antigens derived from transplanted organs, either in the form of intact donor cells or antigenic donor MHC peptides, could become entrapped within the recipient's thymus gland, and as both $CD45RC^{+ve}$ and RC^{-ve} $CD4^{+ve}$ T cell subsets are known to re-circulate through the thymus [312], re-education of these circulating cells in response to donor antigen could occur and result in depletion or anergy of donor reactive clones. This model of tolerance has been termed central tolerance and has been shown to be important in several experimental systems, including tolerance induction in the rat [313]. Cutler and Bell induced tolerance in euthymic neonatal rats by injection of fully allogeneic bone marrow cells from athymic T cell deficient nude rats. Adult recipient animals were found to be chimeric, expressing the MHC and allotype marker of the donor strain, and accepted donor-specific skin allografts. Thymocytes were also shown to be chimeric but failed to respond *in vitro* to alloantigens of the donor-specific haplotype. Additionally, adoptive transfer of peripheral T cells from the tolerant euthymic adult animals to T cell deficient nude recipients bearing skin allografts of the same donor-specific haplotype, failed to initiate graft rejection in the nude recipients. This data suggests that clonal deletion, induced centrally within the thymus was responsible. As discussed earlier, thymectomy performed 3 days prior to introduction of graft alloantigen, resulted in long-term acceptance of cardiac allografts in the OX38 mAb treated animals. Thus, altering the timing of *thymectomy and removing the thymus gland immediately prior

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* All thymectomies were performed using microscopic magnification as described in the Materials and Methods Section 2.7 and as previously published [314]. After completion of these studies, all thymectomised animals were sacrificed and examined by post mortem and none were found to have evidence of incomplete thymectomy.

to transplantation still allowed OX38 mAb induced tolerance. Consequently, central tolerance as an exclusive mechanism responsible for thymus dependent OX38 mAb induced tolerance in this model can be discounted.

The observation that OX38 treated recipients bearing a cardiac allograft, displayed marked depletion of functionally mature $CD4^{+ve}$ T cells, combined with preferentially sparing of RTE, was somewhat surprising. However, preliminary experiments performed in this laboratory by Dr. E Bolton, using CD4 enriched LNCs, has found that following OX38 treatment, there is a high degree of apoptosis (data not shown). Similar experiments using CD4 enriched thymocytes may reveal that thymocytes are less susceptible to anti-CD4 activation induced apoptosis thus explaining the apparent sparing of RTE in this experimental model.

Taken together, these findings suggest a possible role for thymocytes or RTE, as opposed to thymic tissue, in OX38 mAb induced transplant tolerance. Reconstitution of thymectomised anti-CD4 treated animals with $CD4^{+ve}$ T cell enriched thymocytes could re-establish transplant tolerance. However, the adoptive transfer results must be viewed with some caution. Negative selection of CD4 single positive T thymocytes by immuno-magnetic negative beading resulted in cell purities of only 84% single positive $CD4^{+ve}$ $\alpha\beta TCR^{+ve}$ cells, as measured by flow cytometric analysis. The majority of the 'contaminating' cells were CD8, CD4 and $\alpha\beta$TCR negative and may therefore, represent thymic epithelial and/or APCs. These cells could theoretically form satellite thymic elements following transfer although this seems somewhat unlikely. However, for this reason, future reconstitution experiments should include depletion with anti-MHC Class II antibody to minimise this risk and help clarify the importance of RTE cells in this system.

Why should RTE contribute to OX38 induced transplantation tolerance in the rat? One possibility is that RTE may behave as regulatory, or suppressor T cells. It was shown in this chapter that purified $CD4^{+ve}$ thymocytes were less susceptible to inhibition by OX38 mAb than $CD4^{+ve}$ lymphocytes when activated using immobilised anti-TCR antibody. Additionally, anti-CD4 treated and activated thymocytes were found to produce both Th1 and Th2 cytokine message, as detected by RT-PCR. This result is comparable with murine studies, using single positive ($CD4^{+ve}$) thymocytes, which were found to secrete a broader range of lymphokines than peripheral lymphocytes, including interleukins 4, 5, and 10 and γ-interferon [307]. Anti-CD4 treated or untreated RTE cells could aggregate to form a T cell cluster and, by producing a mixture of cytokines, create a local micro environment which favours tolerance and which suppresses potentially alloreactive mature CD4 T cells, in a fashion analogous to that proposed by Waldmann's group. These, workers suggest that naïve Th0 cells aggregate at a cell cluster and produce low levels of IL-2 with moderate secretion of IL-4 and INF-γ. They propose that this particular combination of cytokines promotes anergy in the mature CD4 T cell population, and they have coined the phrase "infectious tolerance" to describe it [206].

Another explanation as to why RTE may be important in anti-CD4 induced tolerance, is that they (perhaps with or without alloreactive CD8 T cells which on their own are incapable of initiating graft rejection) may compete with alloreactive CD4 T cells for locally produced growth factors and or costimulatory molecules expressed on the cell-surface of APCs. Consequently, the CD4 T cells may be deprived of either appropriate pro-proliferative lymphokines and/or second signal costimulation. OX38 treated alloreactive CD4 T cells which would otherwise have been capable of overcoming 'negative signalling' and initiating

graft rejection, may then proceed to a tolerant state and in turn, result in a 'feedback loop', helping to perpetuate further tolerance induction in other emerging T cells.

The importance of the thymus and/or RTE in adoptive transfer studies where adoptively transferred thymectomised animals were found to reject their grafts with kinetics similar to control animals was not investigated further here. The role of the thymus in these experiments may be a result of any or all of the mechanisms discussed above working either independently or together. Further clarification of this complex system will require much more experimental work.

5.4 KEY POINTS

- **Adult thymectomy >40 days prior to OX38 mAb treatment negates the effectiveness of anti-CD4 to induce tolerance to a cardiac allograft and abolishes the ability to confer "infectious" tolerance in adoptive transfer studies.**

- **Thymectomised animals have less circulating CD4 lymphocytes but display the same magnitude of CD4 T cell depletion as euthymic treated animals following OX38 mAb treatment.**

- **In thymectomised anti-CD4 mAb treated animals, there is no evidence for preferential depletion of the CD45RC^{+ve} T cell subset, and in this respect, they contrast with euthymic OX38 mAb treated rats.**

- **Thymectomy on the day of first anti-CD4 treatment (day minus 3 relative to transplant), and reconstitution of adult thymectomised anti-CD4 mAb treated animals with purified single positive CD4^{+ve} thymocytes results in long-term allograft survival, suggesting a role for RTE and not the presence of intact thymic tissue in tolerance induction.**

- **Single positive CD4^{+ve} thymocytes proliferate well in response to immobilised anti-TCR stimulation, irrespective of *in vitro* OX38 pre-treatment, and produce both Th1 and Th2 cytokines as measured by RT-PCR.**

6. Final Discussion

Despite many advances in immunosuppression therapy, such as the combined use of Azathioprine and Prednisolone in the 1960's [315], the introduction of Cyclosporin A in the late 1970's [316] and the development and use of newer agents inhibiting T cell activation and proliferation such as Tacrolimus [317,318,319], Rapamycin [318,319] and Mycophenolate Mophetil [320], in most centres, recipients of first cadaveric renal allografts still only display 3 year graft survival rates of 70% [321].

The use of mAb therapy in clinical transplantation, notably the mouse mAb OKT3, (directed against the conformational epitope formed between CD3ε and either CD3δ or CD3γ) [322], is often effective in reversing acute rejection episodes [323,324,325], but does not induce transplantation tolerance. Similarly, other mAb treatments, such as anti-CD4, which successfully induce transplantation tolerance in rodents, fail, when applied to human transplantation to produce transplant tolerance. One reason why mAb which induce transplantation tolerance in rodents are less effective in humans, may be because there are differences in the tissue distribution of MHC antigens between the two species. In particular, Class II MHC antigens are not expressed constitutively on the vascular endothelium in rodents [326,327], whereas in humans, Class II MHC antigens are constitutively found on vascular endothelium [328]. Nevertheless, understanding the mechanism of action of mAb induced transplantation tolerance in animal models, such as the one described in this study, combined with a better understanding of the physiological differences between the species, may lead to more effective application of immunosuppressive therapy in human transplantation. Ultimately, this understanding, may help achieve the goal of inducing

allograft tolerance in the clinic without the need for continuous administration of non-specific anti-rejection agents.

The results described in this thesis show that a brief pre-operative course of anti-CD4 mAb, (MRC OX38), leads to donor specific transplant tolerance in the Lewis to DA rat strain combination. Transplantation tolerance was associated with partial (50%) depletion of CD4 T lymphocytes, with preferential sparing of RTE ($CD4^{+ve}$ $CD45RC^{-ve}$ $Thy\text{-}1^{high}$). The cytokine RT-PCR data obtained from tolerant and rejecting cardiac allografts and the alloantibody responses of transplanted animals, did not suggest that tolerance achieved with OX38 mAb in this particular experimental model could be explained by a shift from a 'detrimental' Th1 to a 'beneficial' Th2 type of cytokine response. Anti-CD4 mAb was found to influence the residual non-depleted CD4 T cell pool, since cells displayed altered intracellular signalling in response to αβTCR activation and the alteration observed was similar to the signalling defects described previously in anergic T cells [285,286].

The role of the thymus gland in transplantation tolerance in this experimental model was also assessed. Anti-CD4 induced transplantation tolerance and adoptive transfer of tolerance from long-term tolerant animals to untreated thymectomised recipient animals were both found to be thymus dependent. A possible role for RTE in OX38 mAb induced tolerance was indirectly explored by studies using single positive ($CD4^{+ve}$ $\alpha\beta TCR^{+ve}$) thymocytes and LNC. In reconstitution experiments using thymectomised anti-CD4 treated animals, adoptive transfer of single positive thymocytes, restored the ability of OX38 mAb to prolong allograft survival in thymectomised animals. Furthermore, using αβTCR proliferation assays, single positive thymocytes were found to be less affected by anti-CD4 mAb *in vitro* than single positive LNC and thymocytes still produced both Th1 and Th2 cytokine mRNA upon activation.

These data suggest that in the Lewis to DA heterotopic cardiac transplantation model, OX38 mAb may preferentially deplete T effector cells capable of initiating allograft rejection and render the residual T cell pool transiently incapable of normal activation and in a state which may favour anergy induction. RTE may provide the key for lasting tolerance induction in this experimental model by producing both Th1 and Th2 cytokines when in contact with alloantigen. One may speculate that, like the model proposed by Cobbold and collegues [206], a cellular collaborative unit may involve competition for the cell-surface of an APC between OX38 mAb treated RTE, OX38 mAb treated Th1 or Th2 $CD4^{+ve}$ T cells and/or $CD8^{+ve}$ T cells. In the absence of high levels of IL-2, the RTE, by producing low levels of IL-4, may induce the anti-CD4 treated allo-specific mature $CD4^{+ve}$ T cells to become anergic. Furthermore, when IL-2 levels are reduced, secretion of INF-γ and TGF-β may further increase MHC expression and antigen presentation on the surface of the APC, without increasing expression of costimulatory molecules. This would make it more likely that $CD4^{+ve}$ T cells which had 'recovered' from anti-CD4 mAb treatment and/or $CD8^{+ve}$ T cells, would encounter antigen on the surface of an APC but fail to receive appropriate costimulation, driving them towards the anergic state. One can postulate that, if by thymectomy, RTE are removed from this system, then mature $CD4^{+ve}$ T cells which had 'recovered' from anti-CD4 treatment may be the principle cytokine producers at the cell-surface of an APC. After antigen contact, these cells could produce high levels of IL-2 in conjunction with other cytokines and could initiate allograft rejection. Clearly, this scenario entails a considerable degree of speculation, but does provide a possible explanation for the observations made in this thesis.

Further studies to ascertain both the qualitative and quantitative nature of cytokine secretion by RTE, coupled with a better understanding of the influence of the RTE cytokine profile on

mature CD4 T cells, are needed to determine the role of RTE in OX38 mAb induced transplantation tolerance. Such data may then provide a rational basis from which to devise strategies for manipulating human T cell responses following transplantation. Use for example of appropriate anti-CD4 mAb, may allow a 'favourable' cytokine profile to be produced by cells contacting alloantigen, and lead to lasting tolerance, without the need for long-term immunosuppression.

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