Anti-CD2 mediated prolongation of allograft survival

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Abstract

The CD2 molecule is a cell surface glycoprotein expressed on rat T cells which has numerous functions including the provision of a TCR-independent pathway of T cell activation, costimulation in T cell activation via the TCR, intercellular adhesion and the transmission of negative signals. Murine studies have shown that targeting the CD2 molecule with mAb has potent immunological effects including the prolongation of allograft survival. In this project I have assessed the effects of the mouse anti-rat CD2 mAb OX34 and OX55 in transplant models. OX34 proved to be more effective than OX55 in the low responder Lewis to DA strain combination, and this prolonged graft survival was associated with greater depletion of CD4 T cells by OX34. Analysis of the time course and distribution of depletion in different lymphocyte compartments suggested that host factors are also involved in OX34-mediated depletion. Despite the potent effects of OX34 in transplant models it has little effect on *in vitro* allogeneic models compared to those of anti-CD4 mAb. In particular OX34 does not affect proliferation and release of Th_1 cytokines in the MLR and does not prevent anti-TCR-mediated T cell activation. The principal effect of OX34 appears therefore to be depletion of T cells, an effect which is not reproduced in *in vitro* systems. OX34 mAb was also found to have a synergistic action with both the anti-CD4 mAb OX38 and $CTLA_4Ig$. Both of these combinations were able to induce permanent allograft survival in the high responder DA to Lewis combination. Graft survival in these models is associated with abrogation of the anti-globlin response combined with profound and prolonged T cell depletion. Evidence is presented therefore that, in contrast to murine studies, the principle effect of anti-CD2 mAb in rat transplant models is immunosuppression by T cell depletion mediated by the delivery of negative signals.

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Declaration

The data presented in this thesis represent original work performed by the author at the Department of Surgery, Western Infirmary, Glasgow between August 1994 and June 1997. All experiments were performed by the author unless otherwise attributed in the text.

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1 Introduction

1.1 The history of experimental transplantation

Although mediaeval surgeons have been depicted attempting limb transplantation in humans (1) the earliest recorded work in English of systematic attempts at experimental transplantation were made by John Hunter in the 18th Century (2). During his investigations into the process of inflammation Hunter included a number of experiments on tissue and organ transplantation in cocks and humans. Although these experiments generally failed and Hunter did not distinguish between auto, allo and xenotransplantation he firmly established animal experimentation as a tool in transplant research.

In the early 19th Century tumour biologists made many useful observations on the viability of transplanted tumours in rodents. For example it was noticed that adult animals have complete immunity to proliferation of transplanted tumours from a different species and that a spontaneous tumour arising in white mice was transmissible to all other members of an in-bred closed colony but not to other mice (3).

Efforts were made initially to explain tumour immunity by analogy to classical bacterial immunity. Many attempts were made to demonstrate cytolytic antibody in immune animals but no immune serum was shown to be capable of inhibiting tumour growth. This led to the suggestion by JB Murphy in 1926 that graft rejection may be due to a cellular reaction on the part of the host (4).

The first demonstration that successful tumour transplantation depends on donor and host sharing a tissue antigen, which is absent from resistant hosts, was made by Gorer in 1937 (5). Gorer had earlier shown that albino strain mice possess

an antigen (which he called antigen II, later H-2) on their erythrocytes, which is absent from a black strain (6). In a series of tumour transplants between F2 hybrids Gorer showed that the presence of the antigen on the erythrocytes of both hosts and donors was essential for successful transplantation. In these experiments Gorer was fortunate in using mice as experimental models which, very unusually, express MHC antigens on mature erythrocytes. Gorer demonstrated that the barrier to successful transplantation is an antigenic difference between donor and host. This could only be demonstrated at the time using serological methods, Gorer therefore became an advocate of the antibody-mediated model of allograft rejection, a model which persisted until the later work of Medawar.

The techniques of organ grafting were initially perfected by Alexis Carrel. In 1905 Carrel demonstrated the feasibility of organ transplantation by grafting the kidney of a small dog into its neck (7) and later published a large series of renal allografts in cats (8). All animals ultimately died following this procedure, although one animal survived for 29 days. Carrel established beyond doubt that vascularised organ transplantation was a technical possibility and paved the way for organ transplantation in clinical practice.

1.2 The cellular theory of graft rejection

The Second World War provided the impetus for Medawar's research into skin transplantation. The first of Medawar's studies of skin graft rejection was a case report of a patient with extensive burns treated with a combination of skin autografts and allografts in Glasgow Royal Infirmary in 1942 (9). Fifty pinch grafts of each type were used (the donor was the patients brother) and 15 days after the first set of allografts a 'second set' of allografts were taken from the same donor. The autografts therefore provided a control against which to assess changes in the allografts. The

tempo of the first and second set responses suggested an active immune reaction was responsible for graft rejection. Medawar then repeated these experiments in great detail in a large number of outbred rabbits (10). This paper was the first to use graft survival curves and median survival time to illustrate differences in graft survival. It was shown that allografts failed to survive and that the speed of the necrosing process depended on the mass of transplanted skin. Characteristic of the inflammatory process was an intense infiltrate of lymphocytes and monocytes, which were visible to the naked eye in stained sections as a black band within the dermis. This paper also confirmed the specificity of the graft reaction, as a second set of grafts applied from the original donor demonstrated shorter survival. Furthermore the site of grafting the second set did not significantly affect the outcome of the graft, suggesting that the sensitisation was a generalised phenomenon. Medawar concluded that the mechanism of elimination of foreign skin 'belongs to the general category of actively acquired immunity'.

Gorer's demonstration of transplantation antigens and anti-H2 antibodies (11) lead to a belief that transplant rejection was predominantly antibody mediated. Medawar however demonstrated that epithelium cultured by flotation *in vitro* proliferated and coalesced equally on serum from specifically immunised or naïve rabbits (12). Convincing evidence for the role of lymphocytes in graft rejection came with the development of the technique of adoptive transfer of serum and cells between animals of an identical strain. In 1953 Avrion Mitchison showed that accelerated rejection of skin grafts could be transferred passively to naïve mice by the intraperitoneal inoculation of lymph node cells from immune animals (13). Inoculations of serum were ineffective (14). These results were later greatly extended by Billingham using skin grafts in mice (15). These workers found that sensitised regional lymph node cells were particularly effective at transferring immunity.

Another technical advance which was useful in the 'cellular versus humoral' debate was the development of cellulose millipore chambers, which permit diffusion of humoral but not cellular components. It was shown in that tumour allografts, which are normally rapidly destroyed in sensitised hosts, are able to survive indefinitely in such animals if they were cultured '*in vivo*' inside a diffusion chamber (16).

The role of lymphocytes in graft rejection became clear in the 1960s when Govaerts performed a series of kidney allografts in dogs and removed thoracic duct lymph (which contains a very high percentage of small lymphocytes (17)) from these animals after they had rejected their grafts (18). The contained cells were then shown by histological methods to be cytotoxic for a culture of renal epithelial cells from the second kidney of the donor. Further definition of the role of lymphocytes in the initiation of immune responses was made by Gowans who demonstrated that depletion of small lymphocytes from an animal by thoracic duct drainage severely impaired its ability to generate antibody and DTH responses (19) and by Terasaki who fractionated the leukocyte elements of blood into monocytes, polymorphs and lymphocytes and showed that the lymphocyte component was responsible for producing the splenomegaly characteristic of graft-versus-host disease when adult cells were injected into newborn chicks (20).

It was therefore becoming recognised that small lymphocytes are involved in many aspects of immunity and are fundamental to the allograft response. Of considerable importance was the chance discovery of the mixed leukocyte reaction in 1964 (21). During an experiment to assess the morphology of lymphocytes from leukaemic patients cells from two different patients were inadvertently mixed. It was noticed that in this culture some cells grew in size and went into mitosis. These experiments were then extended to include white cell fractions from normal volunteers and it was noted that only lymphocytes from genetically distinct

individuals were able provoke the reaction, and that this blast transformation of lymphocytes could be assessed by the incorporation of tritiated thymidine. As genetic studies showed that the magnitude of the MLR response tended to correlate with the degree of genetic disparity between individuals (22) it was conjectured that this reaction had some similarities to the allograft reaction and could be used as a predictive assay of the likelihood of graft rejection between individual patients (23).

Using vital dyes to quantitate the cytotoxic activity of lymphocytes it was shown by culturing lymph node cells from sensitised rats with donor strain lymphocytes that there was an exponential reduction in the number of surviving target cells with increasing concentration of effector cells (24, 25). From these experiments it was deduced that approximately 1-2% of lymph node cells from an allosensitised animal are capable of specific cytotoxicity and that antiserum has an inhibitory effect on cellular cytotoxicity in keeping with the phenomenon of enhancement. Measurement of cytotoxicity became much easier after the development of a quantitative assay in 1968. Brunner devised a technique of 'labelling' target cells with ^{51}Cr by incubating the cells with radioactive sodium chromate. Lysis of the target cell then causes release of the radiolabel, which can be measured in the supernatant (26).

Proof of the theory that the MLR is a complete model for alloreactivity came with the demonstration that effector cells can also be generated *in vitro*. The specificity of this response was confirmed when it was shown that after culture of cells in the MLR effector cells are generated which are capable of lysing cells expressing the sensitising antigen but not third party cells (27, 28).

1.3 Effector mechanisms in allograft rejection

1.3.1 Lymphocyte subsets in graft rejection

The importance of T cells in allograft rejection was first demonstrated in 1962 when it was noted that mice thymectomised at birth fail to reject skin allografts (29). Thymus grafting in such tolerant mice restored allograft rejection. Further characterisation of the subset of lymphocytes responsible for allograft rejection came with the use of adoptive transfer techniques in irradiated animals (13). Using this technique BM Hall assessed the ability of different lymphocyte subsets to restore the ability to reject a vascularised allograft. Lymphocytes in various doses were prepared from lymph nodes, spleen, thymus and thoracic duct lymph. Affinity purification techniques were used to separate Ig bearing cells. From these experiments it was concluded that recirculating, long lived T cells were primarily responsible for allograft rejection (30). Thymocytes had a poor restorative capacity and removing the thymus from a donor animal increased its ability to transfer graft rejection on a numerical basis, suggesting that recent thymic emigrants diluted the effector cell precursors.

1.3.2 CD4 and CD8 T cell effector functions

After the demonstration in 1975 that T lymphocytes could be divided into two phenotypically distinct subsets which correlate with distinct helper and cytotoxic functions (31), the role of each in graft rejection was investigated. The allograft rejection mechanism is potentially either antigen specific (due to cellular cytotoxicity) or antigen non-specific (due to a DTH response). Early workers had noted a histological similarity between rejected skin allografts and the lesions of delayed-type hypersensitivity, suggesting they are mediated by similar mechanisms

(32). However with the demonstration of lymphocytes directly cytotoxic to allografted cells *in vitro* (33) it was assumed that cytotoxic T lymphocytes are the primary effector cells. The first challenge to this theory of allograft rejection was made by Loveland et al in 1981 (34). Using thymectomised, irradiated and bone-marrow reconstituted (ATXBM) mice they tested the ability of different subsets of lymphocytes to restore allograft rejection. Sensitised donor cells were depleted of either Lyt 1+ or Lyt 2+ lymphocytes using the appropriate monoclonal antibodies prior to adoptive transfer, on the assumption that Lyt 1 corresponded with the helper phenotype and Lyt 2 with the cytotoxic phenotype. Depletion of Lyt 2+ cells had no effect on the ability of donor cells to restore allograft rejection whereas depletion of Lyt 1+ cells from the donor inoculum largely abrogated subsequent graft rejection (35). From these experiments the authors concluded that cytotoxic T cells are not responsible for allograft rejection. (It has subsequently been shown that Lyt1 is a poor marker for T_h cells being expressed at a low level on T_c cells. L3T4 is a more specific phenotypic marker corresponding to CD4 (36)). Further work in this field used either the adoptive transfer of subset-depleted lymphocytes or direct depletion of lymphocytes in experimental animals with monoclonal antibodies. Using a similar technique to Loveland et al Dallman transferred depleted lymphocytes to ATXBM rats prior to skin grafting (37), using the mAb W3/25 as a marker for helper cells and OX8 for cytotoxic T cells. Results accorded with those of Loveland in that removal of cytotoxic T cell precursors from the donor inoculum did not affect its ability to mediate destruction of the graft whereas removal of T helper cells led to prolonged graft survival, suggesting a DTH mechanism of graft rejection. Grafts from animals depleted with OX8 were however infiltrated with OX8+ cells suggesting incomplete depletion of CD8 cells. Lowry used a similar technique with vascularised cardiac allografts in rats and found that whilst W3/25+ cells were the most efficient on a per-

cell basis, OX8+ cells were also able to transfer graft rejection independently (38). Using monoclonal antibodies to deplete either L3/T4+ (T_h phenotype) or Lyt2+ (T_c phenotype) cells *in vivo* Cobbold in 1984 also showed that cells with L3/T4 phenotype were the major promoters of allograft rejection, in that anti-L3/T4 therapy allowed prolonged graft survival whereas anti-Lyt2 had no impact on rejection (39). In a later work however the same authors found a role for T_c cells (40). Firstly they found that a combination of anti-L3/T4 and anti-Lyt2 mAb to be more potent than anti-L3/T4 mAb alone. Secondly it was noted that the accelerated rejection of allografts in primed animals is mediated by T_c cells, in that use of anti-Lyt2 mAb in such animals was more effective than anti-L3/T4. Furthermore this priming of T_c cells was dependent on the presence of L3/T4+ cells. This evidence pointed to a plurality of rejection mechanisms and co-operation between T cell subsets in generating a rejection response.

Much of the work supporting DTH as an exclusive mechanism for allograft rejection is theoretically flawed. Firstly rodents generate poor DTH responses so results using these as experimental subjects cannot be generalised to other animals. Secondly there is no *in vitro* assay of DTH activity so that its involvement can only be inferred by exclusion of other mechanisms. In addition the level of immunosuppression of ATXBM animals and the degree of subset depletion in mAb treated animals was shown to be far from complete, and the direct correlation between phenotype and function to be over simplistic. The T cell deficiency in ATXBM rodents and in nude (athymic) mice is limited to the T_h cell subset and the presence of residual cytotoxic T cell precursors which can be induced with IL2 (41) or cellular helper stimuli (42) has been demonstrated. To overcome the potential problem of incomplete T cell depletion in these animals adoptive transfer experiments have been performed using ATXBM rats grafted with skin from a minor

MHC incompatible donor. The advantage of this technique is that the precursor frequency of Tc for minor MHC products is many orders of magnitude lower than for major MHC products, so the presence of specific CTLp is much less likely. In these experiments Class I MHC antigens act as restriction elements for minor MHC reactive cytotoxic T cells (43). A major role for T_c in these models has been shown by the fact that adoptive transfer of L3/T4+ cells produced a very slow type of allograft rejection (>25 days), which could be restored to normal tempo by the addition of Lyt2+ cells (33). Furthermore Lyt2+ T cells capable of mediating rejection of allogeneic tissue have been cloned (44). In these experiments DTH was excluded as a mechanism as irradiation of hosts (thereby eliminating host helper cells as a source of inflammatory mediators) did not prevent rejection. Perhaps the most compelling demonstration of a role for T_c in allograft rejection came from experiments using allophenic mice (mosaics formed by the fusion of two blastomeres of differing genotype which are therefore tetraparental (45)). If skin from such an animal is transplanted to either pure parental strain, cellular elements within the graft expressing antigens of the other strain are rejected in a highly specific manner. This can be demonstrated using strains of differing colour, where the destruction of foreign melanoblasts is shown by the loss of hair colour in the surviving graft (46).

The debate over the phenotype of cell responsible for allograft rejection changed with the demonstration of MHC restriction in allograft rejection (47), with CD4 cells reacting to class II targets but not class I whilst the reverse is true for CD8 cells (with few exceptions). Furthermore there is no surface marker which uniquely identifies T_h cells. Cytotoxic CD4 T cells and CD8 T cells able to produce IL2 and provide their own help have been identified (47). More illuminating has been the characterisation of cellular functions in allograft rejection. Rosenberg performed a series of adoptive transfer experiments in which purified CD4 or CD8 T cells were

transferred into nude mice allografted with skin of determined antigenicity. The ability of the transferred cells to generate T_h and T_c responses *in vitro* against the specific determinants was tested by limiting dilution analysis. Without exception in a large series of experiments successful graft rejection only occurred in the presence of both detectable helper and cytolytic functions (48) (49). The presence of such 'dual function' lymphocytes has been shown more recently in adoptive transfer experiments using *SCID* (severe combined immune deficient) mice, a very stringent model of immunosuppression, in which either CD4 or CD8 cells can successfully transfer graft rejection (50). It is however probable that situations in which CD8 cells are able to provide their own help for cytotoxic functions are unusual and arise only in contrived laboratory models.

1.3.3 Mechanisms of graft destruction by T cells

There are two principle methods by which T cell subsets kill target cells, both leading to the fragmentation of cellular DNA typical of apoptosis (51). The principle method of cell lysis employed by CTL involves the secretion of the lytic protein perforin which occurs in a specific manner after binding with a cognate target cell. Release of perforin occurs by exocytosis and is a Ca^{2+} dependent process. Secreted perforin alone induces lysis without fragmentation of DNA, and penetration of the cell with serine proteases called granzymes via perforin pores follows to complete DNA breakdown (52). In addition the cell-surface receptor Fas can transduce signals which also lead to apoptotic cell death, which is thought to be a major regulatory mechanism in T cell homeostasis (53). Experiments with perforin knock-out mice revealed residual cytotoxicity against MLR target cells by CTL, which is abolished when FAS deficient cells (from lpr mice) are used, suggesting that CTL can also induce apoptosis by the FAS pathway (54). The contribution of these two pathways

to graft destruction has also been addressed. PCR and immunohistochemistry reveals upregulation of Fas and Fas-ligand in rejecting allografts (55, 56). However the cytotoxicity of graft infiltrating cells cultured from biopsies from human allografts undergoing acute rejection can be inhibited by the addition of concanamycin A (an inhibitor of the perforin/granzyme system) but not anti-Fas antibodies to the culture medium (56). The Fas system may therefore function to regulate the proliferation of T cells in a graft rejection response rather than the lysis of target cells. Further evidence for this comes from experiments where allografts were performed using both FAS-ligand deficient recipients and Fas deficient donor mice; in both groups grafts were rejected at control rates (55). However the immune-privileged status of Sertoli cells of the testis is thought to be due to the induction of apoptosis in reactive T cells by a FAS-dependent mechanism (57).

In vivo CD4 cells have the additional function of providing help for B cells and macrophages. Analysis of the mediators of macrophage function by amplification of the transcripts for iNOS (inducible nitric oxide synthase) and TNFα (Tumour Necrosis Factor α) has shown a marked increase during murine cardiac allograft rejection (55), implying that DTH mechanisms are also induced during the graft response.

Thus the debate over the primary effector cell in allograft rejection has come full circle since Loveland and McKenzie first raised doubts about the exclusive role of cellular cytotoxicity, a theory that was itself a rebuttal of an earlier DTH dependent mechanism postulated by Brent. The argument can to some extent be resolved by adopting the broader perspective that many mechanisms may be involved in allograft rejection, the model under study dictating which is dominant. In the absence of any arm of the immune response in a particular experimental setting

the remaining mechanisms 'take over' the role of graft rejection. However central to all mechanisms is secretion of cytokines, normally a preserve of the CD4 T cell.

1.3.4 Patterns of cytokine secretion by helper T cells

Stimulation of the immune system often leads to a response in which either cellular or humoral components predominate. For example it has been noted that the DTH response to nominal antigen injected subcutaneously can be greatly diminished by intravenous injection of the same antigen and subsequent generation of a humoral response, and the reverse inhibition also occurs (58). Cytokines play a crucial role in determining the dominant type of immunity. During the early stages of an immune response T cells make a wide range of cytokines. *In vitro* studies however have shown that after repeated stimulation T cell clones can be differentiated into two subsets according to their production of cytokines promoting DTH (Th_1) or humoral immunity (Th_2) (59). Th_1 cells typically secrete high levels of IL2, γIFN and TNF-β while Th_2 cells secrete IL4, IL5, IL6 and IL13 (60). In murine studies these T cell clones appear to be phenotypically identical whereas studies in the rat have shown that a high level of expression of the antigen OX22 is typical of the Th_1 subset (61). As outlined in section 1.9.5 the Th_1/Th_2 dichotomy has been studied in detail in the context of allograft tolerance.

1.3.5 Other immune mechanisms of graft destruction

The contribution of T cells to the rejection of allografts has been discussed in detail. Other cellular compartments are also capable of responding to allografts and may play a role in graft rejection.

As outlined in section 1.2 Medawar (12) and Mitchison (13) showed that immune serum is not able to effect graft destruction either in culture or after passive

transfer in murine studies. There are however circumstances in which B cells can be destructive to organ grafts. Sensitisation of high responder strain Lewis rats by skin grafting induces the production of cytotoxic antibody which leads to hyperacute rejection on passive transfer to a naïve host (62). Similarly host sensitisation and the production of cytotoxic antibody is a major problem in clinical renal transplantation (63). Kidney allografts in these recipients are rejected in a hyperacute manner, an outcome which is prevented by performing a cytotoxic antibody 'crossmatch' prior to transplantation. In rat transplant models where host and recipient differ only at the class I locus graft rejection is also mediated by the production of alloantibody (64). However in less contrived fully MHC mismatched allograft models graft rejection can proceed with normal tempo in the absence of B cells (65).

Natural killer cells are a also potent source of cytotoxicity, although their activity does not depend on an MHC interaction and NK sensitive target cells tend to be of tumour origin (66). Analysis of graft infiltrating cells in cyclosporine-treated rats has shown that grafts can survive indefinitely in the presence of high levels of non-specific cellular cytotoxicity (67). NK cells appear not to participate in the rejection of organ grafts although they are known to be involved in the rejection of bone marrow transplants (68).

1.4 The molecular basis of allorecognition

Central to the alloimmune response as demonstrated above is the T cell. The immune response in allograft rejection is initiated when a T cell recognises a foreign protein antigen in the context of an MHC molecule. The structures involved in this process are briefly outlined below.

1.4.1 The T cell receptor complex

The T cell receptor (TCR) for antigen was first identified by generating monoclonal antibodies against surface structures on T cell clones. By subtraction against B-lymphoblastoid clones antibodies targeted against unique T cell structures were produced. Immunoprecipitation experiments then identified a heterodimeric molecule of 90 kD, consisting of a α and a β chain linked by a disulphide bond (69). Each α or β chain resembles an immunoglobulin (Ig) light chain being composed of a series of Ig domains with the addition of a membrane anchor. The cytoplasmic domain of TCR molecules is very short, only 5 to 15 amino acids in length (70). In a similar fashion to Ig molecules certain amino-acid sequences involved in domain-domain interactions are highly conserved (71) whereas the hypervariable sequences are confined to loops projecting from the N terminal end (analogous to the V domain of Ig) (72), where they are available for interaction with the peptide-MHC complex. The TCR dimer is not itself responsible for signal transduction but is non-covalently associated with a complex of invariant polypeptides called the CD3 complex, which subserves this function. The CD3 subunits include three related proteins (γ, δ and ε), which exhibit Ig-like extracellular domains and an unrelated peptide, the ζ chain, which has a very short extracellular region of only nine amino-acids (70). The TCR complex is expressed as four dimers, the clonotypic α/β dimer dictates ligand binding specificity whereas the CD3 dimers γ ε, δ ε and ζζ mediate signal transduction (73).

1.4.2 The Major Histocompatibility Complex

The Major Histocompatibility Complex (MHC) encodes a series of antigens first identified on mouse erythrocytes which, when shared by host and donor

animals, allow the successful transplantation of tumour grafts (5). They were initially referred to as 'transplantation antigens' to reflect their association with allograft rejection before their basic function was understood. Their immunological role became clearer with the demonstration of MHC restriction in 1974 (74). Zinkernagel and Docherty showed that, when specifically immunised, only syngeneic strains generated high levels of cytotoxicity against virus infected target cells. This suggests that cytotoxic T cells only interact with their target cells when they share MHC antigens. The role of MHC products in antigen presentation became clearer when the structure of the MHC class 1 antigen was described in 1987 (75). Soluble HLA-A2 was produced by papain digestion of a human lymphoblastoid cell line which was then crystallised and analysed by X-ray diffraction. This class 1 molecule consist of a membrane-inserted heavy chain divided into three domains (α_1, α_2 and α_3) and a non-covalently associated light chain (β_2 microglobulin). Crystallography showed that HLA-A2 consists of two pairs of structurally similar domains: α_1 has the same tertiary fold as α_2, while α_3 has the same tertiary fold as β_2 microglobulin (β_2 m). The α_1 and α_2 domains each consist of an anti-parallel β-pleated sheet spanned by a long α–helical region. These two domains are paired to create an eight-stranded β–pleated sheet dividing two α–helices, forming an antigen-binding groove. Evidence that this groove is involved in antigen binding comes from an analysis of the position of polymorphic residues in the α_1 and α_2 domains. The majority of these residues lie either on the floor of the groove or the sides of the α helices facing into the groove where they are available for antigen binding (76). Some polymorphic residues are also on the top surface of the α helices facing solvent where they may contact with the T cell receptor. MHC class II molecules are known to have an analogous structure, but are composed of a heavy α chain divided into α_1 and α_2 domains and a lighter β chain, divided into β_1 and β_2 domains, with both chains inserting into the

cell membrane (77). In a similar fashion to class I molecules, an antigen-binding groove is formed by α helices spanning a β-pleated sheet, formed by the membrane-distal α_1 and β_1 domains. In contrast to class I molecules however this groove is open at both ends allowing the presentation of larger peptides (up to 24 amino acids). In addition there is evidence that the class II heterodimer is itself expressed as a dimer at the cell surface (77).

1.4.3 Antigen presentation

The MHC molecules are responsible for presenting protein antigens to T cells, the antigen being degraded and presented as short linear peptides (78). Despite their overall similarity in structure, specific differences account for the distinct functions of the two classes of MHC molecules. MHC class I molecules present peptides produced by degradation of endogenously synthesised proteins (79). In contrast, MHC class II molecules bind peptides generated by a degradation pathway of exogenous protein (80), although many of these peptides are themselves degradation products of other MHC molecules. Furthermore MHC class I molecules present peptides to CD8+ T lymphocytes while class II molecules present antigenic peptides to CD4+ T lymphocytes (81). When a specific T cell interacts with a MHC-peptide complex a signal cascade mechanism is initiated resulting in T cell activation and a specific immune response (82, 83). The two classes of MHC molecules therefore direct different antigenic pools to different pathways: class I molecules interact directly with cytotoxic effector cells to eradicate altered cells while class II molecules present circulating antigen to T helper cells to generate a more generalised immune reaction (52).

1.4.4 CD4 and CD8

CD4 and CD8 were initially described as markers of T cells with distinct effector functions, following which CD4 was found to be expressed by MHC class II-restricted T cells and CD8 by MHC class I-restricted T cells (84). CD4 is a 55-kDa monomeric membrane glycoprotein composed of four extracellular Ig-like domains, a hydrophobic transmembrane region and a long cytoplasmic tail (85). The cytoplasmic tail is associated with the protein tyrosine kinase $p56^{lck}$ whose activity is responsible for initiating the intracellular tyrosine cascade in response to TCR triggering (86). The CD4 molecule forms an aggregate with the TCR complex, for which CD4 acts as the signal transduction mechanism. The CD4 molecule binds to the invariant β_2 domain of MHC class II, where it increases the avidity of the MHC-TCR interaction, leading to increased tyrosine phosphorylation. T cell activation is dependent on the activity of the CD4 coreceptor as shown by the ability of anti-CD4 mAb to inhibit T cell activation (87). CD8 is a disulphide-bonded heterodimer of two polypeptides each with a single Ig-like domain, which binds to an invariant part of MHC class I (the α_3 domain) (88, 89). In a similar manner to CD4, CD8 is thought to stabilise the TCR-Class I MHC complex.

1.5 Costimulation in T cell activation

The mechanism outlined above of cognate recognition of an antigen-MHC complex by the TCR is in itself insufficient to lead to the stimulation of a T cell. The need for multiple signals to induce T cell proliferation was initially predicted on purely theoretical grounds in 1970 (90). Bretscher and Cohn believed that tolerance to self-antigen arose not from genetic coding but by the 'paralysis' of self-reactive lymphocytes. This presumption would require within the lymphocyte the ability to be

either activated or inactivated by immunological stimuli. As available evidence suggested that antibody-forming cells had only one receptor for antigen, this theory required the presence of a 'second signal' to induce activation, whilst absence of that signal would lead to paralysis. The authors explained their theory in terms of the emerging concepts of B-T cell interactions and adduced as evidence the requirement of certain haptens to be conjugated to carrier molecules to induce activation.

The physical requirement for two signals to induce allogeneic stimulation of lymphocytes was demonstrated by Lafferty and co-workers. Experimentally they demonstrated that ultraviolet irradiation of allogeneic stimulator cells in a MLR abrogated the proliferative response (91). It was postulated that a second 'inductive stimulus' was required to activate cells, which was destroyed by UV irradiation. This hypothesis was developed into a more general theory of allostimulation in which the 'inductive stimulus' is provided by haematogenous cells within the graft, rather than parenchymal cells, and removal of these cellular elements, for example by *in vitro* culture prior to transplantation, overcomes the barrier to transplantation (92). The critical role of APC in T cell activation was later demonstrated when the proliferative response of T cells stimulated in the experiments described above could be restored by the addition of culture supernatants of macrophages stimulated by enterotoxin. This soluble costimulator molecule was later shown to be IL1 (93), although in other experimental systems costimulation requires contact with a cell surface molecule.

The model of Bretscher and Cohn predicted that not only should antigen recognition in the absence of a second signal fail to stimulate the lymphocyte, it should also lead to subsequent unresponsiveness to that antigen. Further experiments were performed using purified T cell clones stimulated either by immobilised anti-CD3 antibodies (94) or by antigen presented on chemically modified APC (95). These experiments confirmed the requirement for a second signal in T cell activation,

as addition of allogeneic APC to these cultures at the time of initial presentation allowed cellular proliferation. Furthermore it was noted that T cells stimulated in these experiments lost the ability to respond to specific antigen on later restimulation, even in the presence of normal APC. These cells were shown to be viable by their ability to respond in the presence of IL2. This specific non-responsiveness was termed anergy. A crucial aspect of these experiments is that the costimulatory signal is provided by different cells to those presenting antigen. This suggests a separate signalling pathway to that associated with the TCR. This is in marked contrast to costimulatory activity provided by other molecules such as CD2 (see below), which appear to have a functional interaction with the TCR.

The cellular mechanism by which viable T cells become anergic in this system has been further characterised. Firstly anergy-induction is an active process which requires MHC recognition (96) and leads to a rise in intracellular calcium. Secondly the cellular deficit appears to lie at the level of induction of the IL2 gene, as IL2 mRNA is not detectable in anergic T cell clones (97, 98). Lastly addition of exogenous IL2 at the time of antigen presentation in some systems may overcome the need for costimulatory signals (99).

1.5.1 The CD28/CTLA$_4$-B7 pathway of costimulation

1.5.1.1 Historical background

The CD28 molecule was identified initially by immunisation of rodents with activated T cells and subsequent somatic hybridisation to produce a myeloma clone. The resultant mAb labelled an activation antigen on the T cell surface, which was shown to be able to transmit activation signals (100). Likewise the CTLA$_4$ molecule was identified initially by its presence on activated T cells, by constructing cDNA

libraries of activated T cells followed by subtractive hybridisation against cDNA of resting T cells (101). Similarly the B7 molecule was identified as an activation antigen on B cells as part of a screening process of hybridoma clones after *in vivo* immunisation (102).

1.5.1.2 Structure and expression

CD28 and $CTLA_4$ exist as disulphide-linked homodimeric glycoproteins of 41-43 kDa (103). Before their functional roles were identified it was noted that $CTLA_4$ and CD28 share similar amino-acid sequence homology and overall intron-exon organisation, suggesting they may subserve similar functions (104). CD28 and $CTLA_4$ are coexpressed on activated subsets of both CD4 and CD8 T cells (105). CD28 is constitutively expressed on T cells and is upregulated on activation via the TCR (105). $CTLA_4$ however is not expressed on resting T cells but is induced after activation, reaching maximal levels 48 hours after activation (106). The close relationship between CD28 and $CTLA_4$ is shown by the observation that CD28 expression is crucial for upregulation of $CTLA_4$ and anti-CD28 mAbs alone are capable of accelerating $CTLA_4$ expression (103). Conversely ligation of CD28 with either anti-CD28 mAbs or B7-transfected cells leads to downregulation of CD28 mRNA levels and surface expression (107). Therefore during the time period of maximal $CTLA_4$ expression CD28 expression is declining, this reciprocal expression is important in terms of the function of the two molecules.

1.5.1.3 Regulation of T cell activation by CD28 and $CTLA_4$

As described earlier Jenkins demonstrated that T cells stimulated via the TCR alone became functionally inactivated, and one of the 'second signals' required to prevent this was shown to be mediated by the CD28-B7 pathway. In an early experiment a T cell clone resistant to stimulation via TCR alone was induced to

proliferate on addition of allogeneic B7-expressing B-lymphoblasts. Furthermore addition of anti-CD28 or anti-B7 mAb was able to reverse this proliferation (108). More direct evidence for the involvement of this pathway in T cell activation came with the development of B7-transfectants (109, 110). These cells allowed definition of the binding kinetics between B7 and CD28 and further established the role of B7 in the costimulation and specific upregulation of IL2 transcripts in T cells stimulated via the TCR. In keeping with the predictions of Bretscher and Cohn it has been shown that blockade of the CD28/B7 pathway not only inhibits T cell activation but also leads to specific hypo-responsiveness, in that cells stimulated under these conditions remain unresponsive to specific antigen for variable time periods after the initial exposure whilst retaining the ability to respond to third party antigen (111) (112) (113). However a failure of these experiments based on thymidine incorporation after costimulation with CD28 is that they do not distinguish increased activation from prolonged survival. Whilst the presence of increased IL2 transcripts after activation does indicate increased cellular activation other more recent work suggests that CD28 signalling may function by preventing activation-induced cell death. Firstly anti-CD28 mAb has been shown to inhibit γ-irradiation induced apoptosis and increase expression of the survival gene Bcl-x_l (114); secondly CD28 deficient mice show increased cell death after anti-CD3 mediated activation (115).

In view of the homology in structure between CD28 and $CTLA_4$ it was predicted that $CTLA_4$ would also bind B7. This was shown to be the case when the construct $CTLA_4$Ig (see below) was shown to bind B7-transfectants with 20-fold greater affinity than CD28Ig, and was also shown to inhibit proliferative responses in the MLR, in a similar manner to blockade of CD28 (113) (116). This may result however from blockade of either the CD28 or $CTLA_4$ pathway, as each shares the same B7 family of ligands. More direct targeting of the $CTLA_4$ molecule itself with

antibody however has variable results. Monovalent Fab fragments of anti-$CTLA_4$ mAb, which do not cross-link the $CTLA_4$ molecule, are able to augment T cell proliferation whereas whole anti-$CTLA_4$ mAb are able to inhibit anti-CD3 mediated proliferation even in the presence of optimal CD28-mediated costimulation (117). This suggests that the primary role of $CTLA_4$ may be in negative regulation and would fit neatly with the upregulation of $CTLA_4$ after T cell activation. CD28 and $CTLA_4$ therefore may have opposing effects and the response of a T cell to activation via the TCR depends partly on the dynamic competition between CD28 and $CTLA_4$ mediated signals (118).

1.5.1.4 The B7 family of molecules

A second ligand for $CTLA_4$ was discovered when it was noted that anti-B7 mAb minimally inhibits proliferation in the MLR whereas $CTLA_4$Ig causes maximal inhibition (119), suggesting the presence of further ligands for CD28. The two known B7 molecules, B7-1 (CD80) and B7-2 (CD86), are expressed only on activated APCs and B cells (120). The B7 molecules have a different temporal relationship after B cell activation, in that B7-2 is upregulated very rapidly after activation of the B cell whereas B7-1 expression reaches maximal expression after 48 hours (121).

1.5.1.5 Differential T cell requirements for costimulation

The role of the CD28 pathway in the stabilisation of IL2 mRNA and subsequent promotion of T cell survival has been well demonstrated. More recently however CD28 signalling has been shown to have more diverse effects on T cell cytokine secretion as the absence of CD28-mediated signals induces a bias toward a Th_1 phenotype in naïve T cells. Initial *in vitro* evidence for this came from the demonstration that purified human T cells stimulated by a wide variety of non-cellular stimuli including anti-CD2, anti-CD3, IL2 or lectins only produce IL4 if

anti-CD28 mAb are included (122). Further experiments showed that although purified T cells stimulated by mitogens in the presence of CD28-mediated signals produce both IL2 and IL4, after repeated cycles of CD28 stimulation the levels of supernatant IL4 increased whilst those of IL2 decreased (123). The pathways for production of IL2 and IL4 appear to be closely linked, in that IL2 production is a prerequisite for IL4 production. T cell clones stimulated by specific peptide-MHC complexes in the presence of splenic APC elaborate both IL2 and IL4. Blockade of the CD28 pathway with $CTLA_4Ig$ in this model abrogates production of both cytokines, however addition of exogenous IL2 restores the production of IL4 (124). A positive feed-back circuit also exists to amplify costimulatory signals. IL4 produced by Th_2 cells is able to upregulate the expression of B7-1 and B7-2 by splenic B cells, thereby providing further costimulatory signals to T cells (125). *In vivo* evidence supports the role of CD28 in the generation of Th_2 responses. CD28 knockout mice develop T and B cells normally but have reduced basal levels of Ig and low titres of IgG1, and show diminished Ig class switching after virus infection, but retain normal DTH responses (126). The importance of CD28-mediated signals in T cell-B cell co-operation is further demonstrated by the ability of $CTLA_4Ig$ to prevent *in vivo* priming for humoral responses against protein antigens (127). Furthermore mice transgenic for a soluble form of $CTLA_4$, which blockades CD28-mediated signals, are again able to generate helper-T cells as shown by lymphokine production but have profoundly inhibited antibody production (128). Lastly the agent $CTLA_4Ig$ when administered to mice is able to abrogate the production of antibody to SRBC (129). The generation of Th_2-type responses (in mice and humans) seems therefore to depend crucially on the delivery of CD28-mediated signals. Whether CD28 delivers signals for IL4 production distinct from its role in enhancing IL2 production is not clear.

1.6 The CD2 molecule

As the effect of targeting the CD2 molecule with mAb forms the substance of this thesis this antigen will be described in detail.

1.6.1 Historical background

The CD2 molecule was first identified as an adhesion molecule when anti-CD2 mAb were shown to block rosetting of leukocytes with SRBC, a technique which was used to identify human T cells (130, 131).

1.6.2 Structure and cellular relations

CD2 is a 50-55-kDa glycoprotein (131). Crystalographic studies of soluble forms of rat and human CD2 have revealed a structure composed of two protein domains, an intervening linker region and an extended C terminal tail with overall dimensions of 20x25x75 A (132). NMR spectroscopy confirms that the protein domains are composed of anti-parallel B-pleated sheets, placing the protein within the class of the immunoglobulin superfamily (133). The ligand binding characteristics lie purely with the membrane-distal domain 1 (134). The linker region provides up to 20° of orientational flexibility between domains 1 and 2, which may assist with ligand binding and also adds 17A to the length of the molecule, making the complex between CD2 and its ligand CD58 (humans) or CD48 (rodents) a similar size to the peptide-MHC-TCR complex (135). The C-terminal portion contains a hydrophobic transmembrane portion which anchors CD2 to the cell membrane (136) and a long cytoplasmic tail composed of unique, repeating proline residues. This portion has been shown to be essential for the signalling function of CD2, in that transfected mutants lacking the cytoplasmic portion are unable to subserve a signalling function (137).

The CD2 molecule appears to be able to associate with other cell-surface proteins in a dynamic fashion. The first association noted was with CD3. Immunoprecipitation and deletion experiments of detergent lysates revealed that 40% of human CD2 is associated with the CD3 complex (138). The forces involved are weak however as the experiment was very dependent on the detergent used and the association was not strong enough to permit comodulation. The proximity of CD2 to CD3 suggests that CD2 may be involved in regulation of cellular signals transmitted by CD3. Further evidence for this came with the demonstration that the cytoplasmic portion of CD2 associates with the tyrosine kinases $p56^{lck}$ and $p59^{fyn}$, molecules involved in phosphorylation during signalling events (139). Further immunoprecipitation experiments in both mouse (140) and human (141) revealed weak, non-covalent linkages between CD2 and CD45. CD45 provides tyrosine phosphatase activity which regulates the protein tyrosine phosphorylation characteristic of the cascade of intracellular signalling events (142). The association of CD2 in a loose complex involving CD3 and CD45, together with the presence of a long cytoplasmic tail with a protein tyrosine kinase function, suggest that the CD2 molecule has a more complex function than simple inter-cellular adhesion and may be involved in the regulation of cellular activation.

1.6.3 Distribution

CD2 is expressed on T and NK cells in humans (131), rats (143) and mice (144). Variations exist between species in that CD2 is also expressed on murine B cells (144) and murine and rat macrophages (143). CD2 is one of the earliest markers of thymocyte development, and its expression correlates with the maturational state of the thymocyte, in that the majority of CD4-ve CD8-ve cells express low levels of CD2, double-positive thymocytes express intermediate levels and all single positive

cells express high levels (144). The expression of both CD2 and its ligand CD48 is upregulated on T cell activation, and this upregulation remains stable forming a marker of memory T cells (145). CD2 expression is also widely conserved across species, with homologous molecules identified in pigs (146), sheep (147) and non-human primates (148).

1.6.4 CD2 ligands

The first ligand identified for human CD2 was the SRBC receptor termed T11 target structure (T11TS). Mab binding studies later defined this as identical to LFA-3 (149), later termed CD58, which is widely expressed by both haematopoetic and non-haematopoetic tissues. The ligand for rat CD2 is the structurally related molecule CD48 (150), which has a more limited distribution restricted to lymphoid cells (151). The molecules CD2, CD48 and CD58 are structurally similar and are related to the carcinoembryonic antigen family of IgSF molecules (135). CD48 binds to domain 1 of CD2 and this interaction can be blocked by both OX34 mAb (anti-CD2) and OX45 (anti-CD48) (150). The kinetics of the interaction between CD2 and its ligands have been studied and reveal that the interaction is one of very low affinity, but very fast dissociation rate (152, 153). This may be required to facilitate intercellular contacts in rapidly moving cells and the low affinity may be necessary to prevent the molecules being avulsed from the cell membrane.

1.6.5 Functional properties of CD2

The CD2 molecule has been implicated in a variety of cellular functions, which will be considered separately although they may be different aspects of a single cellular process.

1.6.5.1 Intercellular adhesion

The ability of CD2 to adhere to its physiological ligands in solution and to SRBC has been outlined above. The adhesion function of CD2 was assessed separately from its other functions by transfecting hamster fibroblasts with CD2 cDNA. These cells then showed increased non-physiological conjugate formation with a CD48-expressing mastocytoma cell line, which could be blocked with specific anti-CD2 mAb (154). However the small size of CD2 and its ligand compared to other leukocyte adhesion molecules (such as integrins and selectins) argues against this being its primary role (135).

1.6.5.2 T cell activation

The initial evidence that CD2 has a more active function than simple inter-cellular adhesion came with the recognition that combinations of different anti-CD2 mAbs or anti-CD2 mAb plus soluble LFA-3 could stimulate T cells in both humans (155) and rats (143) in the absence of signalling via the TCR. These studies revealed that there are three functionally significant epitopes on the CD2 molecule, termed $T11_1$-$T11_3$ in the human. $T11_1$ was identified as the ligand-binding site as mAb to this epitope prevented rosetting with srbc. A combination of mAb targeting the non-overlapping epitopes $T11_2$ and $T11_3$ induced marked T cell activation, measured by ^{3}H-Thymidine incorporation and IL2 production (155). Furthermore CD2-mediated signalling required the presence of the CD3-TCR complex, as modulation of this complex completely abrogated CD2-mediated T cell proliferation. Similar results were obtained in the rat, where three non-overlapping epitopes were again identified (143). In a similar fashion mAb which blocked ligand binding (OX34) did not lead to T cell activation whereas a combination of mAb to the remaining epitopes (OX54 and OX55) lead to marked proliferation of target T cells. Further observations were

made regarding mAb-mediated T cell activation in this study, which are relevant to the current project. Firstly optimal CD2-mediated cellular activation appeared to require the presence of accessory cells, and this has been confirmed by other reports (156). This function can be replaced with rabbit anti-mouse polyclonal antibody when CD2-transfected Jurkat cells are activated, suggesting that cross-linking of CD2 molecules may be important. Also, in the presence of cross-linking rabbit anti-mouse antibody OX34 mAb alone becomes mitogenic for Jurkat cells. Lastly CD2-mediated activation requires the presence of the TCR-CD3 complex, as no activation is seen in a TCR^{-ve} Jurkat cell line (143).

Further studies however reported a TCR-independent 'second' pathway of T cell activation via the CD2 molecule (137, 155-160). There are clear theoretical objections to the presence of this pathway of T cell activation, since immune responses are specific and require the presence of structures with clonally distributed variable regions, whilst the CD2 molecule is entirely monomorphic within species. This objection can be overcome by ascribing CD2-mediated activation to *in vitro* artefact with no physiological counterpart. In support of this is evidence demonstrating the interdependence of the CD2 and TCR-CD3 pathways. Experiments with human leukaemic T-cell lines expressing CD2 in the absence of CD3-TCR show the total dependence of CD2 on the presence of the TCR complex (161, 162), although CD2 is able to function in the absence of TCR on NK cells (163). The close physical association between CD2 and TCR suggests that CD2-mediated signalling may be a consequence of the formation of TCR-CD2 aggregates. Furthermore CD2 appears to have an extensive role in regulating signals via TCR-CD3 as outlined in the next section.

1.6.5.3 Costimulation mediated by CD2

In addition to being a route for T cell activation itself, the CD2 molecule provides a means to influence signals transmitted via the TCR-CD3 complex. As outlined in the section above engagement of the TCR-CD3 complex by peptide-MHC or mAb exerting antigen-like effects is not sufficient to induce T cell growth and differentiation. A second costimulatory signal provided by one of a number of cell surface ligands is required. Evidence has accumulated that CD2 can provide such costimulatory signals (164). Firstly murine L cells transfected with CD58 added to anti-CD3 mAb or suboptimal concentrations of phytohaemagglutinin (PHA) induces proliferation of purified human T cells (165). Similarly the response of CD2+ T cell clones of known specificity to a B cell clone is greatly enhanced if that clone is transfected with CD58 (166). Interestingly the same effect was not seen when CD58 was expressed on a third party cell, suggesting that a close proximity between the MHC-TCR conjugate and the CD2-CD58 pathway is needed, in contradistinction to the costimulatory signal provided by the CD28-B7 pathway where no spatial relationship is required between the first and second signals. In the reverse manner CD2-ve T cell clones of known specificity have been shown to have greatly increased proliferative responses after transfection with CD2 (167). Furthermore the cytoplasmic domain of CD2 is required for the integrity of this costimulatory signal, thus differentiating the adhesive and costimulatory functions of CD2. Attempts have been made at the molecular level to define the nature of this CD2-mediated costimulatory signal and experiments in CD2 transfected clones have shown enhanced CD3-mediated phosphorylation of the ZAP-70 family kinase $p72^{syk}$ after CD2 co-stimulation (168). The costimulatory effect of CD2-mediated signals is detectable at the level of induction of the IL2 gene, a process critical for T cell growth (168). CD2-mediated signals have also been shown to trigger lysis of CTL

clones (169), which contrasts with the CD28/B7 pathway, which does not costimulate this activity (170). In a similar manner the activity of a rat NK-cell clone can be regulated with OX34, demonstrating the involvement of CD2 in non-specific cytotoxicity (171).

1.6.5.4 'Negative signals' and apoptosis mediated by CD2

In addition to providing a pathway to activate T cells CD2-mediated signals can also inhibit T cell responses. In a similar experiment to those outlined above a T cell hybridoma was shown to have greatly increased proliferation to antigen presenting B cells after transfection with CD2 cDNA. Addition of anti-CD2 mAb to the culture completely abrogated proliferation of the hybridoma to below that of the untransfected hybridoma and also abolished IL2 production (172). Further evidence that CD2 may provide negative signals came with the development of the LFA-3/IgG_1 fusion protein LFA-3TIP. This protein interacts with the ligand-binding site of CD2 and was shown to inhibit the response of T cells to CD2 independent antigens such as PHA, ConA and tetanus toxoid (173). These experiments demonstrated that a single mAb to the ligand-binding site of CD2 could transmit a 'negative signal' to an activated T cell to prevent proliferation and IL2 production and these experiments were confirmed by other groups (143).

The nature of this negative signal has not been defined but experiments of a very similar nature later showed that CD2 might provide a pathway of apoptosis in activated T cells. During intrathymic T cell development elimination of autoreactive T cell clones occurs by apoptosis and *in vitro* thymocytes undergo apoptosis in response to ligation of surface CD2, CD3 or the Fas antigen (CD95) (174). Subsequent experiments showed that mature peripheral T cells can also undergo programmed cell death by apoptosis mediated through CD2 (175-177). In these experiments resting T cells were stimulated with a mitogenic pair of mAbs to CD2

and introduction of a third mAb resulted in apoptotic cell death of the majority of cells (175, 176). Studies later showed that a single anti-CD2 mAb could also promote apoptosis in cells activated by other means, such as IL2 or anti-CD3 mAb (177). Initial experiments suggested that CD2 provides a distinct pathway of apoptosis separate from the Fas/Fas-L pathway. Particularly strong evidence came from experiments using T cells from patients with lymphoproliferative disease secondary to Fas gene mutations leading to the lack of cell surface expression of Fas molecules. These cells could still be induced to undergo apoptosis via the CD2 pathway (176). Later experiments however have suggested that targeting CD2 with mAb in activated cells leads to upregulation of CD95-L, and furthermore apoptosis in this system can be inhibited by anti-CD95 mAb, suggesting the two pathways are closely related (178). Finally, activated NK cells have also been shown to be susceptible to apoptosis mediated by a single anti-CD2 mAb, by an apparently Fas-independent pathway (179). This provides a possible role for CD2 in NK cells separate from its role in T cells, which appears to be dependent on the presence of the TCR.

1.6.5.5 CD2 and the regulation of cytokine production

As has been outlined above CD2 stimulation by mAb can either promote or inhibit T cell activation depending on the experimental circumstances. In a similar fashion the response of T cells in terms of cytokine production after CD2 stimulation has been investigated. In a series of experiments human T cells were stimulated via CD2, CD3, CD28, or by PHA or staphylococcal enterotoxin. Significantly in view of findings in this thesis dissociation was noted between cellular proliferation and cytokine production. Whilst similar levels of proliferation were noted when cells were stimulated with any combination of mAb to CD2, CD3, CD28, or enterotoxin or PHA alone, the levels of measured cytokine production varied greatly (180). The

most efficient system in terms of production of IL2, γIFN, IL4 and IL10 was stimulation via CD2 and CD28 simultaneously. CD2 stimulation alone caused similar levels of cytokine production to stimulation by the combination of anti-CD3 plus anti-CD28 (except for IL2 production which is absolutely dependent on signalling via CD28), which however produced higher levels of cellular proliferation. Furthermore addition of anti-CD2 mAb to a similar culture abrogated cytokine production without affecting proliferation (181). These results suggest that in the same way that T cell activation can be regulated by second signals so cytokine production is subject to regulation by discrete cellular pathways, including CD2 mediated signals.

CD2 mediated signals have also been shown to regulate the effect of the cytokine IL12. IL12 is secreted by macrophages and plays a central role in the initiation of a Th_1 response by T cells (182). Most notably this involves the stimulation of γIFN production, cellular proliferation and the generation of cytolytic activity. Mab targeting the adhesion domain of CD2 or CD58 has been shown to inhibit IL12-mediated γIFN production by PHA-activated T cells (183). Conversely mitogenic pairs of anti-CD2 mAb costimulate IL12 function to increase γIFN production and proliferation. These results have been interpreted as ascribing a role for the CD2-CD58 ligand pair in ensuring close proximity between T cells and macrophages for the maximal paracrine effect of IL12.

1.6.5.6 CD2 and the regulation of anergy

Another aspect of the ability of CD2-mediated signals to costimulate signalling via the TCR has been explored in the context of T cell anergy. As demonstrated above anergy can be induced in T cell clones by antigen presentation in the absence of costimulation via the B7 molecule. This anergic state can then be reversed by

stimulation with antigen-presenting cells expressing clone-specific MHC and CD2 (184). The conditions required for reversal of anergy in this system were investigated. Firstly blocking mAb to either MHC or CD58 on the APC prevented reversal of anergy, demonstrating both pathways are involved in the process. Secondly a unique observation was made regarding CD2 epitopes. The $T11_3$ epitope (CD2R) in man (but not the rat) is expressed on T cell activation (155). Following induction of anergy (an active process) the CD2R epitope is lost from the T cell clone. After incubation with IL2 for seven days the CD2R epitope is re-expressed, and its re-expression coincides with the cells susceptibility to anergy reversal through CD2-mediated signals. This model suggests a mechanism whereby the CD2 molecule contributes to the anergic state by assuming a phenotype refractory to the transmission of positive costimulatory signals.

A caveat must be made regarding all the above observations on T cell function which are based largely on *in vitro* manipulation of the CD2 molecule with mAb. Despite the implication of CD2 involvement in many aspects of lymphocyte homeostasis CD2 gene knockout mice grow and develop quite normally and are able to generate normal immune responses (185). Qualifications can also be made regarding gene-knockout experiments however as such animals may adapt to the absence of the experimental molecule and therefore elaborate atypical immune responses.

1.7 Strategies to induce allograft tolerance; neonatal tolerance

The first demonstration of immunological tolerance to transplanted tissues was made by Medawar in 1953 (186) in which neonatal mice were made tolerant of cells

of adult mice. This has subsequently been referred to as 'classical' immunological tolerance. Medawar injected a mix of allogeneic kidney, spleen and testis into the abdominal cavity of foetal and neonatal mice. When subsequently challenged with donor specific skin grafts these animals displayed varying degrees of prolonged graft survival. The concept of inducing tolerance in this way was prompted by the observation that skin grafts performed between cattle twins invariably survived, regardless of whether the twins were monozygotic (and therefore syngeneic) or dizygotic. This was interpreted in terms of mosaicism, which had previously been demonstrated for the erythrocytes of twin cattle by Owen (187). Medawar postulated that exposure of an immature immune system to antigen led to subsequent unresponsiveness to that antigen. This theory predicts that foetal or neonatal inoculation of cells leads to a state of chimerism where cells of both donor and host coexist, and the host becomes tolerant of all antigens expressed by the donor cells. This aspect is necessary to explain the observation that neonatal tolerance is not tissue specific. Thus inoculation of bone marrow cells influences the survival of such widely disparate organs as skin and kidney (188), an observation which was explicable after Medawars demonstration that 'transplant antigens' are expressed by both blood leukocytes and tissue parenchymal cells (189). In summarising his observations about neonatal tolerance Medawar made the following additional points (190).

1) The tolerant state is due to an alteration in the host, and not to graft adaptation or passive enhancement.

2) The tolerant state is specific to cells of the donor strain.

3) The tolerant state is systemic.

4) The tolerant state can be brought to an end by the infusion of specifically sensitised syngeneic cells and, more importantly, by naïve syngeneic cells.

This last observation suggests that tolerance is due to a central failure of the immune system, as otherwise the infused cells would come under the same influences which prevent rejection as the hosts own cells. Tolerance was noted to require persistence of the tolerising antigen, and if the chimeric state was lost tolerance also was lost. Medawar outlined two broad possible explanations for neonatal tolerance:

1) Cells preadapted to react upon encountering particular antigen are eliminated from the organism. Tolerance is therefore a property of the organism rather than of the cell.

2) 'Antibody forming cells' can respond to an antigen and undergo a change other than activation or cell death. This implies the existence of a 'tolerant' cell.

Support for the first hypothesis came with the elaboration of the theory of the clonal basis of the immune response (191), whereby clones of immunocompetent cells react to a single antigen only and self-reactive clones are eliminated when prenatal contact is made between the reacting cell and self-components.

The possibility of inducing specific tolerance by introducing foreign cellular antigens into the developing foetal or neonatal immune system and allowing elimination of the donor reactive clones clearly provided support for this model. Later evidence supported the concept of the elimination of reactive clones in models of neonatal tolerance (192, 193).

1.8 Monoclonal antibodies in transplant tolerance

The development of monoclonal antibody technology allowed the production of large amounts of antibody of a single, predetermined specificity. Kohler and Milstein devised a technique of fusing an immortal murine myeloma cell line (usually a non-secreting variant) with spleen cells from an immunised mouse (194,

195). A very high proportion of the resulting tetraploid clones (up to 10%) produced antibody with specificity for the original immunogen, perhaps because actively dividing cells are more prone to fusion. This technique therefore allowed production and perpetuation of a unique clone of antibody producing cells specific to the immunising antigen without the need to precisely define the antigen.

1.8.1 Antibody structure

Antibodies are symmetrical molecules consisting of two identical heavy chains (MW 50000-70000) and two light chains (MW 25000). Immunoglobulins are grouped into five classes: IgM, IgG, IgD, IgE and IgA. The class of immunoglobulin is determined by its heavy chain (C_H), the above classes having μ, δ, γ, ε and α heavy chains respectively. IgM and IgD C_H are encoded by single genes whereas the remaining classes can be divided into subclasses (isotypes), each with its own constant region gene. Light chains exist in two forms, κ and λ. In the mouse and rat there is a single C_κ gene and four C_λ genes; despite the variability of λ chains, 95% of Ig molecules bear κ light chains (196).

IgM exists as a pentamer or hexamer; its multimeric nature gives it greater avidity and facilitates complement activation. It is the oldest immunoglobulin phylogenetically and the first to appear in ontogeny and in an immune response. IgG exists as a monomer and is by far the commonest immunoglobulin in serum. There are four mouse, rat and human IgG subclasses: G1, G2a, G2b and G3 in the mouse, G1, G2a, G2b and G2c in the rat. The names of the IgG subclasses reflect the history of their discovery, which was broadly in order of decreasing serum concentration. The name of an isotype does not therefore imply a structural or functional homology between species (197).

1.8.2 The mechanism of immunosuppression by monoclonal antibody in animal models of transplantation

Five potential mechanisms of monoclonal antibody-mediated immunosuppression have been implicated in animal models:

1) Cellular depletion
2) Transmission of negative signals
3) Ligand modulation
4) Blockade of intercellular adhesion
5) Blockade of costimulatory signals

Each mAb may act by more than one of these mechanisms in any particular model, for example transmission of a dominant negative signal to a cell may lead to cell death and cellular depletion, and ligand modulation may reduce intercellular adhesive forces. Each mechanism will be considered in turn.

1.8.2.1 Cellular depletion mediated by monoclonal antibody

Cellular depletion is an *in vivo* phenomenon whereby the proportion of target cells is reduced after administration of monoclonal antibody. There are three possible mechanisms by which mAb deplete cells *in vivo*: complement-dependent cytotoxicity (CDC), opsonisation of target cells (antibody-dependent cellular cytotoxicity - ADCC) and delivery of a negative signal leading to cell death (apoptosis).

1.8.2.1.1 Complement-mediated lysis

Direct lysis of cells coated with antibody occurs by the classical pathway of complement activation and is reproducible *in vitro*. The first step is binding of a single C1q molecule to the CH_2 domain of the Fc portion of immunoglobulin. This ultimately leads to the conversion of C3 to C3b and activation of the terminal lytic

sequence leading to disruption of the cell membrane (198). IgM is a very potent activator of complement whereas IgG induced activation depends on isotype. In some systems both the mouse and rat IgG1 do not activate complement whereas IgG2a and IgG2b are both complement activating (199). The rat mAb GK1.5 (IgG2b) used in early experimental transplantation is lytic of mouse CD4 cells *in vitro* (200) and depletes CD4 T cells *in vivo* (201). However it was later noted that when chimeric antibodies of GK1.5 were generated with identical epitope specificity but differing isotype the ability to generate CDC *in vitro* does not correlate with *in vivo* depletion (202). In particular the IgG1 isotype of GK1.5 is as effective at inducing depletion as the IgG2b isotype. This suggests that additional mechanisms to CDC are responsible for cellular depletion. In addition mAb may cause cellular depletion in mice with a congenital complement deficiency (203). The depletion mechanism of mAb appears to depend upon the presence of the Fc component as $F(ab)_2$ fragments of GK1.5 prepared with pepsin do not cause cellular depletion (204). Furthermore other mAb of complement-fixing isotype *in vitro* do not cause cellular depletion *in vivo* (205). The bulk of evidence suggests that *in vivo* cellular depletion does not correlate with the ability of a mAb to mediate CDC. It has however been suggested that pairs of mAb targeting distinct epitopes on the CD4 molecule may induce synergistic lysis by CDC which correlates with *in vivo* depletion (206).

1.8.2.1.2 Antibody-dependent cellular cytotoxicity

This process implies the enhanced killing of opsonised cells by killer cells expressing an Fc receptor. Monocytes, macrophages, T cells and Natural Killer cells can all participate in ADCC as they all express Fc receptors. ADCC is widely accepted as an *in vitro* correlate of such Fc-dependent effector mechanisms (199), although ADCC involves cell lysis and ^{51}Cr-release assays do not provide a

measurement of opsonisation and phagocytosis of labelled cells, a process which does not result in release of cell contents into culture supernatant. Little has been published on the ability of xenogeneic mAb to direct ADCC in the rat. A study using isotype switch variants of a murine anti-IL2 mAb showed that the IgG1 isotype is more effective than both IgG2a and IgG2b (207). Experiments have demonstrated the ability of both murine (208) and rabbit (209) mAb but not horse antibody (209) to direct human ADCC and rat mAb is able to direct murine ADCC (202). In these experiments the ability to direct ADCC is very dependent on isotype. Using isotype switch variants of identical epitope specificity, Kipps et al showed that murine IgG2a is very effective at directing human ADCC using blood mononuclear cells as effector cells; IgG2b is less effective and IgG1 is not effective at all (208). In rat ADCC the most effective murine isotype depends on the effector cell used. Splenocyte-mediated ADCC is most effectively enhanced by IgG3 isotype whereas peritoneal macrophage-mediated ADCC is most effectively enhanced by IgG1 and IgG2a isotypes (202).

1.8.2.1.3 T cell homeostasis after T cell depletion

In adult humans the major mechanism of T cell regeneration is via expansion of the peripheral T cell pool. In experimental animals however this role is performed by thymic processes (210)

1.8.2.2 Transmission of negative signals by monoclonal antibody

The ability of cell-surface ligands to deliver negative signals was initially described in functional terms in assays of T cell activation. To fulfil the criteria for delivery of a negative signal a surface antigen-antibody interaction must inhibit T cell activation mediated by a separate pathway in the absence of blockade of the first antigen with its natural ligand. Early experiments showed that the proliferation of T

cells activated by anti-CD3 antibody in the presence of Ia^- (class II negative) accessory cells could be inhibited by the addition of anti-CD4 mAb (211). In a similar fashion Con A mediated activation of a T cell clone (lacking accessory cells) can be inhibited by anti-CD4 mAb (212). More compelling evidence that the CD4 molecule is able to mediate signals distinct from its role as part of the TCR complex came with the production of a CD4+/CD8+ clone (a herpes-virus infected mutant). In this clone only CD8 is physically associated with the TCR complex (and therefore able to interact with MHC molecules) and activation signals mediated via the TCR complex can be inhibited by concomitant signalling via CD4 (213). The CD2 molecule has also been shown to be capable of delivering regulatory signals during T cell activation. More recently with the advent of assays of DNA fragmentation the inhibition of T cell activation mediated by cell surface ligands has been interpreted in terms of the induction of apoptosis (214).

1.8.2.3 Ligand modulation induced by monoclonal antibody

Antigenic modulation is the down-regulation of a cell surface antigen expression induced by exposure to specific antibody and was initially described when it was noted that the susceptibility of a leukaemic cell line to specific anti-sera was reduced by prior exposure of the cell to antibody (215). Other features of this process were later elaborated. Firstly it was noted that the presence of monocytes is an absolute requirement (216). The process of modulation is energy dependent on the part of the cell expressing the antigen and does not occur at 4°C, but does not require energy expenditure on the part of the monocyte, as prior fixing of the monocytes with azide does not influence modulation (217). In addition surface-to-surface contact with monocytes is required (217). The Fc portion of the antibody is required for modulation as $F(ab)_2$ fragments are ineffective (216, 217). It was later shown that secondary labelling with polyclonal antibody could substitute for monocytes in

inducing modulation suggesting that cross-linking of antigen is the crucial step (218). Internalisation of the labelling antibody is not however required as antigen modulation can be stimulated by solid phase antibody (218). Significantly it has also been shown that antigen modulation by specific antibody can also decrease the expression of associated surface molecules. In this way anti-CD4 mAb has been shown to partially comodulate CD2, CD3 (218) and the T cell receptor (219). Antibody-induced modulation of CD2 in the mouse has been shown to be immunosuppressive (220), and particularly potent immunosuppression has been achieved with the modulating murine anti-rat CD4 mAb RIB 5/2 (221). Antigenic modulation is also the primary mechanism of action of the anti-CD4 mAb OKT4 used in primate models of transplantation (222).

1.8.2.4 Blockade of intercellular adhesion

The two pathways of intercellular adhesion most extensively studied are the CD2-CD48/58 pathway and the LFA-1 (CD11a)-ICAM-1 (CD54) pathway. Both of these molecular pairs have been shown to mediate intercellular adhesion when biochemically purified (161), although the distinction between intercellular adhesion and signal transduction is not clear. The role of the CD2 pathway in T cell activation has been extensively discussed and in a similar manner to the bi-directional flow of signals in the CD2-CD48 pathway both LFA-1 expressed on T cells (223) and ICAM-1 on monocytes and endothelium (224) have been shown to mediate regulatory signals in immune responses in addition to intercellular adhesion. Interestingly mAb directed against ICAM-1 (on monocytes) is able to inhibit T cell proliferation mediated by anti-CD3 mAb, presumably via a regulatory effect on monocyte-derived cytokines (224). The potency of blockade of these two pathways on *in vitro* alloreactivity has been demonstrated (225) and recent studies have been performed showing a beneficial effect of anti-LFA-1 therapy in experimental bone

marrow transplantation (226) and both anti-LFA-1 (227) and anti-ICAM-1 mAb (228) have been assessed in phase I trials in human renal transplantation.

1.8.2.5 Blockade of costimulatory signals

T cell activation requires antigen-MHC recognition plus non-specific costimulatory signals (90), and the absence of these signals may lead to clonal anergy (98). Early demonstrations of this phenomenon used fixed anti-CD3 mAb in the absence of accessory molecules to induce anergy (94). A similar effect has been demonstrated by activating T cells whilst blockading costimulatory pathways with monoclonal antibody. Pathways successfully targeted in this way include CD2 (see below), CD28 (see below), gp39-CD40 (229) and LFA-1 (230).

1.9 Anti-CD4 monoclonal antibodies in animal models of transplantation

As the bulk of research efforts in the use of mAb in transplantation have focussed on the CD4 molecule the key developments in this field will be outlined here.

The efficacy of anti-CD4 mAb in influencing alloimmune responses was first realised when it was demonstrated that the murine anti-rat CD4 mAb W3/25 is able to abrogate the mixed lymphocyte reaction without killing participating cells (231). More extensive studies were performed with the rat anti-murine CD4 mAb GK1.5, which was shown to inhibit *in vitro* proliferation and class II directed cytolysis (200).

1.9.1 General immunological effects of anti-CD4 monoclonal antibody therapy

The earliest observation made on the effect of the *in vivo* use of anti-CD4 mAb was its ability to prevent humoral immunity. Initially anti-Thy-1 mAb was shown to prevent the generation of agglutinating serum to SRBC administered at the same time (39). It was later shown by ELISA that therapy with the depleting anti-CD4 mAb GK1.5 prevented both the IgM and IgG response to various antigens administered intravenously including SRBC and KLH (232), and BSA (201). Antibody production stimulated by T-independent antigens (eg LPS) was unaffected. A crucial aspect of this suppression of humoral immunity was the observation that treated animals also failed to generate an antiglobulin response to the administered antibody. This finding was extended to show that both GK1.5 and OX38 induce immunological tolerance to themselves, which correlates with their ability to induce cellular depletion, as low doses, which are insufficient to induce depletion, also allow the development of an anti-globulin response (233, 234). That cellular depletion is not the only mechanism involved in tolerance induction to protein antigens is shown by the fact that an antiglobulin response was generated against an anti-CD45 mAb administered at the same time as GK1.5 (233). Abrogation of the anti-globulin response is not however a prerequisite for an immunosuppressive effect as the depleting murine mAb BWH4 is unable to induce tolerance to itself whilst having a potent effect on allosensitisation (235). Benjamin looked carefully at the issue of anti-globulin response after mAb therapy and confirmed that certain anti-CD4 mAb are able to induce tolerance to their own isotypic and idiotypic determinants and can also induce tolerance to co-administered non-cell binding mAb. Other cell binding mAb however are very immunogenic and anti-CD4 therapy is unable to prevent the generation of an anti-idiotypic immune response (236). Anti-CD4 mAb also prevents

the induction of a humoral response to alloantigens after the transplantation of both skin grafts (237) and vascularised cardiac allografts (234, 238).

Later experiments showed that the ability of anti-CD4 mAb to block the generation of humoral immunity to a wide variety of antigens is not matched by a similar inhibition of cellular immunity. The mAb GK1.5 for example is able to inhibit both the primary and secondary CD4-driven induction of CD8+ cytotoxic T cells directed against viral antigens (239) but is unable to abrogate the production of specific CTL generated against alloantigens presented by skin (238) or cardiac allografts (237). The mechanism of antigen recognition differs in these two models in that viral antigens are presented in the context of self-class II molecules whereas class I and class II transplant antigens are recognised directly by host T cells at a higher frequency, which may be a more potent stimulus. Other experimental evidence also exists showing that allolytic CD8 T cells can function in the absence of CD4-mediated help (240). The consistent observation however that anti-CD4 mAb is able to influence humoral immunity to a much greater degree than cellular immunity provided an early indication of a dichotomy amongst CD4 helper T cells and suggests that different helper cell populations are involved in the provision of help for humoral and cellular immunity.

1.9.2 Influence of anti-CD4 therapy on allograft survival

The potent effect of anti-CD4 mAb on humoral and cellular responses prompted their use in animal models of transplantation. Early work by Cobbold showed that anti-CD4 mAb is able to marginally prolong the survival of skin allografts, which is extended by the addition of anti-CD8 mAb (39). The first indications of the complexity of mAb therapy came with the demonstration that a mAb (W3/25) with potent *in vitro* effects (231) and immunosuppressive effect in

EAE (205) does not influence allograft survival (241) and mAb with potent *in vivo* effects on humoral immunity (GK1.5) also did not influence skin allograft survival (237). However tolerance to vascularised cardiac allografts has been achieved with depleting anti-CD4 monotherapy in the mouse using GK1.5 (242) and the YTS family of mAbs (243) and in the rat using OX38 (234). More stringent models of allograft rejection such as skin grafts or high responder rat strains have required the combination of anti-CD4 mAb therapy with other modalities such as Cyclosporine (244) or anti-CD3 mAb (245). Results with rodent models cannot easily be extrapolated to primates and humans however, where anti-CD4 mAb has a less marked immunosuppressive effect and in general promotes a host anti-globulin response (246, 247).

An important aspect of therapy with mAb is the timing of administration in relation to the exposure of the animal to antigen. This is important because CD4 T cells have differential roles in the sensitisation and effector phases of allograft rejection (235) and it is known that primed CD8 cells can function in the absence of CD4 derived help (40, 240). The inhibitory effect of anti-CD4 mAb is most potent if binding with CD4 occurs prior to activation via the CD3-TCR complex (248) and *in vitro* studies of T cell activation have confirmed that prior incubation with anti-CD4 is necessary to inhibit stimulation via the TCR (143). Anti-CD4 mAb administered during the sensitisation phase in a system using skin graft priming is effective in abrogating accelerated graft rejection but has no effect when administered more than seven days after the priming event (249) when a mature B cell response has evolved which is no longer dependent on T cell help. This is in contrast to cyclosporine and anti-IL2 mAb both of which are effective when administered during the effector phase of an immune response (250). The crucial time point for ligation of CD4 with antibody is when host T cells encounter graft antigen. Experimental evidence

suggests that sensitisation to an organ allograft occurs by the migration of graft leukocytes to host regional lymph nodes via lymphatics (92). Monoclonal antibody therapy administered at the time of solid organ transplantation therefore will label T cells prior to their encounter with graft APCs in regional lymph nodes. That early post-operative anti-CD4 mAb therapy is effective in preventing allostimulation has been demonstrated by Sayegh (251).

1.9.3 Depleting and non-depleting anti-CD4 antibody regimens

A common effect of anti-CD4 therapy is target cell depletion. Two mAbs used in early experiments, GK1.5 and OX38, both cause cellular depletion and early publications attributed their immunosuppressive effect to target cell depletion (39, 242). This belief was reinforced when it was shown that cellular depletion caused by OX38 correlated with administered dose and low doses failed to cause either cellular depletion or prolong graft survival (234), and antibody regimes using combinations of two different anti-CD4 mAbs produced profound cellular depletion and allowed permanent allograft survival in high-responder rat strain combinations (244). Furthermore using the GK1.5 family of isotype switch variants it was shown that the ability to cause cellular depletion coincided with the ability to influence immune responses, although the effect on allograft survival was not tested (202). That cellular depletion is not however the sole cause of the *in vivo* effect of anti-CD4 mAb was shown when it was demonstrated that:

1) Cellular depletion is not of itself sufficient to induce permanent allograft survival
2) Depletion is not necessary for some anti-CD4 mAbs to be effective.

Numerous lines of evidence suggest that cellular depletion alone is not sufficient explanation for the effect of anti-CD4 mAbs. The two murine anti-CD4

mAbs W3/25 and BWH-4 cause a similar degree of cellular depletion but only BWH-4 is effective in prolonging allograft survival (235). Secondly the anti-CD4 mAb pair of OX35 and OX38 causes profound depletion and is effective in inducing transplant tolerance, its effect however can be completely abrogated by pre-operative thymectomy without any effect on lymphocyte populations (244). Attempts to induce allograft tolerance without causing cellular depletion are obviously attractive, as they do not entail the prolonged non-specific immunosuppression of depleting protocols. As has been explained above the ability to cause cellular depletion rests partly in the Fc portion of the antibody molecule. Initial attempts to devise a non-depleting mAb therefore used $F(ab)_2$ fragments of the depleting antibody GK1.5. This was shown to be effective in inhibiting humoral immunity without causing cellular depletion (204). Similarly the complement-fixing (IgG2b) murine antibodies YTS191.1 and YTA3.1 have been used in combination in 'sub-lytic' doses to induce tolerance to human γ-globulin without causing depletion (206). Following this work mAbs were devised which do not deplete T cells. An early example is the rat anti-mouse CD4 mAb KT6. This (IgG2a) mAb competes for binding with the depleting antibody YTS191 suggesting the significance of specific epitopes in influencing ligand function. KT6 causes blockade of CD4 without modulation and has been shown to be successful in promoting allograft survival (252). Other non-depleting mAb have been devised which function by causing modulation of the CD4 molecule. The rat antibody YTS177.9 modulates the CD4 molecule and allows tolerance induction to intravenously administered peptides and skin grafts differing at multiple minor transplant antigens (253). These regimes are in general not as effective at inducing transplant tolerance as depleting regimes and do not alone allow permanent survival of cardiac allografts. Prolonged CD4 blockade may be a more effective mechanism of immunosuppression than ligand modulation as modulation alone may allow

internalisation of the mAb and re-expression of unlabelled CD4 (254). More potent immunosuppression has been obtained in the rat using the murine non-depleting mAb RIB5/2. This antibody modulates the CD4 molecule and is able to prevent the induction of an anti-globulin response when given in high doses for prolonged periods. It has been demonstrated to allow transplant tolerance to rat skin grafts when used in high doses (255), to prevent host sensitisation by skin-grafting (249), and to induce tolerance to cardiac allografts when used in a single low dose in combination with an infusion of donor splenocytes (221).

1.9.4 Differential effects of anti-CD4 mAb on CD4 subsets

A notable effect of all depleting regimes of anti-CD4 mAb is the incomplete depletion of CD4 T cells. This is true of regimes in both rats (234) (256) and mice (39, 257, 258) where increasing the dose of mAb rarely depletes CD4 T cells beyond 95%. Naïve and memory cell populations appear to respond differently to anti-CD4 mAb labelling *in vivo*. Weyand demonstrated that although the mAb GK1.5 is able to abolish primary anti-viral cytolytic responses secondary responses resulting from earlier priming remained intact, suggesting that memory cells reside within the undepleted CD4 population (239). Further *in vitro* evidence showed that activation of CD4 T cells via the TCR protected cells from the inhibitory effects of anti-CD4 mAb (259) and *in vivo* studies showed that undepleted cells after therapy with GK1.5 contained a high percentage of cells expressing the IL2-receptor activation marker (260). More specifically CD4 cells of the Vβ6 family, when stimulated by the superantigen M1s-1, are spared depletion by GK1.5 therapy when assessed by the Vβ6-specific antibody RR4-7 (260).

CD4 T cells can be further divided into Th_1 and Th_2 subsets on the basis of function and cytokine production (60). Th_1 cells produce the signature cytokine γIFN

and provide help for macrophage-dominant DTH-type responses whereas Th_2 cells produce IL4, the dominant cytokine stimulating B cell activity. In a similar manner to naïve and memory cells these two subsets have been shown to respond differently to anti-CD4 mAb. An analysis of cytokines produced in MLR experiments has shown that after the initial culture levels of IL4 are similar in W3/25 labelled and unlabelled cultures but after a period of rest followed by restimulation IL4 levels (detected by RT-PCR of mRNA) were greatly increased in cultures containing W3/25 and undetectable in unlabelled cultures (261). The authors interpreted these findings as suggesting that Th_1 cells require greater numbers of TCR-MHC interactions to be activated and are therefore more susceptible to anti-CD4 mAb-mediated blockade. Similarly CD4 T cells stimulated by anti-CD3 mAb show an inability to generate Th1 cytokines when labelled with GK1.5 (262). Some transplant models have also been able to demonstrate a relative sparing of Th_2 function after anti-CD4mAb therapy. The non-depleting mAb BWH-4 has been used in a model of accelerated graft rejection and has been shown to abrogate the production of IL2 but preserves IL4 elaboration at both the gene and protein level (263, 264), although immune deviation is incomplete as an explanation of transplant tolerance (see below).

1.9.5 Induction of non-classical tolerance by anti-CD4 mAb therapy in transplant models

In order to meet the criteria for donor-specific immunological tolerance it is necessary to demonstrate that animals with long surviving grafts will accept further grafts from the donor strain but reject grafts from a third-party strain. Many models of transplant tolerance induced by anti-CD4 mAb have met this criterion (221, 234, 265). However in contrast to 'classical immunological tolerance', where tolerant

animals do not produce immunological responses to donor type cells (188), alloreactive lymphocytes are detectable in these animals (253, 266) and typically react normally to donor-type cells in the MLR (234) (265). This has been termed 'split' or 'functional' tolerance and has also been demonstrated in transplant protocols using organ culture (267) and donor-specific blood transfusion (266). This dissonance between *in vitro* and *in vivo* assays of tolerance has been described earlier when it was also noted that assays of proliferation in the MLR and CML failed to predict the development of neonatally induced tolerance (268). Fathman addressed the issue of cells from tolerant animals responding normally in the MLR and suggested that animals become tolerant to peptides presented during tolerance induction, which will include MHC molecules presenting tissue-specific peptides (269). As clonal deletion has been excluded as a mechanism of tolerance induction in these models the peripheral mechanisms of tolerance including anergy, suppression and immune-deviation have been explored, all of which have been implicated in different systems.

1.9.5.1 Anergy in anti-CD4 mAb transplant models

As alloreactive lymphocytes represent a small proportion of the entire T cell repertoire, precise (monoclonal) mechanisms have been devised to investigate anergy amongst the graft-reactive cells. If donor and host animals differ at the Mls 1^a locus the fate and function of Mls 1^a reactive Vβ6+ T cell clones can be monitored with clonotypic antibodies. The first demonstration of clonal anergy using this method was in a bone marrow transplant model when the Mls 1^a-reactive Vβ6+ clone was shown to be anergic when stimulated by an anti-Vβ6 mAb (270). In a similar fashion the Vβ gene segments encoding reactivity with the class II antigen IE have been defined (Vβ5, 11 and 17). In a pancreatic islet cell transplant model using the

depleting mAb GK1.5 Alters and colleagues demonstrated that lymphocytes from B6 mice (IE-) tolerant to IE+ islets showed greatly diminished proliferation to anti-Vβ11 mAb (256). Furthermore this anergic state could be reversed by the addition of IL2. Anergy of a similar type has been demonstrated in CD8 T cells after treatment with anti-CD3 mAb (271).

1.9.5.2 Immune suppression in anti-CD4 mAb transplant models

Much of the work on suppressor T cells has been done by Waldmann and colleagues. This group have used a model of murine allogeneic skin transplantation across a multiple minor antigen mismatch under cover of non-depleting anti-CD4 and CD8 mAbs. An early indication that cells from tolerant hosts are able to influence naïve cells came with the observation that infusion of naïve cells is unable to break tolerance ('dominant-tolerance') (272). Adoptive transfer experiments also showed that tolerance in the T cell population is stable and long lasting, this would require either that the initial tolerised cells survive indefinitely or that new thymic emigrants come under the influence of tolerant cells and are themselves tolerised. To explore this last point further adoptive transfer experiments were performed of naïve cells into tolerant animals whose T cells are transgenic for the human CD2 gene. Two features suggested that the new cells had themselves become tolerant:

1) After a period of co-habitation in the tolerant host the host T cells were depleted with an anti-human CD2 mAb without affecting the tolerant state
2) Cells from the chimeric mouse could be infused into a second T cell depleted host and not break tolerance.

Waldmann termed this phenomenon 'infectious tolerance' (273). This would imply that naïve T cells receive signals from tolerant T cells at close proximity at the

site of antigen presentation. To test this hypothesis the same group sought evidence of 'linked suppression'. They found that CBA (H-2^k) mice tolerant to a minor-mismatched skin graft could then accept a third party graft transgenic for the class I molecule K^b and the tolerised minor antigens but would reject the K^b antigen when expressed on a separate graft. This was interpreted as meaning tolerant T cells recognising the tolerised minor antigens expressed on class II molecules then become tolerant to foreign class I molecules expressed on the same APC (274). Similar results were obtained by another group who showed that tolerance to APC-depleted (but not naïve) hearts could be transferred by splenocytes from rats made tolerant with anti-CD4 mAb (275). Waldmann has suggested two possible mechanisms by which naïve T cells in these models come under the influence of tolerant T cells (276). In a passive model tolerant anergic cells would accumulate at the site of antigen presentation resulting in diminished collaborative units. This would mean that new naïve cells would encounter foreign antigen isolated from T cell help and would themselves become tolerant (the 'civil-service' model). In an active system tolerant T cells would influence naïve cells by secreting tolerance-inducing cytokines.

1.9.5.3 Patterns of cytokine secretion in anti-CD4 mAb transplant models

Early indicators of diminished expression of cytokine receptors in tolerant cardiac allografts compared to acutely rejecting allografts came from an analysis of IL2-receptor expression (277). This was matched by a failure of cultured graft infiltrating cells to produce IL2 despite normal IL2 gene expression. Graft tolerance in this model could be abrogated by administration of exogenous IL2, suggesting a selective failure of IL2 translation. Following this publication many groups have explored the differential production of cytokines in tolerant and rejecting allografts. As has been outlined in section 1.9.4 CD4 T cells can be sub-divided into Th_1 and

Th_2 subsets which correlate broadly with cytokine secretion favouring help for cell-mediated or humoral immunity respectively. This dichotomy is most clearly seen in the response of both humans and mice to chronic parasitic infections (60). There is extensive evidence that during rejection of organ transplants all cytokines may be expressed within the graft, representing the redundancy of immune pathways in graft rejection (278). In keeping with this allografts can be rejected in IL2 knockout mice despite detectable IL4 expression in the graft (279) and γIFN knockout mice similarly reject allografts despite having diminished DTH responses (280). Numerous transplant models have shown a preferential decrease of Th_1-associated cytokines during the induction of adult tolerance including anti-CD4 mAb (281) and anti-TCR mAb (282) regimes and donor specific transfusion (DST) (283), however the association of upregulated Th_2 cytokines with allograft tolerance is more problematic. In a model of transplant tolerance mediated by DST Josien noted a broad reduction of transcripts for both Th_1 and Th_2 cytokines (284) and in a transplant model where rejection is known to be mediated by alloantibody Tweedle noted a preferential decrease in Th_2 cytokine-gene transcripts in tolerant cardiac allografts (285). An interesting aspect of the Th_1/Th_2 dichotomy has been the observation by Waldmanns group that infectious tolerance by adoptive transfer in his model of tolerance to minor mismatched allografts can be abrogated by the addition of anti-IL4 mAb at the time of cell infusion. As tolerant lymphocytes in his model do not produce increased levels of IL4 Waldmann has surmised that the anti-IL4 antibody neutralises IL4 produced by naïve cells in the process of tolerance induction after contact with host lymphocytes. This would then allow these cells to develop Th_1 functions (286). Although the bulk of evidence suggests that differential Th_1/Th_2 functions are detectable in transplant models it does not support a simple association of preferential Th_2 utilisation leading to allograft tolerance.

1.10 Anti-CD2 monoclonal antibodies in experimental transplantation

1.10.1 Anti-CD2 monoclonal antibody therapy in murine models of transplantation

Compared to the body of work published on the effect of anti-CD4 mAb relatively little work has been done on anti-CD2 mAb therapy. Early studies were performed in the mouse using the anti-CD2 mAb 12-15 (287). An interesting aspect of 12-15 mAb is the discordance between its *in vitro* and *in vivo* effects. Numerous experiments have demonstrated its inability to affect the MLR (288) (289), or the *in vitro* induction of allo-CTL (290). Despite the involvement of the CD2-CD48 pathway in the augmentation of CTL lysis (discussed in Chapter 2), anti-CD2 mAb is not able to inhibit *in vitro* lysis by either allo-CTL or a CTL clone (154). Some workers have shown a slight inhibition of anti-CD3 mediated proliferation by the mAb 12-15 with associated reduction in IL2 and IL4 production (291) whilst others have been unable to demonstrate this effect (290). *In vivo* experiments however have shown the potency of anti-CD2 in various aspects of T cell immunity. *In vivo* priming for TNP-specific CTL (288) and allo-CTL (289) (292) is inhibited by concurrent administration of anti-CD2 mAb. Anti-CD2 is also able to delay the isotype switch from IgM to IgG after injection of the antigen oxazalone, although this effect is slight compared to the effect on CTL induction (293).

The *in vivo* effect of anti-CD2 mAb therapy in mice however does not extend to the induction of transplant tolerance; when used as monotherapy it is only able to prolong cardiac allograft survival (292). In this model mAb therapy (with the rat mAb 12-15) is most effective when administered preoperatively, and has no effect

when administered four days after transplantation. This inability to influence an established rejection process and the effect on CTL induction rather than lysis suggests that anti-CD2 in this model affects the afferent limb of alloimmunity. A consistently detectable effect of anti-CD2 therapy in the mouse is down-modulation of the CD2 molecule, which occurs without comodulation of CD4, CD8 or CD3 (288). Further analysis using Fab and $F(ab)_2$ fragments demonstrates that the Fc component is crucial for both antigen modulation and the immunosuppressive effect (289). In these models of murine anti-CD2 therapy there is no evidence of cellular depletion. Although anti-CD2 mAb is unable to influence the proliferation of lymphocytes stimulated *in vitro* with anti-CD3 mAb, when lymphocytes are taken from animals treated *in vivo* with anti-CD2 mAb, proliferation subsequently induced *in vitro* by anti-CD3 mAb is inhibited (294). This may be due to more efficient antigen modulation after *in vivo* administration of mAb (295). Furthermore inhibition of proliferation in these assays was accompanied by altered cytokine production; IL2 and IL4 production was diminished whilst the production of the immunosuppressive cytokine TGFβ was increased (294). A contrary effect on IL4 production has been noted in the context of transferable suppression. CD2 mAb treated and immunised animals produce CD4 T cells which are able to inhibit the *in vitro* generation of CTL against the original immunogen (289). Culture supernatants in these assays show a consistent increase in IL4 levels compared to controls, suggesting that anti-CD2 mAb therapy has favoured the development of a Th2 phenotype. This is in contrast to blockade of costimulation by the CD28-B7 pathway, where antibody therapy favours the development of a Th1 phenotype (122), and demonstrates a qualitative difference between the mechanisms of these two costimulatory pathways.

1.10.1.1 Combination monoclonal antibody therapy in the mouse

Whilst anti-CD2 mAb therapy alone is not able to induce allograft tolerance in the mouse it is more effective when combined with other therapies. The earliest work combined anti-CD2 with anti-CD3 mAb (296) to induce allograft tolerance. In this regime anti-CD2 was administered prior to anti-CD3 and prevented anti-CD3 mediated cytokine release. Combination with anti-CD4 was ineffective. Further experiments in mice have shown that anti-CD2 is also able to synergise with FK506 (297), anti-CD48 (290) and anti-CD28 (291) therapy to induce allograft tolerance. Experiments using anti-CD2 mAb therapy with human cell cultures have also shown that interference with more than one accessory T cell pathway is necessary to suppress proliferative responses (225). Taken together these results imply that numerous intercellular pathways are available to T cells for the provision of costimulatory signals and blockade of one pathway alone is insufficient to 'isolate' a T cell.

1.10.2 Anti-CD2 monoclonal antibody therapy in rat models of transplantation

Little work has been published using anti-CD2 mAb in the rat and a consistent picture of its effect is hard to obtain. An early work demonstrated the inability of OX34 to influence the MLR (298), which was later contradicted by two other groups showing a variable inhibition (299, 300). Both of these publications also demonstrated a minor inhibition of anti-TCR mediated T cell activation by anti-CD2 mAb in the rat. Similarly conflicting evidence exists regarding the effect of anti-CD2 mAb in allograft models. The mAbs OX34 (IgG2a) and OX54 (IgG1) were assessed by Hirahara using a vascularised cardiac allograft in a low responder (Lewis-DA) strain combination. OX34 in this study was able to induce permanent allograft

survival whereas OX54 was only able to slightly prolong graft survival (299). Using OX34 in an identical strain combination Sido was only able to prolong allograft survival to a MST of 45 days (300). These groups also give conflicting interpretations of flow cytometry data. Hirahara showed a predominant depletion of CD4 T cells with some modulation of CD2 from remaining cells whilst Sido reported down-modulation of CD2 to less than 20% of naïve levels without significant cellular depletion, in a similar manner to anti-CD2 therapy in the mouse (301).

1.10.3 Mechanisms of action of anti-CD2 mAb therapy in rodent studies

The small number of publications available does not describe a clear pattern for the efficacy of anti-CD2 mAb therapy at modulating immune responses *in vivo* in the rat and also suggests a different mechanism of anti-CD2 mAb therapy in rats and mice. However some indication of the mechanisms involved can be deduced. The synergy in action between anti-CD2 mAb and anti-CD48 mAb in the mouse (290) suggests that simple blockade of the CD2-CD48 ligand pair is an inadequate explanation. Further evidence in the rat has shown that the mAb OX45 is very effective at modulating CD48 from the surface of rat LNC but does not prolong allograft survival or influence proliferative responses to various mitogens, again suggesting that interference with the CD2-CD48 pathway does not explain the potency of anti-CD2 mAb (301). A similar series of experiments performed in the mouse has shown that while modulating anti-CD48 mAb is unable to influence proliferative responses it does inhibit the *in vitro* generation of allo-CTL to a greater extent than anti-CD2 mAb, again suggesting a mechanism other than simple ligand-receptor blockade (302). Furthermore the ability of anti-CD2 mAbs binding domains other than the CD48-binding domain (eg OX54 and OX55) to influence immune

responses suggests there is no absolute requirement for ligand-receptor blockade (303). A different interpretation of these findings is that there are further unidentified ligands for the CD2 molecule.

A consistent finding in murine studies is modulation of the CD2 antigen. This requires intact mAb (including the Fc portion) and epitope specificity appears less important than isotype (304). Down modulation of CD2 in this model is associated with upregulation of other adhesion molecules, which may contribute to an inappropriate activation environment where adhesion receptors are upregulated yet fail to engage their ligands. As described above the picture in rat models is less clear, with both cellular depletion and CD2 modulation being described. Using OX34 therapy in a model of adjuvant arthritis in Lewis strain rats however Hoffman also described selective CD4 T cell depletion associated with diminished disease severity (305). In a recent paper analysing the *in vivo* effect of the mAbs OX34, OX54 and OX55 Sido noted that in the high responder transplant combination DA to Lewis that modulating OX34 and OX55 were most effective in prolonging allograft survival and that graft rejection in animals treated by these two modalities coincided with clearance of mAb from the serum and re-expression of modulated CD2 molecules (303). A further observation was made in this paper regarding differential effects on B and T cell immunity. Sido demonstrated that despite inhibiting the generation of CTL to KLH, anti-CD2 mAb therapy was unable to prevent the generation of a humoral response to the same antigen (303), suggesting a minor role for CD2 in T cell help for humoral responses, in agreement with murine studies.

Little detail is available regarding the maintenance of transplant tolerance induced by anti-CD2 mAb therapy. Kreiger et al showed by in situ hybridisation that both Th_1 and Th_2 cytokines are up regulated in rat cardiac allografts made tolerant with OX34 therapy compared to isografts (306). In a murine model using an anti-

CD2 mAb to obtain tolerance to pancreatic islet allografts Kapur noted a reduction in expression of both the CTL-associated cytokine granzyme B and IL10 in tolerant grafts (307), arguing against immune deviation as a mechanism of allograft tolerance. Recent murine studies using a combination of anti-CD2 and anti-CD3 mAb have demonstrated a role for IL4, as addition of anti-IL4 mAb to the treatment regime abrogates tolerance and this regime is ineffective in IL4 knockout animals (308).

No evidence is available in transplant models on the transmission of negative signals via CD2 as a mechanism of immune tolerance. This phenomenon has been demonstrated in polyclonal *in vitro* systems where T cell activation and cytokine secretion mediated by a CD2 mAb pair can be inhibited by a third anti-CD2 mAb (181).

1.10.4 Anti-CD2 mAb therapy in primate models

Following the efficacy of anti-CD2 mAb therapy in rodent models its use in both humans and baboons has been attempted. The construct LFA3TIP, which competitively inhibits the CD2-CD58 interaction, has been shown to prolong baboon cardiac allograft survival from ten to eighteen days (309). In a human series reported from Belgium forty patients were randomised to either Cyclosporine-based triple therapy alone or triple therapy plus the rat IgG2b anti-human CD2 mAb BTI-322. A reduction in acute rejection episodes at nine months was noted without any adverse side effects (310).

1.11 Blockade of the CD28-B7 pathway in experimental transplantation

Whilst the relationship of the CD2 pathway to the TCR complex is unclear and signalling by these two pathways does not fit neatly into the two signal paradigm devised by Bretscher and Cohn, the CD28-B7 pathway, in contrast, appears to be a more purely costimulatory pathway so that attempts to blockade CD28-B7 with $CTLA_4Ig$ have been performed with the hope of fulfilling Bretscher and Cohn's predictions of clonal anergy.

1.11.1 Construction of $CTLA_4Ig$

The human $CTLA_4Ig$ construct was originally elaborated by Linsley et al (116). cDNA coding for the extracellular domain of $CTLA_4$ was extracted from total RNA of a human leukaemia cell line. This fragment was then ligated with cDNA coding for the hinge, CH2 and CH3 domains of human Ig Cγ1 and inserted into a mammalian expression vector, CDM8, which was transfected into COS cells. The resulting supernatant contains a product which binds to B7 with 20-fold greater affinity than CD28Ig (116) and binds efficiently to human, rat and murine B7 (311).

1.11.2 Immunological effect of $CTLA_4Ig$ therapy

Compared to the extensive published literature on the effect of anti-CD2 in allo-immune responses there is comparatively little information regarding the use of $CTLA_4Ig$. Early *in vitro* studies demonstrated that $CTLA_4Ig$ is able to inhibit the MLR (116, 312) but does not inhibit proliferation induced by the more potent mitogens Con A and immobilised anti-CD3 mAb (313). Analysis of cytokine production in these experiments showed that the production of both IL2 and IL4

mirrored the degree of cellular proliferation, arguing against a differential subset effect. In comparing *in vivo* and *in vitro* effect Baliga noted that whilst $CTLA_4Ig$ is very effective at inhibiting the MLR when added directly to the culture, even high dose *in vivo* therapy fails to inhibit the proliferation of cells from treated animals (313). In a similar manner to some experiments with anti-CD2 mAb, $CTLA_4Ig$ is able to prevent the *in vivo* priming of hapten-induced (TNP) CTL induction and *in vitro* restimulation of primed CTL and also inhibits the afferent limb of contact sensitivity but does not prevent the more potent *in vivo* priming of allo-CTL in murine models (313). $CTLA_4Ig$ has no effect on lysis of established murine CTL when added to cultures. These results suggest there is a hierarchy of immunological stimuli with allo-immunisation the most difficult to circumvent and that *in vivo* responses have other pathways of costimulation in addition to the CD28-B7 system. Workers using the rat model however have shown inhibition of *in vivo* allo-CTL induction by $CTLA_4Ig$ (314).

Other *in vivo* studies established that $CTLA_4Ig$ therapy in SRBC or KLH treated mice is able to inhibit the production of antibody-forming cells (127). Likewise $CTLA_4Ig$ inhibited the formation of alloantibody in a rat renal allograft model (314). This contrasts with anti-CD2 mAb, which has a more potent effect on T cell than B cell responses.

Unlike mAb therapy directed at T cell antigens these potent effects on T and B cell responses in rodents are achieved without major effects on T cell subsets and ligand expression. Analysis of the T cell antigens CD4, CD8, CD2 and CD3 antigens showed no change after $CTLA_4Ig$ therapy (313), as did analysis of class II MHC expression in splenocytes from treated animals (127).

1.11.3 $CTLA_4Ig$ therapy in transplant models

In rodent transplant models conflicting results have been reported on the efficacy of $CTLA_4Ig$ therapy. It is able to induce indefinite graft survival and donor specific tolerance after xenogeneic pancreatic islet grafting in the mouse (315), but is only able to prolong non-vascularised cardiac allograft survival (MST 35 days) (313). Early reports suggested that $CTLA_4Ig$ is unable to produce tolerance to rat vascularised cardiac allografts (in the high responder Brown Norway to Lewis combination) (312) however later reports demonstrated tolerance to murine vascularised cardiac allografts (316) and rat renal allografts (317).

An important aspect of the regimes used in these experiments is the timing of the dose of $CTLA_4Ig$. Some workers administered the construct (50μg/mouse or 0.5mg/rat given intravenously) at the time of transplantation (313, 316) whilst others have demonstrated an advantage in delaying administration until 48 hours post-operatively (314, 318). Delaying administration until days 4 and 5 (319) or day 7 (313) has no effect on allograft survival demonstrating that $CTLA_4Ig$ is unable to reverse an established rejection process. As the half-life of $CTLA_4Ig$ in mice is 2.8 days (127) the bulk of $CTLA_4Ig$ administered at day 0 would still be circulating after 48 hours. There are two possible explanations for the enhanced effect of delayed therapy:

1) There is evidence that alloimmune responses are most effectively stimulated by dendritic cells, and Larsen has shown that the migration of dendritic cells to the spleen peaks two days after transplantation (320)

2) Differential expression of B7 molecules may influence the effect of $CTLA_4Ig$ therapy as it is known that B7-1 expression is maximal 48 hours after stimulation of APC (121).

The second explanation is the most likely, as it is specific to $CTLA_4Ig$ whereas an advantage in delaying treatment has not been noted with other immunosuppressive modalities. In keeping with this finding is the observation that perfusion of the donor heart with $CTLA_4Ig$ prior to transplant does not affect allograft survival, suggesting that $CTLA_4$ ligands are not available prior to induction of an immune response (316).

A notable effect of $CTLA_4Ig$ therapy is its ability to synergise with other modalities to induce transplant tolerance in more stringent experimental models. In high responder Lewis rats $CTLA_4Ig$ therapy alone is unable to induce permanent allograft survival. Permanent survival of cardiac allografts in these animals however can be obtained by combining $CTLA_4Ig$ with either anti-CD4 mAb (321) or donor-specific blood transfusion (318), and of renal allografts by combining $CTLA_4Ig$ therapy with low dose Cyclosporine A (322). Tolerance to highly immunogenic secondary skin grafts in mice can be obtained by combining $CTLA_4Ig$ with donor-specific transfusion (323) and to small bowel allografts in Lewis rats by combining $CTLA_4Ig$ with anti-CD4 mAb (324). Combining $CTLA_4Ig$ with depleting regimes of anti-CD4 and anti-CD8 mAb has also proved successful in prolonging survival of cardiac xenografts (325). In view of the redundancy of costimulatory pathways in T cell activation an attractive therapeutic strategy is to combine $CTLA_4Ig$ with blockade of other accessory T cell molecules. In this way $CTLA_4Ig$ has been shown to combine with blockade of the LFA-1 pathway to induce long-term acceptance of pancreatic islet allografts (326) and with anti-CD2 mAb to induce tolerance to non-vascularised murine cardiac allografts (291). A particularly potent effect has been obtained by combining $CTLA_4Ig$ with blockade of the CD40-gp39 pathway, which is an important costimulatory pathway in the development of B cell responses. This

regime has been shown to induce long-term survival of allogeneic skin grafts in mice (327).

An interesting use of $CTLA_4Ig$ has been the transfection of myoblasts with cDNA coding for $CTLA_4Ig$. When allogeneic myoblasts are transfected in this way and transplanted into live animals a small proportion develop stable secretion of low-dose $CTLA_4Ig$. This has been shown to extend survival of pancreatic islet allografts (MST 32 days) without other treatment (328).

Recently mAb targeting the CD28 molecule has been used in experimental transplantation in an attempt to overcome the dual blockade by $CTLA_4Ig$ of the CD28 and $CTLA_4$ molecules. By allowing signalling via the $CTLA_4$ molecule it was hoped that the negative regulatory function of this molecule could be retained whilst inhibiting the costimulatory function of CD28. Using the modulating mAb JJ319 in the DA to Lewis cardiac allograft model Dengler et al noted a very similar prolongation of allograft survival to that induced by $CTLA_4Ig$ alone (329). This group also noted a conflict between the *in vivo* and *in vitro* effect of JJ319. This mAb was shown to costimulate T cell activation in both the MLR and mitogen-mediated activation when added directly to culture plates whereas lymphocytes taken from a JJ319 treated animal showed reduced proliferative responses in identical experiments. This finding may be explained by more efficient CD28 modulation after *in vivo* therapy preventing transmission of a positive signal.

1.11.4 Mechanism of tolerance induction by $CTLAI_4g$ therapy

There are few publications exploring tolerance mechanisms induced by $CTLA_4Ig$ so a consensus view is difficult to obtain. In a similar manner to other regimes tolerance in these models can be assessed under the general headings of deletion, anergy and suppression.

1.11.4.1 Deletion

In the models where $CTLA_4Ig$ produces allograft tolerance it does so by a non-classical mechanism. There is no published data demonstrating deletion of alloreactive cells in tolerant animals whereas there is data demonstrating functioning alloreactive cells in these models in terms of proliferation (318) and cytokine production (317).

1.11.4.2 Anergy

In view of the predictions of Bretscher and Cohn's two-signal model of T cell activation and the demonstrations of clonal anergy in *in vitro* systems this is an attractive explanation for immune tolerance in transplant models. Unfortunately in the transplant setting anergy has not been formally demonstrated. In the early experiments of Linsley et al showing inhibition of antibody responses to SRBC even high dose $CTLA_4Ig$ therapy did not induce tolerance as most animals displayed enhanced responses when retested after 25 days (127). Similarly $CTLA_4Ig$ therapy in models of solid organ transplantation displays evidence of activation of alloreactive cells. Numerous groups have demonstrated a reduction in the MLR at early time points after transplantation (when cultures may be contaminated by residual $CTLA_4Ig$), which have not been reproduced in animals with long surviving grafts (314, 318). Analysis of effector cell function in animals made tolerant to allografts with $CTLA_4Ig$ has also shown that despite survival of the graft donor specific CTL are produced with similar titrations to rejecting controls (291). This is a significant observation as studies performed early after transplantation have shown the ability of $CTLA_4Ig$ to prevent the induction of allo-CTL (see above). This anomaly suggests that:

1) CTL are produced with slower kinetics in tolerant animals

2) CTL produced under these circumstances are unable to effect tissue damage *in vivo*.

This demonstration of effector cells in tolerant animals suggests that tolerance in these models is maintained by immune regulation within the allograft.

1.11.4.3 Suppression

Histological examination of tolerant allografts in $CTLA_4Ig$ treated animals has shown that despite preservation of graft function and architecture a cellular infiltrate, predominantly of mononuclear cells, still occurs (314). Sayegh and co-workers have sought evidence for immune-deviation in the pattern of cytokine secretion by these cells. Immunohistology of grafts from treated mice shows diminished staining for γIFN with increased staining for IL4 compared to rejecting controls. The difficulty in extrapolating results from *in vitro* systems is demonstrated by the fact that, in contrast to the above result, T cell hybridomas only produce IL4 after costimulation via CD28 (122). Analysis of immunoglobulin isotypes provides further evidence for differential subset activation in $CTLA_4Ig$ treated animals, as cardiac allografts show increased staining for the Th_2-associated IgG1 isotype (317). Support for the Th_2 dominance in $CTLA_4Ig$ treated animals comes from experiments in the EAE model, where a similar phenomenon has been demonstrated by immunohistology in the nervous system of treated rats (330). Diminished elaboration of Th_1 cytokines in the allograft could reflect either failed expansion of the Th_1 subset or diminished secretion of Th_1 cytokines by reactive T cells. To answer this question a more precise definition of the mechanisms underlying $CTLA_4Ig$ induced tolerance in a monoclonal system has been devised using pigeon cytochrome *c* as a nominal antigen and the fate of Ag reactive Vβ3 and Vα11 T cells followed with specific

mAb. Using this technique Judge et al have shown that both mechanisms may be involved, as expansion of reactive cells is reduced by approximately 50% (excluding deletion as a mechanism) with diminished cytokine secretion by the remaining cells (331). Despite the evidence of Th_2 dominance in some transplant models treated with $CTLA_4Ig$, adoptive transfer of cells from tolerant animals fails to influence allograft survival in naïve recipients (312), arguing against a potent suppressive effect.

Contrary to the predictions of Bretscher and Cohn's 'two signal' model therefore there is no clear evidence for anergy in models of transplant tolerance induced by $CTLA_4Ig$ and immune regulation appears to be a more likely mechanism. In a similar manner to other regimes evidence for different mechanisms can be discerned, which may depend on the model under study.

1.12 High and low responder rat strains in transplant models

The distinction between high and low responder rat strains was originally made in experiments of allograft survival induced by donor specific transfusion and passive enhancement by transfer of immune serum. It was noted that both techniques were able to induce permanent allograft survival in DA strain rats but not in Lewis rats (332). Experiments using numerous other regimes have confirmed that rat strains differ in their ease of tolerance induction. Rats of $RT1^a$ haplotype (DA and ACI) are consistently the easiest in which to induce tolerance and have been used in mAb models of transplant tolerance including anti-CD4 (234) and anti-CD2 (299) mAb. High responder rat strains include Lewis ($RT1^l$) and PVG-$RT1^u$. These strains have historically been very resistant to tolerance induction, and have usually required multiple treatment modalities. Tolerance to DA kidney allografts has been obtained

in these animals by using a combination of Cyclosporine A with either anti-CD4 or anti-TCR mAb (245) whereas the more immunogenic lung allograft is not tolerised in Lewis rats by a combination of Cyclosporine A and $CTLA_4Ig$ (333). Attempts by conventional mechanisms (DST or anti-CD4 mAb therapy) to induce tolerance to cardiac and skin allografts in Lewis rats were unsuccessful. Success was achieved by either combining potent immunosuppressive regimes or with myeloablation. Frede et al achieved allograft tolerance in the cardiac model in Lewis rats by combining donor-specific transfusion with Cyclosporine A and Rapamycin (334) whilst Zeng et al used a toxic regime of total lymphoid irradiation, anti-thymocyte globulin and donor blood transfusion (335). Simpler regimes to induce tolerance have been devised which involve combining $CTLA_4Ig$ with other modalities. Firstly Lin et al noted in the Brown Norway to Lewis combination that donor blood transfusion combined with $CTLA_4Ig$ two days post operatively is successful in the majority of recipients (318), whilst Fathman's group successfully obtained allograft tolerance in Lewis rats to both hearts (321) and small bowel (324) by combining $CTLA_4Ig$ with anti-CD4 mAb. Most of these regimes are inconsistent in their effect, inducing tolerance in only a minority of recipients. A similar effect can be achieved in Lewis rats by a prolonged (6 week) regime of post-operative anti-CD4 mAb, which again produces tolerance in a minority of recipients (336). In the high-responder strain combination of DA ($RT1^a$) to Lou ($RT1^c$) skin graft tolerance has been achieved in a minority of recipients by combining a prolonged post-operative regime of two anti-CD4 mAb (OX35 and OX38) with Cyclosporine A, (although other reports suggest that class I A^c is the low responder phenotype to A^a-see below).

Three areas of research have been performed to distinguish the immunological responses of high and low responder strain rats to account for the difference in allograft survival.

1) Ilano et al noted that OX35 (anti-CD4 mAb) is able to induce cardiac allograft tolerance in DA rats when used per-operatively whereas high dose, prolonged therapy is required in high responder Wistar Furth ($RT1^u$) rats (337). No differences were detectable in alloreactivity of tolerant animals; in particular unseparated cells from both strains reacted normally in MLR and reconstituted rejection in irradiated hosts. However when cells from naïve animals were divided into CD4 and CD8 subsets a differential activity of the CD8 subset was noted. CD8 cells from DA strain rats were unable to respond independently in the MLR to foreign cells and were unable to reconstitute rejection in irradiated hosts, whereas CD8 cells from Wistar Furth rats were able to perform both functions without CD4 T cell help, suggesting that CD8 T cells from high responder strain rats are able to generate their own helper activity.

2) A number of groups have worked with PVG congenic animals in which response to the class I A^a is genetically controlled, PVG-$RT1^c$ being a low responder phenotype and PVG-$RT1^u$ the high responder phenotype. Using R1 ($A^aB^cC^cD^c$) and R8 ($A^aB^uC^uD^u$) recombinants, Butcher et al analysed their respective responses to class I (A^a) disparate grafts (low responders fail to reject these grafts). This group demonstrated that the low responder phenotype fails to generate alloantibody and CTL to the A^a antigen (338). Using the same strain combinations Stepkowski et al found by limiting dilution analysis that the deficit in the low responder animals is at the level of helper-T cell precursors, the frequency of cytotoxic-T cell precursors being equal in the two strains (339).

3) Another group have analysed the response of high and low responder strain rats to mAb therapy. Yin et al noted that the anti-CD4 mAb OX38 induces cardiac allograft tolerance in the Lewis to DA combination but not the reverse (336). On analysing the kinetics of lymphocyte depletion induced by OX38 in the two strains it

was noted that allograft tolerance correlated with more profound and prolonged CD4 T cell depletion in DA strain rats. In DA strain rats a preoperative dose of 4x5mg/kg OX38 induced over 90% depletion of CD4 T cells with return to 50% of pre-treatment levels 14 weeks post operatively, in contrast in Lewis rats the same dose of OX38 induced only 80% depletion with return to 50% of pre-treatment levels after one week. The difference in allograft survival therefore may reflect more prolonged mAb-induced immunosuppression in the low responder strain.

The work of Butcher et al with congenic PVG rat strains suggests that the potent response of high responder rats is genetically controlled at the level of both the responder and stimulus. However the work of Yin et al and other observations made in this thesis suggests that the high responder status of Lewis rats is a more generalised phenomenon which also applies to non-MHC antigens.

1.13 Aims of this project

Extensive knowledge of the effect of anti-CD4 mAb therapy in rodent models of solid organ transplantation has been obtained by many different laboratories and it has largely been by using the CD4 antigen as a target molecule that attempts have been made to obtain transplant tolerance with mAb. These experiments have met with success in rodent models which has not been reproduced in higher animals. Partly for this reason research efforts have recently turned away from mAb to smaller molecules as induction therapy in experimental and clinical transplantation. Numerous other surface antigens are involved in T cell activation beside CD4, and the effect of targeting these ligands with mAb has not been explored to the same degree. The CD2 molecule has a complex relationship with the T cell receptor and multiple functions in T cell homeostasis and provides a promising target for immunotherapy. The effect of targeting CD2 with mAb has been explored in murine

models largely by the group of JS Bromberg in Charlestown, Carolina, USA. This group have reported that anti-CD2 mAb therapy in the mouse has an immunosuppressive effect dependent on CD2 modulation without causing significant cellular depletion (292, 304). This effect is not however potent enough to allow permanent allograft survival in mice without the addition of anti-CD3 mAb, with which anti-CD2 mAb has a special relationship in murine systems. Consequently these experiments do not allow an analysis of the effect of targeting the CD2 molecule alone, so that results are difficult to interpret at the cellular and molecular level. The rat therefore would seem to be a more promising model with which to explore therapy targetting this molecule, as the group of Hirahara in Tokyo has shown its ability to induce permanent allograft survival in low responder strain rats (299).

This project aimed therefore to use two different anti-CD2 mAb, OX34 and OX55, in rat cardiac allograft experiments. Firstly cardiac allografts were performed in low responder DA rats using induction therapy with various doses of OX34 and OX55. These experiments investigated the requirements for ligand saturation, modulation and cellular depletion to induce permanent allograft survival in these animals. This optimal dose of OX34 was then combined with other therapies in the high responder Lewis strain, and again the effect on CD2 expression and cell populations was investigated. In these experiments the response of the treated rats to the injected mAb was also assessed in terms of the generation of an anti-globulin response and the clearance of antibody.

Having established the potency of anti-CD2 mAb in allograft models a series of experiments were performed to reproduce these effects *in vitro,* to explore the mechanism of action of the mAb. These included examining the ability of anti-CD2 mAb to act as a mitogen, and its effect on the allogeneic MLR and mitogen-induced

cytokine release. Throughout these experiments the effect of anti-CD2 mAb was compared directly with that of anti-CD4 mAb and $CTLA_4Ig$ in similar assays.

Lastly having established models of allograft tolerance in both DA and Lewis strain rats the immunological mechanisms maintaining tolerance were investigated. Investigations included an assessment of the donor-specific effector mechanisms in tolerant animals, histology of the tolerised grafts and measurement of the response of cells from tolerant animals to donor antigen.

The data regarding the use of anti-CD2 mAb in rat models is compared to previous work in the mouse and rat, to obtain a consensus view on the potential of CD2 as a target molecule in the transplant setting.

2 Materials and Methods

2.1 Media used with lymphocyte preparations

2.1.1 Wash medium:

Hanks Buffered Salt Solution (HBSS) (GibcoBRL Life Technologies, Paisley, UK) supplemented with 2% heat-inactivated (HI) Foetal Calf Serum (FCS) (Sigma Biosciences, Poole, Dorset, UK), 100 U/ml Penicillin, 100μg/ml Streptomycin and 10mM Hepes solution (all Gibco BRL), was used in all preparative procedures for cells, unless otherwise stated.

2.1.2 Culture medium:

RPMI 1640 (Gibco BRL) supplemented with 2mM L-glutamine (Gibco BRL), 100 U/ml Penicillin, 100μg/ml Streptomycin, and $5x10^{-5}$ 2-Mercaptoethanol (FCS) (Fischer Scientific, Loughborough, UK) was used as a culture medium for cells, supplemented with HI normal syngeneic rat serum or FCS as stated.

2.2 Maintenance of hybridomas

Hybridoma cells producing OX34 (IgG2a mouse monoclonal directed at the ligand-binding epitope of domain 1 of the CD2 molecule (133, 150, 152, 340)), OX55 (IgG1 mouse monoclonal which binds non-competitively with OX34 to an epitope on domain 1 of CD2 not involved in the CD2-CD48 interaction (133)), OX38 (IgG2a mouse monoclonal directed at the CD4 molecule (340)), and R73 (IgG1 mouse monoclonal directed at a constant determinant of the α/β TCR (341))

were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in complete RPMI + 10% FCS in 5% CO_2 at 37°C.

The Il2-dependent CTLL cell-line and the Natural-Killer cell-sensitive lymphoid Y3 cell line were also obtained from European Collection of Animal Cell Cultures (Salisbury, UK) and cultured as above.

2.2.1 Monoclonal antibodies used in *in vitro* and *in vivo* experiments

Agent	Isotype	Target Molecule
OX34	Murine IgG2a	CD2
OX55	Murine IgG1	CD2
OX38	Murine IgG2a	CD4
R73	Murine IgG1	αβTCR
$CTLA_4Ig$	Human IgG1	B7
ESH8	Murine IgG2a	Human factor VIII
OX1	Murine IgG1	CD45

Table 2.1 Reagents used in transplant experiments

2.2.2 Preparation of monoclonal antibodies.

Hybridoma cells were washed in serum-free medium and resuspended at $2x10^6$/ml before injecting 0.5 ml into pristane primed BALB/c mice to produce ascites. Ascites was purified with a protein A column and quantified by radial immuno-diffusion assay using Nanorid RID plates (The Binding Site, Birmingham, UK) according to the manufacturers instructions. Purified mAb was then diluted in PBS to a working concentration of 5 or 10mg/ml. The control mouse IgG2a antibody ESH8 (anti-human factor VIII) was obtained from the Scottish Antibody Production Unit, Lanarkshire, Scotland. The construct $CTLA_4Ig$ was a gift of Dr. Linsley, BMS Seattle (116).

2.2.3 Characterisation of monoclonal antibody

After production by the techniques described in materials and methods, the titre of monoclonal antibodies was characterised by flow cytometry using serial tenfold dilutions of mAb wash medium incubated with lymph node cells (LNCs) as targets. The construct $CTLA_4Ig$, which does not bind resting LNCs, was characterised using ConA blasts (which express the activation antigen B7) as a target cell.

2.3 Experimental animals

Adult Lewis (LEW, $RT1^l$), DA ($RT1^a$) and PVG ($RT1^c$) were obtained from Olac (Bicester, Oxon, UK). Donor and recipient pairs were mismatched at both major and minor histocompatibility loci.

2.3.1 Heterotopic heart transplantation.

Procedures were performed under halothane anaesthesia. The donor rat was heparinised (200u) before exsanguination by division of the abdominal vessels. The donor heart was exposed and the pulmonary artery and aorta were divided and all other vessels ligated. The donor heart was drained of blood and stored at 4°C before transplanting to the abdominal vessels of the recipient rat in the manner described by Ono and Lindsey (342). Survival of cardiac allografts was assessed by alternate day palpation and rejection defined as cessation of beating.

For confirmation of transplant tolerance identical second grafts or third party cardiac allografts were harvested in the same manner and anastomosed to the carotid artery and internal jugular vein.

2.4 Preparation of cell suspensions

Lymph node cells were prepared by harvesting lymph nodes from the rat cervical and mesenteric lymphatic plexuses into wash medium using sterile techniques. Lymph nodes were compressed through a wire mesh sieve to disrupt the node and release free cells. Cell suspensions were then decanted and washed three times in wash medium to remove debris before resuspending at the working concentration.

Splenocytes were prepared by harvesting a rat spleen using sterile techniques into wash medium. The spleen was then disrupted in wash medium with sterile plastic forceps to release free cells. Red blood cells within the cell suspension were lysed by adding 5mls of sterile water to the resuspended cell pellet followed immediately by 5mls of 2N saline. Cells were washed three times in wash medium and debris discarded.

Peripheral blood lymphocytes were prepared either by harvesting blood from an anaesthetised rat by direct cardiac puncture into a syringe containing 0.5mls 1:1000 heparin or by the expression of small quantities of blood from the rat tail after transection of tail tip of an anaesthetised rat. Whole blood was then diluted 1:1 with PBS before layering onto 5mls 75% Percoll with 25% balanced salt solution containing 1% Hepes. Tubes were centrifuged at 2000 revs/min for 20 minutes. Lymphocytes were harvested by aspiration from the plasma: Percoll interface. Red blood cells were then lysed by hypotonic shock and cells washed and resuspended as described above.

Peritoneal macrophages were prepared by injecting 20 mls of culture medium into the peritoneal cavity of a freshly killed rat. The abdomen was then opened and medium aspirated. Cells were isolated by centrifugation at 4°C and identified by flow cytometric methods with the macrophage-specific antibody ED1.

2.4.1 Preparation of purified CD4 T cells

LNCs were prepared from two naïve rats, washed and suspended in 1ml wash medium as described above. This cell suspension was then incubated for 30 minutes on ice with pure OX8 and OX12 antibody (50µl of 10mg/ml) to saturate CD8 and B cells. Cells were then washed three times, counted and resuspended at 10^8/ml. Goat anti-mouse IgG-coated magnetic beads (Biomag, Advanced Magnetics Inc., UK) were then prepared (1ml per 10^3 cells) by washing three times in HBSS. Biomag beads were then added to the cell suspension and incubated for 20 minutes on ice with periodic shaking. Bound cells were then separated by magnetic traction, retained and resuspended in 2 mls wash medium (to dislodge trapped cells). After repeated separation of cells supernatants containing CD4 cells were pooled together and re-incubated with Magnabeads and the process repeated. Purified CD4 cells were then washed, counted and analysed by flow cytometry to assay degree of purity.

2.4.2 Preparation of Concanavalin A transformed lymphoblasts (Con A blasts)

Splenocytes were prepared as above and resuspended in culture medium at 2.5 x 10^6/ml. 10 mls of cells were then placed into 50ml culture flasks with 50µl ConA at 1mg/ml. Cells were then incubated in 5% CO2 at 37°C for 72 hours before using as target cells.

2.5 Assay of antibody-induced cellular activation

Lymph node cells were prepared and harvested as above. Monoclonal antibodies (either R73 or OX34) were used to induce lymphocyte activation either fixed to a plate surface or in solution. Ninety-six flat-bottomed well plates (Life

Technologies) were coated with 50µl/well polyclonal rabbit anti-mouse serum at 10µg/ml and incubated at 37°C for 2 hours in a sterile environment. Plates were then washed three times with PBS before adding 30µl/well R73/OX34 at 5µg/ml in PBS. Parallel plates were set up containing the same antibodies in solution at 20µg/ml. Wells containing PBS only were used as controls. Plates were then incubated for a further two hours before further washing. Unbound binding sites were then saturated by overnight 'blocking' with 1% mouse serum at 100µl/well at 4°C. Plates were again washed before adding $2x10^5$ purified CD4 T cells in 200µl culture medium per well. Plates were incubated at 37°C for 24 hours before adding tritiated thymidine (1µCi/well) in serum-free medium and further culture for eighteen hours. Cells were harvested and thymidine incorporation measured by quantitation of β emission.

For determination of the influence of second antibodies on proliferation by anti-TCR mAb LNCs were incubated with a concentration range of antibody in culture medium for 20 minutes on ice and washed to remove excess antibody prior to adding to wells.

2.6 The mixed lymphocyte reaction (MLR)

Spleen cells were used as stimulators in one-way MLRs. Prior to adding to U-bottomed 96-well plates (Life Technologies) splenocytes were resuspended at $2x10^6$/ml in culture medium and subjected to irradiation (2000 rads). Responder cells were allogeneic lymph node cell suspensions at $4x10^6$ per ml in culture medium. Control wells were cultured without stimulator cells (negative control) or with responders only containing ConA (5µg/ml) (positive control). 100µl of each cell suspension were co-cultured in 5% CO_2 at 37°C for three, four or five days before pulsing for 18 hours with tritiated thymidine (1µCi/well). Early experiments revealed

that cellular proliferation in these reactions increased over the period three to five days. However the thymidine incorpoartion after three days was sufficient to allow discrimination between experimental conditions so this time point was used in the majority of experiments to save on incubation time. Cellular incorporation of thymidine was measured by quantitation of β emission. Identical cultures were set up and cell culture supernatants retained for evaluation of cytokine concentration.

For evaluation of the effect of monoclonal antibody on proliferation induced by the MLR reagents were added at stated concentrations to responding lymphocytes for 30 minutes on ice and washed to remove excess antibody prior to adding to wells containing stimulator cell populations.

2.7 IL2 assay.

IL2 concentration in culture supernatants was assayed using the IL2 dependent murine cell line CTLL. $5x10^3$ CTLL cells were plated in 100μl culture medium with 100μl serial dilutions of MLR culture supernatant or IL2 standards. Plates were cultured for 24 hours before pulsing with tritiated thymidine (1μCi/well) and incorporation measured as above. Values for IL2, expressed as units/ml, were derived from a standard curve of human recombinant IL2 (Glaxo Laboratories, Geneva, Switzerland). Specificity of the assay was confirmed by the ability of anti-IL2 and the inability of anti-IL4mAbs to block CTLL proliferation.

2.8 γIFN assay

γIFN concentration in culture supernatants was assayed using an ELISA system. Ninety-six well flat bottom plates (Immulon 4, Dynatech, Billingshurst, UK.) were coated with 100μl of a 1:1000 dilution of rabbit anti-mouse γIFN serum in

0.1M $NaHCO_3$ (cross reactive with rat γIFN, a gift of Dr John Tite, Wellcome Research Laboratories, Beckenham, UK) overnight at 4°C. All subsequent incubations were at 37°C in 5% CO_2 alternating with extensive washing using PBS/0.05% Tween 20. Plates were 'blocked' with 200μl/well 10% FCS prior to the addition of MLR culture supernatants or recombinant rat γIFN standards (Life Technologies) followed by 100μl/well biotin conjugated mouse monoclonal anti-rat γIFN, DB1, (a gift of Dr P van der Meide, BPRC, Rijswijk, The Netherlands) for one hour. 1:100 dilution extravidin peroxidase (Sigma Chemical Co Ltd, Poole UK) was then added at 100μl/well followed by 100μl/well TMB peroxidase substrate (Dynatech Laboratories) for 30 minutes at room temperature. The reaction was stopped by the addition of 0.2M H_2SO_4 and optical density read at 450nm with a Dynatech MR 5000-plate reader. Values for γIFN were derived by comparison with a standard curve constructed from serial dilutions of γIFN standards.

2.9 Preparation of target cells for cytotoxicity assays.

Strain specific ConA blasts were prepared as above. Blasts were washed to remove FCS and then resuspended at 2.5 $x10^7$/ml in serum-free culture medium. Blasts were then incubated with 5 MBq ^{51}Cr for 1 hour with periodic shaking in a water-bath at 37°C. Target cells were then washed five times in wash medium to remove free isotope before suspending at 10^6/ml in culture medium.

2.10 *In vitro* generation of cytotoxic T lymphocytes (CTLs)

Specific CTL were generated in a 'bulk' MLR. In order to ascertain the optimum responder: stimulator ratio for CTL generation LNCs were suspended at 2,

4 and $8x10^6$/ml and were incubated in equal volumes with irradiated DA splenocytes at 10^6/ml in 20mls complete RPMI medium in 5% CO_2 at 37°C for 5 days.

2.11 CTL-mediated cytotoxicity assay

To assess the activity of generated CTLs serial dilutions of effector cells from the bulk MLR culture (starting ratio 200:1) were then incubated with 10^4 target cells (^{51}Cr-labelled strain-specific ConA blasts) in 200µl complete medium in 96 well V-bottomed plates (Bibby Sterilin, Stone, Staffordshire) for 6 hours before harvesting 100µl supernatant. γ emission from released ^{51}Cr was measured using a 1282 Compugamma (LKB Wallac). Maximum release from lysed cells was measured following the addition of 100µl Triton X and spontaneous release measured in wells containing labelled targets only. Percentage cytotoxicity is expressed as 100x (experimental-spontaneous cpm/maximum-spontaneous cpm). These experiments revealed that maximal CTL generation occurred with a responder: stimulator ratio of 2:1 in the bulk MLR, which was used in all subsequent experiments (figure 2.1).

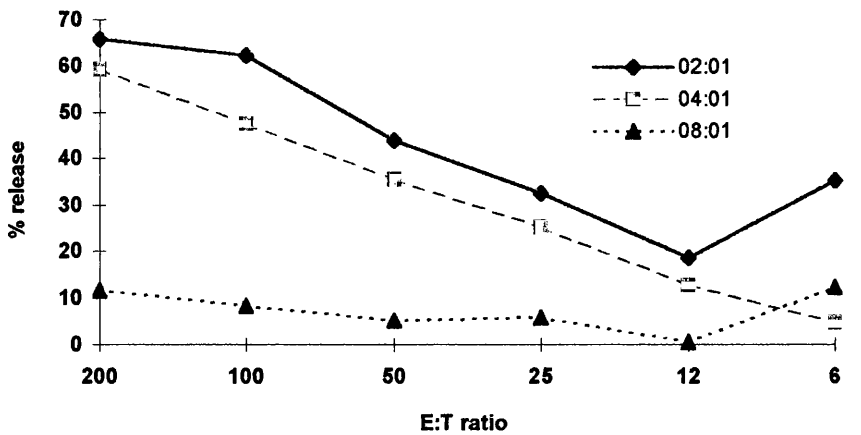

Figure 2.1 *In vitro* generation of allo-CTL.

Specific cytotoxicity of Lewis allo-CTL generated in bulk MLR with DA strain stimulators at effector: stimulator ratios of 8, 4 and 2

2.11.1 NK cell-mediated cytotoxicity assay

A 'bulk MLR' was used as a source of effector cells as above. The assay was performed as described for CTL above, but using ^{51}Cr-labelled NK-sensitive Y3 targets cells.

2.12 Flow cytometric analysis of lymphocyte antigen expression after *in vivo* monoclonal antibody therapy

The monoclonal antibody under study was injected at the required dose into the dorsal vein of the penis at stated time points. Lymphocyte populations were then harvested as above at different time points after administration. Cells were washed and suspended at 10^7/ml in PBS/0.2% FCS. For assessment of ligand expression by two-colour analysis 100µl cells were incubated for 30 minutes on ice with either 10µl mAb directly conjugated to fluorochrome, or with a 10µl purified primary antibody followed by 20µl polyclonal rabbit anti-mouse antibody bound to fluorochrome. Cells were washed repeatedly between incubations in PBS and prior to FACS analysis (Coulter EPICS). Initial control experiments were performed to assess non-specific binding of the isotype-matched control antibody (IgG2a) ESH 8 to murine lymphocytes and maximal binding by the pan-specific murine anti-CD45 antibody OX1 (Figure 2.2).

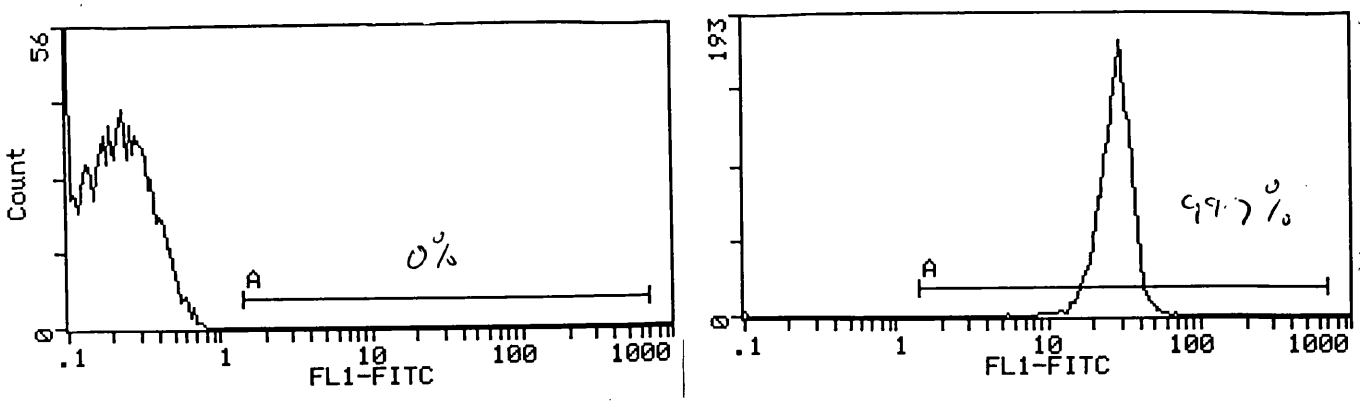

Figure 2.2 Positive and negative controls in flow cytometry experiments.

Flow cytometry experiments were performed according to the method in the text using a) the irrelevant murine antibody ESH 8 and b) the lymphocyte pan-specific antibody OX1 followed by rabbit anti-mouse FITC.

2.13 Flow cytometric analysis of elimination of circulating antibody after intravenous administration

The presence of circulating antibody in treated rats was determined by a non-quantitative flow cytometric method. Monoclonal antibody treated rats were tail-bled into heparin-containing eppendorf tubes at set time points after administration of antibody. Samples were centrifuged at low speed and serum extracted and diluted 1:1 with PBS. 100µl dilute serum was then incubated on ice with 100µl strain-specific LNCs at 10^7/ml for 30 minutes. After three washes antibody binding to lymphocytes was detected by further incubation with FITC-conjugated rabbit anti-mouse antibody and FACS analysis. Figure 2.3 shows control experiments where naïve rat serum or 20 µl 1:1000 OX34 were used in the first incubation.

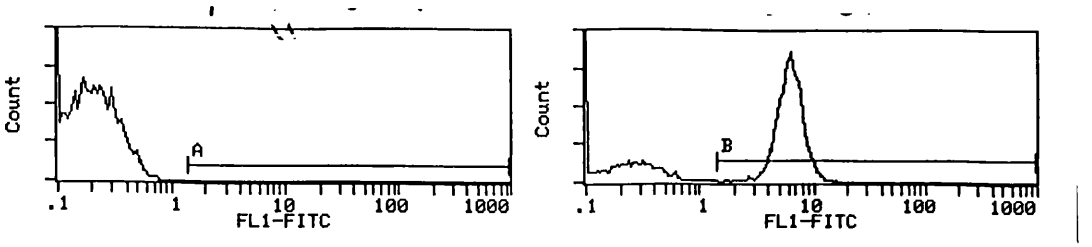

Figure 2.3 Positive and negative controls in flow cytometric detection of circulating mAb in rat serum.

Lewis LNCs were incubated with a) naïve Lewis serum or b) 20µl 1:1000 OX34 before secondary labelling with FITC-conjugated rabbit anti-mouse antibody.

2.14 Flow cytometric detections of circulating antiglobulin response after intravenous administration of monoclonal antibody

The anti-globulin response of mAb treated rats was detected by a non-quantitative flow cytometric method. Serum from mAb-treated rats was prepared as above. Fresh LNCs (100µl containing 10^6 cells in PBS) were incubated with 10µl OX34 or OX38 at 5mg/ml on ice for 30 minutes followed by washing. These anti-body labelled cells were then used as targets and incubated with 100µl dilute (1:1) serum from rats treated as above. Cells were washed and the presence of anti-mouse Ig was determined by tertiary labelling with 20µl FITC-labelled mouse anti-rat IgM and IgG antibody. Figure 2.4 shows control experiments demonstrating a) labelling of target cells with appropriate OX34 or OX38 target antibody, b) control

experiments where OX34-labelled Lewis LNCs are incubated with naïve rat serum before tertiary labelling with FITC-conjugated mouse anti-rat IgG and IgM, c) control experiments where OX38-labelled Lewis LNCs are incubated with naïve rat serum before tertiary labelling with FITC-conjugated mouse anti-rat IgG and IgM. These experiments show that approximately 20% of OX34-labelled LNCs are labelled with mouse anti-rat IgM after incubation with naïve rat serum, this probably represents labelling of IgM-expressing B cells.

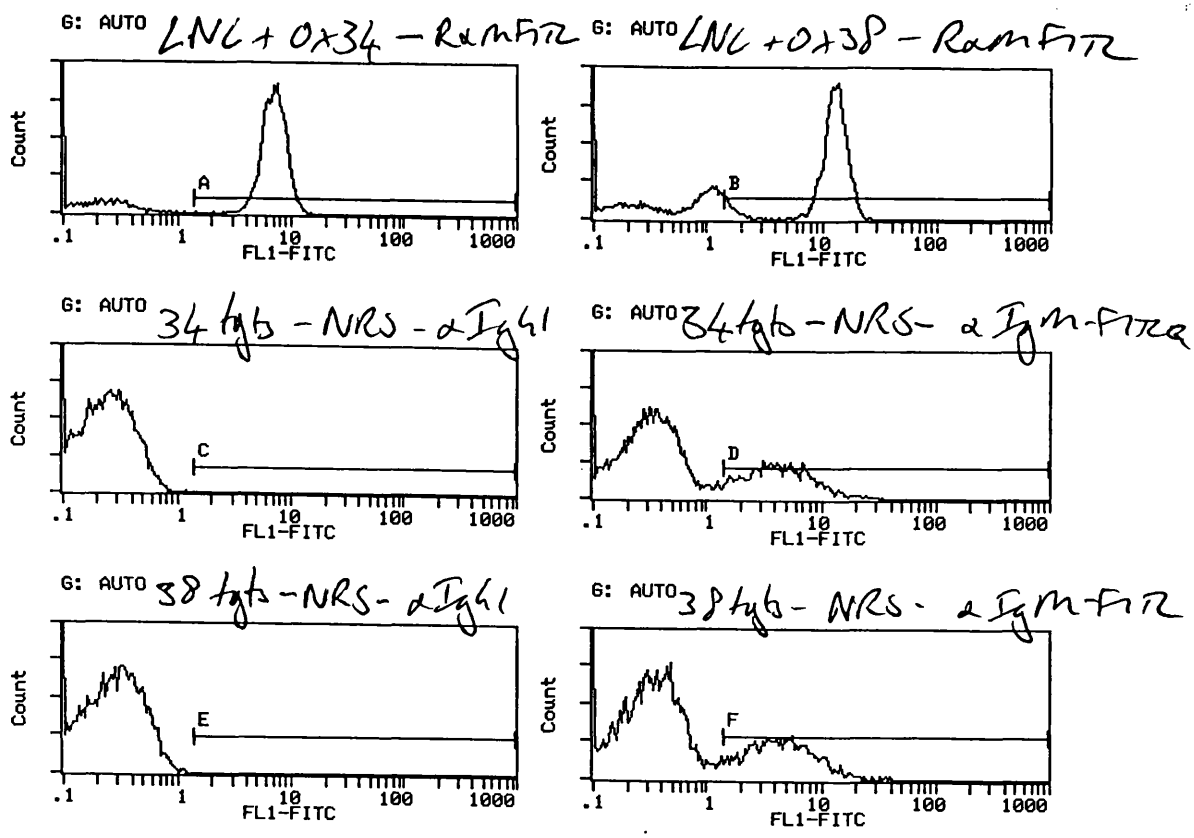

Figure 2.4 Flow cytometric detection of circulating antiglobulin response.

Control experiments showing a) labelling of target cells with OX34 and OX38, b) background labelling of OX34-labelled targets with anti-IgG and anti-IgM, c) background labelling of OX38-labelled targets with anti-IgG and anti-IgM

2.15 Determination of total circulating leukocyte number

This method was used to obtain the absolute numbers of circulating lymphocytes after administration of antibody, whereas flow cytometry was used to assess relative proportions of lymphocyte subsets. Rats were tail-bled into heparin-containing eppendorf tubes; blood was then diluted 1:20 with Turck's solution (2% acetic acid in water plus 5-6 grains of gentian violet per 100 mls). The sample was then shaken gently for one minute to lyse red cells. Leukocytes stain deep violet black and can be counted on a haemocytometer slide.

2.16 Antibody-mediated, complement-dependent cytotoxicity assay

The ability of OX34 and OX38 to lyse lymphocytes by complement-mediated mechanisms was assessed by an *in vitro* ^{51}Chromium release assay. DA strain splenocyte ConA blasts were prepared and incubated with ^{51}Chromium and used as target cells as described above. 50µl of target cells at 10^6/ml were then incubated with 50µl of serial tripling dilutions of serum or antibody (initial concentration 20µg/ml) in 96 V well plates at room temperature for 30 minutes. After washing, 100µl of fresh DA serum was added as a source of complement. Cells were incubated for 2 hours at 37°C before harvesting 100µl of supernatant for γ counting and estimation of cytotoxicity using the formula:

$$\% \text{ specific } ^{51}\text{Cr release} = \frac{\text{experimental-spontaneous}}{\text{maximum-spontaneous}} \times 100$$

Heat inactivated (56°C for 30 minutes) normal Lewis rat serum was used to determine spontaneous release; maximum release was determined by detergent lysis of target cells.

2.17 Monoclonal antibody-mediated antibody-dependent cellular cytotoxicity

This process depends upon recognition of antibody coating rat lymphocytes by Fc receptors on phagocytic cells. FACS analysis revealed that the NK-sensitive rat lymphoma cell line Y3 is labelled by both OX34 and OX38. To assess the ability of phagocytic cells to recognise mouse mAb a standard cytotoxicity assay was performed using antibody-labelled Y3 cells as targets. Y3 cells were labelled with ^{51}Cr as described above and resuspended in a minimal volume of culture medium. 500μl of cells were then incubated on ice for 30 minutes with 20μl OX34 or OX55 (5mg/ml). In the absence of a suitable assay for macrophage function the ability of NK cells (which express an identical FcγR111) to participate in ADCC was assessed. Effector cells were obtained from Lewis LNCs after 5 days of culture in a 'bulk' MLR with DA splenocytes as described above. Effector cells were suspended initially at $2x10^6$/ml and antibody-coated Y3 cells at 10^4/ml. Serial dilutions of 100μl effector cells (starting 200:1) were then incubated with 100μl target cells in V-bottomed plates and ^{51}Cr release measured as described.

2.18 Statistical analysis

2.18.1 Parametric data

Results of thymidine incorporation assays, chromium release assays and ELISA plates were analysed by Students t test using integral software with Microsoft Excel (Microsoft Corp).

2.18.2 Non-parametric data

Survival data from transplant experiments is displayed graphically using Kaplan-Meyer survival curves. Comparison between curves was performed by the logrank test. Calculations were performed manually with the aid of a pocket calculator according to the formula laid out in Introduction to Medical Statistics, (Bland, M).

3 Effect of monoclonal antibody therapy on survival of cardiac allografts in high and low responder strain rats.

3.1 Study objective:

1) To establish the effect of pre- and post- operative therapy with the anti-CD2 mAb OX34 and OX55 on cardiac allograft survival in the Lewis to DA transplant model.
2) To establish the minimal dose of OX34 required to induce permanent allograft survival in DA strain rats and to compare this with therapy with anti-CD4, anti-TCR and $CTLA_4Ig$.
3) To assess the effect of therapy with anti-CD4, anti-CD2, and $CTLA_4Ig$ on cardiac allograft survival in high responder Lewis rats.
4) To assess the effect of combination therapy with anti-CD2 plus either anti-TCR, anti-CD4 or $CTLA_4Ig$ on cardiac allograft survival in the high responder rat strains Lewis and PVG.
5) To establish the optimum dose of combination therapy with anti-CD2 plus either anti-CD4 or $CTLA_4Ig$ that prolongs cardiac allograft survival in Lewis strain rats.

3.2 Study design:

Cardiac allografts were performed between reciprocal pairs of DA and Lewis strain rats and from DA to PVG rats. Monoclonal antibodies were administered to

rats either pre- or post- transplantation at the stated doses. Survival of cardiac allografts induced by each regime was recorded and compared graphically. Figures on graphs represent doses of mAb and figures in brackets represent dosing schedule relative to time of transplantation.

3.3 Survival of cardiac allografts in untreated animals

In order to establish the kinetics of unmodified cardiac allograft rejection grafts were performed in naïve animals. Unmodified high and low responder rat strains were shown to reject cardiac allografts in a similar time span (6-8 days). Control experiments were performed only at the beginning of the project and control curves appearing in subsequent survival graphs refer to this experiment.

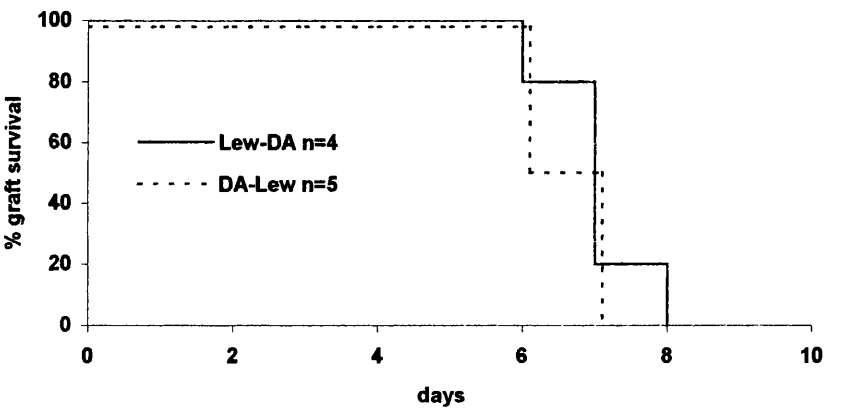

Figure 3.1 Survival of cardiac allografts in untreated control rats

Graph shows post-operative survival of reciprocal cardiac allografts in unmodified DA and Lewis rats.

3.4 Experiments to assess the ability of monoclonal antibody therapy to prolong allograft survival in low responder rats.

3.4.1 Effect of OX34 and OX55 therapy on survival of Lewis allografts in DA strain rats

Firstly mAb to CD2 was used in transplant experiments in the Lewis to DA model. Antibody was administered intravenously prior to transplantation. Increasing doses of OX34 were used to find an optimum dose that consistently induces prolonged allograft survival. OX34 administered at 5mg/kg intravenously on two occasions preoperatively induced permanent allograft survival in 80% of recipients (figure 3.2). Increasing the dose of OX34 did not increase the survival rate as a small percentage of rats always rejected their allografts (figure 3.3). In a similar manner OX55 was administered preoperatively and was noted to be less effective on a dose-for-dose basis (figure 3.3).

We then asked if OX34 therapy is as effective when administered post-operatively, after the induction of an immune response. A similar dose to one that induced long-term allograft survival when administered pre-operatively was much less successful when administered two days later (figure 3.4).

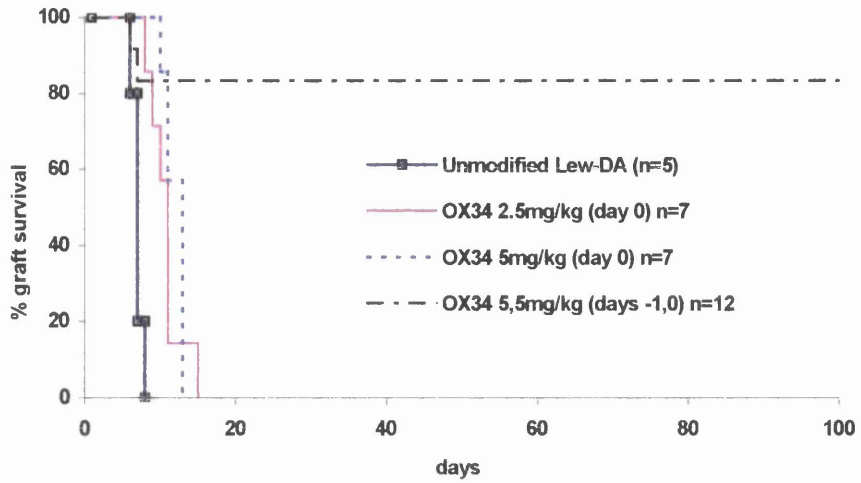

Figure 3.2 Effect on graft survival in DA rats of pre-operative OX34 therapy

Survival of Lewis cardiac allografts in DA strain rats treated pre-operatively with increasing doses of OX34 (OX34 2x5mg/kg v unmodified $p<0.01$, logrank test).

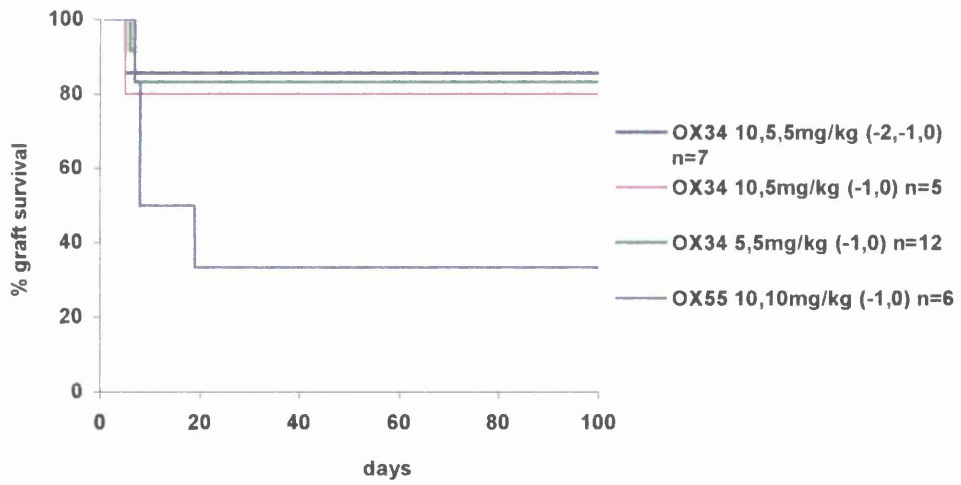

Figure 3.3 Effect on graft survival in DA rats of pre-operative OX55 or high dose OX34 therapy

Survival of Lewis cardiac allografts in DA strain rats treated pre-operatively with high dose OX34 (10-20mg/kg) or OX55 (20mg/kg) therapy (OX34 [all doses] v OX55 $p<0.05$, logrank test). Figures in brackets refer to treatment day relative to day of transplant.

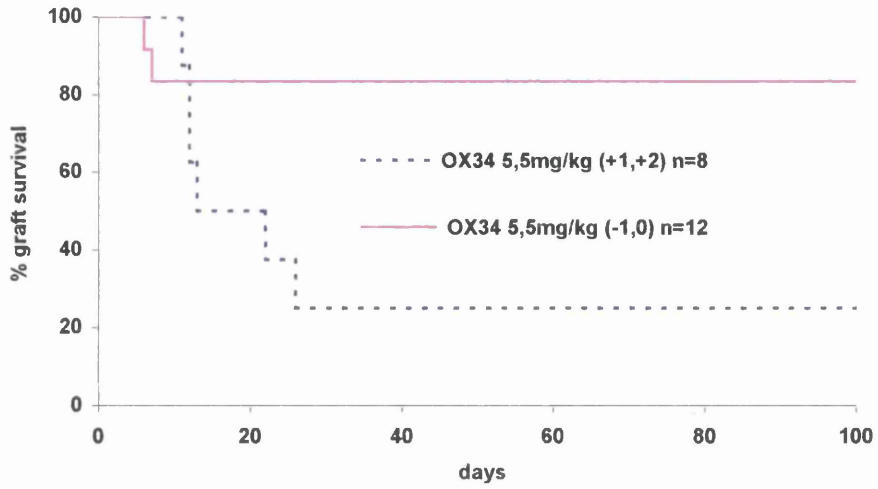

Figure 3.4 Effect on graft survival in DA rats of post-operative OX34 therapy

Survival of cardiac allografts in DA rats treated with OX34 (10mg/kg) pre- or post-operatively (pre- v post-operative therapy, $p<0.05$ logrank test). Figures in brackets refer to treatment day relative to day of transplant.

3.4.2 Anti-CD2, anti-CD4, anti-TCR and $CTLA_4Ig$ therapy all induce long-term allograft survival in DA rats

The effect of $CTLA_4Ig$, anti-TCR and anti-CD4 mAb therapy on allograft survival in DA strain rats was then assessed in comparison to anti-CD2 mAb therapy. OX38 was administered at a dose of 10,2,2,2 mg/kg (the optimum dose in this strain combination (343)) on consecutive days prior to transplant and 0.5 mg/rat $CTLA_4Ig$ was administered day 2 post-operatively. All modalities induced similar prolongation of allograft survival (figure 3.5).

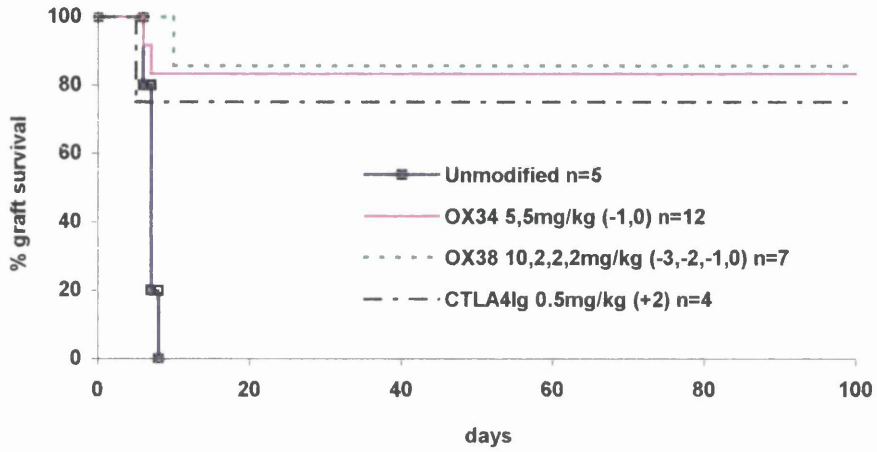

Figure 3.5 OX34, OX38 and $CTLA_4Ig$ induce permanent graft survival in DA rats

Survival of Lewis cardiac allografts in DA strain rats treated with OX34, OX38 or $CTLA_4Ig$ monotherapy. Figures in brackets refer to treatment day relative to day of transplant.

3.5 Experiments to assess the ability of targeting accessory molecules to prolong allograft survival in high responder rats.

In a similar manner to above the reagents under study were administered to high responder Lewis rats grafted with DA strain hearts to assess their influence in this more rigorous model of allograft rejection. OX34 and OX38 were administered over consecutive days pre-operatively whilst $CTLA_4Ig$ was administered two days post-operatively. All reagents tested produced some prolongation of graft survival although none were able to induce permanent engraftment. OX34 2x5mg/kg, MST=20 days; OX38 10+3x2mg/kg, MST=18 days and $CTLA_4Ig$ 2mg/kg, MST=19 days (figure 3.6).

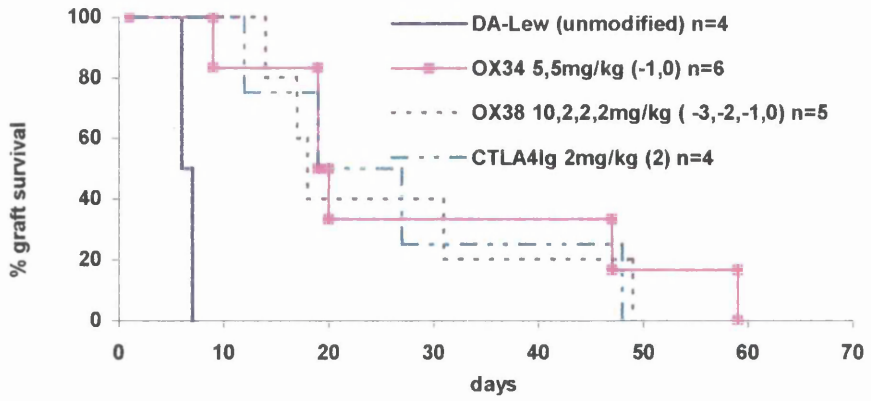

Figure 3.6 OX34, OX38 and CTLA$_4$Ig fail to induce permanent allograft survival in Lewis rats

Survival of DA allografts in Lewis rats treated with OX34, OX38 or CTLA$_4$Ig monotherapy at doses which induce permanent graft survival in DA rats. Figures in brackets refer to treatment day relative to day of transplant.

3.5.1 Combination therapy in the prolongation of allograft survival in high responder strain rats.

Each of the modalities tested produced a similar profile of survival curve in Lewis rats, with no grafts achieving permanent allograft survival. Combinations of mAb were therefore tested in an attempt to induce allograft tolerance in high-responder rats.

3.5.1.1 Anti-CD2 plus anti-TCR monoclonal antibody therapy in Lewis and PVG strain rats

As can be seen from the above experiments anti-TCR, CD2 and CD4 mAb and CTLA$_4$Ig therapy induced long-term allograft survival in the majority of DA strain rats but had less impact in Lewis rats. In view of the published murine studies combining anti-CD3 with anti-CD2 mAb therapy this combination was then assessed in the DA to Lewis cardiac allograft model. Even high doses of both modalities induced an MST of only 19 days; identical to the MST induced by 'standard dose'

(2x5mg/kg) OX34 monotherapy and failed to induce permanent engraftment in any recipient (figure 3.7).

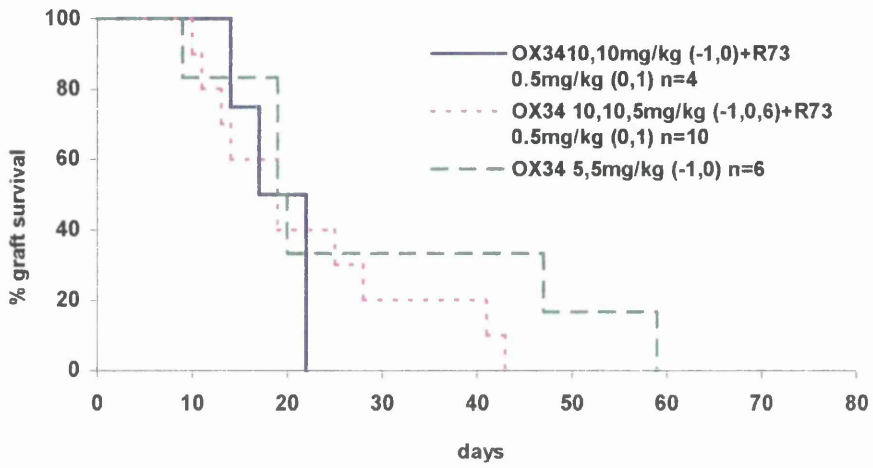

Figure 3.7 Combination therapy with OX34 and R73 in Lewis rats

Survival of DA allografts in Lewis rats treated with combination therapy with OX34 and R73. Figures in brackets refer to treatment day relative to day of transplant. Control animals treated with OX34 monotherapy (experiment performed jointly with J Casey).

We then asked if the failure of this mAb combination to improve allograft survival was an effect peculiar to the DA to Lewis strain pair. We therefore assessed the effect of the same mAb strategy in the DA to PVG (RT-1^c) strain combination. Increasing doses of each mAb were administered and R73 was administered both pre- and post-operatively. Even by using OX34 at twice the 'standard dose' (2x10mg/kg) and R73 (2x2mg/kg) the MST was only marginally prolonged (14 days) with no allograft surviving beyond 20 days (figure 3.8).

3.5.1.2 Anti-CD2 plus anti-CD4 monoclonal antibody therapy in Lewis strain rats

In view of the failure of anti-TCR mAb to combine with anti-CD2 mAb therapy to prolong allograft survival in high responder strain rats it was then decided

to combine anti-CD2 therapy with anti-CD4 therapy. When standard doses of OX34 (2x5mg/kg) and OX38 (10,2,2,2 mg/kg) were administered preoperatively to Lewis rats receiving DA allografts median survival is prolonged to 49 days with 50% of treated rats going on to permanent allograft survival. Increasing the dose of both reagents substantially (OX34 3x5mg/kg plus OX38 3x10 mg/kg pre-operatively) produced a modest increase in allograft survival (MST 100 days) with 8 of 15 rats permanently accepting their allografts (figure 3.9).

3.5.1.3 Anti-CD2 monoclonal antibody plus $CTLA_4Ig$ therapy in Lewis strain rats

In a similar manner anti-CD2 mAb therapy was then combined with $CTLA_4Ig$ therapy in the high responder DA to Lewis combination. Standard doses of each modality (OX34 2x5mg/kg pre-operatively plus $CTLA_4Ig$ 2mg/kg post-operatively) extended median allograft survival to 49 days with 5 of 11 rats permanently accepting their allografts. Increasing doses of both modalities were then assessed for their effect on the proportion of rats permanently accepting their allografts. Firstly a second dose of $CTLA_4Ig$ was administered at the time of transplantation in addition to the post-operative dose at day 2. This regime did not improve allograft survival, inducing a median survival of 46 days with 2 of 8 animals permanently accepting their allografts. The dose of OX34 was then doubled to 2x10mg/kg given pre-operatively along with a standard post-operative dose (2mg/kg) of $CTLA_4Ig$. Using this regime median survival was extended to over 100 days with 4 of 6 animals permanently accepting their allografts (figure 3.10).

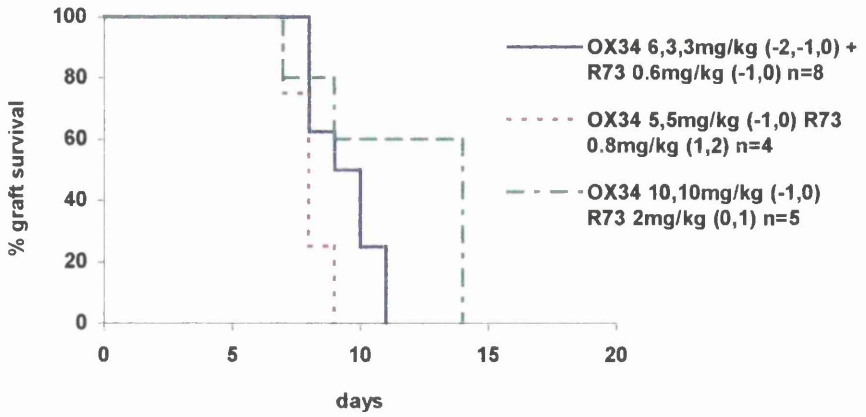

Figure 3.8 Combination therapy with OX34 and R73 in PVG rats

Survival of DA allografts in PVG rats treated with combination therapy with OX34 and R73. Figures in brackets refer to treatment day relative to day of transplant (experiment performed jointly with J Casey).

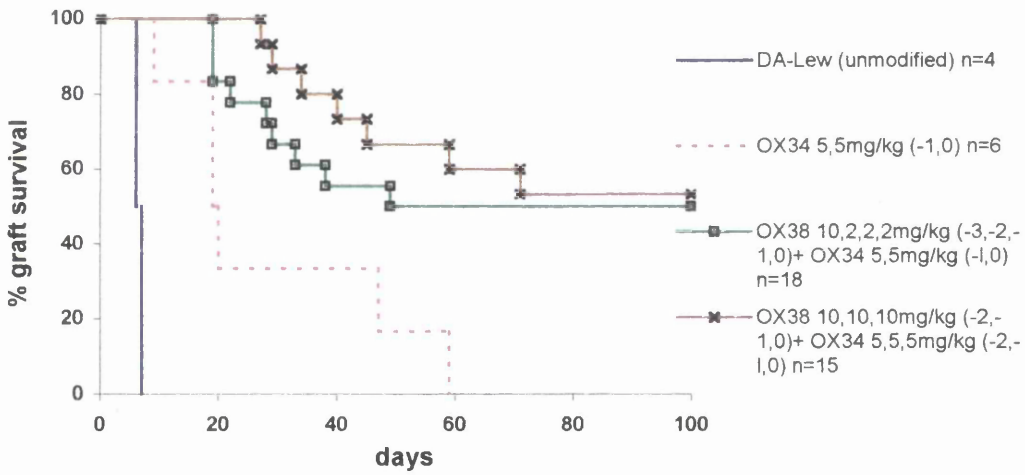

Figure 3.9 Combination therapy with OX34 and OX38 in Lewis rats

Survival of DA cardiac allografts in Lewis rats treated with combination OX34 and OX38 monoclonal antibody therapy. Control rats treated with OX34 monotherapy (OX34 5,5mg/kg v OX34 5,5mg/kg + OX38 10,2,2,2mg/kg $p<0.05$, logrank test). Figures in brackets refer to treatment day relative to day of transplant.

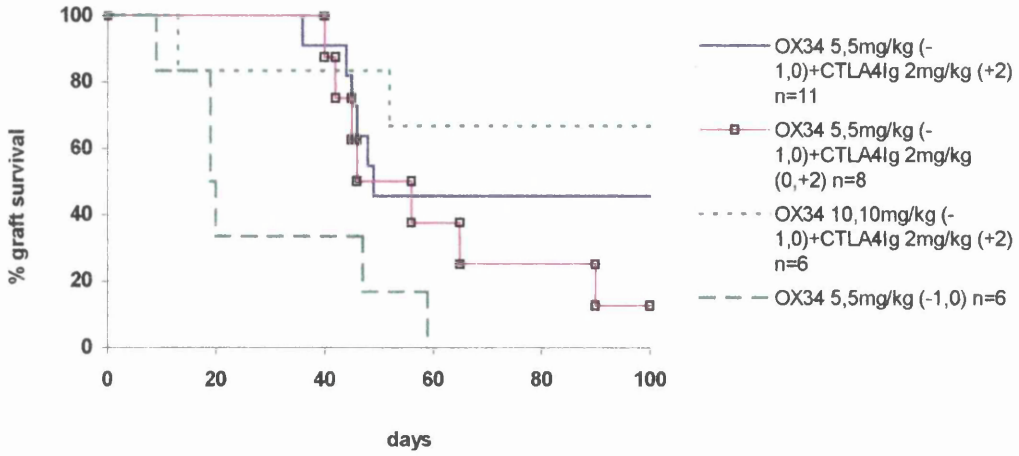

Figure 3.10 Combination therapy with OX34 and $CTLA_4Ig$ in Lewis rats

Survival of DA cardiac allografts in Lewis rats treated with combination therapy with OX34 $CTLA_4Ig$. Control animals treated with OX34 monotherapy (OX34 5,5 mg/kg v OX34 5,5mg/kg + $CTLA_4Ig$ 2mg/kg $p<0.05$, logrank test). Figures in brackets refer to treatment day relative to day of transplant.

3.6 Conclusions and discussion of results

3.6.1 Monoclonal antibody therapy in low responder DA rats.

Initial control experiments revealed that naïve Lewis and DA strain rats reject reciprocal allografts in a similar time span, with no heart graft surviving beyond 8 days.

Survival studies revealed that OX34 is able to induce permanent allograft survival of Lewis allografts in DA strain rats at an optimum dose of 2x5 mg/kg given pre-operatively (10 of 12 rats permanently accepted their allografts). This is a lower dose than that used by Hirahara (3x7mg/kg) although this regime was administered via the peritoneal route (299). Lower doses of OX34 (2.5mg/kg and 5 mg/kg) induced only minimal prolongation of allograft survival suggesting that a threshold level of immunosuppression is required to prevent acute rejection. Later studies

revealed that these doses of mAb induced similar initial levels of cellular depletion (see Chapter 5), suggesting this is not the only mechanism involved in their immunosuppressive effect. Higher doses of OX34 produced very similar survival curves, in that a consistent proportion of allografts (10-20%) was rejected, even when 2x10 mg/kg was administered. Furthermore these rejections always occurred within the time span of unmodified acute allograft rejection (7-11 days). Late graft failure did not occur with these regimes (graft failure within the first 72 hours in naïve animals are regarded as technical failures and excluded from analysis in these experiments). These findings are consistent with the hypothesis that acute rejection in these animals is an all-or–nothing response, and a threshold level of immunosuppression is required to prevent the response. A possible explanation for the consistent proportion of rats able to reject allografts despite OX34 therapy is failure of the injection technique, although this is unlikely as the procedure was performed on anaesthetised rats.

Post-operative doses of OX34 are less effective at inducing allograft survival with only 3 of 12 grafts being permanently accepted after this regime. Other workers using anti-CD2 mAb in the rat (299) and mouse (292, 344) models have noted similar findings (although Hirahara was able to induce permanent graft survival in DA rats using a regime of 7mg/kg consecutively for 8 days post-operatively (299)). This however contrasts with the well-described benefits of administering $CTLA_4Ig$ post-operatively (318). As discussed in the introduction there are two possible explanations for the beneficial effect of post-operative therapy with $CTLA_4Ig$: either the upregulation of B7-2 (121) or the migration of allo-APC (320) to regional lymph nodes, both of which processes reach maximal levels 48 hours after transplantation. The observation that anti-CD2 mAb is most effective when given at the time of transplantation suggests that the process of allosensitisation begins immediately after

revascularisation, so that the theory of sensitisation begining after migration of allo-APC to the spleen is not supported by evidence presented here. (The unusual observation therefore that $CTLA_4Ig$ is most effective when administered post-transplantation is most likely explained by the upregulation of B7-2). The spread of graft failures after post-operative therapy with OX34 (days 11-26) contrasts with the all-or-nothing response of pre-operative therapy, and suggests that OX34 has limited success at reversing an established immune response. This is in keeping with *in vitro* findings described later showing that OX34 is effective during the inductive phase of an immune response, but has little effect on established effector mechanisms.

Pre-operative therapy with OX55 is less effective than OX34 at inducing permanent allograft survival, even when used at higher doses. Of the group of six treated rats four grafts were rejected. Three of these occurred under 10 days, and one graft was rejected at day 20, the only late graft failure noted in DA rats after pre-operative therapy. The use of OX55 in transplant experiments has only been reported in a single publication previously, where it was noted to have minimal effect on graft survival in high responder Lewis rats (303).

Also reported is the effect of therapy with OX38 and $CTLA_4Ig$ in the low responder Lewis to DA combination. The use of these therapies in rat transplant experiments is well recorded (234, 314, 345). The pattern of survival curves obtained with these modalities conforms to that of OX34, in that a small percentage of allografts is rejected in the first twelve days post-transplant.

3.6.2 Monoclonal antibody therapy in high responder Lewis and PVG rats

Therapy with OX34, OX38 and $CTLA_4Ig$ at doses that are able to produce permanent allograft survival in DA rats is only able to prolong allograft survival in

Lewis rats. The survival curves obtained for the three modes of therapy in Lewis rats differ to those obtained in DA rats but are very similar to each other, in that there is a gradual attrition of all allografts during the period 10-60 days. This is an interesting observation as data discussed in this thesis suggests that these three modalities have different mechanisms of action, yet produce very similar effects at the level of allograft survival in the two rat strains examined. The large number of late graft failures seen in Lewis rats differs from the all-or-nothing response seen with DA strain animals. This may be interpreted as an inability of DA strain rats to mount an immune response to allografts after exposure to graft antigen during a period of immunosuppression by a variety of modalities, whereas the same modalities in Lewis rats are able to prevent acute allograft rejection for the duration of their effect, but the rats retain the ability to mount an immune response once the effect of the administered therapy has declined. Further evidence of the importance of the duration of immunosuppression is presented in Chapter 5.

As described in the introduction murine studies have shown a beneficial effect of combining anti-CD2 mAb with anti-TCR mAb (296) (anti-CD2 mAb alone is ineffective in producing permanent allograft survival in mice). The same tactic was therefore tried in high responder rats. No survival advantage was noted with this combination in either Lewis or PVG strain rats, even with high dose therapy, demonstrating that synergy between monoclonal antibody therapies targeting distinct cell surface ligands is not readily transferred between species.

In contrast a combination of 'standard' doses of OX34 (2x5mg/kg) and OX38 (10,2,2,2mg/kg) has a marked effect on allograft survival in Lewis rats, inducing a MST of 49 days with 50% of allografts achieving permanent engraftment. The relationship between these two therapies may be additive, whereby the effect of the two therapies combined (at doses X+Y) is equal to the effect of a dose 2X or 2Y of

single therapy, or synergistic, whereby the effect of doses X+Y exceeds the effect of either 2X or 2Y. In order to establish precisely the nature of the relationship between the effect of OX34 and OX38 it would be necessary to perform experiments using both OX34 and OX38 alone at doses of 2x5mg/kg + 10,2,2,2mg/kg (=26mg/kg). This experiment has not been performed so we are unable to say unequivocally that the effect between these two therapies is synergistic rather than additive. There is indirect evidence however that the combined effect is synergistic:

1) High dose OX34 therapy (20mg/kg) used in combination with R73 confers no survival benefit in Lewis rats.
2) High dose OX38 therapy (30mg/kg) has a minimal survival advantage over standard dose when used in combination with OX34 in Lewis rats.
3) High dose OX34 therapy (20mg/kg) has only a small survival advantage over standard dose when used in combination with $CTLA_4Ig$ in Lewis rats.
4) In Chapter 5 the interaction between OX34 and OX38 or $CTLA_4Ig$ is seen to produce more profound T cell depletion than seen when OX34 is used alone. Evidence is also presented in DA rats that T cell depletion caused by OX34 is not dose dependent, so that the extra depletion caused by a combination of reagents must be due to a functional interaction.
5) A further functional interaction is seen between OX34 and $CTLA_4Ig$ which leads to abrogation of the anti-globulin response to OX34 and prolonged cellular depletion.

In a similar manner to the combined effect of OX34 and OX38 a survival advantage is noted by combining OX34 with $CTLA_4Ig$ therapy in Lewis rats. Furthermore the trend is one of prolonged survival with increasing dose of OX34 in

that $CTLA_4Ig$ 2mg/kg +OX34 2x5mg/kg induces a MST of 49 days and $CTLA_4Ig$ 2mg/kg + OX34 2x10mg/kg induces a MST of >100 days (although the proportion of permanently engrafted animals is not greatly increased and the dose effect does not reach statistical significance). Again this argues against a simple additive immunosuppressive effect of combining the two therapies. Chapter 5 describes a mechanism of synergy whereby $CTLA_4Ig$ therapy induces prolonged survival of OX34 mAb within Lewis rats, and increasing the initial dose of OX34 would therefore allow a higher dose of OX34 to remain circulating during the period when Lewis rats treated with OX34 alone are able to eliminate OX34 and mount a rejection response (day 12-60). This prolonged circulation of OX34 may be crucial in allowing the development of transplant tolerance in Lewis rats.

4 The comparative effect of anti-CD2 monoclonal antibody on *in vitro* lymphocyte functions

4.1 Study objective

Data from transplant experiments showed anti-CD2 mAb to be very effective at prolonging allograft survival in rat models, particularly when used in combination with other modalities. The biological effects of anti-CD4 mAb and $CTLA_4Ig$ have been discussed in the introduction in terms of the selective inhibition of signal 1 and signal 2 pathways. In this section inhibitory effects on lymphocyte function are demonstrated for these two modalities which can be interpreted as a consequence of blockade of these two pathways. Experiments are demonstrated which show that the effects of anti-CD2 mAb cannot readily be fit into the same two-signal paradigm. In order to elucidate its mechanism of action a series of experiments were performed examining the effect of anti-CD2 mAb on *in vitro* T cell functions and these effects were compared to those of anti-CD4 mAb and the chimeric protein $CTLA_4Ig$. This series of experiments aimed therefore to dissect the steps of allostimulation using *in vitro* assays and to examine the influence of anti-CD2 mAb at each stage using models of:

1) Direct antibody-mediated T cell activation
2) Alloantigen driven cellular proliferation
3) Alloantigen driven cytokine elaboration
4) Generation of allospecific CTL
5) Amplification and function of Natural Killer cell activity

Secondly experiments were performed to explore the ability of anti-CD2 mAb to cause lysis of T cells *in vitro*. These include a) an assessment of the ability of anti-CD2 mAb to cause complement-mediated cytolysis (CDC) and b) an assessment of the ability of anti-CD2 mAb to promote antibody-dependent cellular cytotoxicity (ADCC).

4.2 Study design

1) The mitogenic ability of anti-CD2 and anti-TCR mAb was examined using plate-bound mAb and mAb in solution as described in materials and methods to stimulate purified CD4 T cells. Cells were cultured for 24 hours prior to the addition of ^{3}H-Thymidine.

2) In order to investigate the influence of mAb on cellular proliferation induced by allogeneic cells standard MLR plates were set up as described in materials and methods using reciprocal DA and Lewis strain cell populations. ESH8, $CTLA_4Ig$, anti-CD2, anti-CD4 and anti-TCR mAb at a range of doses were incubated with responder cells prior to adding to stimulator cells. Control wells contained responder and stimulator cells (medium only), responder cells without stimulator cells and responder cells plus ConA. Plates were cultured for three days unless stated in the text.

3) In order to investigate the influence of mAb on cellular proliferation induced by anti-TCR mAb, anti-TCR plates were prepared as described in materials and methods. Responder cells were pre-incubated with mAb preparartions at doses stated in the text on ice to allow labelling without ligand modulation. Cells were washed prior to adding to mAb-lined wells. Proliferation was measured by thymidine incorporation in the standard manner.

4) Culture supernatants were retained from the above experiments to assess the influence of the reagents tested on elaboration of the cytokines IL2 and γIFN.

5) To assess the influence of mAb on the *in vitro* generation of allo-CTL 'bulk' MLR cultures were established as described in materials and methods, using DA strain splenocytes as stimulator cells and Lewis LNCs as responders. A responder: stimulator ratio of 2:1 was used to generate specific CTL. Anti-CD2 and anti-CD4 mAb were added at 20μg/ml at the beginning of these cultures to assess inhibition of CTL generation. ^{51}Cr labelled DA splenocytes were used as target cells in subsequent cytotoxicity assays.

6) To assess the effect of monoclonal antibody on the lytic activity of allo-CTL induced in the 'bulk' MLR parallel, unmodified cultures were set up to produce CTL. Cell cultures were then washed and incubated with 20μg mAb prior to mixing with target cells as described in materials and methods.

7) To assess the effect of monoclonal antibody on NK cell function 'bulk' MLR cultures were set up to produce NK cells (non-specific cytotoxicity of cell cultures is greater after a period of allostimulation presumably due to the generation of LAK cells). Monoclonal antibody was added to cultures to assess influence on NK cell generation. NK effectors generated in unmodified cultures were incubated with mAb prior to mixing with Y3 target cells as described in materials and methods.

8) The ability of OX34 and OX38 to lyse lymphocytes by complement-mediated pathways was assessed by an *in vitro* ^{51}Chromium release assay using labelled DA strain ConA blasts as target cells. Serum from either a naïve Lewis rat or one undergoing acute allograft rejection of a DA strain heart was heat- inactivated and used as controls (the acute phase serum therefore provided a source of cytotoxic antibody for use as a positive control). Target cells were incubated with serial

tripling dilutions of control serum or monoclonal antibody (initial concentration 20μg/ml) and an equal volume of fresh DA serum used as a source of complement.

9) In the absence of a suitable assay for macrophage function the ability of OX34 (IgG2a) and OX55 (IgG1) to promote ADCC by NK cells (which also express FcγR111) was assessed. Effector cells were obtained from Lewis LNCs after 5 days of culture in a 'bulk' MLR with DA splenocytes. Effector cells were then incubated at different dilutions with Y3 blast cells labelled with either OX34 (IgG2a), OX55 (IgG1) or unlabelled.

4.3 Analysis of the effect of monoclonal antibody on assays of T cell function: T cell activation

The ability of anti-CD2 mAb to activate T cells directly and to influence proliferation of T cells activated by other means was examined.

4.3.1 Anti-CD2 monoclonal antibody as a mitogen

Firstly experiments were performed to explore the abilty of OX34 to induce T cell activation. The CD2 molecule is known to provide a pathway for T cell activation in experimental systems, usually when a combination of monoclonal antibodies is used. T cell activation was measured in a series of six experiments where purified Lewis strain CD4 T cells were incubated for 24 hours in flat bottom wells containing either plate-bound or soluble OX34 or the anti-TCR mAb R73 and proliferation measured 18 hours later as described in materials and methods. Table 4.1 shows that R73 is mitogenic both in solution and when immobilised on the well surface. OX34 is not mitogenic for T cells under the same circumstances.

	Medium	Soluble OX34	Immobilised OX34	Soluble R73	Immobilised R73
cpm (mean)	1946	3583	1594	12141	1133908
SD	312	822	253	2671	5891
p value		<0.01	<0.05	<0.001	$<1x10^{-7}$

Table 4.1 Direct mitogenic effect of OX34 compared to R73

Mean thymidine incorporation by Lewis strain CD4 T cells stimulated by soluble and plate-bound monoclonal antibody (20μg/ml) (mean of six experiments, analysis by unmatched t test) (experiments performed with Dr Hilary Marshall).

4.3.2 Effect of anti-CD2 monoclonal antibody on the mixed lymphocyte reaction

In vitro assays of alloreactivity were performed to establish if the effect of anti-CD2 mAb on transplant survival in different rat strains is reproducible in *in vitro* systems. Mixed lymphocyte reactions were performed and the effect of blockade of cellular ligands examined. Lewis and DA strain LNCs were used as responders and irradiated DA and Lewis strain splenocytes as stimulators. Firstly the reagents under study were assessed in a one way-MLR for their effect on allogeneic stimulation induced in Lewis LNCs by DA splenocytes. Antibodies were used at the saturating concentration of 20μg/ml as determined by flow cytometry, and were added to cells when co-culture commenced (figure 4.1).

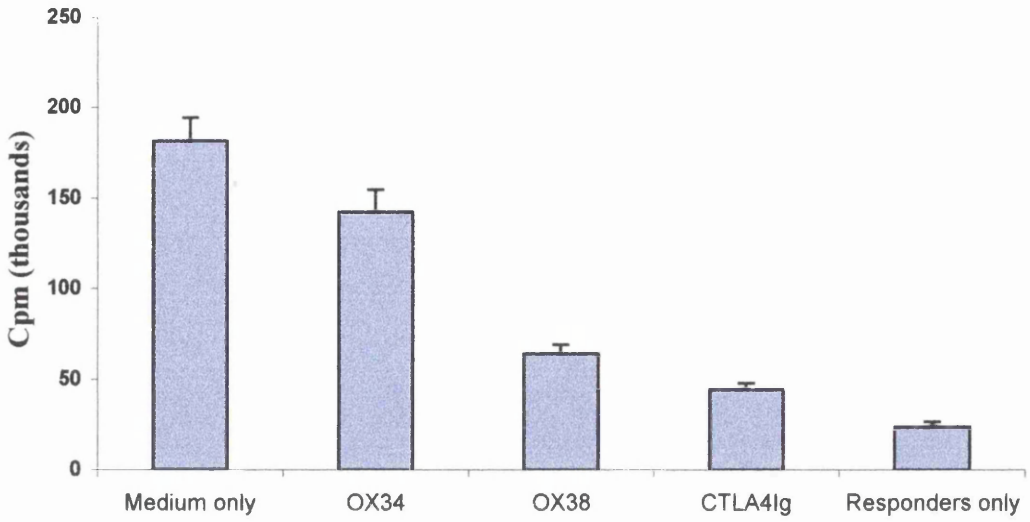

Figure 4.1 Effect of OX34 compared to OX38 and $CTLA_4Ig$ on proliferation in MLR experiments

Mean thymidine incorporation by Lewis strain LNCs (one experiment) stimulated by DA splenocytes on addition of monoclonal antibody (20 μg/ml) at beginning of culture. Control wells contain mixed lymphocytes alone and responder cells alone (6 wells per reagent; medium v OX34 $p<0.001$, unmatched t test).

This single experiment showed a marked ability of anti-CD4 mAb and $CTLA_4Ig$ to inhibit the MLR but also showed a lesser inhibition by anti-CD2 mAb in this strain combination and at this dose. Experiments were then performed with reciprocal strain combinations and dose ranges of OX34, OX55 and $CTLA_4Ig$ to explore this effect further.

Firstly, using DA and Lewis splenocytes and LNCs, MLRs were performed with the addition of OX34 in decreasing doses from 10μg/ml. Figure 4.2 shows the mean of three experiments using DA LNCs as responders and of six experiments using Lewis LNCs as responders (proliferation measured at day 3 in all experiments), confirming the inability of OX34 to influence the MLR.

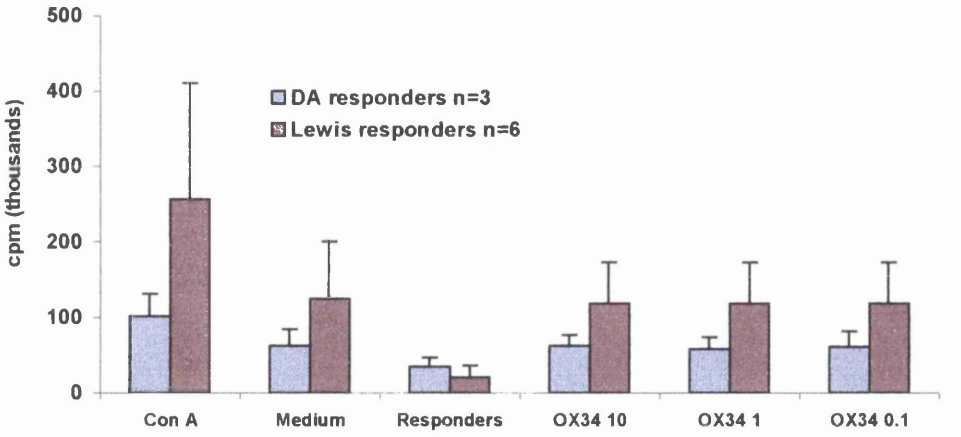

Figure 4.2 Dose effect of OX34 in MLR experiments

Mean thymidine incorporation by DA LNCs (three experiments) and Lewis LNCs (six experiments) stimulated by reciprocal strain splenocytes on addition of OX34 (μg/ml) at beginning of culture. Control wells contain responder cells plus ConA, mixed lymphocytes alone or responder cells alone (medium v OX34 10μg/ml n/s in both series).

Secondly parallel plates were set up to assess the ability of OX55 to influence the proliferation of Lewis LNCs stimulated by irradiated DA splenocytes. Figure 4.3 shows the mean results of a four experiments with proliferation measured at day three.

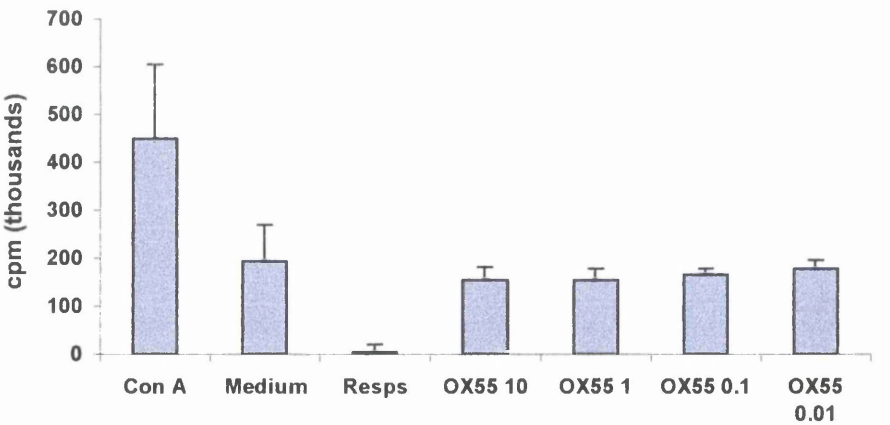

Figure 4.3 Dose effect of OX55 in MLR experiments

Mean thymidine incorporation by Lewis LNCs stimulated by DA splenocytes on addition of OX55 (μg/ml) at beginning of culture. Control wells contain responder cells plus ConA, mixed lymphocytes alone or responder cells alone (mean of four experiments, medium v OX55 10μg/ml n/s).

4.3.3 Effect of $CTLA_4Ig$ on the mixed lymphocyte reaction

$CTLA_4Ig$ therapy has been shown to have differential effects on allograft survival in high and low responder strain rats, and initial experiments demonstrated its ability to influence the MLR using Lewis strain responder cells. Further experiments were therefore performed to confirm the ability of $CTLA_4Ig$ to inhibit T cell activation in this system and to explore any strain specific effect of this inhibition. $CTLA_4Ig$ at a range of doses (μg/ml) was added at the beginning of culture using the same lymphocyte preparations as above from DA (three experiments) and Lewis (four experiments) rats and the proliferation obtained under these conditions expressed as the proliferation index (thymidine incorporation expressed as percentage of that obtained in unmodified reaction). Figure 4.4 demonstrates that addition of $CTLA_4Ig$ to an MLR inhibited proliferation of lymphocytes from both high and low responder strain rats equally.

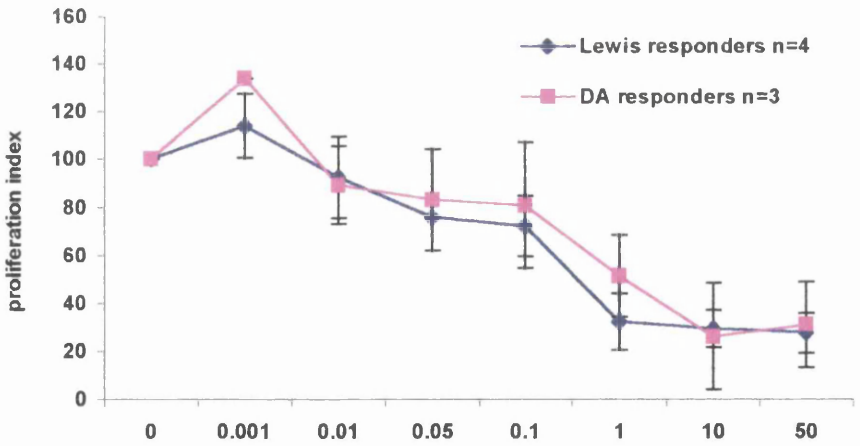

Figure 4.4 Dose effect of $CTLA_4Ig$ in MLR experiments using DA and Lewis strain responders

Mean relative proliferation of DA LNCs (three experiments) and Lewis LNCs (four experiments) on incubation with reciprocal strain splenocytes with addition of $CTLA_4Ig$ (μg/ml) at beginning of culture (experiment performed with Dr I Newman)

Both OX38 and $CTLA_4Ig$ therefore have demonstrable effects on lymphocyte function in *in vitro* models of alloreactivity that parallel their *in vivo* effect. In view

of their potent effect on allograft survival the absence of effect of OX34 and OX55 is surprising. Lymphocyte proliferation in the mixed lymphocyte reaction is functionally analogous to stimulation by alloantigens *in vivo* in that both TCR mediated signals and costimulatory signals are required. To further examine the effect of mAb in terms of this signal 1/signal 2 paradigm the effect of the reagents under study on T cell activation mediated by antibody to the T cell receptor were then assessed .

4.3.4 Analysis of the effect of monoclonal antibody on assays of T cell function: anti-TCR antibody mediated activation

In these experiments Lewis strain LNCs (2 x 10^5/well) were preincubated with the reagent under study (10μg/ml) on ice for 30 minutes prior to adding to flat-bottomed wells coated with anti-TCR antibody as described in materials and methods. Cells were cultured for 24 hours initially, and then for a further 18 hours after the addition of thymidine. Figure 4.5 shows the mean results from three experiments. Of the reagents tested only OX38 is able to partially suppress lymphocyte proliferation in this model.

Further experiments were then performed using a dose range of OX34 and figure 4.6 shows the mean of three experiments confirming the inability of this mAb to influence T cell activation in this system. These data provide further evidence that although CD2 is associated with the TCR complex anti-CD2 mAb does not influence TCR mediated signalling.

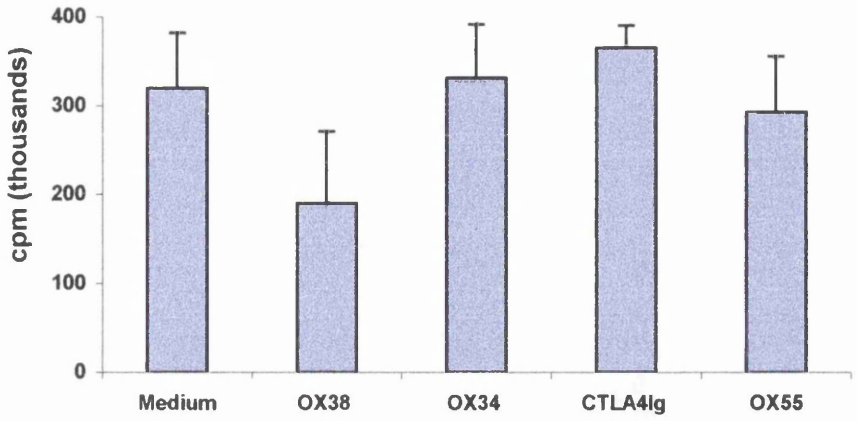

Figure 4.5 OX34 and $CTLA_4Ig$ are unable to inhibit T cell activation via TCR

Thymidine incorporation by Lewis LNCs pre-incubated with mAb (μg/ml) and stimulated via the T cell receptor with fixed R73 mAb for 24 hours (mean of three experiments; medium v OX38 $p<0.05$, unmatched t test).

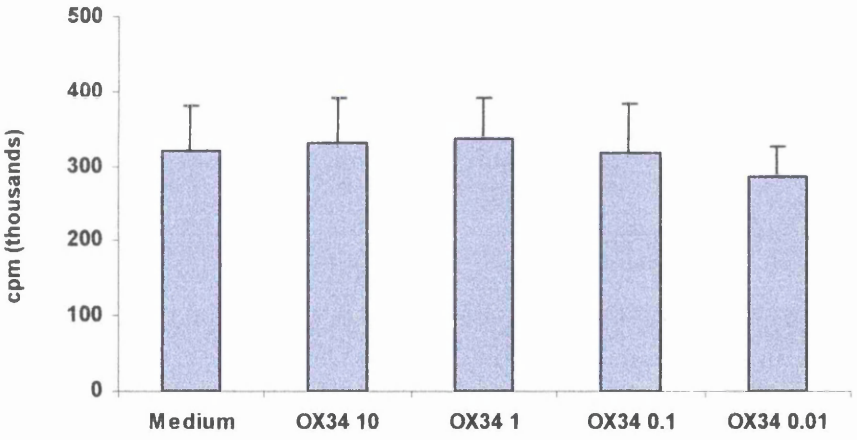

Figure 4.6 Dose effect of OX34 in anti-TCR mAb mediated proliferation experiments

Thymidine incorporation by LNCs pre-incubated with OX34 (μg/ml) and stimulated via the T cell receptor with fixed R73 mAb for 24 hours (mean of three experiments).

4.4 Analysis of the effect of monoclonal antibody on assays of T cell function: cytokine production

The above experiments used the incorporation of ^{3}H Thymidine as an indicator of cellular proliferation, and show that OX34 is neither able to stimulate T cell activation itself nor suppress T cell activation caused by other means. However in addition to proliferation, T cell activation leads to the production of cytokines and the generation of effector functions. I therefore wished to explore the production of cytokines by lymphocytes stimulated by the above methods, to confirm that continued cellular proliferation in the presence of anti-CD2 mAb was accompanied by normal cytokine production. The key cytokines involved in the generation of cell-mediated immunity are IL2 and γIFN. Supernatants from the above experiments were preserved and the production of these cytokines assayed.

4.4.1 Influence of monoclonal antibody on IL2 production in MLR supernatants

IL2 was measured using the IL2-dependent CTLL cell line. $5x10^{3}$ CTLL cells were cultured in 200μl complete medium containing serial dilutions of supernatant from the mixed lymphocyte reactions and anti-TCR reactions outlined above. Cells were cultured for 24 hours before the addition of ^{3}H Thymidine as described in materials and methods. Incorporation was compared to a standard curve obtained using human IL2. Figure 4.7 shows the mean results from three experiments where IL2 was measured in MLR supernatants after the addition of medium alone, OX38, OX34, OX55 or ESH8 (10μg/kg) or a culture in the absence of stimulators. The IL2 concentration in supernatants of responder cells stimulated by ConA alone (control well in MLR experiments) is excluded from this analysis as levels greater than

1000u/ml are obtained. This graph demonstrates that IL2 production broadly mirrors cellular proliferation, and that in addition to its inability to influence proliferation in the MLR anti-CD2 mAb has minimal influence on IL2 production.

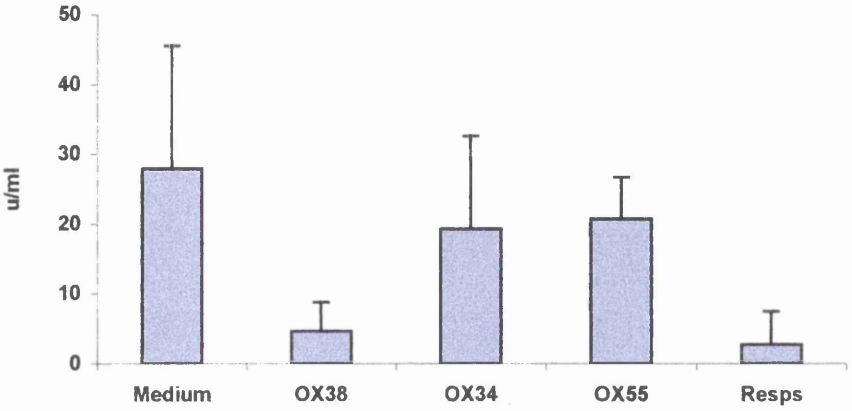

Figure 4.7 Effect of mAb on IL2 production by lymphocytes in the MLR

Estimation of IL2 concentration in MLR supernatant after addition of mAb (10μg/ml) at beginning of culture. Control wells contained mixed lymphocytes in medium or responder cells alone (mean of four experiments, medium v OX38 $p<0.05$, unmatched t test).

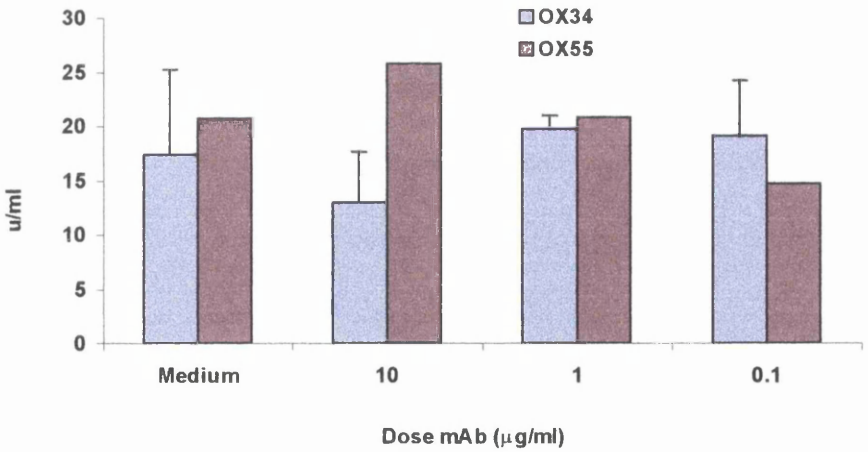

Figure 4.8 Effect of dose range of anti-CD2 mAb on IL2 production by lymphocytes in the MLR

Estimation of IL2 concentration in MLR supernatant after addition of dose range of OX34 (mean of three experiments) or OX55 (mean of two experiments) mAb (μg/ml) at beginning of culture. Control wells contained mixed lymphocytes in medium alone.

The influence of anti-CD2 mAb on IL2 elaboration was further demonstrated by the addition of a dose range of mAb to MLR experiments as above. The IL2 concentration in supernatants of these experiments is shown in figure 4.8.

4.4.2 Influence of monoclonal antibody on γIFN production in MLR supernatants

In a similar manner supernatants from the above experiments were assayed for their γIFN content. The data shown are obtained from reactions where antibody was added at 10μg/ml to the culture. Not all supernatants were retained for analysis so the data are incomplete. 100μl culture supernatants were assayed by an ELISA system using polyclonal anti-γIFN as described in materials and methods and compared to a standard curve obtained using stock γIFN of known concentration. Table 4.2 suggests that γIFN production mirrors closely cellular proliferation, in that both OX38 and $CTLA_4Ig$ are able to inhibit its production by alloantigen stimulated lymphocytes, whereas anti-CD2 mAb appears to have little effect.

Added reagent	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Mean
medium	46.6	31.2	101.7		59.8
OX38	14.1				14.1
OX55	73.4	87.4			80.4
OX34	35.5	70.3	88.3	85.9	70.0
$CTLA_4Ig$	9.8				
responders alone	17.2	13.3			15.2

Table 4.2 Influence of OX34, OX55, OX38 and $CTLA_4Ig$ on γIFN production in the MLR

100μl culture supernatants were preserved from experiments described in Section 4.4.1 and γIFN concentration measured by an ELISA system compared to γIFN standard solution (U/ml).

4.4.3 Cytokine production by anti-TCR stimulated lymphocytes

Cytokine production in MLR supernatants measured above appears to mirror very closely the degree of lymphocyte proliferation, and is not inhibited by anti-CD2 mAb. In the same way IL2 and γIFN production by cells stimulated with anti-TCR antibody was assessed. Supernatants were retained from three of the experiments where Lewis LNCs were stimulated by immobilised anti-TCR antibody after incubating with reagents under study at 10μg/ml. IL2 and γIFN in these supernatants was assayed by the same methods as above. Despite achieving high levels of proliferation, supernatants of T cells activated by anti-TCR mAb contained very low levels of IL2 (<2u/ml) so that comparisons between groups are not accurate using this CTLL assay and results are not shown. Figure 4.9 shows that in a similar manner to above γIFN production however followed closely the degree of cellular proliferation, and of the reagents tested anti-CD4 mAb produced the greatest inhibition of γIFN elaboration.

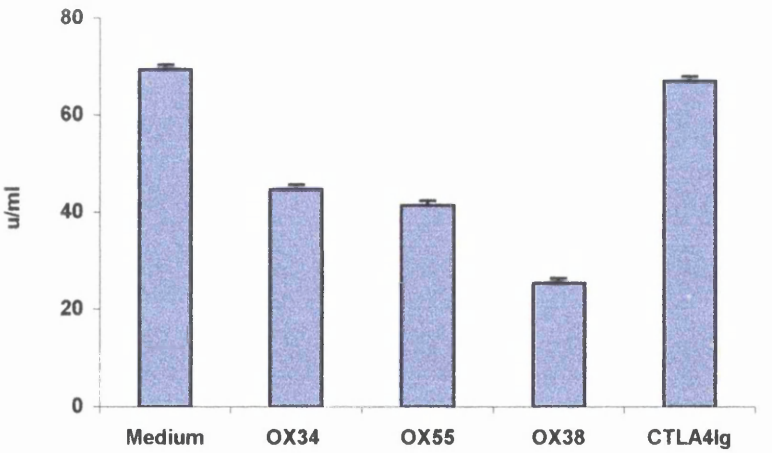

Figure 4.9 Influence of OX34, OX55, OX38 and CTLA$_4$Ig on γIFN production by anti-TCR stimulated lymphocytes

Estimation of γIFN concentration in supernatant of anti-TCR stimulated Lewis LNCs (mean of three experiments) preincubated with mAb (10μg/ml). Cells cultured for 24 hours, control wells contained cells and medium only (medium v OX38 $p<0.05$, unmatched t test).

To further investigate the influence of anti-CD2 mAb on elaboration of γIFN by anti-TCR stimulated LNCs supernatants were taken from a series of TCR plates where responder cells had been incubated with a dose range of OX34 prior to incubation. Figure 4.10 confirms that OX34 has minimal influence on γIFN production in this system.

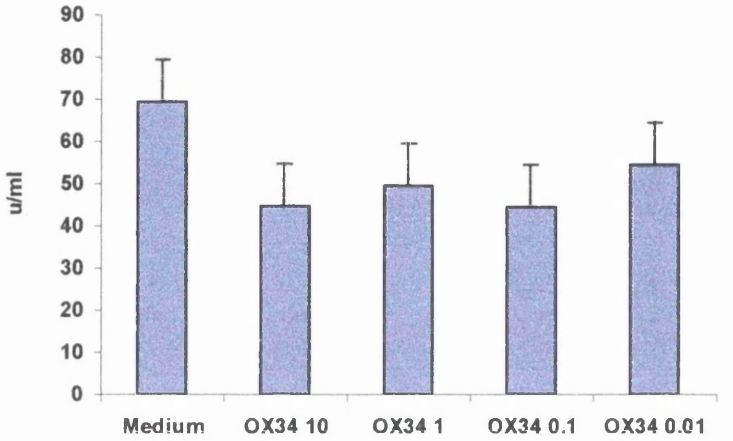

Figure 4.10 Influence of dose range of OX34 on γIFN production by anti-TCR stimulated lymphocytes

Estimation of γIFN concentration in supernatant of anti-TCR stimulated Lewis LNCs (mean of three experiments) pre-incubated with dose range of OX34 mAb (μg/ml). Cells cultured for 24 hours, control wells contain cells and medium only.

4.5 Analysis of the effect of monoclonal antibody on assays of T cell function: lymphocyte effector functions

Results demonstrated above show that antibody to the CD2 molecule has little detectable effect on lymphocyte proliferation in the models described and has minimal influence on the elaboration of the signature Th_1 cytokines IL2 and γIFN by activated lymphocytes. These results do not accord with the potent *in vivo* effect of anti-CD2 mAb. I next wished to explore the possibility that activated CD4 T cells which are labelled with anti-CD2 mAb and able to secrete Th_1 cytokines normally

are still unable to provide help for the generation of antigen-specific cell mediated immunity. To this end a system of *in vitro* generation of cytotoxic T lymphocytes (CTL) that could be manipulated with different reagents was devised. Lewis strain LNCs were cultured in 20 mls complete medium with irradiated DA splenocytes for five days in a 'bulk' MLR. Earlier experiments had shown this to be an effective method of generating allo-CTL (figure 2.1). The influence of mAb on this system was assessed by adding antibody to the culture medium at 10 μg/ml at the beginning of the culture. The cytotoxicity of the generated effectors was then measured in a standard ^{51}Chromium-release assay using DA strain ConA blasts as target cells at decreasing effector: target ratios. This experiment was repeated three times using both anti-CD4 and anti-CD2 mAb in the bulk culture medium. OX38 effectively abolished the generation of CTL in this system and OX34 also consistently diminished the potency of CTL generation. A typical result is shown in figure 4.11.

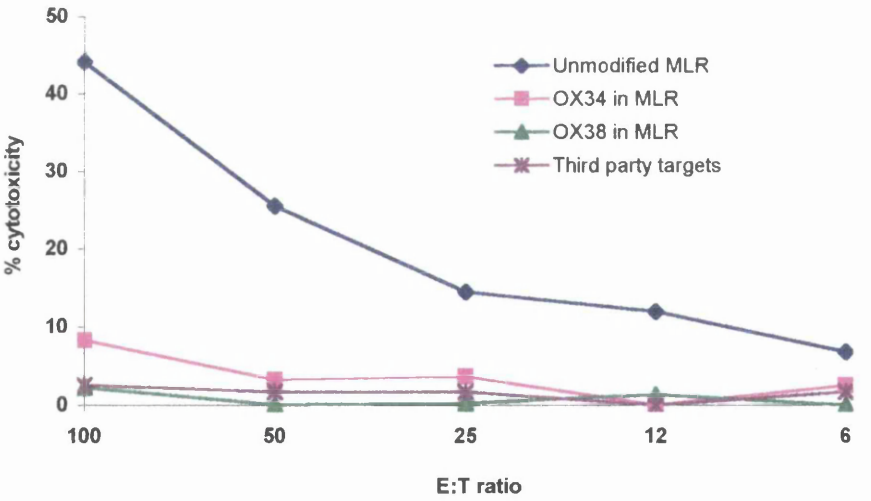

Figure 4.11 Effect of OX34 on the *in vitro* induction of CTL

Specific cytotoxicicty of Lewis CTL generated in 'bulk' MLR with DA splenocytes on addition of mAb (10μg/ml) at beginning of culture.

This result suggests one of two broad alternative explanations; either that anti-CD2 mAb prevents CD4 T cells providing help for the generation of CTL or that anti-CD2 mAb inhibits the activity of CTL generated in this system (as OX34 is free

in solution during the MLR culture any CTL generated will be labelled with antibody). To explore the latter possibility as an explanation for the failure to generate specific cytotoxicity in this system a parallel experiment was designed in which CTLs were generated in an unmodified bulk MLR and then incubated with antibody briefly before addition to DA strain targets in the ^{51}Chromium-release assay. This experiment was repeated three times and on each occasion neither anti-CD4 mAb nor anti-CD2 mAb were able to influence the activity of CTL generated in an unmodified MLR. Figure 4.12 shows a typical result.

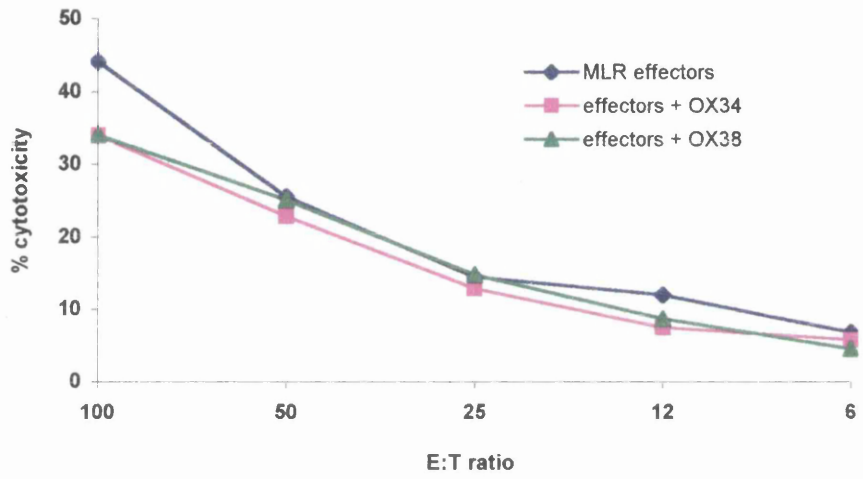

Figure 4.12 Effect of OX34 on the lytic capacity of allo-CTL

Specific cytotoxicity of Lewis CTL generated in 'bulk' MLR with DA splenocytes compared to similar effectors labelled with mAb (10μg/ml) prior to addition to DA targets.

Of the above experiments only the generation of CTL in the MLR is affected by OX34. This suggests that OX34 may exert its inhibitory effect on cell-mediated immunity *in vitro* at a stage between the recognition of alloantigen by CD4 T cells (and subsequent activation and cytokine release) and the provision of cognate signals to CD8 cells. This mechanism is discussed further at the end of the chapter.

4.6 Analysis of the effect of monoclonal antibody on assays of Natural Killer cell functions

The data presented above demonstrate that mAb to the CD2 molecule has effects on T lymphocytes which are less easily demonstrated than those of anti-CD4 mAb and $CTLA_4Ig$. The CD2 molecule is also however expressed on NK cells in addition to T lymphocytes in the rat. We therefore then assessed the effect of anti-CD2 mAb on non-specific cytotoxicity. The same 'bulk' MLR as above was used as a source of NK cells and the influence of mAb during the period of alloactivation was assessed by incubation with NK sensitive Y3 cells (figure 4.13).

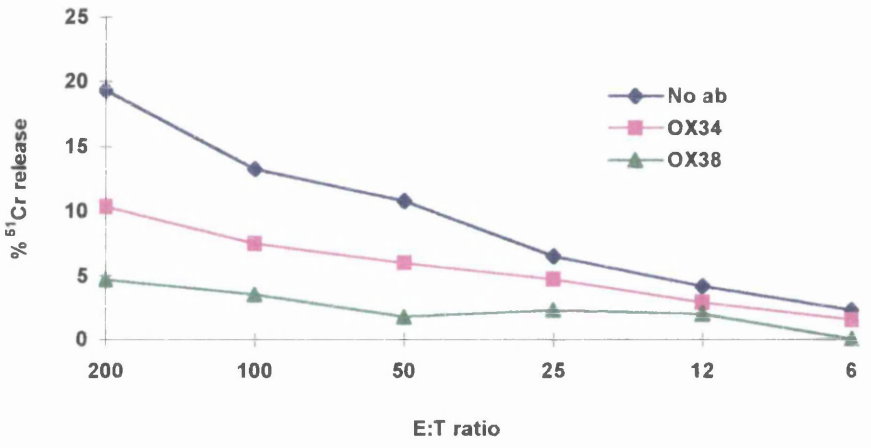

Figure 4.13 Effect of OX34 on the *in vitro* amplification of NK activity

Cytotoxicity against Y3 target cells of effector cells generated in 'bulk' MLR on addition of mAb (20μg/ml) at beginning of five-day culture.

OX34 has an apparent inhibitory effect on the amplification of NK activity in the MLR, but not to the same degree as OX38. This experiment was repeated with identical results. We then assessed the effect of mAb on the lytic activity of effector cells generated in an unmodified MLR against Y3 targets. After five days of culture cells were washed and aliquots incubated briefly with OX34 before addition to ^{51}Chromium labelled Y3 cells in a standard NK cell assay as described in materials

and methods (figure 4.14). In contrast to its inhibitory effect on the generation of NK activity, OX34 had no inhibitory effect on the lytic activity of activated NK cells. The apparent increase in cytotoxicity by OX34-labelled NK cells may be due to soluble OX34 acting as an opsonin on Y3 cells. This experiment was repeated with similar results.

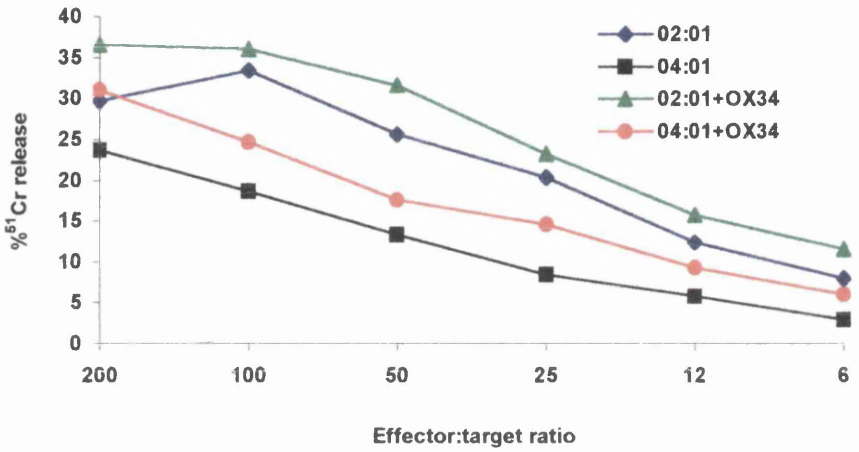

Figure 4.14 Effect of OX34 on the lytic capacity of NK cells

Cytotoxicity against Y3 target cells of effectors generated in a bulk MLR at responder: stimulator ratios of 2 and 4. Effector cells are unmodified or labelled with OX34 and washed prior to adding to target cells.

4.7 Analysis of the ability of monoclonal antibody to lyse T cells by complement fixation

Figure 4.15 shows high levels of cytotoxic antibody in the serum taken from a rat undergoing acute rejection of a DA allograft, demonstrating that this system is capable of measuring CDC. Both OX34 and OX38 are unable to lyse blast cells in this system.

4.8 Analysis of the ability of monoclonal antibody to lyse T cells by promoting ADCC

Figure 4.15 shows that mouse mAb does not activate rat complement. Evidence was sought for the participation of OX34 and OX55 in antibody-dependent cellular cytotoxicity (ADCC), another Fc dependent function. As can be seen from figure 4.16 both antibodies are very effective opsonins of DA blast cells in this system.

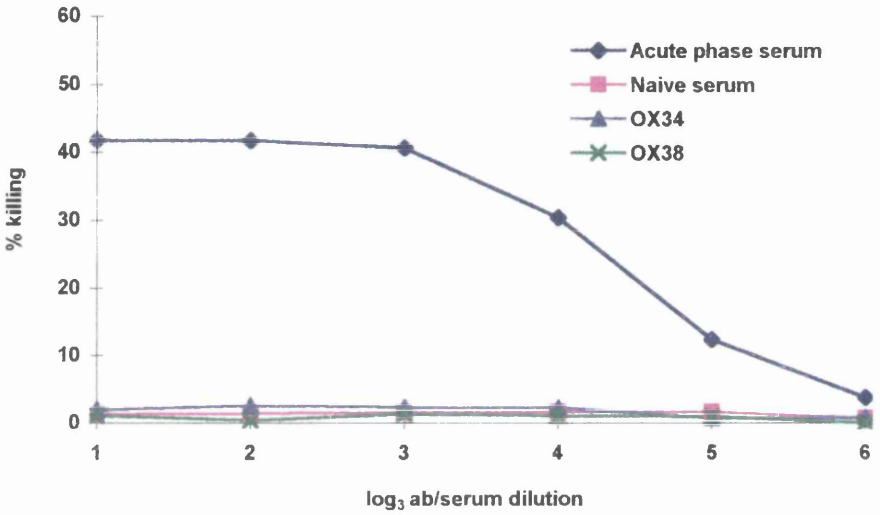

Figure 4.15 Assessment of the ability of mouse mAb to fix rat complement *in vitro*

Assessment of *in vitro* complement-mediated cytotoxicity of DA blast cells by OX34 and OX38 mAb (starting concentration 20 μg/ml) compared to DA-sensitised and naïve rat serum.

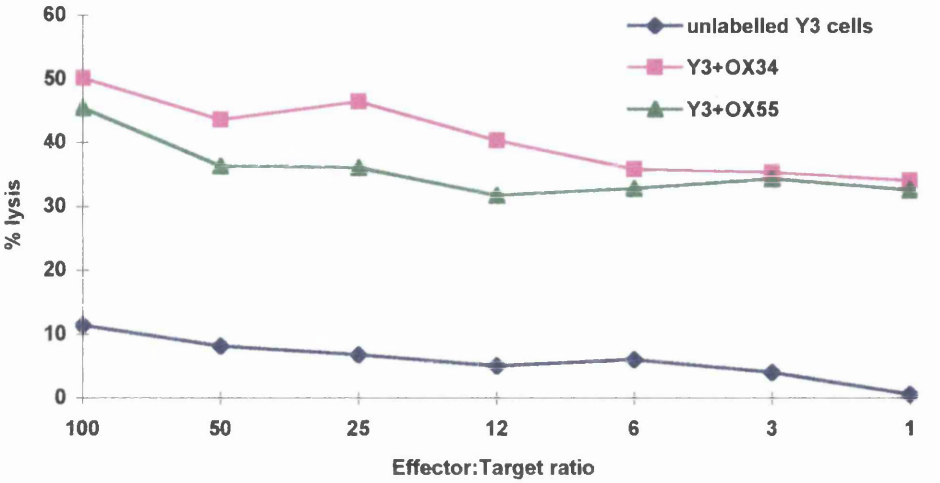

Figure 4.16 Assessment of the ability of mouse mAb to promote ADCC by NK cells *in vitro*

Lysis of Y3 target cells by MLR effector cells. Targets were labelled with mAb and washed prior to incubation with effector cells.

4.9 Conclusions and discussion of results

4.9.1 Direct mitogenic effect of monoclonal antibody

Anti-TCR mAb is noted to be mitogenic for T cells both when fixed to the well surface and when in solution. The potent effects of anti-TCR in this context have been well described previously and are widely used as a research tool (346). In contrast OX34 does not appear to be mitogenic for T cells either when in solution or when immobilised on the plate surface.

4.9.2 Influence of monoclonal antibody on mixed lymphocyte reactions

Figure 4.1 concurs with previous reports that both anti-CD4 mAb and $CTLA_4Ig$ are able to inhibit cellular proliferation in the MLR (116, 231). Alloactivation in this system is analogous to the transplant model, in that signal 1

(via the TCR) and signal 2 (via costimulatory molecules) are required. The effects of anti-CD4 and $CTLA_4Ig$ on this system can be interpreted in terms of the blockade of these two signals. In the first MLR experiment there was also a small degree of inhibition by OX34. Subsequent assays however reported in figure 4.2 show over a series of experiments using different responder and stimulator cell types and a dose range of reagents that neither OX34 nor OX55 had a net effect on cellular proliferation in this system. This suggests that either CD2-mediated functions are not influenced by anti-CD2 mAb labelling or that they are not crucial in cellular activation induced by allostimulation. Experiments reported in section 5.6.7 reveal that the CD2 ligand is profoundly modulated in cell cultures containing either OX34 or OX55, establishing that normal expression of the CD2 molecule is not necessary for allostimulation in this system. Also notable is the clear dose-response relationship of $CTLA_4Ig$ in these experiments, suggesting that costimulatory signals can provide a quantitative regulation of T cell activation, rather than a simple 'on-off' signal. Furthermore despite the variable proliferative responses obtained by DA and Lewis strain responder cells (cells from Lewis strain rats tend to produce higher levels of thymidine incorporation on stimulation with DA strain cells than the reverse situation), the relative inhibition of this response by $CTLA_4Ig$ is similar in both rat strains.

The lack of effect of anti-CD2 mAb in MLR experiments is surprising in view of the potent effect of such reagents in transplant models. Anti-CD4 mAb and $CTLA_4Ig$ have potent effects on both systems, which are consistent with their postulated effects on signal 1 and signal 2 in T cell activation. The presence of normal lymphocyte activation by allogeneic cellular methods despite labelling with anti-CD2 mAb (and the modulation of the CD2 molecule shown in Chapter 5)

suggests that these reagents exert their biological effect by mechanisms other than interference with T cell activation signals.

4.9.3 Influence of monoclonal antibody on anti-TCR mediated T cell activation

Stimulation of T cells by anti-TCR mAb has a very potent mitogenic effect that overcomes the need for costimulatory signals (346, 347) and allows exploration of the influence of reagents on T cells stimulated purely via the T cell receptor (signal 1). It is notable in these experiments that higher levels of thymidine incorporation are obtained after 24 hours than are obtained after three days of culture in the MLR. In these experiments OX38 mAb is partially able to inhibit T cell activation, whilst $CTLA_4Ig$, OX34 and OX55 have no effect. This is consistent with the theory that anti-CD4 mAb interferes with signalling via the TCR whilst $CTLA_4Ig$ blockades costimulatory pathways, and therefore does not influence this system. The inability of anti-CD2 mAb to inhibit the proliferation of T cells activated in this way provides further evidence that although CD2 is physically associated with the T cell receptor and signalling via the two pathways is closely linked, the biological effects of anti-CD2 mAb noted in transplant experiments can not be explained by interference with TCR-mediated signalling.

4.9.4 Influence of monoclonal antibody on mitogen-induced cytokine elaboration

In proliferation experiments reported above (both MLRs and anti-TCR mediated) the degree of cytokine (IL2 and γIFN) elaboration by activated cells is broadly in keeping with the degree of ^3H incorporation. The exception to this is the very low level of IL2 noted in the supernatant of LNCs stimulated by anti-TCR mAb

(<2 u/ml), despite very high levels of thymidine incorporation. A possible explanation for this finding is that the high level of cellular proliferation results in consumption of IL2. However it has also been noted in these experiments that LNCs stimulated purely by ConA achieve similarly high levels of thymidine incorporation but also produce high concentrations of IL2 in culture supernatants (>1000u/ml). There is not therefore a direct correlation between cellular activation and IL2 concentration in culture supernatant. Previously published work has shown measureable IL2 production in culture supernatants of CD4 T cells activated by mAb to the CD3 molecule if the cells are also labelled with anti-IL2R antibody (347). In these experiments there was also no direct correlation between IL2 production and thymidine incorporation. Other work using anti-CD4 mAb in mitogen assays using both anti-TCR mAb (262) and the allogeneic MLR (261) has shown a differential effect on cytokine production, causing a reduction in γIFN production whilst sparing IL2. For this reason we assessed the effect of anti-CD2 mAb on mitogen-induced production of IL2 and γIFN in addition to its effect on proliferation, to exclude the possibility that anti-CD2 mAb exerts its effect by selective inhibition of Th_1 cytokine elaboration. In contrast to the widely disparate levels of IL2 production detectable after anti-TCR mAb and ConA mediated T cell activation, the levels of γIFN produced in anti-TCR reactions and the levels of both cytokines produced in MLR cultures appears to follow closely the degree of cellular proliferation and inhibition by mAb. Anti-CD4 mAb therefore inhibits IL2 production in the MLR to a similar degree as it is able to inhibit proliferation, and both anti-CD4 mAb and $CTLA_4Ig$ are able to inhibit γIFN production in the same way. (The effect of $CTLA_4Ig$ on IL2 production in the MLR was not assessed). In contrast only anti-CD4 mAb is able to inhibit γIFN production by anti-TCR stimulated T cells. This is in keeping with earlier reports demonstrating the direct activation of T cells in this system without

the requirement for costimulatory signals (211, 347). It is easier to draw conclusions from these experiments on the effect of anti-CD2 mAb on IL2 production in mitogen assays rather than γIFN. For example the data demonstrated show an absence of effect on IL2 production using a wide dose range of OX34 used in MLR assays. The data for γIFN is however less easy to interpret. The number of assessments of γIFN in MLR supernatants is small so a consistent picture has not been obtained, although γIFN levels are much higher than in supernatants of MLRs with added anti-CD4 mAb and $CTLA_4Ig$. In contrast dose-effect curves have been obtained for the effect of OX34 mAb on γIFN by anti-TCR stimulated T cells giving a consistent result. Whilst γIFN concentration is lower in all wells containing OX34, the inhibition at each concentration does not reach statistical significance at any particular dose. It is possible therefore that OX34 mAb does have a small effect on γIFN production in this system. However in keeping with the readily demonstrated inhibition of Th_1 cytokines in the models in which OX38 and $CTLA_4Ig$ have an effect, the absence of inhibition of proliferation by OX34 is matched by a similar inability to prevent Th_1 cytokine release.

4.9.5 Influence of monoclonal antibody on CTL generation and function

Investigations so far reveal a paradoxical combination of profound *in vivo* immunosuppression by anti-CD2 mAb therapy and relative silence in *in vitro* assays. We therefore explored a further step in the alloactivation of T cells to explore the possibility that anti-CD2 mAb may influence the provision of cellular help by means other than inhibition of the elaboration of Th_1 cytokines, which has been readily demonstrated for both anti-CD4 mAb and $CTLA_4Ig$. Initial experiments described in materials and methods have shown the 'bulk' MLR to be an effective means of

generating specific CTL without the requirement for exogenous IL2. On repeating these experiments with mAb added to the culture it was noted that anti-CD4 mAb is able to abolish the generation of CTL in this system and that anti-CD2 mAb has a similar, although less marked effect. Murine studies have also shown that anti-CD2 mAb can inhibit the *in vitro* induction of CTL (225, 290), although these experiments did not analyse the stages of this process in terms of proliferation and cytokine production. Furthermore neither mAb has any effect on the cytotoxic potential of CTL after they have been generated in an unmodified MLR. This is expected for anti-CD4 mAb, as CTL rarely express the CD4 molecule. CTL (predominantly CD8+) are labelled with anti-CD2 mAb but previous publications have also demonstrated the inability of OX34 to inhibit CTL lysis (154). These findings for anti-CD2 mAb are contradictory. We have shown that anti-CD2 mAb labelling of CD4 T cells does not prevent activation and cytokine release at the level of allo-MHC recognition, and anti-CD2 mAb labelling of mature CTL does not prevent interaction with allo-MHC bearing target cells. The mechanism of the effect of anti-CD2 mAb in this *in vitro* system must therefore be either:

1) by interfering with the recognition of allo-MHC by CTL precursors
2) by inhibiting the provision of CD4-derived helper signals other than IL2 and γIFN to CTLp and prevention of CTL induction.

An isolated effect on CTLp may be because either a) CTLp are inherently more sensitive to an effect of anti-CD2 mAb (for example susceptibility to mAb induced cell death) or b) the low frequency of CTLp may lead to a dose-related exaggeration of anti-CD2 mAb mediated effects.

Regardless of the mechanism involved in the inhibition of induction of allo-CTL by OX34, this finding is the only *in vitro* counterpart noted to the effects in intact animals. Due to the profound depletion of T cells in OX34 treated animals it is

not possible to compare meaningfully the influence of OX34 on the generation of allo-CTL during the induction phase of graft tolerance in an intact animal with CTL induction during unmodified acute rejection.

4.9.6 Influence of monoclonal antibody on NK cell function in *in vitro* assays

The 'bulk' MLR used to generate CTL was also used as a source of NK effectors as these cells show greater NK activity than naïve cells (possibly a result of cytokines in the culture medium producing LAK cells). In a similar manner to the inhibition of generation of specific cytotoxicity in this system, anti-CD4 mAb is able to reduce the non-specific cytotoxicity of MLR effector cells, presumably by reducing the availability of cytokines in the culture medium. It was also noted however that OX34 is able to reduce the generation of NK activity, although to a lesser degree than OX38. This is difficult to explain in similar terms as we have demonstrated that OX34 has minimal effect on the elaboration of IL2 and γIFN in the MLR. However OX34 may interfere with intercellular signals other than IL2 and γIFN. This would concur with the earlier demonstration of the inhibition of CTL generation, which also cannot be explained by inhibition of IL2 and γIFN production. Another possibility is that OX34 labelling of CD2-bearing NK cells during the period of alloactivation and cytokine release leads to accelerated NK cell death. This has been demonstrated with human NK cells, where cross-linked anti-CD2 mAb are able to activate resting NK cells but induce apoptosis in IL2-primed NK cells (179).

In agreement with murine studies prior labelling with anti-CD2 mAb does not inhibit the lytic activity of non-specific effector cells (154). In the three sets of experiments performed, incubating MLR effector cells with OX34 prior to adding to

Y3 targets slightly increased the levels of NK activity. There are two possible explanations for this:

1) OX34 acts as stimulus to a CD2-mediated pathway of NK cell activation (this phenomenon has also been noted *in vitro* experiments where the mAb is cross-linked by secondary antibody (169, 171))
2) Residual mAb in the washed OX34-labelled effector cells bound to NK targets (Y3 cells are also labelled by OX34) and acted as an opsonin to increase ADCC (see below).

The observed effects of OX34 on NK cell function however cannot explain the potent *in vivo* effects of OX34 on graft rejection, as this process occurs largely independently of NK cells.

4.9.7 Mechanisms of monoclonal antibody-mediated cellular lysis

Studies outlined in Chapter 5 reveal that OX34 mAb causes profound T cell depletion when administered to rats intravenously. As discussed at length in the introduction there are three possible mechanisms by which monoclonal antibodies may cause cellular depletion in animal systems:

1) by direct complement-mediated cytolysis (CDC)
2) by promoting antibody-dependent cellular cytotoxicity (ADCC) and opsonisation by cells expressing the Fc receptor
3) By delivery of a 'negative signal' to the target cell resulting in cell death.

Evidence is presented in Section 5 showing that OX34 therapy preferentially and more rapidly depletes the CD4 than CD8 subset, suggesting that the CD4 T cell is uniquely sensitive to OX34 binding and favours the participation of the 'negative signal' hypothesis for the mechanism of action of OX34. However cellular assays are also demonstrated in this section to assess the ability of mAb to participate in the first two mechanisms described above.

Figure 4.15 demonstrates clearly that neither OX34 nor OX38 have an intrinsic ability to lyse cells by complement fixation. This is an expected result, as evidence discussed in the introduction shows that mAb are inconsistent in their ability to fix foreign complement, and the evidence of the preferential depletion of CD4 cells makes a non-specific system such as CDC unlikely. For the mAb which have been shown to be capable of fixing complement this ability has been reproducible in experiments such as the one performed here (202, 206). The ability to fix complement is dependent on the Fc portion of the mAb and is also isotype dependent. In rats IgG1 is thought not to fix complement, (OX55 (IgG1) was not tested in this system) whilst IgG2a is a complement fixing isotype (202).

Another possible mechanism for cellular depletion is by non-specific killing of antibody-coated lymphocytes by cells expressing Fc receptors. This process may promote either cell lysis, as both NK cells and some T cells have been shown to be capable of ADCC (348) or opsonisation of target cells by monocytes and macrophages. As discussed ADCC has been assessed in this project by the ability of MLR effector cells to participate in this process. The experiment described in figure 4.16 shows low levels of non-specific cytotoxicity for unmodified MLR effector cells against Y3 target cells. There is however a marked increase in cytotoxicity after incubation with both OX55 (IgG1) and OX34 (IgG2a). Furthermore the level of cytotoxicity diminishes only slightly with decrease of the effector: target ratio. This indicates that maximal lysis of target cells is obtained at the minimal effector: target ratio, indicating the efficiency of ADCC promoted by OX34 and OX55 mAb in this system. Similar results have been demonstrated for ADCC by mouse macrophages directed by the rat anti-CD4 mAb GK1.5 (202). Fc receptors of rat NK cells therefore are capable of interacting with mouse Ig, despite the inability of mouse Ig

to fix rat complement. This is explained by the different loci on the Fc portion of an IgG molecule which are responsible for these two functions.

The very potent ability of mouse IgG2a to promote ADCC in this way can be incorporated into a possible mechanism for *in vivo* cellular depletion. It is possible that intravenous injection of OX34 leads to labelling of lymphocytes with mouse mAb, which promotes an interaction with host macrophages. This theory would not however explain the selective effects of OX34 mAb described in the next section.

5 The comparative *in vivo* effects of anti-CD2 monoclonal antibody therapy

5.1 Study objective

Section 1 established that anti-CD2 mAb has a potent effect on allograft survival and Section 2 demonstrated unpredicted effects on *in vitro* immune responses, which suggest a different mechanism of action to both anti-CD4 mAb and $CTLA_4Ig$ therapy. In this section I therefore performed a further series of experiments to investigate the effect of anti-CD2 mAb on lymphocyte populations *in vivo* and again compared these effects to those induced by anti-CD4 mAb and $CTLA_4Ig$ under the same conditions. Firstly experiments were performed on lymphocytes extracted from various rat lymphoid organs to determine the tissue distribution of CD2 and B7 in naïve animals. I also explored the expression of B7 in rats undergoing acute allograft rejection and compared this to that of animals with long surviving allografts. The remainder of this section is divided into investigations of the pharmacokinetics and pharmacodynamics of anti-CD2 mAb therapy.

5.1.1 Pharmacokinetics of anti-CD2 mAb therapy.

This entails investigation of the extent of distribution of anti-CD2 mAb into lymphoid compartments after parenteral administration and the mechanism and kinetics of elimination of the foreign immunoglobulin by the rat host. The time course of mAb elimination is compared to the duration of immunosuppression and allograft survival in transplanted rats. The kinetics of elimination of OX34 and OX38 at therapeutic doses is compared in DA and Lewis rats.

5.1.2 Pharmacodynamics of anti-CD2 mAb therapy.

This entails investigation of the effect of administration of anti-CD2 mAb on lymphocyte population and antigen expression. The predominant effects noted after anti-CD2 mAb therapy in the rat are lymphocyte depletion and antigen modulation. These two effects are studied in detail and comparison is made between the effects in high and low responder strain rats. In addition the depleting and modulating effects of the different modalities under study are compared in an attempt to correlate prolongation of allograft survival with a measurable immunological effect.

5.2 Study design

1) To establish the extent and magnitude of CD2 and B7 expression lymphocytes were prepared from naïve peripheral blood, lymph nodes, spleen, thymus and peritoneal lavage in the manner described in materials and methods. Lymphocytes were incubated with relevant fluorescein-conjugated test mAbs prior to FACS analysis. To demonstrate B7 expression PBLs were prepared from a naïve rat, a rat acutely rejecting a cardiac allograft and also from Lewis rats with long surviving DA allografts after OX34-based therapy. These lymphocyte preparations were labelled with $CTLA_4Ig$ prior to secondary labelling and FACS analysis.

To investigate the tissue distribution of anti-CD2 mAb and $CTLA_4Ig$ after parenteral therapy DA strain rats were injected with OX34 at various doses and $CTLA_4Ig$ at 2.5mg/kg. Lymphocytes were then prepared from peripheral blood, lymph nodes, spleen and thymus and labelling with OX34 and $CTLA_4Ig$ was assessed by FACS analysis.

2) The kinetics of elimination of OX34 was investigated in Lewis and DA strain rats and compared to that of OX38. Monoclonal antibody was administered to

both rat strains at the dose used to induce cardiac allograft survival in the Lewis to DA model (OX34 2x5mg/kg, OX38 10,2,2,2mg/kg on consecutive days). The presence of residual mAb was assessed at days seven, fourteen and twenty-one on PBLs and in serum by flow cytometry. Rats were tail-bled into heparin-containing eppendorf tubes and samples were centrifuged at low speed and serum extracted and diluted 1:1 with PBS. 100μl dilute serum was then incubated on ice with 100μl naïve strain-specific LNCs at 10^7/ml for 30 minutes. After three washes antibody binding to lymphocytes was detected by further incubation with FITC-conjugated rabbit anti-mouse antibody and FACS analysis.

The generation of an anti-globulin response to the mouse immunoglobulin was assessed by a flow cytometric method. Serum from mAb-treated animals was prepared as above. Fresh LNCs (100μl containing 10^6 cells in PBS) were incubated with 10μl OX34 or OX38 at 5mg/ml on ice for 30 minutes followed by washing. These antibody-labelled cells were then used as targets and incubated with 100μl dilute (1:1) serum from rats treated as above. Cells were washed and the presence of anti-mouse Ig was determined by tertiary labelling with 20μl FITC-labelled mouse anti-rat IgM and IgG antibody. The influence of co-administered $CTLA_4Ig$ on the generation of an anti-globulin response and mAb elimination was demonstrated by similar techniques.

3) To explore the pharmacodynamics of OX34 mAb therapy a cohort of DA rats was injected with 2x5mg/kg OX34. At each set time interval post injection six rats were sacrificed and PBLs and LNCs prepared for FACS analysis. The frequency of B cells and CD4 and CD8 T cells was then measured from each compartment. In a similar manner the effect of OX55 mAb on lymphocyte populations was measured 24 hours after intravenous administration of the therapeutic dose (2x10mg/kg) for comparison of the depleting effects of the two mAb in view of the lesser ability of

OX55 to influence graft survival. The effect of OX34 mAb therapy on NK cell numbers is not directly measurable due to the absence of a specific NK cell marker for the rat. Therefore the NK cell frequency is estimated by calculating the non-B non-T cell population in a large number of rats at various time points after OX34 mAb therapy (this population also contains a small number of monocytes and γδT cells). In addition the effect of OX34 therapy on NK cell function was measured by assessing the non-specific cytotoxicity of lymphocyte preparations from OX34 treated and naïve rats. A comparison was also made of the depleting effect of OX34 (2x5mg/kg) and OX38 (10,2,2,2mg/kg) in DA and Lewis rats (n=3). In this experiment relative depletion measured by flow cytometry is correlated to absolute reduction in circulating leukocyte numbers measured by microscopy.

4) The depleting effect of mAb combinations was assessed in a similar manner in Lewis rats. Reagents were administered at the optimum dose used to induce prolonged allograft survival and lymphocytes were prepared from peripheral blood at set time points for estimation of lymphocyte frequencies.

5) The saturation of CD2 ligands with OX34 mAb after intravenous administration of low and high dose OX34 was assessed by harvesting undepleted LNCs and PBLs 24 hours after OX34 therapy. The extent of OX34 labelling of cells was then determined by secondary labelling with FITC conjugated rabbit anti-mouse antibody (20μl in 100 μl of cells). The presence of unsaturated OX34 binding sites was then assessed by the addition of a further 10 μl OX34 (5mg/ml) to 100μl of cells on ice *in vitro*. After washing these cells were again incubated with 20μl FITC conjugated rabbit anti-mouse antibody. The mean channel fluorescence of bound OX34 of *in vivo* and *in vitro* treated samples were then compared by FACS analysis.

6) The modulating effect of anti-CD2 mAb therapy was measured by both *in vitro* and *in vivo* methods. PBLs were prepared from DA rats treated with OX34 and

OX55 and levels of CD2 expression measured by flow cytometry. The modulating effect of OX34 was also compared directly to that of OX38 in Lewis rats treated with both mAb. To assess the modulating effect of OX34 in the absence of cellular depletion purified CD4 T cells were prepared and incubated with OX34 *in vitro*. 5 mls of CD4 T cell suspension were incubated at $4x10^6$/ml in culture medium for 18 hours in 5% CO_2 at 37°C with mAb (OX34 or OX38) at 20μg/ml. After incubation cells were washed to remove excess antibody and resuspended on ice. Labelling of residual molecules with OX34 and OX38 was then detected using FITC-conjugated rabbit anti-mouse antibody and compared to the labelling of naïve lymphocytes after incubation on ice for 30 minutes with the same concentration of mAb as above.

5.3 Distribution of the CD2 molecule

To establish the distribution of the CD2 molecule amongst lymphoid cells in the rat cells were prepared from lymph nodes, peripheral blood, spleen and thymus of a naïve rat as described in materials and methods and incubated with OX34 followed by fluorescein-conjugated rabbit anti-mouse antibody. This shows that CD2 is expressed at varying levels on cells from lymph nodes, spleen and circulating blood lymphocytes. In addition CD2 is expressed at a lower level on virtually all thymocytes (figure 5.1). Assessment of differential expression shows that CD2 is expressed on all T lymphocytes expressing the αβ TCR but not on B lymphocytes (figures 5.2, 5.3). There is also an αβTCR- subset which is labelled by OX34 on *in vitro* culture, which contains γδT cells and NK cells (figure 5.2). This subset is most notable amongst splenocytes where it forms 10% of the cell population. Amongst T cells CD2 is expressed equally on CD4 and CD8 cells (in figure 5.4 mean channel fluoresence of OX34 labelling of CD4 cells=2.6, and of CD8 cells=2.4). Analysis of labelling with OX34 by peritoneal macrophages (ED1+) shows labeling of both ED-

1 and OX34 at a very low level, which may represent non-specific labelling by Fc receptors (figure 5.5).

5.4 Distribution of the B7 molecule

The construct $CTLA_4Ig$ binds to the B7 molecule, which is expressed on APCs and activated B cells. B7 is expressed at a low level in the resting state and is upregulated on activation of both cell types. To demonstrate binding of $CTLA_4Ig$ to B7 in the resting and activated state PBLs were prepared from both a naïve rat and a Lewis rat 50 days post transplant which was undergoing acute allograft rejection after failed therapy with OX34 and $CTLA_4Ig$. Cells were incubated with $CTLA_4Ig$ (20μl 1:100) and labelling measured with FITC-conjugated sheep anti-human antibody. As can be seen in figure 5.6 resting lymphocytes express B7 at a low level on a small minority of non-T cells. Both the number of cells and the intensity of B7 labelling is upregulated in LNCs taken from an animal undergoing acute allograft rejection, but is still largely restricted to the non-T cell subset. (figure 5.6). B7 expression (determined by $CTLA_4Ig$ labelling) was also determined in Lewis rats with healthy allografts at 21, 40 and 150 days post-transplant after therapy with OX34 and $CTLA_4Ig$. Interestingly these animals show a profile of $CTLA_4Ig$ labelling similar to naïve rats (figure 5.7). A similar finding is noted in tolerant Lewis rats with DA allografts after therapy with OX34 and OX38.

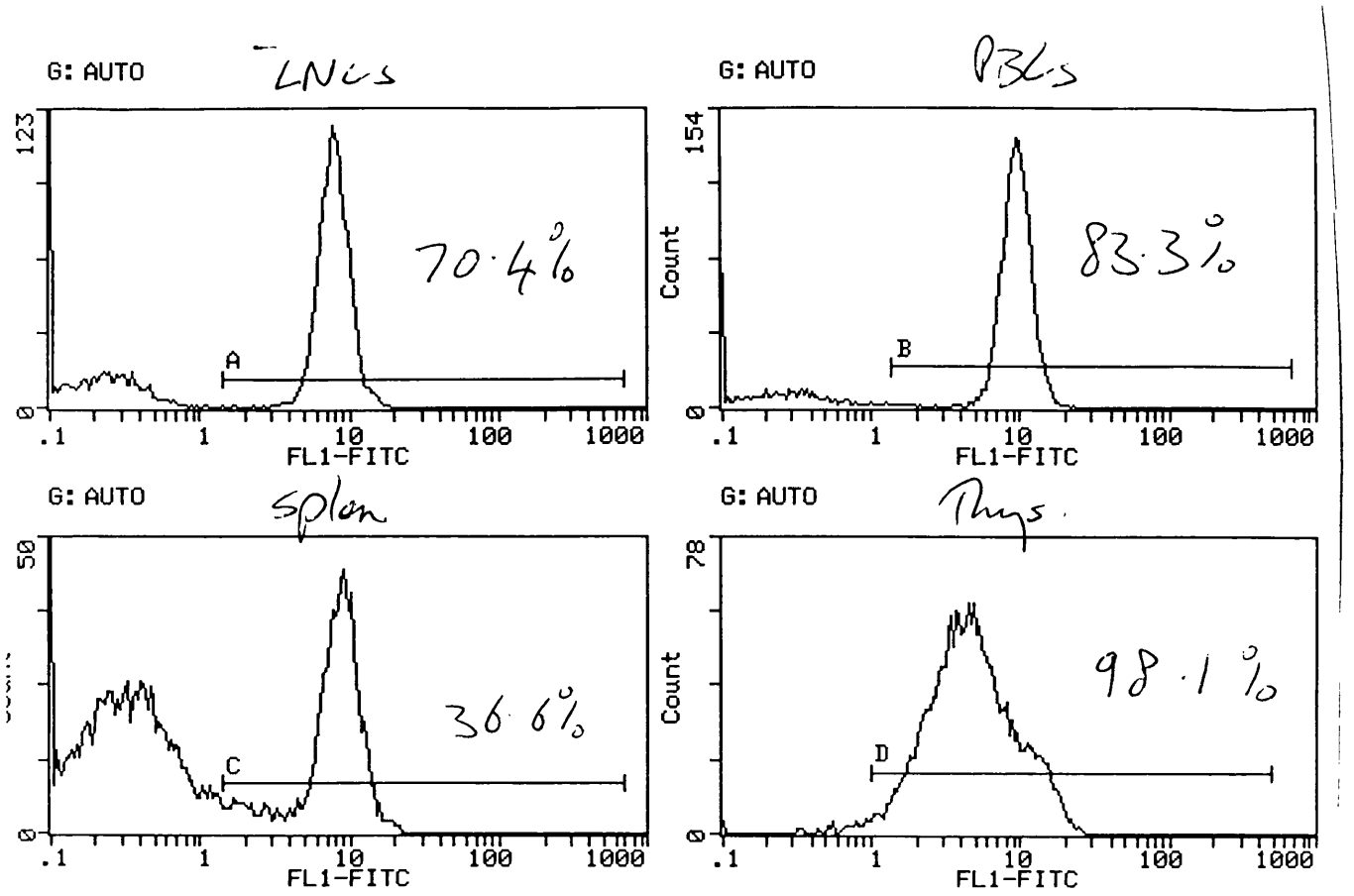

Figure 5.1 Distribution of the CD2 molecule on lymphoid cell subsets

Cells were prepared from lymphoid organs of a naïve rat using the standard method. Cells were incubated with OX34, washed and secondarily labelled with FITC rabbit anti-mouse Ig.

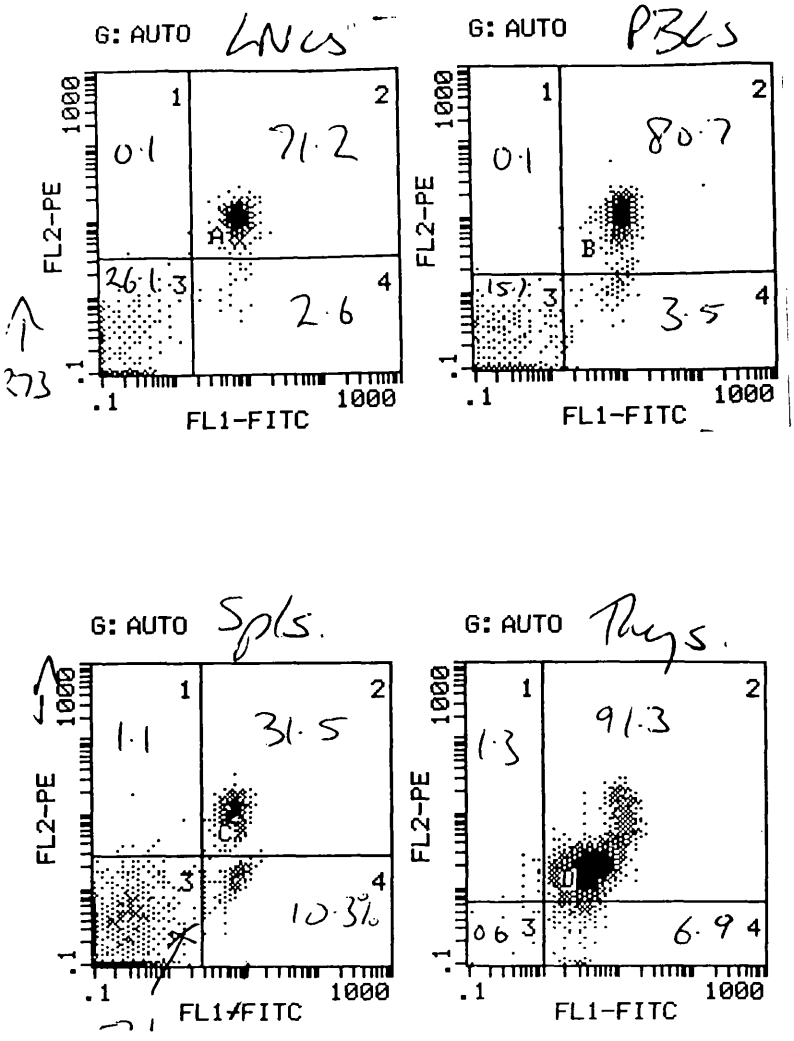

Figure 5.2 Distribution of CD2 on T cells

Cells were prepared from lymphoid organs in the standard manner and incubated with FITC-conjugated OX34 followed by PE-conjugated R73.

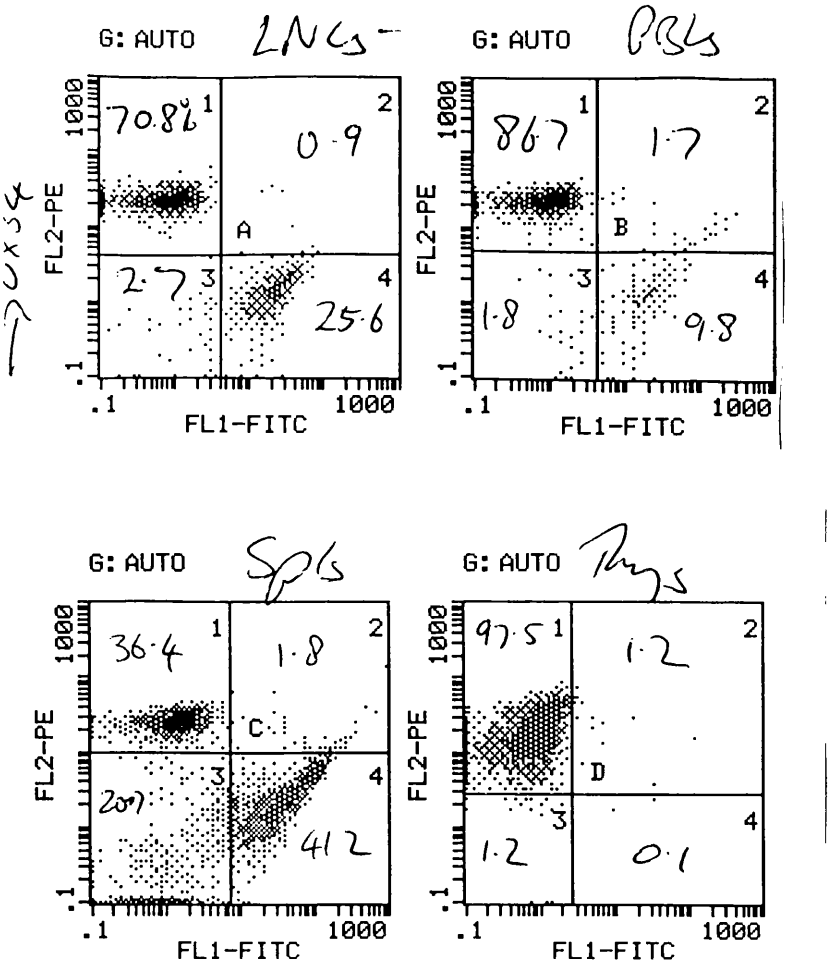

Figure 5.3 CD2 is not expressed by B cells

Cells were prepared from lymphoid organs in the standard manner and incubated with OX34 followed by PE-conjugated rabbit anti-mouse Ig and finally FITC-conjugated OX12.

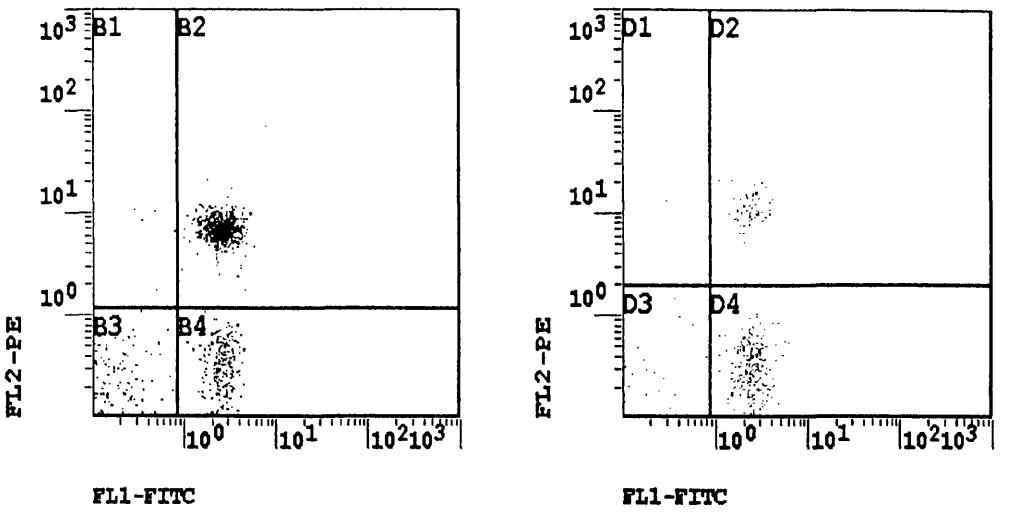

Figure 5.4 CD2 expression on T cell subsets

Lymph node cells were incubated with FITC-conjugated OX34 followed by either a) W3/25-PE or b) OX8-PE.

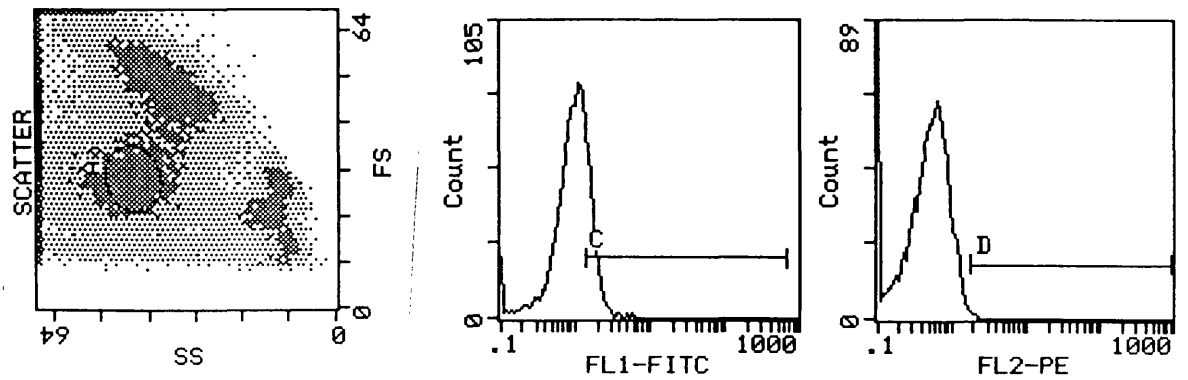

Figure 5.5 Labelling with OX34 by peritoneal macrophages

A suspension of peritoneal cells was divided into two aliqouts and incubated with either a) FITC conjugated ED-1 or b) FITC conjugated OX34.

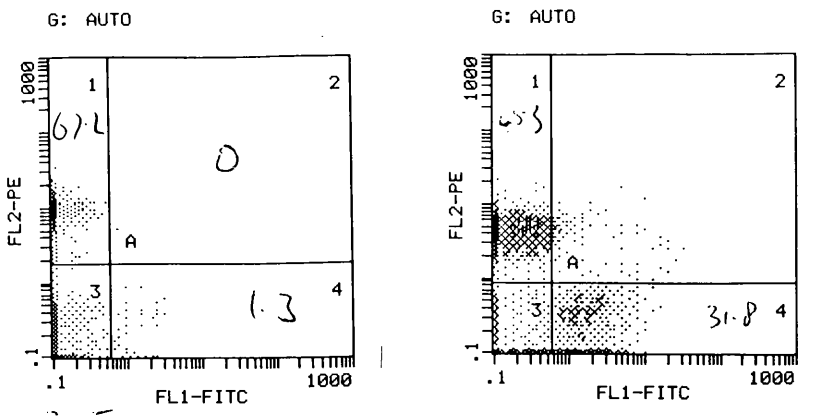

Figure 5.6 B7 expression on non-T cells from a rat rejecting an allograft

LNCs were taken from a naïve rat 24 hours after administration of $CTLA_4Ig$ (2.5mg/kh) and a transplanted Lewis rat rejecting a DA allograft after therapy with OX34 and $CTLA_4Ig$. Cells from the transplanted rat were incubated with $CTLA_4Ig$ and all cells secondarily labelled with FITC-conjugated rabbit anti-human antibody and finally R73-PE.

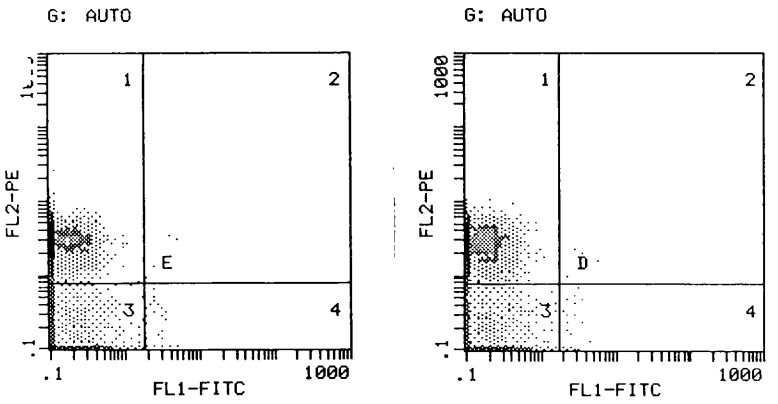

Figure 5.7 B7 expression on non-T cells in tolerant Lewis rats

LNCs were taken from Lewis rats with long-surviving DA allografts after therapy with either a) OX34 and OX38 or b) OX34 and $CTLA_4Ig$. Cells were incubated with $CTLA_4Ig$ followed by FITC-conjugated rabbit anti-human antibody and finally R73-PE.

5.5 Pharmacokinetics of monoclonal antibody therapy

5.5.1 Distribution of OX34 after intravenous administration

To establish the extent of OX34 labelling of lymphoid cells *in vivo* after intravenous injection, 10 mg/kg OX34 was administered to a DA rat 24 hrs prior to harvesting LNCs, PBLs, splenocytes and thymocytes. The extent of OX34 labelling of cells was then determined by secondary labelling with FITC conjugated rabbit anti-mouse antibody. Cells were then incubated with further OX34 *in vitro* to detect unlabelled binding sites. OX34 binding sites on LNCs, PBLs and splenocytes were all saturated after intravenous injection at this dose (figure 5.8) whereas thymocytes were labelled at a lower level. This suggests the presence of a blood-thymus barrier to the passage of OX34, as *in vitro* incubation with further antibody substantially increased labelling. Therapy with lower doses of OX34 (2.5mg/kg and 5mg/kg) fails to saturate all OX34 binding sites on LNCs (figure 5.9).

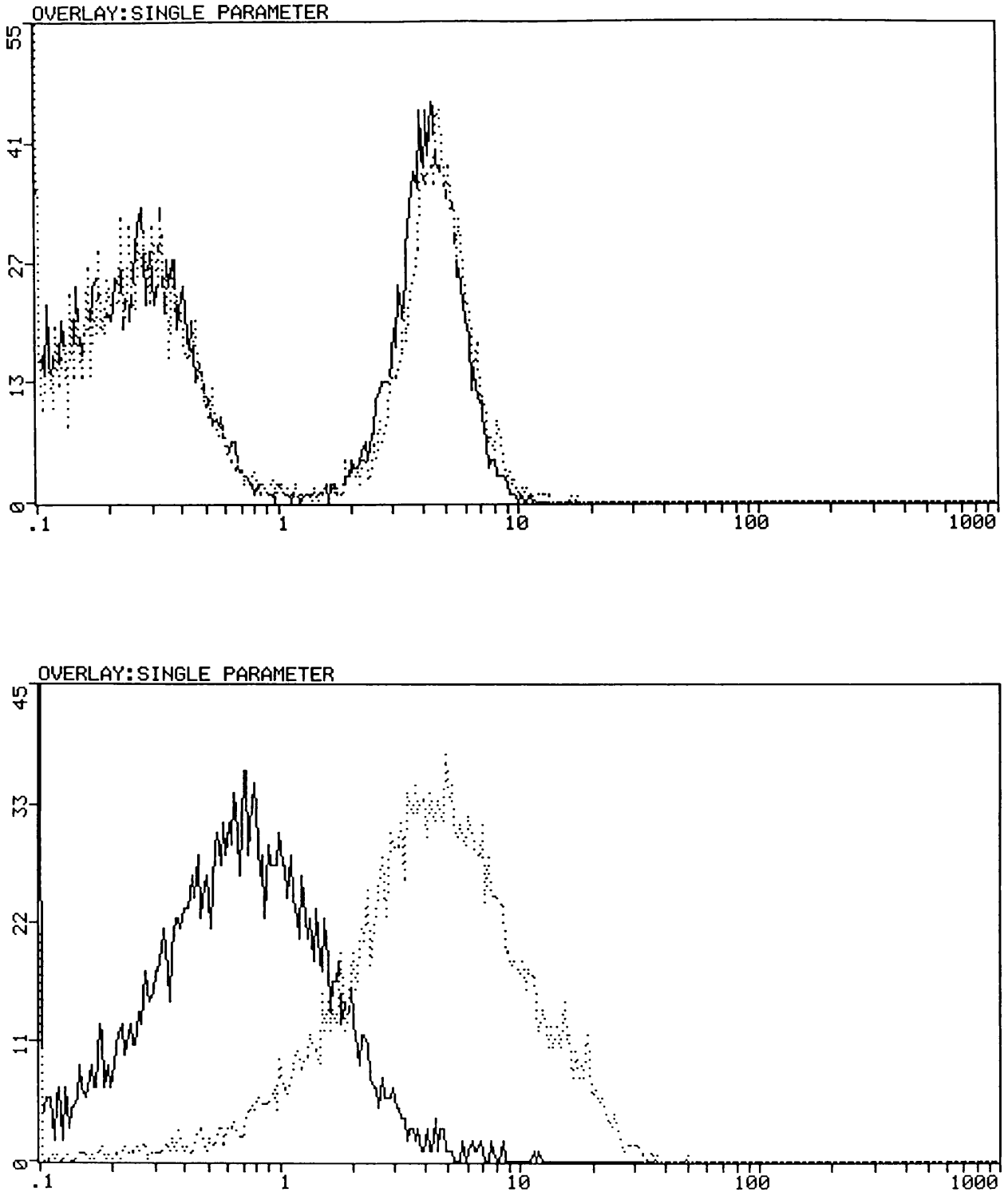

Figure 5.8 Saturation of binding sites on LNCs and thymocytes after OX34 therapy

LNCs (top) and thymocytes (bottom) were prepared from a DA rat 24 hours after intravenous injection of OX34 (10mg/kg). Cells were incubated with rabbit anti-mouse FITC (solid line) or OX34 followed by rabbit anti-mouse FITC (dotted line).

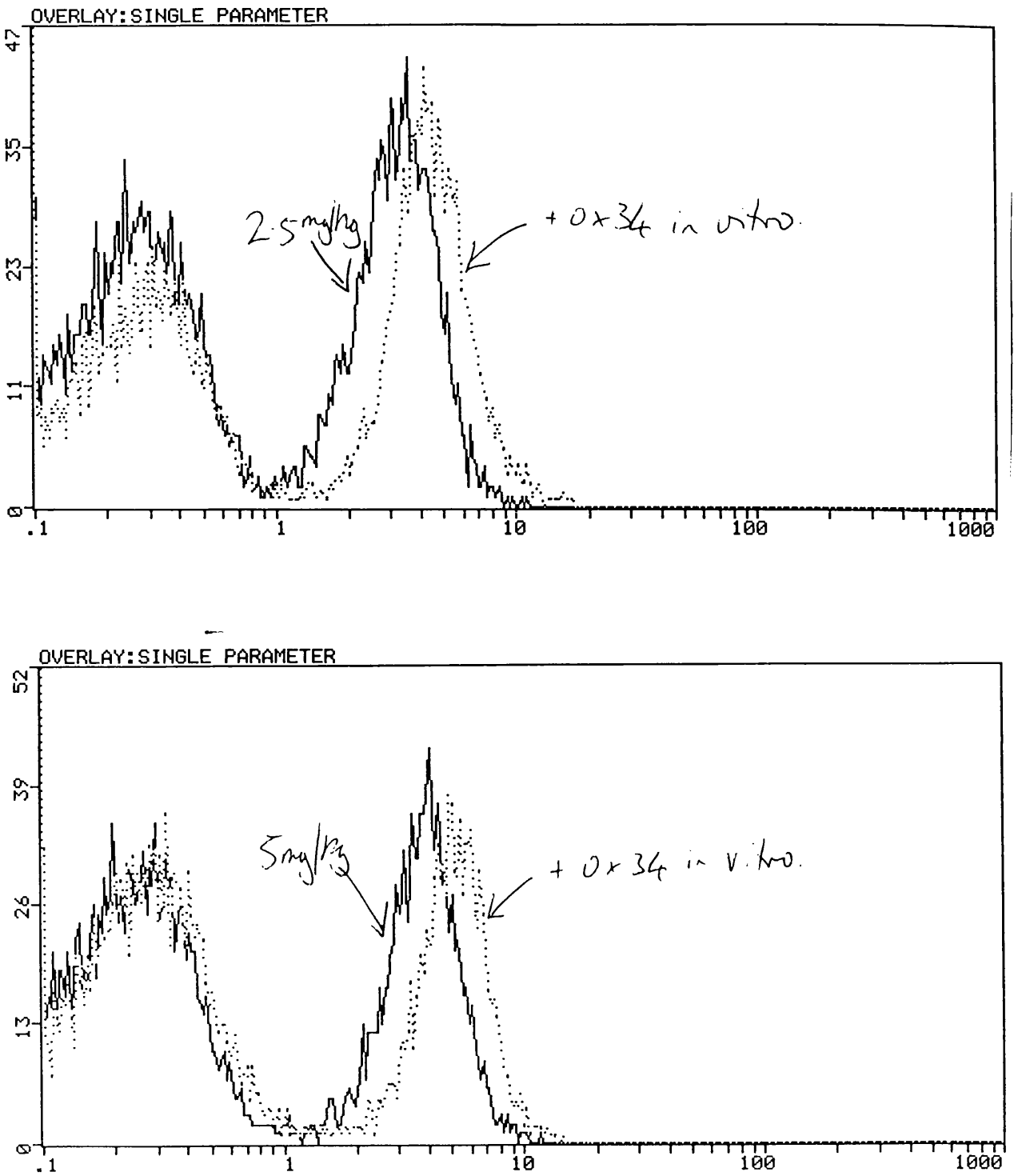

Figure 5.9 Saturation of binding sites on LNCs after low dose OX34 therapy

DA rats were injected intravenously with OX34 2.5mg/kg (top) or 5mg/kg (bottom). Cells were incubated with rabbit anti-mouse FITC (solid line) or OX34 followed by rabbit anti-mouse FITC (dotted line).

5.5.2 Elimination of OX34 and OX38 by DA and Lewis strain rats after intravenous injection

We then asked if the difference in survival rates recorded between DA and Lewis strain rats may be associated with a more prolonged duration of action of either monoclonal antibody in DA compared to Lewis rats. We therefore wished to assess the kinetics of elimination of monoclonal antibody from the serum of treated rats and the generation of an antibody response to the foreign mouse protein. Lewis and DA rats (3 per group) were each injected intravenously with OX34 (10mg/kg) or OX38 (16mg/kg). PBLs were harvested at days 7, 14 and 21 and tested for mAb labelling with FITC conjugated rabbit anti-mouse antibody. Serum was taken at similar time points and the presence of residual circulating mAb determined by incubating serum (diluted 1:1 in PBS) with fresh Lewis LNCs and using rabbit anti-mouse FITC as a secondary label. In a similar manner the presence of an anti-globulin response was assessed by a non-quantitative flow cytometric method. Fresh LNCs were labelled with OX34 or OX38, these cells were then incubated with serum from rats treated as above and the presence of anti-mouse Ig determined by tertiary labelling with FITC-conjugated mouse anti-rat IgG and IgM, as described in materials and methods. The results are summarised in Table 5.1. Examples are also given showing that DA strain rats do not generate anti-globulins when treated with either OX34 or OX38 (Figure 5.10), Lewis rats however generate IgG anti-globulins against OX38 at day 14 (two of three rats) (Figure 5.11) and against OX34 by day 21 (three of three rats) (Figure 5.12).

n=3 per group		Day 7		
		Cell-bound mAb	Circulating mAb	Anti-Ig response
DA	OX34	+	+	-
	OX38	+	-	-
Lewis	OX34	+	+	-
	OX38	-	-	-

n=3 per group		Day 14		
		Cell-bound mAb	Circulating mAb	Anti-Ig response
DA	OX34	+	+	-
	OX38	+	-	-
Lewis	OX34	+	-	-
	OX38	-	-	+

n=3 per group		Day 21		
		Cell-bound mAb	Circulating mAb	Anti-Ig response
DA	OX34	+	-	-
	OX38	-	-	-
Lewis	OX34	-	-	+
	OX38	-	-	+

Table 5.1 Elimination of OX34 and OX38 by DA and Lewis rats

Correlation between duration of cell-bound and circulating OX34 and OX38 mAb with the generation of an anti-globulin response in Lewis and DA rats.

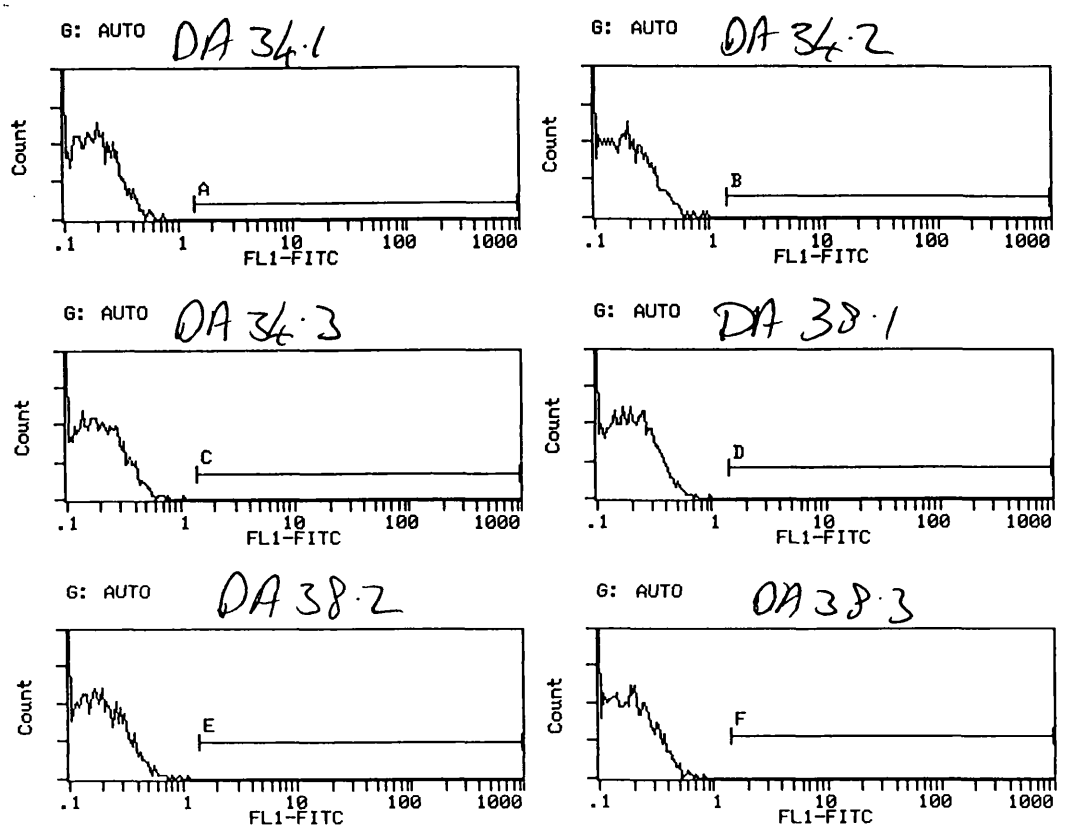

Figure 5.10 Failure of DA strain rats to produce IgG antiglobulins against either OX34 or OX38 fourteen days after administration.

Serum from DA strain rats treated with OX34 (1-3) or OX38 (4-6) was incubated with specific target cells before tertiary labelling with mouse anti-rat IgG (see materials and methods for experimental details and controls).

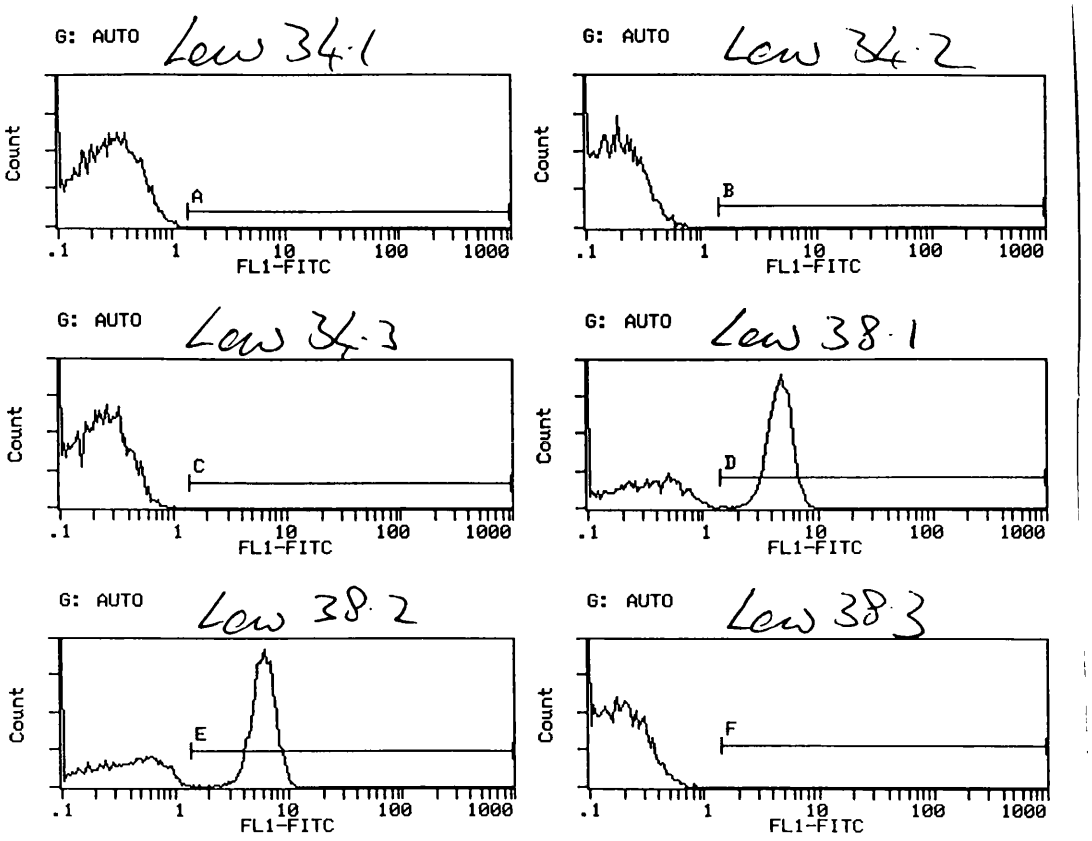

Figure 5.11 Lewis strain rats produce IgG antiglobulins against OX38 but not OX34 fourteen days after administration.

Serum from Lewis strain rats treated with OX34 (1-3) or OX38 (4-6) was incubated with specific target cells before tertiary labelling with mouse anti-rat IgG (see materials and methods for experimental details and controls).

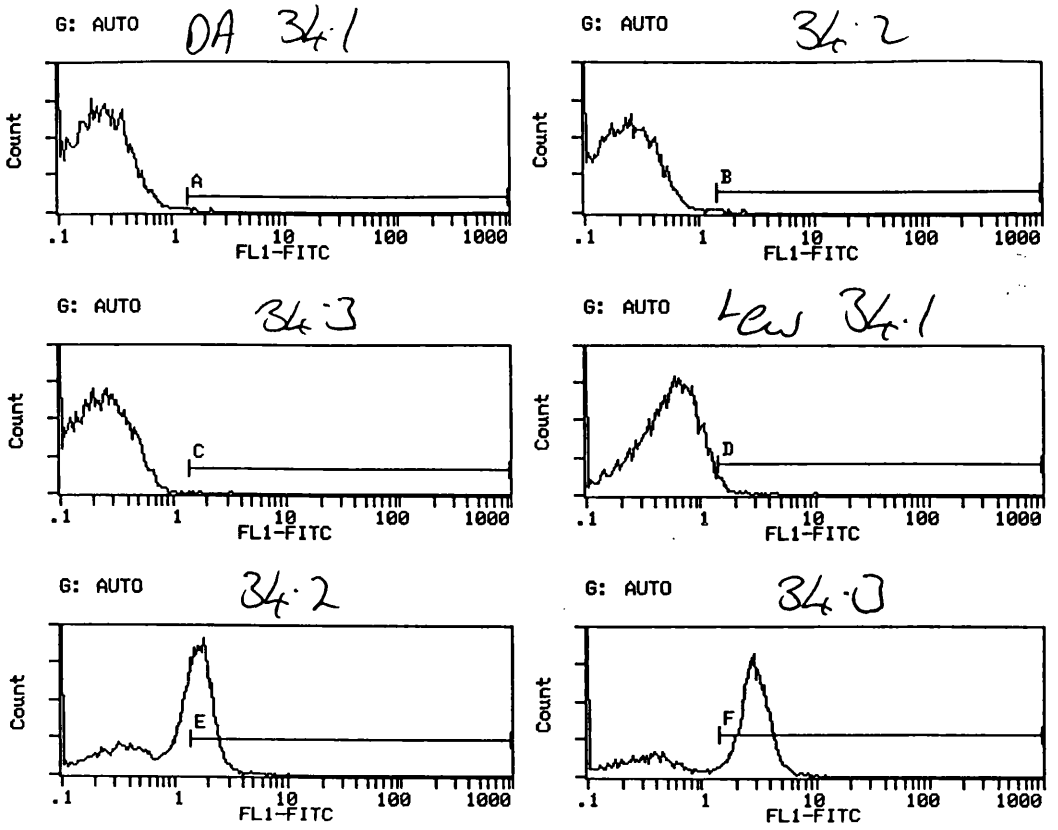

Figure 5.12 Lewis strain rats but not DA strain rats produce IgG antiglobulins against OX34 twenty-one days after administration.

Serum from DA (1-3) and Lewis (4-6) strain rats treated with OX34 was incubated with specific target cells before tertiary labelling with mouse anti-rat IgG (see materials and methods for experimental details and controls).

5.5.3 Distribution of $CTLA_4Ig$ after intravenous injection

In a naïve animal labelling of a small minority of non-T cells from peripheral blood and lymph nodes is detectable 24 hours after intravenous injection of $CTLA_4Ig$. Figure 5.6 shows a FACS analysis of LNCs taken from a rat 24 hours after administration of 2.5mg/kg $CTLA_4Ig$ intravenously. $CTLA_4Ig$ is detected with FITC-conjugated sheep anti-human antibody.

5.5.4 Elimination of OX34 by Lewis strain rats after intravenous injection in combination with $CTLA_4Ig$

As has been demonstrated this combination of treatment modalities is effective in prolonging allograft survival in the high responder DA-Lewis strain combination. When used as monotherapy in Lewis strain rats OX34 is eliminated from the circulation by day 14 and from the cell surface by day 21, and this elimination is associated with the development of an anti-OX34 IgG response. The CD28:B7 pathway has been shown to provide costimulation in the development of B cell responses so we therefore explored the possibility that $CTLA_4Ig$ may influence the development of anti-OX34 immunoglobulin in this model and the subsequent elimination of monoclonal antibody.

Firstly three untransplanted Lewis rats were treated with OX34 at the same dose as above (2x5mg/kg) followed by $CTLA_4Ig$ (2.5mg/kg) two days later. Rats were tail-bled at set time points and serum and cells were harvested and assayed for residual mouse monoclonal antibody and anti-OX34 immunoglobulin by tertiary labelling with mouse anti-rat immunoglobulin (IgM and IgG) as shown in Table 5.2.

Lewis rats, n=3	Day 14, 21 and 28		
	Cell-bound mAb	Circulating mAb	Anti-Ig response
OX34 (2x5mg/kg) + $CTLA_4Ig$ (2.5mg/kg)	+	+	-

Table 5.2 Effect of $CTLA_4Ig$ on elimination of OX34 in Lewis rats

Persistence of circulating and cell-bound OX34 mAb and failure of anti-OX34 response in Lewis rats treated with $CTLA_4Ig$.

This experiment confirms that persistence of OX34 in Lewis rats is extended when OX34 is administered in conjunction with $CTLA_4Ig$.

Secondly we explored the correlation between the generation of an anti-globulin response, the elimination of monoclonal antibody, the persistence of cellular depletion and ligand modulation, and allograft survival in high responder Lewis rats. Six Lewis rats receiving DA heart transplants were treated intravenously with OX34 (2x10 mg/kg given pre-operatively) and $CTLA_4Ig$ (2.5mg/kg given 2 days post-operatively). Animals were tail-bled at days 21, 40 and 65 and serum separated. The presence of residual monoclonal antibody was determined by incubating with fresh LNCs and anti-OX34 antibody was detected using OX34 labelled LNCs as target cells as described previously. Lymphocyte sub-population profiles were assessed by flow cytometry (see Section 5.6.1). Of this particular cohort of six transplanted rats one allograft was rejected at day 13 whilst the others went on to permanent allograft survival.

Day	21		40		65	
Graft surviving	Yes n=5	No n=1	Yes n=5	No n=1	Yes n=5	No n=1
αIg response	-	+	-	+	-	+
Circulating antibody	+	-	+	-	-	-
Cellular depletion	+	-	+	-	-	-

Table 5.3 Abrogation of anti-globulin response by $CTLA_4Ig$ and graft survival

Allograft survival following OX34 and $CTLA_4Ig$ therapy in Lewis rats is associated with abrogation of the anti-globulin response, leading to persistence of OX34 mAb and continued cellular depletion.

In this experiment the generation of an anti-globulin response in one of six rats correlates with elimination of mouse monoclonal antibody, the recovery of the T cell population and allograft rejection. This suggests that $CTLA_4Ig$ prevents the generation of an antiglobulin response to mouse protein allowing prolonged antibody-mediated cellular depletion, which is associated with allograft survival. The failure of $CTLA_4Ig$ to prevent an antiglobulin response in one of six rats may suggest that a threshold dose of $CTLA_4Ig$ is required or may represent a failure of experimental technique. The remaining five rats appear to eliminate mouse monoclonal antibody at a slower pace by mechanisms that do not involve antiglobulin.

5.6 Pharmacodynamics of OX34 monoclonal antibody therapy

5.6.1 Effect on lymphocyte populations of intravenous OX34 therapy in DA rats.

To determine the effect of OX34 therapy on lymphocyte populations OX34 was administered intravenously at 5mg/kg on two consecutive days to a cohort of untransplanted DA rats. At each predetermined time point post-injection six rats were sacrificed and PBLs and LNCs harvested for analysis.

5.6.1.1 Estimation of T cell frequency.

Firstly cells were incubated with FITC-conjugated R73 (anti-TCR) to establish the frequency of T cells in the lymphocyte population (figure 5.13).

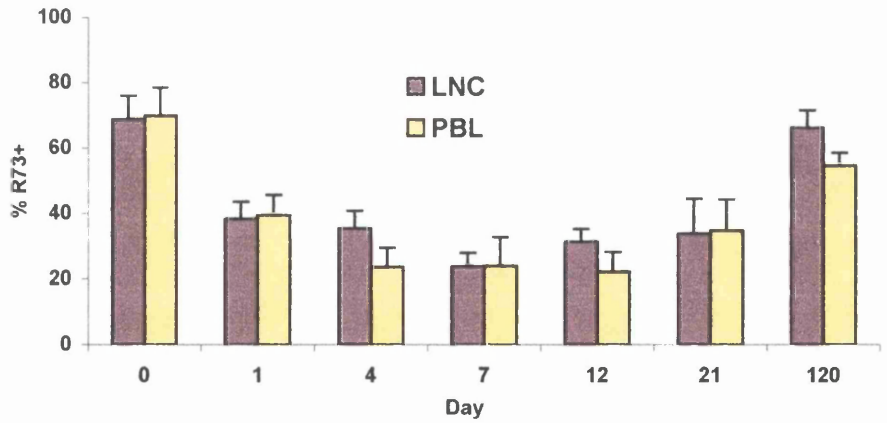

Figure 5.13 T cell depletion by OX34

Frequency of T cells in DA LNCs and PBLs following therapy with OX34 (2x5mg/kg) (n=6).

OX34 treatment caused depletion of T cells from lymph nodes and peripheral blood. R73+ T cells form approximately 70% of LNCs and PBLs in a naive animal.

This figure declined progressively over the first 7 days after injection to reach a minimal level of 24% in both populations. Some recovery was seen by day 21 and even at day 120 levels of R73 expression had not returned to normal (66% and 54% respectively).

5.6.1.2 Estimation of CD4:CD8 ratio

In a similar manner relative depletion of specific CD4 and CD8 subsets was assessed using fluorochrome conjugated W3/25 and OX8 respectively. The frequency of CD8 T cells remained broadly constant throughout the period of the study (13-16%) despite OX34 therapy. The ratio of CD4 to CD8 cells was then established for each time point and is illustrated in figure 5.14.

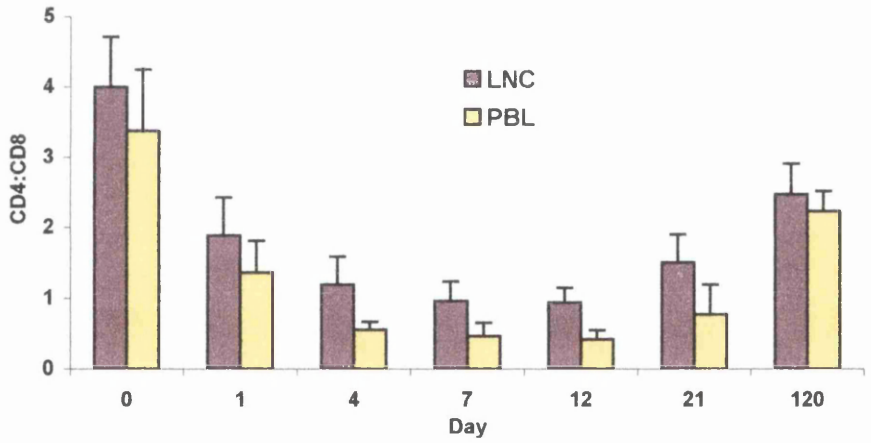

Figure 5.14 Preferential depletion of CD4 T cells by OX34
CD4:CD8 ratio in LNCs and PBLs of DA rats (n=6) treated with OX34 (2x5mg/kg).

In an unmodified animal the ratio of CD4 to CD8 T cells is approximately 3.5:1. OX34 therapy preferentially depletes the CD4 subset so that the CD4 frequency falls from 54% of both LNCs and PBLs to a minimum of 14% and 5% respectively at day 12 (CD4: CD8 ratio 0.9 and 0.4 respectively). The depletion of CD4 cells appears to be less marked amongst LNCs than PBLs and recovered more

fully over time. Again however by day 120 recovery of the CD4 population was still not complete (CD4 frequency 47% and 38% respectively).

5.6.1.3 Estimation of B cell frequency after OX34 therapy

Analysis with the B cell marker OX12 shows that the decreasing frequency of T cells is matched by an apparent relative expansion of the B cell subset. PBLs were prepared from the cohort of DA rats treated with OX34 (2x5mg/kg) used in the above experiments and stained for B cells at various time points. Figure 5.15 shows the reciprocal relationship of B and T cell populations after OX34 therapy, and also shows that the non-B, non-T cell (R73-/OX12-) population remains unchanged despite OX34 therapy.

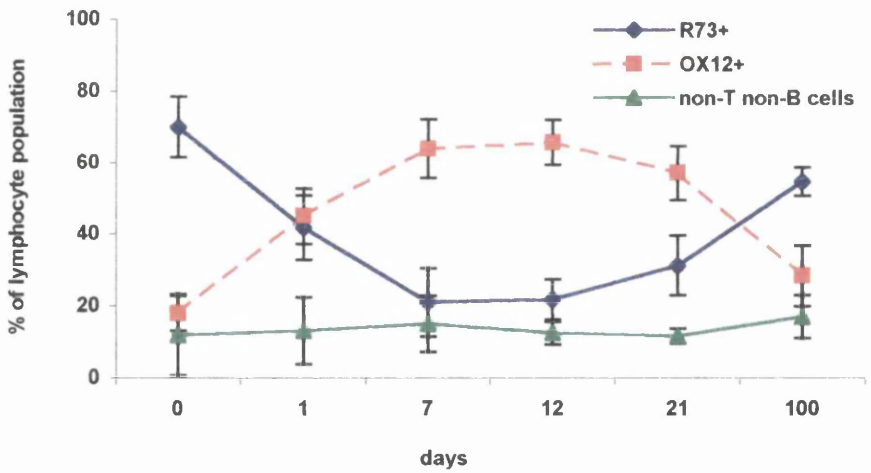

Figure 5.15 Relative preponderance of B cells after OX34 therapy

Percentages of T, B and unlabelled cells amongst PBLs after administration of OX34 (2x5mg/kg) to DA rats (n=6).

5.6.1.4 Influence of dose of OX34 on cellular depletion

Doses of OX34 lower than 2x5mg/kg fail to prevent acute allograft rejection in DA strain rats. We therefore investigated if this difference in survival is associated with a lesser degree of cellular depletion by lower doses of mAb. Two DA rats were

treated with OX34 2.5 mg/kg and four rats with 5mg/kg. After 24 hours PBLs were taken and lymphocyte subset frequencies determined in the standard manner. Figure 5.16 shows the lymphocyte proportions in comparison to the data shown above for naïve DA rats and DA rats treated with OX34 2x5mg/kg. The degree of depletion of lymphocyte subsets is broadly similar in animals treated with each dose.

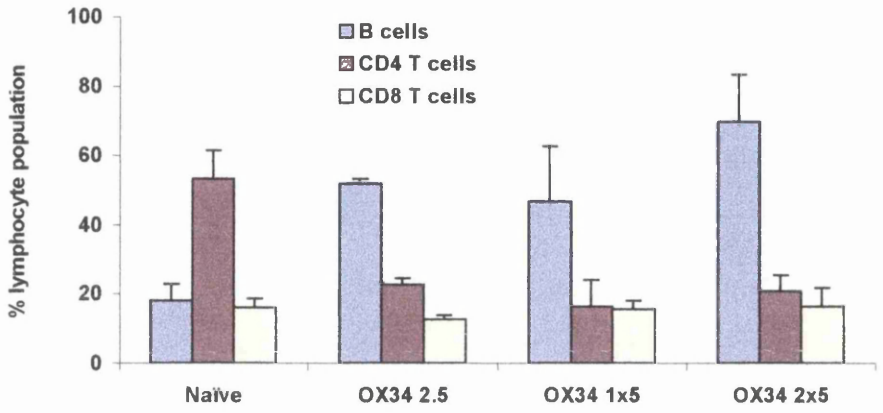

Figure 5.16 Effect of dose of OX34 on lymphocyte depletion

Relative lymphocyte frequencies in peripheral blood of naïve DA strain rats (n=6) and DA rats 24 hours after administration of OX34 2.5mg/kg (n=3), OX34 5mg/kg (n=4) and OX34 2x5mg/kg (n=6).

5.6.1.5 Estimation of NK cell frequency after OX34 therapy

The lack of a specific NK cell marker in the rat prevents precise delineation of the effect of anti-CD2 therapy on NK cell numbers. The cell population which is both R73-ve and OX12-ve contains the NK cell and γδ T cell fractions. This population appears to remain constant in size (approximately 10%) despite OX34 therapy in DA rats (Figure 5.15). Natural killer cells in the rat express the CD2 molecule and would therefore be expected to be labelled by OX34 mAb after intravenous administration. However when OX34 labelling of non-depleted cells is measured at various time points after intravenous OX34 therapy in a large number

(24) of DA rats it is notable that the proportion of cells labelled with OX34 coincides very closely in size with the remaining R73+ T cell fraction (Table 5.4).

n=24	R73+	RaM+
LNC (SD)	32.52 (10.22)	32.37 (11.38)
PBL (SD)	30.87 (13.54)	30.52 (11.27)

Table 5.4 Estimation of labeling with OX34 and R73 on residual cells after OX34 therapy

Mean percentage (SD) of DA PBLs labelled with either R73 or rabbit anti-mouse antibody at a range of time points following administration OX34 (n=24).

5.6.1.6 Estimation of NK cell function after OX34 therapy.

Despite the greatly depleted T cell numbers assay of non-specific cellular cytotoxicity in a DA strain rat after intravenous administration of OX34 shows very little effect, suggesting that NK cells have not been significantly depleted by OX34. This is in keeping with the apparent inability of OX34 to label NK cells. To estimate NK cell function splenocytes were prepared from DA strain rats treated with 2x5mg/kg OX34 and compared to those from a naïve DA strain rat in a cytotoxicity assay using the ^{51}Chromium labelled NK-sensitive Y3 cells. Figure 5.17 shows that non-specific cellular cytotoxicity is minimally affected in this model. An interpretation of these findings is given at the end of the chapter.

5.6.2 Comparison of the depleting effect of OX55 and OX34 in DA strain rats

As has been demonstrated OX55 is less effective at prolonging allograft survival than OX34 even at higher doses (OX55 10mg/kg given days-1, 0 relative to transplant produces permanent engraftment in 2 of 6 rats). We hypothesised that this difference in efficacy can be explained by a difference in the ability of the two mAb

to cause cellular depletion. Figure 5.18 shows the frequencies of B cells and T cell subsets in PBLs of 6 DA rats treated with OX55 at the above dose on day 1 compared with the depletion obtained in 6 DA rats treated with OX34 (2x5mg/kg).

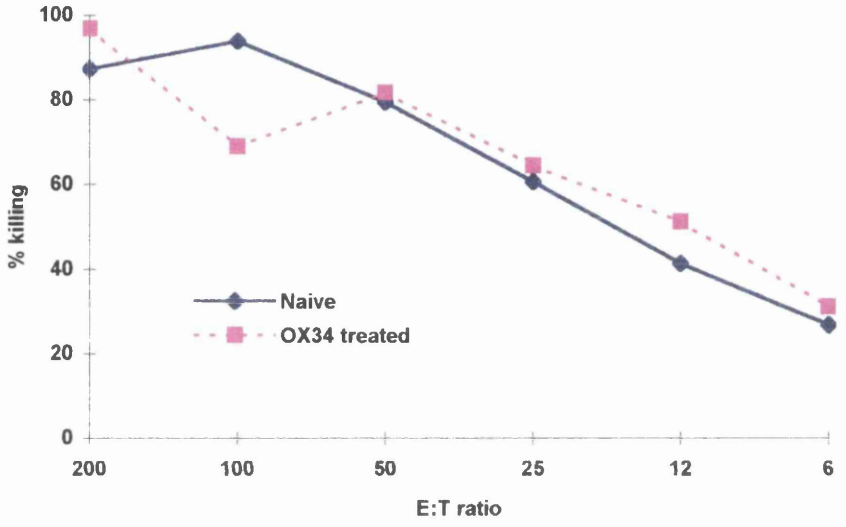

Figure 5.17 Effect of OX34 therapy on NK cell function

Non-specific cellular cytotoxicity of PBLs from a DA rat 24 hours after intravenous administration of OX34 (2x5mg/kg) compared to a naïve rat.

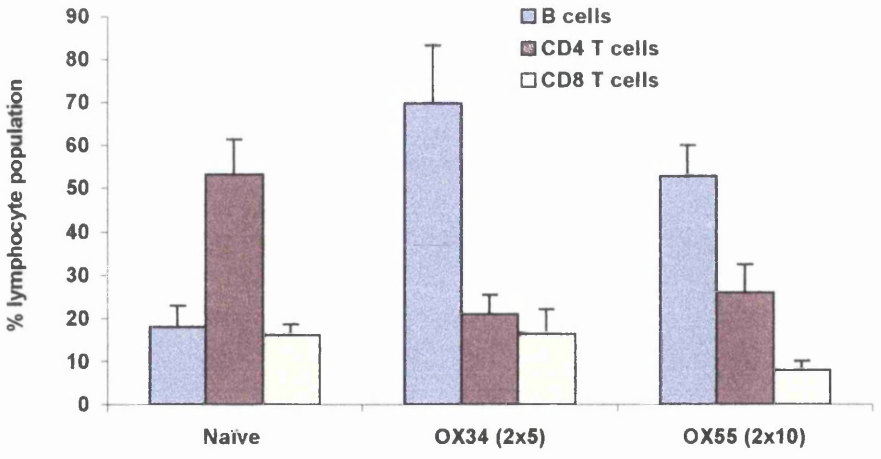

Figure 5.18 Depleting effect of OX55 therapy

Estimation of subset frequency in PBLs after 24 hours in DA rats treated with OX34 (2x5mg/kg) or OX55 (2x10mg/kg) (n=6 per group).

OX55 produces a similar degree of T cell depletion as OX34 but analysis of T cell subsets shows that the CD4 subset is not preferentially depleted. The CD4:CD8 ratio in the experimental groups used above is shown in Table 5.5.

n=6/group	Naive	OX34 (2x5mg/kg)	OX55 (2x10mg/kg)
CD4: CD8 ratio	3.30	1.28	3.25

Table 5.5 Differential effect of OX55 therapy on T cell subsets

CD4: CD8 ratio in the residual T lymphocyte population in PBLs after therapy with OX34 and OX55 compared to naïve animals.

5.6.3 Comparison of the depleting effect of OX34 with OX38 in high and low responder strain rats.

In the above experiments relative frequencies of T cell subsets were measured, which do not give an indication of total leukocyte depletion. For this reason a further series of experiments was performed in which groups of animals were injected intravenously with mAb and then tail-bled at set time points. The total circulating leukocyte count was then measured by staining with Turck's solution and microscopy as described in materials and methods at time periods beginning at four hours after injection. The same sample was then subjected to analysis by flow cytometry to define subset frequencies. In these experiments both high and low responder strain rats were used to assess any difference in the level of depletion obtained. In addition the depletion caused by OX34 was compared to that produced by OX38. Lewis and DA rats (3 per group) were each injected intravenously with OX34 (10mg/kg) or OX38 (16mg/kg). Total leukocyte counts are shown in figures 5.19 and 5.20.

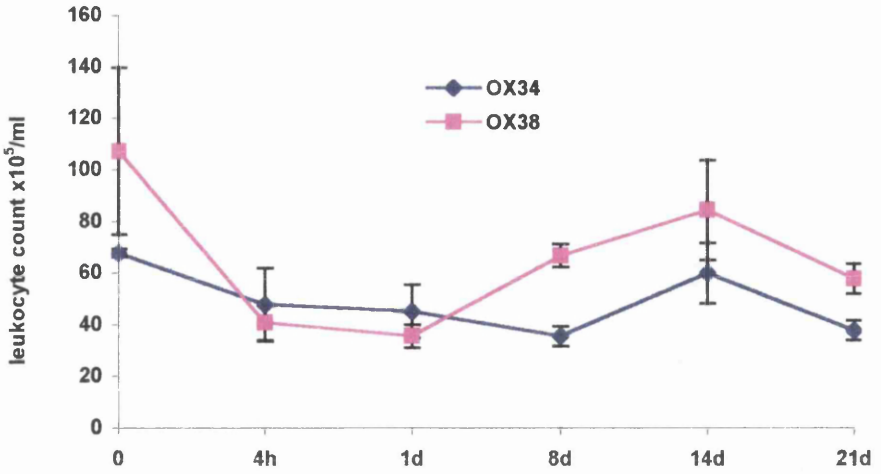

Figure 5.19 Total circulating leukocyte count in DA rats treated with OX34 or OX38

Whole blood was removed from naïve or treated DA rats (n=3) after therapy with OX34 or OX38. Total leukocyte count was determined by labelling with Turks solution.

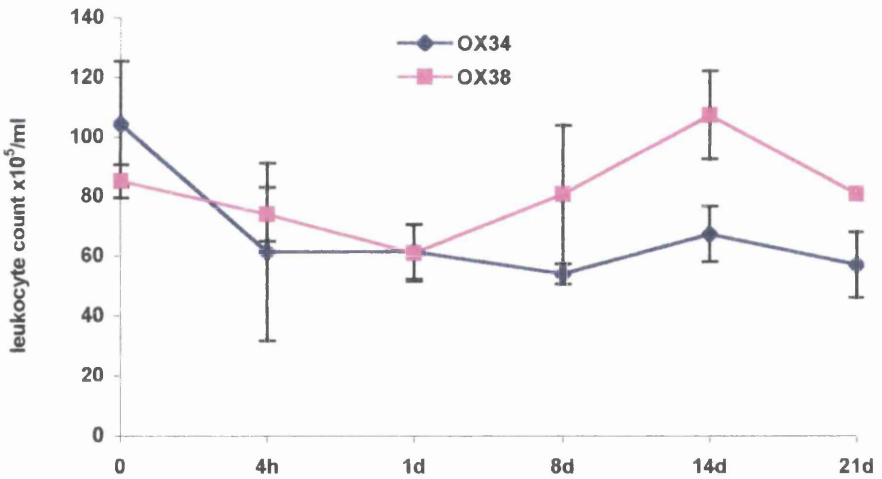

Figure 5.20 Total circulating leukocyte count in Lewis rats treated with OX34 or OX38.

Whole blood was removed from naïve or treated Lewis rats (n=3) after therapy with OX34 or OX38. Total leukocyte count was determined by labelling with Turks solution.

5.6.3.1 Comparison of the depletion of T cell subsets in DA and Lewis rats induced by OX34 and OX38

Both mAb cause leukocyte depletion in both rat strains, with an apparently steeper decline in DA rats. The total leukocyte depletion masks a predominant depletion of CD4 T cells, which is greater in rats treated with OX34 than OX38 and is maintained for the duration of the experiment (figures 5.21 and 5.22). The frequency of CD8 T cells shows minimal change in either rat strain treated with either modality, with values between 16% and 20%. The pattern of CD4 T cell depletion caused by OX34 and OX38 is similar in both rat strains. However when analysed closely CD4 depletion in DA strain rats is consistently greater than in Lewis rats (table 5.6), so that approximately twice the number of CD4 T cells remain after OX34 therapy in Lewis rats.

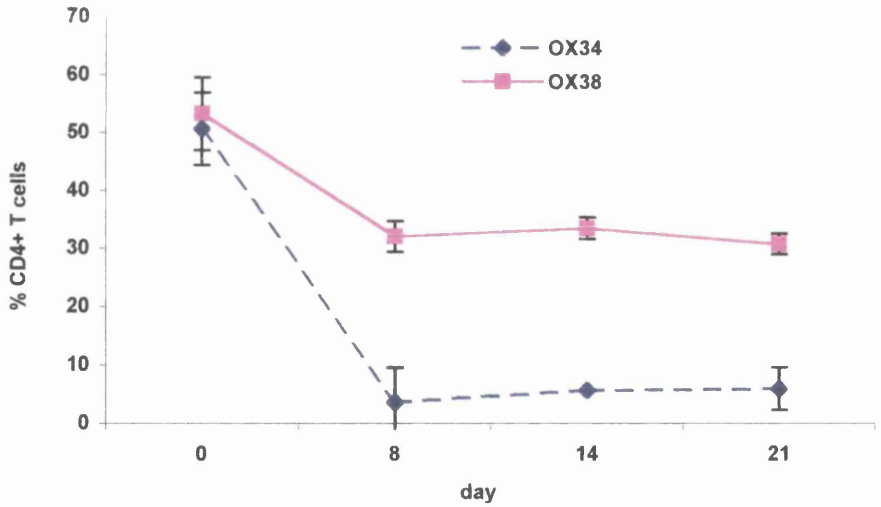

Figure 5.21 Depletion of CD4 T cells in DA rats treated with OX34 or OX38

Cells were prepared from DA rats treated with OX34 (2x5mg/kg) or OX38 (10,2,2,2mg/kg) and the percentage of CD4 T cells determined by labelling with W3/25.

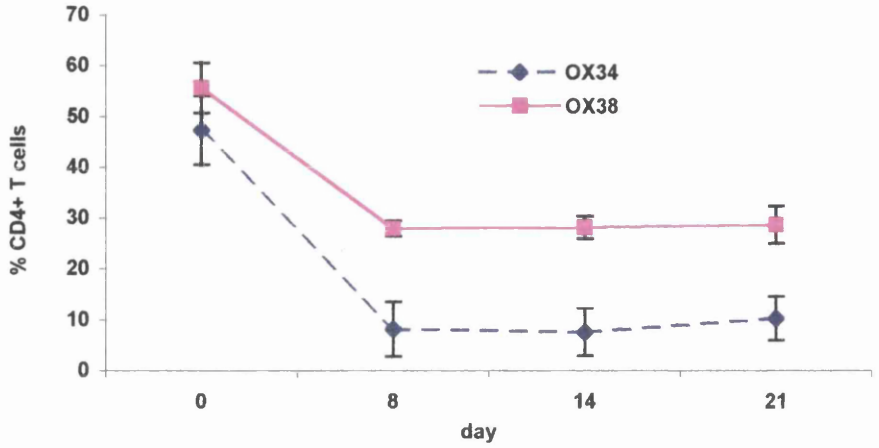

Figure 5.22 Depletion of CD4 T cells in Lewis rats treated with OX34 or OX38

Cells were prepared from Lewis rats treated with OX34 (2x5mg/kg) or OX38 (10,2,2,2mg/kg) and the percentage of CD4 T cells determined by labelling with W3/25.

Day	Lewis	DA	p
7	8.2 (5.37)	3.6 (5.81)	0.14
14	7.6 (4.66)	5.6 (4.21)	0.25
21	10.3 (4.33)	5.9 (3.66)	0.19

Table 5.6 Comparison of CD4 number in Lewis and DA rats after therapy with OX34

Residual CD4 T cell percentages (SD) in Lewis and DA strain rats (n=3) treated with OX34 (2x5mg/kg) (t test for unpaired data).

5.6.4 Effect on lymphocyte populations of intravenous $CTLA_4Ig$ therapy.

Fundamental to an examination of the mechanism of action of $CTLA_4Ig$ is an estimation of its effect on lymphocyte subset frequencies. To explore this effect $CTLA_4Ig$ was administered intravenously to two rats at a dose of 2.5mg/kg. PBLs and LNCs were prepared 24 hours later and R73, W3/25 and OX8 labelling was assessed by flow cytometry. The proportion of CD4 and CD8 T cells was identical to that in a naïve animal. $CTLA_4Ig$ therapy would be expected to influence

predominantly the B cell population, which expresses B7. PBLs and LNCs from the above two rats were stained with OX12-FITC to assay the B cell fraction which was then compared to the results from naïve DA rats. Table 5.7 shows that the B cell fraction appears to remain largely unchanged despite $CTLA_4Ig$ therapy at this time point.

OX12+ cells	Naïve (n=6)	Treated 1	Treated 2
LNC	24.55 (SD 5.34)	29.2	27.9
PBL	19.69 (SD 4.52)	15.9	19.9

Table 5.7 Estimation of B cell frequency at 24 hours in untransplanted DA rats treated with $CTLA_4Ig$ (2.5mg/kg)

5.6.5 Dose effect on saturation of the CD2 molecule after OX34 therapy

Survival data presented in Chapter 3 show that a threshold level of OX34 therapy (2x5mg/kg) is required to induce long-term allograft survival, whilst lower doses fail to prevent acute allograft rejection. Further evidence detailed in figure 5.16 shows that the same doses which lead to widely different results in transplant experiments produce very similar levels of cellular depletion. We therefore investigated the saturation of CD2 molecules using low and high dose OX34 as an alternative explanation for this dose effect. Figure 5.8 shows that LNCs taken from a rat 24 hours after administration of OX34 (2x5mg/kg) show no increased labelling with FITC-conjugated rabbit anti-mouse Ig after *in vitro* incubation with further OX34. In contrast LNCs from a rat treated with lower doses of OX34 (2.5 and 5 mg/kg) show increased labelling with OX34 after *in vitro* culture with OX34, suggesting that the lower dose did not lead to saturation of all OX34 binding sites (figure 5.9). Therefore a small number of unbound CD2 molecules appears sufficient to initiate acute allograft rejection.

5.6.6 Effect of anti-CD2 monoclonal antibody on CD2 expression

The level of CD2 antigen expression shown in figure 5.23 on cells from an OX34 treated rat is lower than that of a naïve animal (mean channel fluoresence of OX34 labelling is 8.94 and 4.28 on LNCs of naïve and OX34-treated rats respectively), demonstrating that labelling of CD2 molecules with OX34 *in vivo* leads to down-regulation (modulation) of the CD2 molecule on undepleted cells. In a similar manner the ability of OX55 to cause antigen modulation was assessed. Six rats were treated with OX55 (2x10mg/kg) and LNCs and PBLs harvested after 24 hours. These cells were labelled with FITC-conjugated rabbit anti-mouse Ig either with or without prior incubation with OX55 *in vitro* to assess saturation of CD2 molecules with OX55 mAb in addition to antigenic modulation. A very different pattern of modulation is seen with OX55 compared to OX34 (figure 5.24). Incomplete saturation of CD2 molecules is obtained at this dose of OX55. This finding is inconsistent, with two of the six rats showing complete saturation of CD2 molecules, however OX34 at a similar dose uniformly saturates CD2 molecules at this time point. Secondly intravenous OX55 induces a greater degree of antigenic modulation than OX34.

5.6.7 CD2 modulation induced by *in vitro* culture with monoclonal antibody

In order to investigate the modulation of the CD2 molecule in the absence of cellular depletion PBLs and LNCs were taken from a rat and the T cell fraction purified as described in materials and methods. Purified T cells were then suspended in culture medium at 37°C for 18 hours with mAb at 20μg/ml. Labelling with OX34 was then detected in a similar manner using FITC conjugated rabbit anti-mouse antibody. OX34 caused a more profound modulation of the CD2 molecule *in vitro*

with little labelling with mouse antibody detectable after 18 hours incubation (figure 5.23).

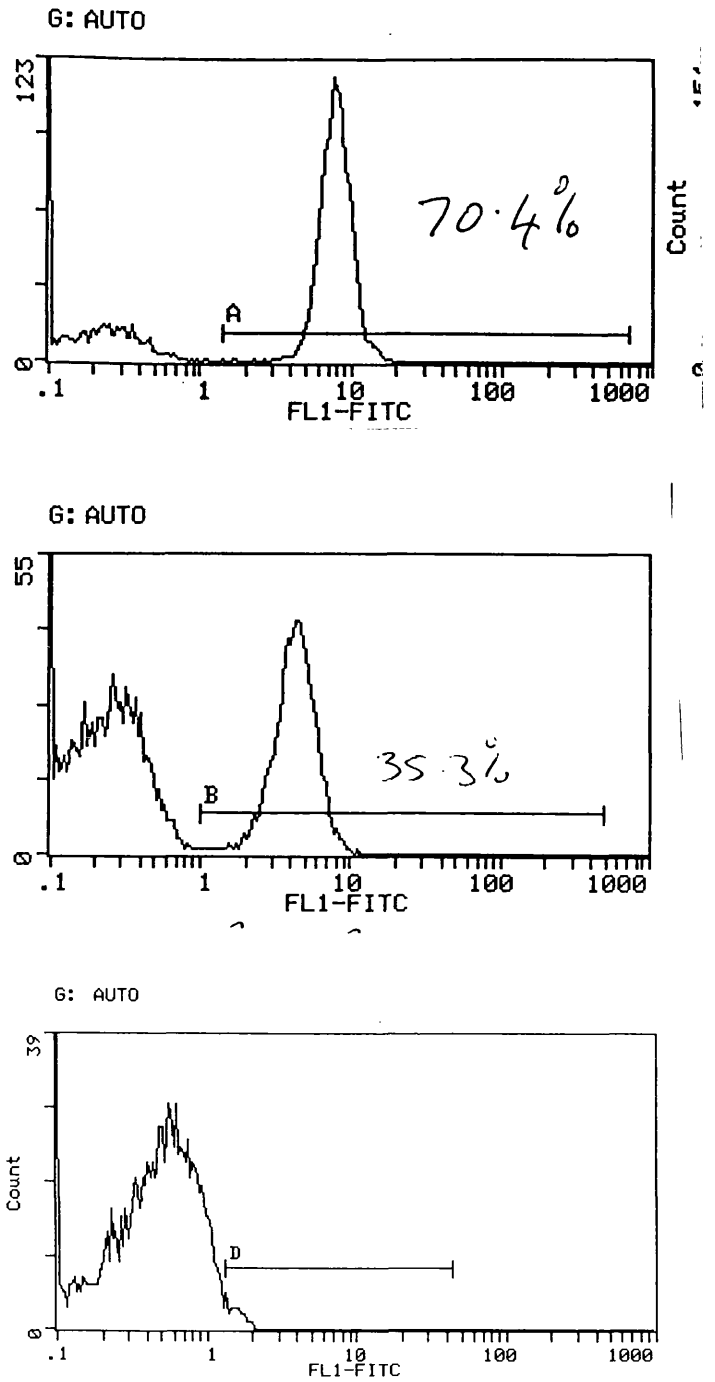

Figure 5.23 OX34 mAb-mediated modulation of the CD2 molecule

LNCs were prepared from a naïve DA rat (top), a similar rat 24 hours after intravenous injection of OX34 (10mg/kg) (middle) and LNCs from a naïve rat were incubated with OX34 (20μl) *in vitro* for 18 hours at 37°C (bottom). Naïve cells were incubated with OX34 to establish normal CD2 expression; all cells were then labelled with FITC-conjugated rabbit anti-mouse antibody.

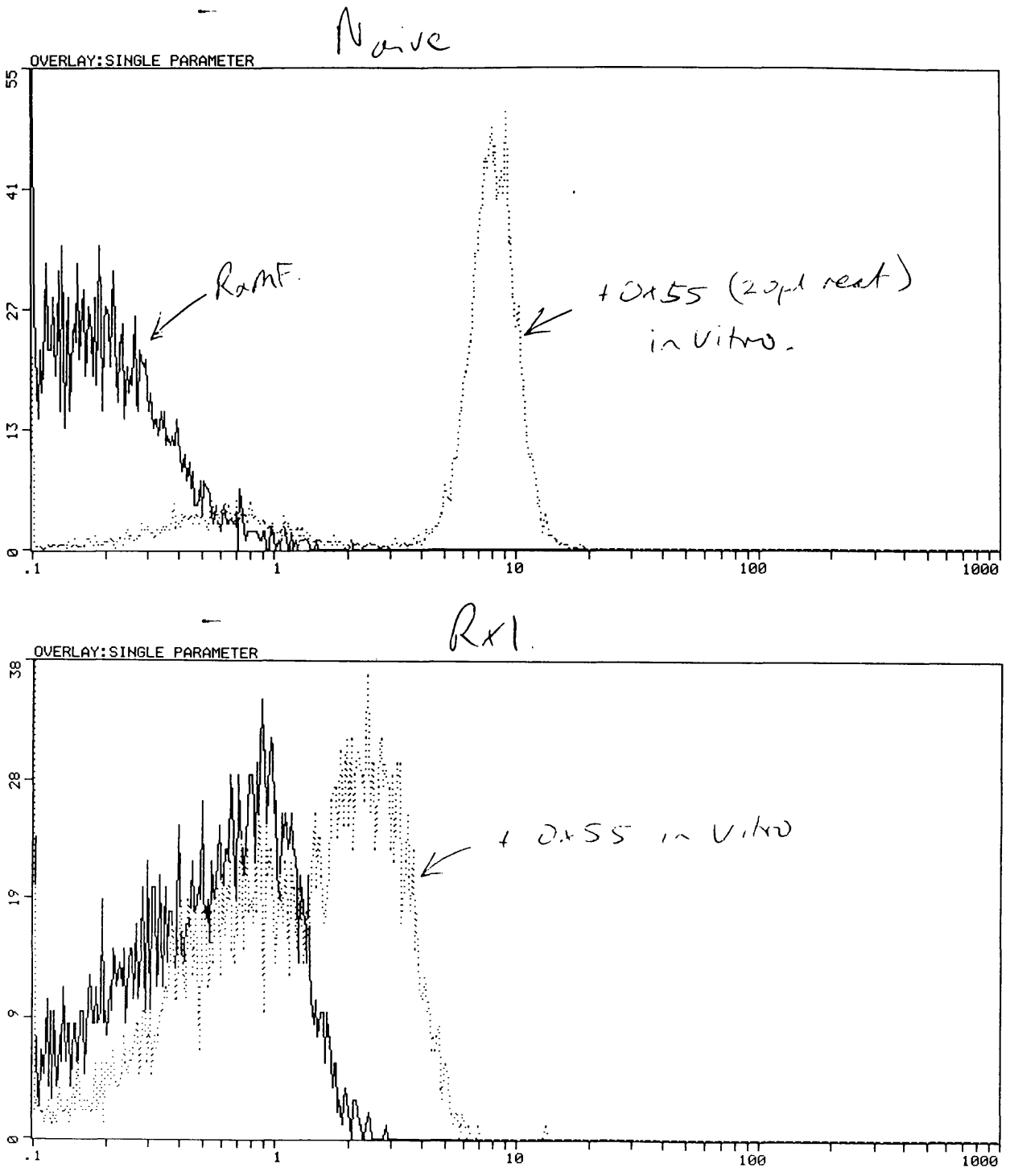

Figure 5.24 Expression of the CD2 molecule after intravenous therapy with OX55

LNCs were prepared from a naïve DA rat (top) and a similar rat 24 hours after intravenous injection of OX55 (20mg/kg) (bottom). Cells were labelled directly with FITC-conjugated rabbit anti-mouse antibody (solid line) or with OX55 followed by FITC-conjugated rabbit anti-mouse antibody (dotted line).

5.7 Effect on lymphocyte populations of combined OX34 and OX38 monoclonal antibody therapy

The combination of OX34 (2x5mg/kg) and OX38 (16mg/kg) has been shown to be more effective in prolonging allograft survival in the high responder DA to Lewis strain combination compared to either monoclonal antibody alone. The above evidence shows that both monoclonal antibodies cause T cell depletion, particularly of the CD4 subset. We therefore wished to examine the effect of combining both antibodies on lymphocyte subsets and in particular to assess the time course of CD4 and CD8 T cell depletion. A combination of the minimal dose allowing tolerance induction in the Lewis-DA combination (OX38 10,2,2,2 mg/kg and OX34 2x5mg/kg) produces permanent engraftment in 50% of transplants in the DA to Lewis combination. Five animals treated in this way were tail-bled at set time points and PBLs were separated for analysis. B cells and CD4 and CD8 T cell subsets were identified as described in materials and methods. As can be seen in figure 5.25 the antibody combination causes a profound, progressive depletion of T cells, particularly the CD4 subset, which reaches a maximum at 14 days. As noted before the frequency of CD8 cells initially shows a slight relative increase presumably due to more rapid CD4 T cell depletion. Subsequently however the CD8 T cell frequency declines, an effect not noted with either modality used as monotherapy. There is an initial relative preponderance of B cells to T cells, however the B cell element then returns toward a more normal fraction of the lymphocyte population despite continued T cell depletion. The remainder of the circulating lymphocyte population is then composed of the TCR-ve/CD4-low cell discussed below.

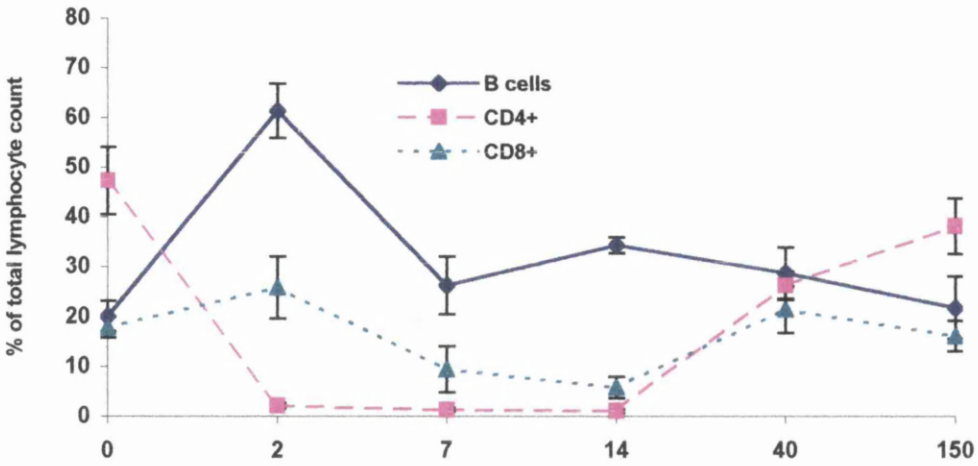

Figure 5.25 Lymphocyte depletion in Lewis rats treated with OX34 and OX38

PBLs were prepared from Lewis rats (n=5) treated with OX34 (2x5mg/kg) and OX38 (10,2,2,2mg/kg) and specific subset frquencies determined by labelling with fluorochrome-conjugated OX12, W3/25 and OX8 (figures denote day post-injection).

When compared to the depletion caused by either monoclonal antibody used alone in Lewis rats the antibody combination induces greater depletion of CD4 T cells and also appears to cause depletion of the CD8 subset. The depletion of T cell subsets in Lewis rats caused by OX34 (2x5mg/kg) and OX38 (10,2,2,2mg/kg) individually has been described above. Figures 5.26 to 5.28 compare the depletion of T cell subsets caused by these two regimes in Lewis rats when used separately or in combination at days 7 and 14 after intravenous injection in untransplanted rats.

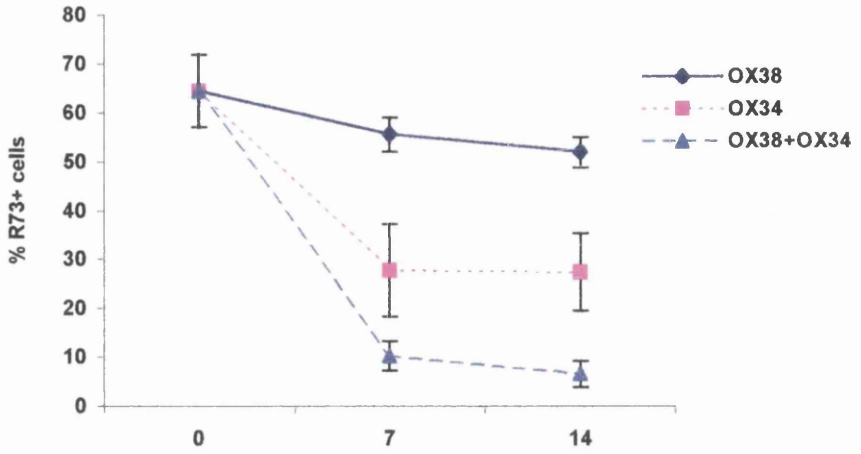

Figure 5.26 T cell depletion in Lewis rats treated with OX38, OX34 or both modalities

PBLs were prepared from Lewis rats treated with OX38 (10,2,2,2mg/kg), OX34 (2x5mg/kg) or both modalities combined and the T cell frequency determined by labelling with R73-FITC. Day 0 control represents R73 labelling of a large number of historical controls.

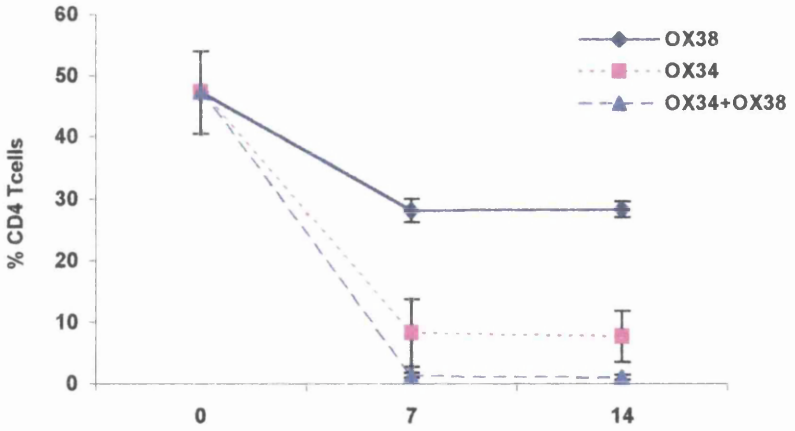

Figure 5.27 CD4 T cell depletion in Lewis rats treated with OX38, OX34 or both modalities

PBLs were prepared from Lewis rats treated with OX38 (10,2,2,2mg/kg), OX34 (2x5mg/kg) or both modalities combined and the CD4 T cell frequency determined by labelling with W3/25-PE. Day 0 control represents R73 labelling of a large number of historical controls.

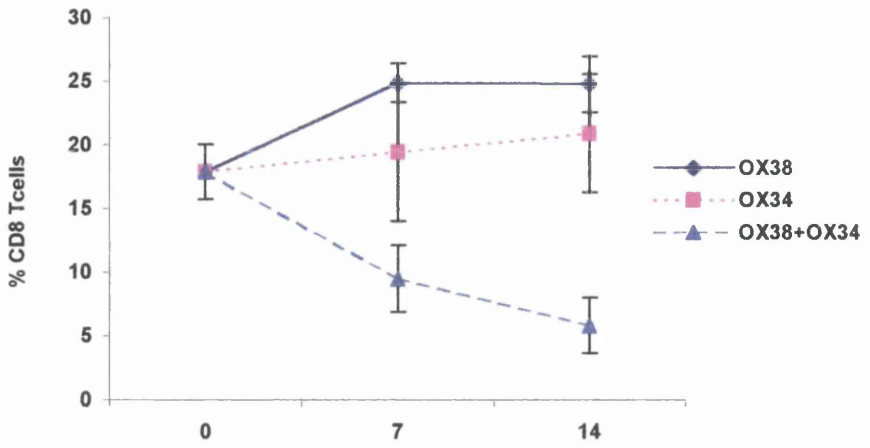

Figure 5.28 CD8 T cell population in Lewis rats treated with OX38, OX34 or both modalities

PBLs were prepared from Lewis rats treated with OX38 (10,2,2,2mg/kg), OX34 (2x5mg/kg) or both modalities combined and the CD8 T cell frequency determined by labelling with OX8-PE. Day 0 control represents R73 labelling of a large number of historical controls.

The combination of OX34 and OX38 therefore causes a profound degree of CD4 T cell depletion and also depletes CD8 T cells, an unexpected finding in view of the inability of OX34 monotherapy to deplete CD8 T cells. The degree of depletion caused by the combination of OX38 and OX34 (R73 +ve T cells are reduced to <10% of circulating mononuclear cells by day 14) is matched by a relative proliferation of other cellular elements. In contrast to DA strain rats or Lewis rats treated with either modality alone B-lymphocytes do not predominate in the remaining lymphocyte population. A population of cells is detectable in large numbers (approximately 40% of circulating mononuclear cells) during the period of T cell depletion, presumed to be monocytes, which is αβTCR-, CD4+ (low level) and expresses immunoglobulin κ-light chain at lower levels than B-lymphocytes and is not labelled by OX34 (figure 5.29). This population of cells is detectable in both DA and Lewis strain rats treated with OX34 and OX38 monotherapy but is present in

relatively fewer numbers (10-15%) than in Lewis rats treated both modalities, possibly due to the more marked T cell depletion.

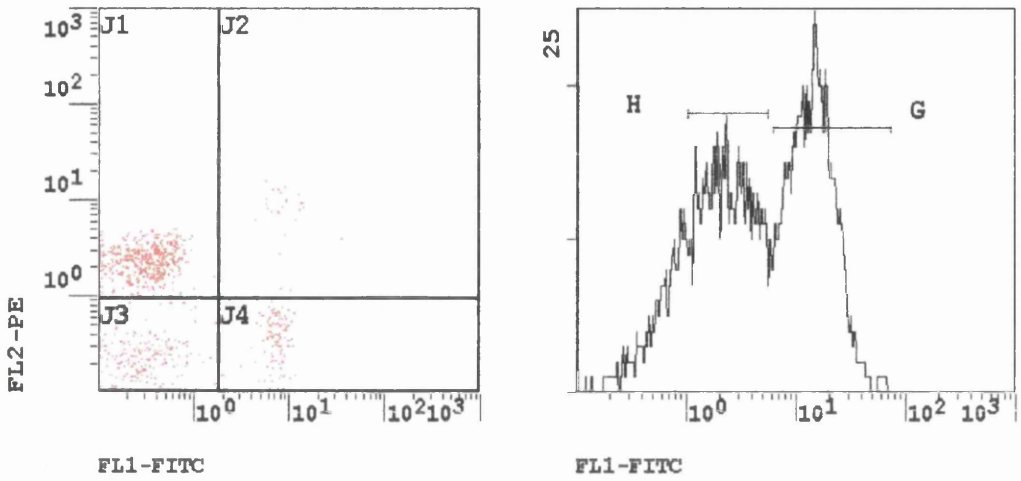

Figure 5.29 Proportion of monocytes in Lewis rats treated with OX34 and OX38

Circulating cells were prepared from Lewis rats 14 days after therapy with OX34 (2x5mg/kg) and OX38 (10,2,2,2mg/kg). Cells were labelled with a) directly-conjugated R73-FITC and W3/25-PE or b) directly-conjugated OX12-FITC (group H=35.2%, group G=42.2%).

5.7.1 Modulation of leukocyte antigens in Lewis rats by combined OX34 and OX38 monoclonal antibody therapy

It has been noted that both OX34 and OX55 are capable of causing modulation of their respective cellular ligands, and other experiments (not shown) indicate that OX38 is also capable of inducing modulation of the CD4 molecule. Combining the two treatments allows comparison of the degree and time course of antigen modulation and re-expression caused by these two antibodies. PBLs were prepared at days 7 and 14 from-tail bled rats treated with both OX38 (10,2,2,2mg/kg) and OX34 (2x5mg/kg) and from naïve Lewis rats. Cells were then incubated *in vitro* with PBS,

OX38 or OX34 at saturating concentrations. Labelling with mouse antibody was then detected with FITC conjugated rabbit anti-mouse antibody (figure 5.30). The labelling of cells incubated with PBS and rabbit anti-mouse *in vitro* represents both OX38 and OX34 bound to cells after intravenous injection.

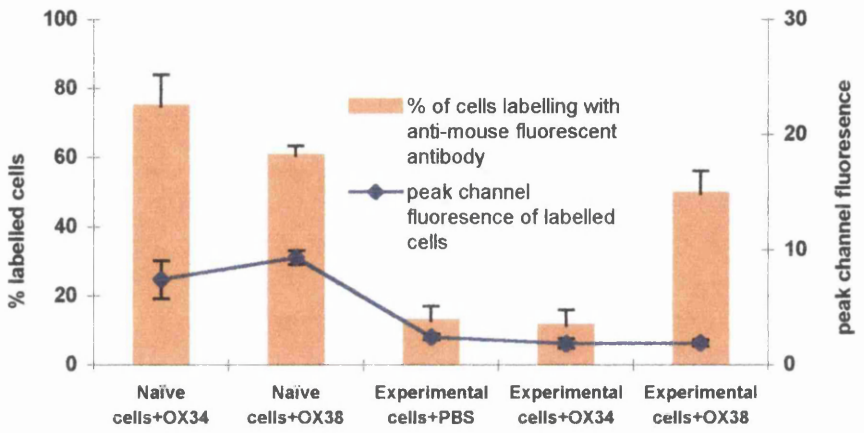

Figure 5.30 Differential re-expression of CD2 and CD4 after dual therapy with OX34 and OX38 in Lewis rats

PBLs were prepared from Lewis rats 7 days after injection with both OX34 (2x5mg/kg) and OX38 (10,2,2,2mg/kg). Cells were incubated with either OX34 or OX38 prior to secondary labelling with FITC-conjugated rabbit anti-mouse antibody.

As can be seen from figure 5.30 reincubating PBLs from a treated animal at day 7 with OX34 did not increase labelling with rabbit anti-mouse FITC, suggesting that CD2 molecules are still saturated with OX34 at this point. In contrast re-incubating with OX38 markedly increased the percentage of cells labelled with rabbit anti-mouse to approximately 45%. Clearly by day 7 CD4 molecules are no longer saturated with OX38 mAb. The combination of monoclonal antibodies therefore appears to cause saturation and modulation of CD2 and CD4 molecules, with the CD4 molecule being re-expressed by day 7 whilst CD2 is still saturated and modulated, and remains so at day 14. Curiously despite the increased proportion of PBLs taken at day 7 from a treated Lewis rat labelling with OX38 the mean peak channel fluorescence remains low after re-incubation with OX38 and rabbit anti-

mouse FITC (1.9) compared to the mean peak channel fluorescence of naïve PBLs incubated with OX38 and rabbit anti-mouse FITC (9.34), suggesting that this cell population expresses CD4 at a low level. The large number of cells labelled with OX38 (45%) at a time point when T cells are still profoundly depleted (<10%) suggests that this increased labelling on *in vitro* culturing with OX38 may be due to the TCR–ve, CD4-low cell (monocytes) described above.

5.8 Effect on lymphocyte populations of combined $CTLA_4Ig$ and OX34 monoclonal antibody therapy

This combination of treatment modalities was also effective in inducing permanent allograft survival in the high responder DA to Lewis strain combination. In a similar manner to above the depleting effect of this treatment regime was assessed. Six Lewis rats receiving DA transplants were treated intravenously with OX34 (2x10mg/kg) pre-operatively and $CTLA_4Ig$ (2.5mg/kg) two days post-operatively. Animals were tail-bled at set time points and lymphocyte subsets assayed in the standard manner. Of this cohort of six rats five permanently accepted their allografts whilst one rat rejected its graft at day 13. An analysis of total circulating leukocyte count reveals that permanent engraftment is associated with prolonged leukocyte depletion whereas graft rejection is associated with leukocyte proliferation (figure 5.31).

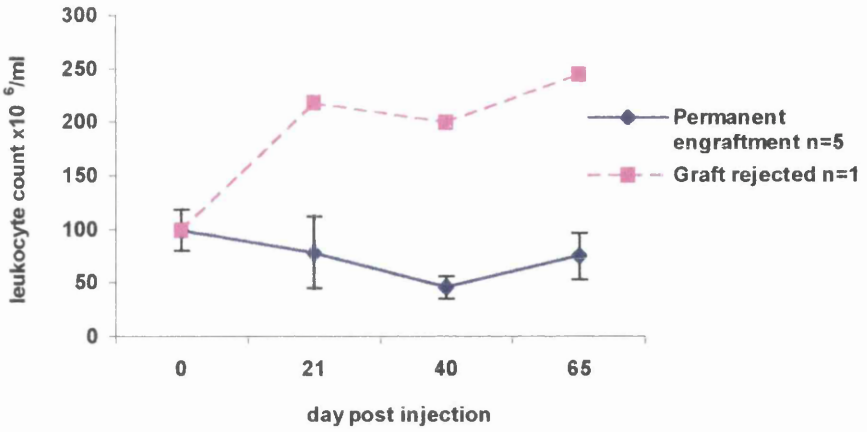

Figure 5.31 Total circulating leukocyte count in Lewis rats treated with OX34 and $CTLA_4Ig$.

Whole blood was removed from transplanted Lewis rats after therapy with OX34 (2x10mg/kg) and $CTLA_4Ig$ (2.5mg/kg). Total leukocyte count was determined by labelling with Turks solution.

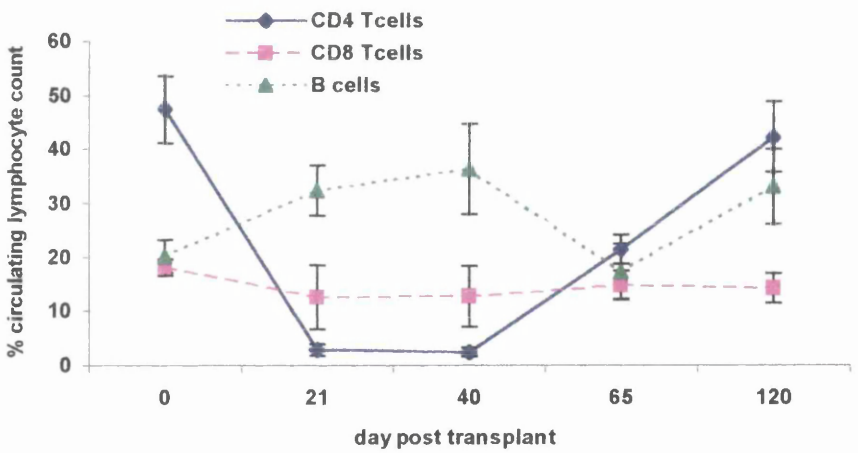

Figure 5.32 Lymphocyte subset frequencies in transplanted Lewis rats (n=5) treated with OX34 and $CTLA_4Ig$

Lymphocyte subset frequencies were determined in PBLs of transplanted Lewis rats (n=5) treated with OX34 (2x10mg/kg) and $CTLA_4Ig$ (2.5mg/kg) at various time points after therapy. Frequencies were determined by labelling with directly conjugated W3/25, OX8 and OX12.

An analysis of lymphocyte subsets in rats that accepted their allografts shows that in a similar manner to the other regimes explored above the combination of

OX34 (2x10mg/kg) and $CTLA_4Ig$ (2.5mg/kg) causes a predominant depletion of CD4 T cells and a relative dominance of the B cell subset (figure 5.31).

5.8.1 Comparative CD4 T cell depletion in Lewis rats after OX34-based regimes

It is notable in these experiments that CD4 T cells are profoundly depleted (<5% of circulating lymphocytes), to a greater degree than obtained by OX34 as monotherapy. This greater depletion may be accounted for by the higher dose of OX34 used (2x10mg/kg compared to 2x5mg/kg). This is unlikely however because as demonstrated above intravenous OX34 at a dose of 2x5mg/kg induces ligand saturation with unbound monoclonal antibody still circulating in the serum at day seven. The increased depletion must therefore be a consequence of $CTLA_4Ig$ therapy, although levels are not as low as those obtained by combining OX34 with OX38. Depletion also appears to be more long lasting with the OX34 and $CTLA_4Ig$ combination, which may be a consequence of abrogation of the anti-globulin response as outlined in Section 5.5.4.

Day post-injection	OX34 (2x5mg/kg) n=3	OX34(2x5mg/kg)+ OX38(10,2,2,2mg/kg) n=5	OX34(2x10mg/kg)+ $CTLA_4Ig$(2.5mg/kg) n=5
1		2.15 (0.5)	
7	8.2 (5.37)	1.34 (0.42)	
14	7.6 (4.67)	1.02 (0.33)	
21	10.33 (4.3)		2.9 (1.11)
40		26.23 (2.85)	2.38 (0.77)
65			21.36 (2.70)

Table 5.8 Comparison of CD4 T cell depletion by OX34-based regimes

Residual percentage of CD4 T cells in Lewis rats after treatment with monoclonal antibody regimes (SD).

In this treatment regime the CD2 molecule remains partially modulated from the cell surface for the duration of cellular labelling with OX34 (>40 days), in a

similar manner to that seen when OX34 is used as monotherapy or in combination with OX38.

5.9 Conclusions and discussion of results

5.9.1 CD2 antigen expression in lymphocyte subsets

Figure 5.1 demonstrates the extent of CD2 labelling by OX34 on cells from lymphoid organs. OX34 labels LNCs and PBLs to a similar degree (there is consistently a marginally greater proportion of CD2+ cells in peripheral blood than lymph nodes). CD2 is expressed on a lower number of splenocytes due to the spleen's relatively greater number of B cells. Thymocytes are shown universally to express CD2. The broad peak of OX34 labelling by these cells reflects the increased expression of CD2 with advancing maturity of thymocytes. Figure 5.3 confirms that OX34 and OX12 mAb label mutually exclusive subsets of lymphocytes. The possible exception to this is the small 'tail' of the lymphocyte cluster amongst splenocytes which is OX34+ and OX12+. These cells may represent monocytes, which express CD2 at a low level in addition to immunoglobulin light-chain (143). Figure 5.2 shows that CD2 is expressed on all αβ TCR+ lymphocytes (labelled with R73). There is also a small cluster of CD2+ cells (highest in the spleen) which are αβ TCR-. This population represents either NK cells, γδ T cells or a combination of the two types. Human (131) and mouse NK (154) cells are known to express the CD2 molecule, whereas work in the rat has only identified CD2 on leukaemic cell lines with NK-like activity (171). Whilst murine γδT cells express CD2 (349), no data is available regarding CD2 expression on γδT cells in the rat. Amongst T cells CD2 is expressed equally by CD4+ and CD8+ cells (figure 5.4). This finding is important in

view of the preferential depletion of CD4 T cells by OX34, which cannot therefore be explained in terms of lower labelling of CD8+ cells by OX34. Figure 5.5 demonstrates that peritoneal macrophages are labelled with OX34 to a lesser degree than T lymphocytes, which may represent non-specific labelling by Fc receptors.

5.9.2 B7 antigen expression on activated, but not tolerant B cells

Figure 5.6 shows that, as expected, resting lymphocytes are not labelled with $CTLA_4Ig$. Similarly labelling of B7 is not detected in a rat undergoing acute allograft rejection at day 7, but is detectable by day 50 in a cohort of rats with late rejection of their allografts after therapy with OX34 and $CTLA_4Ig$. This late expression is not in keeping with the reported upregulation of B7 48 hours after alloactivation as discussed in the introduction (102). Cells were not tested for B7 expression at other time points during the rejection response so the normal pattern of B7 expression by B cells has not been defined. However at the time points tested (days 21, 40 and 150) no upregulation of B7 is noted in Lewis animals with surviving grafts after therapy with OX34 and $CTLA_4Ig$ or in tolerant Lewis rats after therapy with OX34 and OX38 (figure 5.7). Whilst this cannot be interpreted as a mechanism of tolerance in these animals as the full details of B7 expression have not been defined in this project the failure of upregulation of B7 on tolerant cells of transplanted animals is consistent with their failure of IL2 secretion on *in vitro* restimulation in the MLR.

5.9.3 Distribution of OX34 monoclonal antibody after intravenous administration

This section describes the pharmacokinetics of OX34 after intravenous administration and compares the mechanisms of elimination of mAb in DA and

Lewis strain rats. The kinetics of elimination of OX34 are also examined in Lewis rats treated with combination therapy.

Figure 5.8 shows that OX34 administered at 2x5mg/kg (the minimal dose that induces permanent allograft survival in DA strain rats) saturates all CD2 ligands on LNCs. Similar saturation of OX34 binding sites was obtained with splenocytes and PBLs. Thymocytes however are labelled at a lower level after OX34 therapy at this dose. This suggests the presence of a blood-thymus barrier to the passage of OX34 after intravenous administration, which either prevents or delays the passage of OX34 molecules. Figure 5.9 shows that lower doses of OX34 (2.5 and 5mg/kg) that fails to induce permanent allograft survival in DA strain rats also fails to saturate all CD2 molecules. Evidence is demonstrated in Section 5.6.1.4 that anti-CD2 mAb therapy-mediated T cell depletion at early time points after administration is not dose dependent. It is possible therefore that incomplete saturation of CD2 molecules after sub-optimal therapy with OX34 is sufficient to allow depletion of some labelled cells, whilst the remaining unbound CD2 molecules on undepleted T cells retain the functional capability to initiate graft rejection in these animals.

5.9.4 Elimination of mAb in high and low responder strain rats.

These experiments explored two hypotheses:

1) That the increased efficacy in transplant experiments of both OX34 and OX38 in DA compared to Lewis rats is related to a more prolonged duration of action of both mAb in the DA strain.
2) That OX34 mAb has a longer duration of action than OX38 after *in vivo* therapy in both strains.

Table 5.1 provides evidence in favour of both of these hypotheses. Both OX34 and OX38 are eliminated more quickly by Lewis than DA strain rats, and this

elimination is associated with the generation of an anti-globulin reaction in Lewis rats (demonstrated in figure 5.11). It is notable however that both OX34 and OX38 are eliminated from the serum of Lewis rats before the antiglobulin response is detectable. This does not preclude the involvement of neutralising anti-mouse antibody in these rats however, as the initial antibody produced will be bound to OX34 or OX38 in rat serum and therefore not detectable in this assay. An antiglobulin response was not detectable in OX34-treated DA rats despite the preponderance of the B cell fraction noted in Section 5.6.1.3 (figure 5.10). Both IgG and IgM were tested for in these experiments (except at day 21, when IgM was not tested for due to unavailability of the conjugated antibody). No IgM production was detected by either rat strain. The inability of DA strain rats to generate an anti-globulin response is interesting in view of their ability to eliminate both mAb tested in these experiments. The half-life of native IgG is in the region of 20 days; DA rats have completely eliminated both OX34 and OX38 from the circulation 21 days after their administration. This suggests that DA strain rats have an alternative means of eliminating foreign mAb; this may involve modulation and internalisation of the mAb by T cells or may be an Fc-mediated effect of macrophages, as we have demonstrated the ability of rat Fc receptors to recognise mouse Fc (although the different rates of elimination of OX34 and OX38 argue against this). In these experiments all mAb are eliminated from the circulation more rapidly than from the cell surface. This is to be expected, as mAb remaining in the circulation would be able to label CD2 molecules re-expressed on the cell surface after modulation.

Also noted in these experiments is that OX38 is eliminated more rapidly than OX34 by both Lewis and DA strains. Both mAb were administered at the optimum dose used in transplant experiments (OX34, 10mg/kg; OX38, 16mg/kg) and the time taken for their elimination is the reverse of the dose used. In Lewis rats the

elimination of mAb is associated with the earlier production of anti-OX38 IgG (day 14) than anti-OX34 IgG (day 21). These findings raise two further questions:

1) Is OX38 more immunogenic than OX34, despite sharing the same isotype?
2) How is OX38 eliminated more rapidly than OX34 in DA rats (in which an anti-globulin response is not detectable)?

Evidence presented in Section 5.7 shows that circulating monocytes are labelled by OX38 but not OX34, despite the ability of peritoneal macrophages to label with OX34. It is therefore possible that OX38 is bound by macrophages leading to preferential processing and facilitation of a B cell response. In support of this hypothesis is the marked proliferation of monocytes noted in Lewis rats treated with OX34 and OX38, in which OX38, but not OX34, is eliminated rapidly from the cell surface. Both mAb are of IgG2a isotype, so are likely to be processed by macrophages at a similar rate by Fc-mediated mechanisms. Another explanation is that OX34 induces a greater suppression of CD4-derived T cell help required for an active immune response by more profound CD4 T cell depletion. The different rate of elimination of the two mAb by DA strain rats however cannot be explained in terms of anti-globulin. In these animals therefore OX34 must persist longer than OX38 due to a greater suppression of CD4 T cell-derived help by OX34 for an active immunological process which does not involve antibody.

5.9.5 Elimination of mAb in high responder strain rats treated with OX34 and $CTLA_4Ig$

Table 5.1 shows that Lewis rats injected with OX34 normally develop an anti-globulin response to mouse protein. Coadministration of $CTLA_4Ig$ to these animals prevents the development of either an IgM or IgG response to the antibody, and leads

to its prolonged circulation (table 5.3). In this cohort of six rats, five failed to generate anti-globulin, had persistent circulation of OX34 mAb and cellular depletion and went on to permanent graft survival. These rats were able to eliminate OX34 by means other than the production of anti-globulin, although this elimination was delayed to between 40 and 65 days. As other experiments have shown that $CTLA_4Ig$ does not cause depletion of T cells (Section 5.6.4), this prolonged depletion must be due to the persistence of OX34 in the circulation. It is interesting that elimination of OX34 by Lewis rats treated in this way takes longer than in DA rats treated with OX34 alone, in both of which groups OX34 is cleared without anti-globulin. This adds weight to the theory that an alternative active process is responsible for clearing antibody in these animals, which is also sensitive to the immunosuppressive effects of OX34 and $CTLA_4Ig$.

The abrogation of the antiglobulin response to mouse mAb by $CTLA_4Ig$ is in keeping with the reported dependence of T cell-B cell interactions on functional CD28-B7 costimulation. The importance of CD28 costimulation in IL4 secretion (122-124) and the ability of blockade of CD28 by $CTLA_4Ig$ to prevent IL4 release and antibody production (116, 127, 129) has been discussed at length in the introduction.

5.9.6 Monoclonal antibody mediated cellular depletion

The following sections discuss in detail the effects on lymphocyte populations of intravenous administration of the anti-CD2 mAb OX34 and OX55, the anti-CD4 mAb OX38 and $CTLA_4Ig$ and compare these effects in DA and Lewis strain rats.

A notable effect of mAb treatment is depletion of T cells from all lymphocyte compartments. Figure 5.13 shows that the frequency of T cells amongst LNCs and PBLs of DA rats falls from approximately 70% to a minimum of 24% after 7 days. A

similar degree of depletion is noted in splenocytes, but depletion was not assessed in the thymus. The larger part of the cellular depletion appears to occur within the first 24 hours of administration; there is also however a progressive loss of T cells over the next seven days in these experiments. Experiments described in figures 5.19 and 5.20 using measurement of the total circulating leukocyte count confirms that depletion of leukocytes in the circulation begins within the first four hours after administration of antibody. The finding that there is a progressive loss of T cells after an initial sharp fall is difficult to explain. During the period of progressive lymphocyte loss there is excess mAb in the circulation and complete saturation of CD2 molecules on circulating lymphocytes. The late depletion of a small number of T cells may therefore be due to an intrinsic resistance to depletion by these cells. This is in keeping with the finding that in all of our experiments using mAb alone and in combination total depletion of target cells is never achieved. There must therefore be a small subset of cells that is resistant to depletion, although this resistance can be overcome with continued exposure. The presence of an anti-CD4 mAb resistant CD4 T cell population has been recorded previously (260, 350). An alternative explanation for the progressive loss of T cells during a period of mAb excess is that the process of cellular depletion requires engagement of the mouse mAb with host Fc receptors, and these Fc receptors are initially saturated so that further depletion requires expression of further Fc receptors. Indeed evidence is presented in this thesis that a host Fc-mediated mechanism is involved in an interaction with OX34 to deliver a negative signal to CD4 T cells in *in vitro* experiments.

The recovery of the T cell population after mAb-mediated depletion appears to be slow, in that 100 days after administration of OX34 to DA rats T cells are still depleted despite the elimination of circulating mAb by day 21. In the introduction to

this thesis evidence has been presented that in rodents lymphopoiesis is driven mainly by the thymus, rather than by expansion of peripheral cells (210). A possible explanation for prolonged depletion therefore is that, despite the presence of the blood-thymus barrier described above (which provides an incomplete barrier to the passage of antibody), mAb are able to persist for longer within the thymus and able to exert a more long lasting effect.

The reduction in the T cell fraction is matched by a relative preponderance of B cells shown in figure 5.15. The B cell fraction may have increased because of either:

1) A simple reduction in the T cell number, so that B cells form a larger percentage of the remaining cells
2) A combination of reduction in the T cell number with proliferation of B cells.

Figures 5.19 and 5.20 describe the findings of experiments where the total circulating leukocyte count in rats treated with OX34 and OX38 was compared to the subset frequencies measured by flow cytometry. The dramatic fall in the T cell number is accompanied by a smaller fall in circulating leukocyte count, suggesting that other components of the leukocyte series are proliferating as T cells are depleted. In addition figure 5.15 shows that the non-B, non-T cell fraction remains constant after OX34 therapy, so that the larger fraction of B cells is unlikely to be explained by a simple reduction in the number of T cells. However it is not possible to conclude categorically that the B cell fraction proliferates after OX34 therapy as absolute numbers of lymphocyte subsets have not been measured.

5.9.7 Differential depletion of T cell subsets by anti-CD2 mAb

In Section 5.3 it was demonstrated that the CD2 molecule is expressed equally on CD4 and CD8 T cells. If depletion of OX34 labelled cells is exclusively an Fc-

mediated phenomenon then depletion of these two subsets of cells could be expected to be equal. However figure 5.14 demonstrates that OX34 preferentially depletes the CD4 subset, so that after therapy there are fewer CD4 than CD8 T cells. In a DA rat treated with OX34 the frequency of CD4 T cells falls from 54% and 53% to 14% and 5% in LNCs and PBLs respectively 12 days after administration. During the same period the frequency of CD8 cells changes from 14% and 16% in a naïve animal to 15% and 12% in a treated animal in the same compartments. The CD8 compartment therefore appears to remain largely unchanged by OX34 therapy. However because of the fall in numbers of CD4 T cells it is impossible from this data to conclude that OX34 has no effect on CD8 T cells, but its effect is far less than on CD4 T cells. Factors within the CD2-expressing cell therefore appear to be crucial in the mechanism of mAb-mediated depletion.

In contrast to this figure 5.14 provides evidence that factors outside the cell also influence mAb-mediated depletion. Figure 5.13 shows a similar level of total T cell depletion in lymph nodes and peripheral blood whilst figure 5.11 shows that the preferential depletion of CD4 T cells is more marked amongst PBLs (the CD4: CD8 ratio falls from 3.5 to 0.9 and 0.4 in LNCs and PBLs respectively). As we have demonstrated that LNCs are saturated with mAb 24 hours after administration of intravenous OX34, this disparity in depleting effect cannot be explained by the greater access of PBLs to mAb in the serum. Furthermore lymphocytes continually circulate between different lymphocyte compartments, so that a dynamic equilibrium is established between CD4 and CD8 T cells moving freely between compartments and the continued depletion of T cell subsets at different rates within those two compartments. The difference in degree of CD4 depletion between the two compartments may therefore be due to the greater access of circulating PBLs to effector mechanisms (for example in the spleen or liver). This finding provides

evidence that factors outside the cell are also important in the mechanism of T cell depletion. A possible resolution of the apparently conflicting evidence of these two graphs is that T cells are depleted by Fc-mediated mechanisms that are specific for the CD4 T cell.

5.9.8 Dose effect in mAb-mediated depletion

Figure 5.16 demonstrates that over the dose range tested relative depletion of lymphocyte subsets is equal after 24 hours. The lower doses tested (2.5 and 5mg/kg) fail to saturate all CD2 binding sites and also do not lead to permanent allograft survival when given to transplanted rats. A number of conclusions can be drawn from this:

1) Saturation of all binding sites is not necessary for mAb-mediated depletion
2) The initial level of cellular depletion is not a crucial determinant of long-term allograft survival.

Whilst the initial level of cellular depletion may not be a determinant of prolonged graft survival it remains plausible that the duration of depletion is important. The evidence of prolonged depletion caused by mAb combinations supports this theory. According to this model of tolerance induction therefore the size of the initial dose is important not for its initial effect on lymphocyte numbers but because it allows longer circulation of mAb and longer cellular depletion. This then allows the development of a tolerant regime in the rat, and the period of cellular depletion required for tolerance induction is longer in Lewis than DA strain rats.

5.9.9 Comparison of depleting effects of OX34 and OX55

Evidence has been presented that OX55 produces a similar total level of T cell depletion as OX34. In the data shown in figure 5.18 total T cell count falls from

approximately 70% of PBLs in a naïve animal to 39% 24 hours after administration of OX34. In a similar time span in animals treated with OX55 the total T cell count falls to 34%. Despite this similarity in total depletion OX55 does not preferentially deplete CD4 T cells in the same manner as OX34 (the CD4:CD8 ratio in OX55 treated rats is 3.25, similar to that of a naïve animal). There is therefore much stronger evidence that OX55 is able to deplete CD8 T cells than OX34 (the frequency of CD8 T cells falls from 16% to 8% in OX55 treated rats). The difference in effect between OX34 and OX55 may be explained by the following possibilities:

1) The different isotypes of the two mAb may direct different effector functions in lymphocyte depletion
2) The different epitopes on the CD2 molecule bound by the mAb may dictate the response of the lymphocyte to mAb binding (OX34 labels the CD48-binding domain of CD2 whereas OX55 labels an epitope at the base of domain 1 (135))
3) Differences in binding kinetics of the two mAb may lead to a different response to binding by the lymphocyte as it has been shown that 10-fold higher concentrations of OX55 than OX34 are required to saturate CD2-binding sites, suggesting that OX55 has lower affinity for the CD2 molecule (298).

No evidence is presented in this thesis in support of any mechanism to distinguish between the depleting effects of OX34 and OX55. However there is a common thread of indirect evidence in all the data presented in this thesis that an Fc-mediated dominant negative signal is involved in the effect of OX34, and the gateway of this signal appears to be the ligand-binding domain of the CD2 molecule expressed on a CD4 T cell.

5.9.10 Comparison of the depleting effects of OX34 and OX38 in DA and Lewis rats

The potent effect of OX34 in depleting CD4 T cells is seen when its effect is compared directly with the depleting anti-CD4 mAb OX38. Figures 5.21 and 5.22 show that in both Lewis and DA strain rats OX34 produces a greater degree of depletion than OX38. In addition the trend is for both mAb to produce greater depletion in DA than Lewis strain rats, although the difference is small and does not reach statistical significance. It is unlikely therefore that a difference in mAb-mediated depletion is sufficient explanation for the difference in allograft survival in the two strains.

5.9.11 Assessment of the depleting effect of $CTLA_4Ig$

In comparison to the gross effects that anti-CD2 and anti-CD4 mAb have on lymphocyte populations $CTLA_4Ig$ has no detectable impact in these assays. This is to be expected as earlier studies have shown only low-level labelling of activated B cells by $CTLA_4Ig$, so lymphocyte depletion will not occur in a naïve rat. Table 5.7 shows that the B cell fraction is unaltered by $CTLA_4Ig$ therapy. The possibility remains however that activated B cells or APC are adversely affected by labelling with $CTLA_4Ig$, although this phenomenon has not been explored in this thesis.

5.9.12 The effect of anti-CD2 mAb on NK cells

The effect of OX34 therapy on NK cell numbers and function in these experiments can only be inferred indirectly due to the absence of a specific NK cell marker in the rat. The size of the NK cell subset can be estimated by measuring the non-B, non-T cell fraction, which contains both NK cells and γδT cells. In a naïve rat

this subset forms approximately 10% of PBLs (R73-/OX12- cells, see figure 5.15) and also forms a distinct subset demonstrable by flow cytometry as the cluster of cells which is OX34+ but R73-. In the FACS image displayed in Section 5.3 the number of cells labelled with OX34 after *in vitro* culture is therefore 5% greater than the total number of αβT cells labelled with R73. Four findings are described in the text from which the fate of NK cells and γδT cells after OX34 therapy can be deduced:

1) The size of the non-B, non-T cell compartment remains constant despite OX34 therapy (figure 5.15)
2) The total number of residual cells after OX34 therapy which are labelled with OX34 equals the number of cells labelled with R73 (Table 5.4)
3) There is no reduction in NK cell activity after OX34 therapy (figure 5.17)
4) There is a small population of cells in a naïve rat which is OX34+ but R73- (figure 5.2).

These findings lead directly to a number of conclusions:

1) The total number of NK cells plus γδT cells remains approximately constant,
2) In an OX34-treated animal the only cells that remain labelled with OX34 are αβT cells (ie any other cellular compartment which expresses CD2 must either be completely depleted by OX34 therapy or not label with OX34),
3) NK cells are not functionally depleted.

The only explanation that accounts for this series of findings is that NK cells are not labelled by OX34 and therefore remain undepleted and that γδT cells are completely depleted by OX34 therapy and are therefore not detectable. The evidence that rat NK cells express the CD2 molecule is based on assays of leukaemic cell

cultures (171), these findings suggest however that rat NK cells are not labelled with OX34.

5.9.13 Anti-CD2 monoclonal antibody mediated ligand saturation

The data in this section demonstrate that OX34 administered at a dose which induces permanent allograft survival in DA rats also saturates CD2 ligands immediately after intravenous injection. In contrast a lower dose (2.5mg/kg) which does not prevent acute allograft rejection also fails to saturate all OX34 binding sites after intravenous injection. This therefore leaves unbound CD2 molecules which appear to retain the functional capability to participate in the generation of the allograft response. This is an important finding in view of the equal levels of T cell depletion obtained by high and low dose OX34 therapy, and suggests two distinct roles for the CD2 molecule. Evidence has been presented that the CD2 molecule can provide a pathway for delivery of a 'negative signal' to CD4 T cells resulting in cell depletion. The findings reported in this section show that undepleted T cells after OX34 therapy are unable to generate a rejection response if their CD2 molecules remain saturated with OX34 whereas a similar number of undepleted T cells retaining unbound CD2 molecules after lower dose therapy are able to participate in the generation of immunological rejection. This suggests a role for the CD2 molecule in the allo-immune response distinct from its role as a mediator of negative signals. These findings demonstrate a plurality of functions for the CD2 molecule, with the dominant effect being determined by the conditions of ligand engagement.

The inability of OX55 to induce saturation of CD2 molecules at the high dose of 2x10mg/kg may be explained by the requirement for ten-fold greater concentration of OX55 than OX34 to induce ligand saturation (298).

5.9.14 Effect of OX34 monoclonal antibody on CD2 expression

The data demonstrated in figure 5.23 show that OX34 saturation of CD2 molecules leads to antigenic modulation of undepleted cells. This modulation of the CD2 molecule may explain the inability of these remaining cells to mount an immune response. It is noteable however that OX55 induces almost total modulation of the CD2 molecule, despite being a less potent antibody in terms of cellular depletion and tolerance induction than OX34 (Sido et al noted a comparable degree of modulation by OX34 and OX55, which was however more prolonged for OX34 (303)). This suggests that both mechanisms may be important aspects of anti-CD2 mAb-mediated immunosuppression. We have demonstrated that cellular depletion alone is insufficient to prevent acute allograft rejection when a low dose of OX34 is used, and evidence has been presented here that OX55 can induce near total modulation of the CD2 molecule yet is an inefficient treatment in transplant models, inducing permanent allograft survival in only two of six transplanted rats. A hypothesis which encompasses both of these mechanisms is that a degree of cellular depletion is required to prevent allograft rejection and the undepleted cells are in some way disabled by saturation with anti-CD2 mAb and by modulation of the CD2 molecule.

Figure 5.23 reveals that in contrast to expected results CD2 modulation by OX34 is more marked after *in vitro* incubation than *in vivo* administration. This is unexpected as published work has shown antigen modulation to be an Fc-mediated event which requires the presence of monocytes (216-218), which are abundant in *in vivo* systems, and this finding contradicts published work showing equal *in vitro* and *in vivo* CD2 modulation by OX34 (303). This provides a further confounding observation in attempts to explain the depleting effect of mAb. We have shown that:

1) Fc-dependent processes are probably involved in *in vivo* cellular depletion and that rat NK cells (and therefore macrophages) can recognise mouse Fc
2) Fc-dependent ligand modulation occurs normally in *in vitro* assays.

Despite these findings lymphocytes can be cultured in *in vitro* assays with OX34 mAb and suffer Fc-dependent modulation of CD2 yet appear to survive and proliferate as normal and secrete cytokines when stimulated by mitogen. Two theories may be invoked to explain this paradox:

1) The unexpectedly profound degree of CD2 modulation induced by OX34 in *in vitro* cultures may ironically prevent delivery of a negative signal via the CD2 molecule and therefore allow survival of the cells in culture.
2) There may be a feature of the Fc-mediated signal which is specific to an intact animal to explain the apparent health of OX34-treated lymphocytes in culture. The crucial step may involve contact between antibody-coated lymphocytes and fixed-tissue macrophages in the liver and spleen.

Furthermore these results demonstrate that lymphocytes can be activated *in vitro* by allogeneic cells despite complete modulation of the CD2 molecule. This result also contradicts the *in vivo* finding that unbound CD2 molecules provide a crucial accessory function in allosensitisation to a solid organ allograft, as saturating doses of OX34 prevent acute graft rejection. Allosensitisation differs in these two systems however as T cell numbers are greatly reduced in an OX34-treated rat.

5.9.15 Effect of combined OX34 and OX38 therapy on lymphocyte populations

The survival data described in Chapter 3 have shown this mAb combination to be very effective at inducing transplant tolerance in the high responder Lewis strain. Attempts were therefore made to define the effects of combined therapy on lymphocyte populations. The mAb combination produces a very profound degree of CD4 T cell depletion with the CD4 level reduced to 1% of the lymphocyte population by day 14. In a similar manner to OX34 when used alone the depletion of CD4 cells is progressive, increasing with time. Our earlier observation that mAb-mediated depletion is not dose dependent suggests that the effect of combined OX34 and OX38 therapy is synergistic at the level of T cell depletion. It has been shown in our work that OX34 alone does not deplete all CD4 T cells and similarly reports using OX38 in the rat do not achieve 100% reduction of all T cells (234). Both anti-CD4 (211) and anti-CD2 mAb (172, 174-178) are capable of transmitting negative signals to T cells. Synergistic T cell lysis in this model may be explained by undepleted T cells becoming susceptible to cell death on receiving additional negative signals. The mAb combination also has unexpected effects on other lymphocyte subsets. The CD8 population also appears to be depleted by the mAb combination. This is unexpected as neither modality used alone has any detectable effect on CD8 cells. This effect may be spurious and reflect proliferation of other cellular elements within the circulation. This combination of mAb induces proliferation of a cellular subset not identified in large numbers in other transplant experiments. These cells are characterised by the low expression of CD4 and κ-light chain typical of monocytes. The proliferation of monocytes in these animals may be a response to the profound CD4 T cell depletion, and their presence does not compromise the function of the allograft. It seems likely that the presence of these

cells in such large numbers (40% of circulating lymphocytes) accounts for the apparent decline in the numbers of CD8 T cells.

5.9.16 Effect of combined OX34 and OX38 on lymphocyte antigen expression

It has been noted that OX34 and OX38 are able to induce saturation and modulation of the CD2 and CD4 molecules after intravenous administration. Combining the two modalities in a single animal allows comparison of the kinetics of modulation of both molecules. As can be seen in figure 5.30, seven days after administering the combined therapy, all OX34 binding sites on OX34-treated rats remain saturated, and the peak channel fluorescence remains low in keeping with the modulation of the CD2 molecule. In contrast reincubation of these cells with OX38 leads to further labelling of CD4 molecules, so that 50% of the cell population are labelled with FITC-conjugated anti-mouse antibody, compared to <10% of cells taken directly from a treated animal (where T cells are depleted to <10% of circulating lymphocytes). Furthermore the peak channel fluorescence of these labelled cells is much lower than that of naïve cells labelled with OX38. These two findings suggest that the re-expressed OX38 binding sites seven days after OX38 therapy are not on T cells, but are more likely to be on cells of the monocyte-macrophage series. This rapid processing of OX38 by monocytes may account for the enhanced ability of Lewis rats to generate anti-globulins to OX38 compared to OX34.

5.9.17 Effect of combined OX34 and $CTLA_4Ig$ on lymphocyte populations and antigen expression

This section describes a number of findings. Firstly the combination of mAb produces both more profound and more long-lasting depletion of CD4 T cells than OX34 when used alone. The more prolonged depletion has been accounted for by the abrogation of the anti-globulin response described in Section 5.5.4. More difficult to explain is the more profound depletion of CD4 T cells seen with this combination, as $CTLA_4Ig$ does not cause cellular depletion (see Section 5.6.4). A possible explanation for the synergistic depletion caused by OX34 and OX38 is that a combination of two mAb in close proximity to the T cell receptor, both of which are capable of causing depletion, leads to increased cell death. This phenomenon has been described before in the context of anti-CD4 mAb (206) and is discussed in the introduction. In contrast $CTLA_4Ig$ has been shown not to cause T cell depletion, so that synergistic lysis cannot be adduced as an explanation for the profound depletion noted. An alternative explanation is likely to involve apoptosis. As discussed in the introduction signalling via the CD28 molecule has been shown to be able to inhibit apoptosis induced by both γ-irradiation (114) and anti-CD3 (115) mAb. Our work has demonstrated that a dominant effect of OX34 in animal systems is a CD2-mediated negative signal on CD4 T cells. The enhanced effect of combining the two therapies may therefore be explained by $CTLA_4Ig$ preventing the CD28-mediated pathway of rescue from apoptosis induced by OX34 binding to CD2.

6 Analysis of alloreactivity in transplanted animals treated with anti-CD2 monoclonal antibody

6.1 Study objective

This section outlines investigations performed to explore aspects of the immune environment in animals with long surviving allografts, in order to explore the mechanism of action of OX34-based immunosuppressive regimes.

6.1.1 Assessment of transplant tolerance.

As survival data shows anti-CD2 mAb is effective at prolonging allograft survival in the low responder strain combination. We have also devised two new strategies to induce permanent allograft survival in high responder Lewis rats by combining anti-CD2 mAb with anti-CD4 mAb and $CTLA_4Ig$. Various mechanisms may be involved in allowing permanent engraftment of tissue grafts in rodents including both graft adaptation by loss of immunogenic APC and development of true transplant tolerance. Before analysing alloreactivity in transplanted animals it is necessary therefore to demonstrate that transplanted animals have become tolerant to donor tissue by performing second donor-specific and third party allografts.

6.1.2 Analysis of mechanisms of transplant tolerance.

Having demonstrated donor specific tolerance in two of the transplant models under study (third party grafts were not successfully performed in the DA-Lewis model treated with OX34 and $CTLA_4Ig$ due to insufficient numbers of surviving

animals) we then turned to *in vitro* experiments to further examine the mechanisms of tolerance. Transplant tolerance is maintained by three principal mechanisms:

1) Clonal deletion of alloreactive cells
2) Clonal anergy
3) Immune regulation.

We explored features of the immune environment in tolerant animals and attempted to find evidence for the above mechanisms by performing the following experiments:

1) Measurement of proliferation of lymphocytes from tolerant animals on *in vitro* stimulation by splenocytes from donor strain and third-party animals.
2) Analysis of cytokine production by lymphocytes stimulated as above.
3) Analysis of antigen-specific cellular cytotoxicity.
4) Analysis of antigen-specific cytotoxic antibody production.
5) Microscopy of explanted hearts.

6.2 Study design

1) To assess the state of responsiveness of animals with permanently surviving allografts animals were retransplanted after a period exceeding one hundred days with a second allograft placed in the neck as described in materials and methods. The criterion for allograft rejection is cessation of beating as for primary grafts.

2) To assess mechanisms of alloreactivity in tolerant animals MLRs were performed in which LNCs from tolerant and naive rats were incubated with either medium alone, medium containing Con A, donor-strain splenocytes or third party PVG splenocytes. Proliferation was measured by incorporation of thymidine after 72 hours of culture by the standard method. Results from different experiments can be expressed numerically using the proliferation index (PI), which takes into account

background proliferation of cells in medium alone. A direct comparison can then be made between cells from different groups of animals analysed at different time points. Supernatants of these reactions were retained for comparative assessment of cytokine (IL2 and γIFN) production by cells from tolerant and naïve animals.

3) Direct assessment of alloreactivity in high responder Lewis rats with permanently surviving allografts was performed by assessing the production of cytotoxic T cells and cytotoxic antibody in tolerant animals. These measurements were performed in the rats treated with OX34 and OX38, the model in which donor-specific tolerance has been proved. Allo-CTL production was measured in tolerant rats more than 100 days post-transplant only, as measurements at earlier time points would involve sacrificing rats before tolerance has been established and whilst the animals are still depleted of T cells (comparison of CTL induction between T cell depleted and naïve rats would yield little useful information). Splenocytes were prepared from mAb treated, tolerant rats, unmodified rats undergoing acute allograft rejection (ten days post-transplant) and from naïve rats. Strain-specific and third party splenocyte ConA blasts were prepared as above and labelled with ^{51}Cr for use as target cells. 100μl serial dilutions of effectors (starting $2x10^7$/ml) in serum-free culture medium were incubated with 100μl target cells containing 10^4 blasts in V-bottomed plates. Incubations were for 4 hours in 5% CO_2 at 37°C. 100ml culture supernatant was then harvested for estimation of γ-emission.

4) Cytotoxic antibody activity was also measured in tolerant Lewis rats 100 days post transplant. Serum was prepared from tolerant rats, unmodified rats undergoing acute allograft rejection (seven days post-transplant) and from naïve rats by tail-bleeding and centrifugation. Serum was heat-inactivated by heating in a water bath for 30 mins at 56°C. Strain-specific and third-party target cells were prepared from splenocytes as described above. 50μl of tripling dilutions of serum in culture

medium were added to V-bottomed plates followed by 50µl of target cells containing 5 x 10^4 blasts. After incubation for 30 mins in 5% CO_2 at 37°C 100µl of reconstituted guinea-pig complement was added to each well and the plates were then incubated for a further one hour. Control wells contained 1) targets in medium alone 2) targets + 150µl Triton and 3) targets plus complement alone. Plates were then centrifuged briefly to deposit cells and 100µl of supernatant harvested for estimation of γ-emission as previously.

5) The presence of IgG and IgM antibody was also measured by a flow cytometric technique at the same time point. DA strain LNCs were used as target cells and incubated with serial dilutions of serum from tolerant animals. Production of anti-DA IgG and IgM was then detected by secondary labelling with mouse anti-rat IgG and IgM fluorescent antibody. Mean channel fluorescence of the T cell peak was measured for each sample. Serum was also taken from transplanted rats treated with OX34 and OX38 at days seven and forty post-transplant to ensure detection of earlier peaks of antibody production.

6.3 Demonstration of transplant tolerance.

Firstly DA strain rats which had been treated with OX34 prior to transplanting with Lewis strain hearts were retransplanted with donor specific Lewis hearts or third party PVG hearts. As can be seen in figure 6.1 donor strain hearts are accepted whilst third party PVG grafts are rejected, although at a slower tempo than in naïve animals.

In a similar manner Lewis rats treated with OX34 and OX38 prior to transplanting with DA strain hearts were also rechallanged with donor specific and third party grafts (third party grafts were not successfully performed in the DA-Lewis model treated with OX34 and $CTLA_4Ig$ due to insufficient number of

surviving animals). Again donor specific tolerance was demonstrated with third party grafts being rejected at a tempo closer to that expected in a naïve animal (figure 6.2).

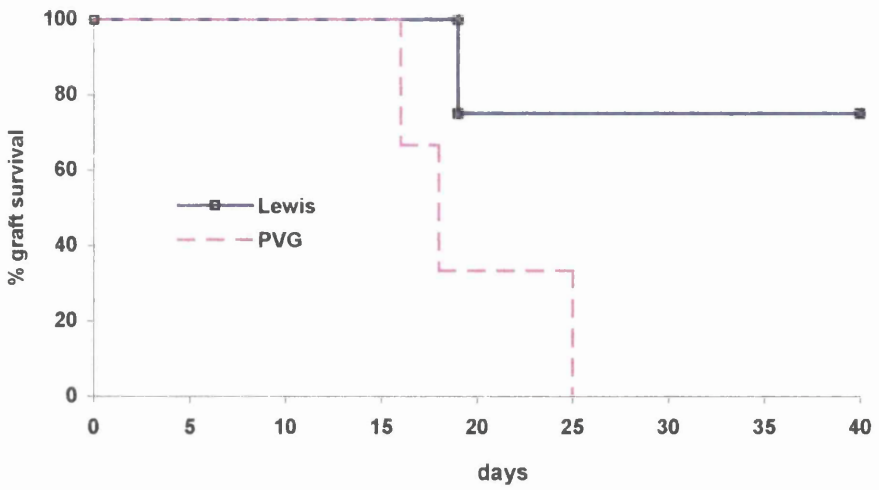

Figure 6.1 Survival of second transplants in DA strain rats treated with OX34 (2x5mg/kg) preoperatively

Animals were retransplanted with either donor-specific Lewis hearts (n=4) or third party PVG hearts (n=3). (Lewis v PVG $p<0.1$, logrank test).

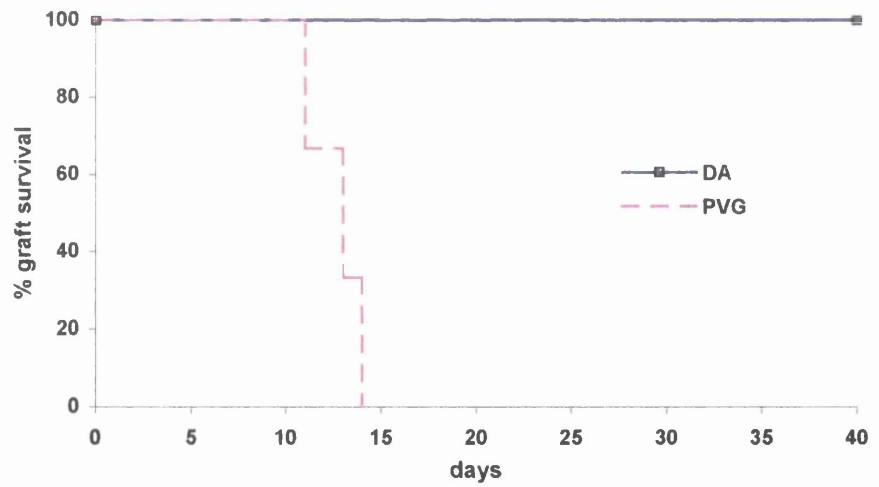

Figure 6.2 Survival of second transplants in Lewis strain rats treated with OX34 and OX38

Tolerant rats treated with OX34 (2x10mg/kg) and OX38 (10,2,2,2mg/kg) preoperatively were retransplanted with either donor-specific DA hearts (n=4) or third-party PVG hearts (n=3) (DA v PVG $p<0.05$, logrank test).

6.4 Analysis of mechanisms of transplant tolerance in DA strain rats

Having demonstrated donor specific tolerance in two of the transplant models under study we then turned to *in vitro* experiments to further examine the mechanisms of tolerance.

6.4.1 Assessment of alloreactivity of lymphocytes from DA strain rats treated with OX34 on *in vitro* stimulation.

As DA strain rats treated with OX34 (2x5mg/kg) with long-term surviving Lewis allografts failed to reject a further Lewis allograft we performed mixed lymphocyte reactions to assess if this unresponsiveness could be demonstrated *in vitro*. Two series of experiments were performed, and cells from tolerant DA strain rats were consistently able to proliferate on stimulation with donor strain cells. Figure 6.3 shows the results comparing the proliferation of five tolerant and three naïve rats in a single experiment.

6.4.2 Analysis of cytokine production in mixed lymphocyte reactions using tolerant DA strain responders

Further evidence for alloresponsive cells amongst lymphocytes from DA rats made tolerant to transplanted Lewis strain hearts by pre-operative OX34 therapy was sought by analysing cytokine production in the above mixed lymphocyte reactions. Supernatants from the first experiment were retained and the production of the cytokines IL2 and γIFN was assayed by the methods described in materials and methods. Figure 6.4 shows an analysis of IL2 and γIFN present in supernatants of an MLR using Lewis strain splenocytes as stimulators and naïve and tolerant DA strain

LNCs as responder cells. In this experiment cells from both tolerant and naïve animals proliferate less on stimulation with Lewis strain cells than with PVG strain cells, and cells from tolerant animals are more responsive to all stimuli.

These results are in agreement with the proliferation data and suggest that despite the tolerant DA strain rats inability to reject a donor strain heart, lymphocytes from these animals are still able to respond to donor antigen *in vitro*, by cellular proliferation and cytokine production.

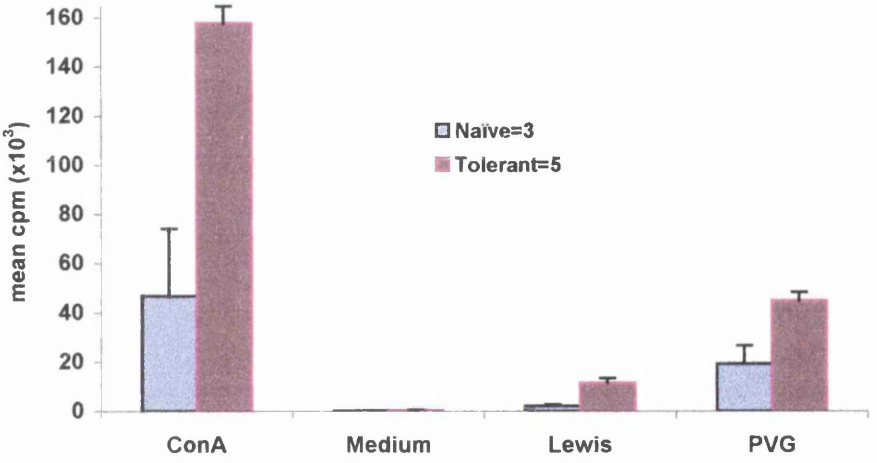

Figure 6.3 Proliferation of cells from naïve and tolerant DA rats after therapy with OX34 on *in vitro* stimulation with donor antigen

Mean thymidine incorporation from a single experiment of naive DA LNCs and DA rats transplanted with Lewis allografts after pre-operative therapy with OX34 (2x5mg/kg). Stimulator cells are donor-specific Lewis and third-party PVG strain splenocytes. Control wells contain responder cells plus ConA or responder cells alone.

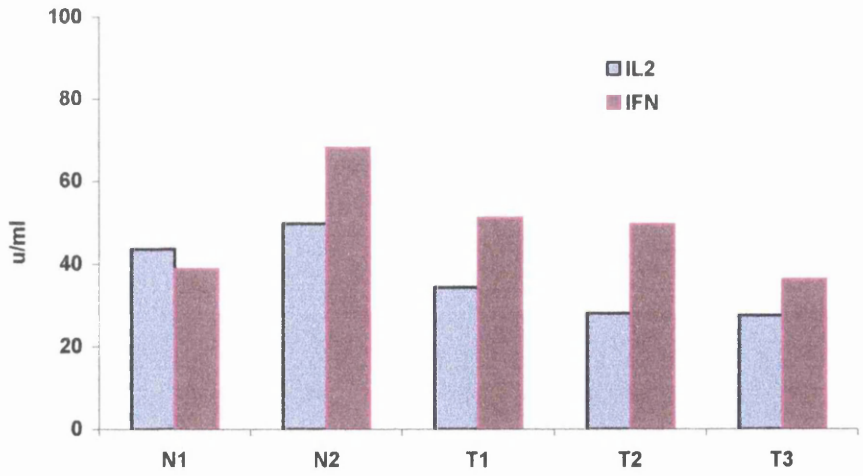

Figure 6.4 Production of Th_1 cytokines by cells from tolerant and naïve DA rats on *in vitro* stimulation with donor antigen

Analysis of IL2 and γIFN concentration in supernatant of a single MLR using LNCs from naive DA rats and DA rats transplanted with Lewis allografts after pre-operative therapy with OX34. Stimulator cells are Lewis splenocytes; differences are not significant.

6.4.3 Assessment of alloreactivity of lymphocytes from DA strain rats treated with $CTLA_4Ig$ on *in vitro* stimulation.

Long-term allograft survival of DA strain rats treated with $CTLA_4Ig$ and transplanted with Lewis strain hearts has been achieved in this project although true tolerance was not demonstrated by performing second grafts. Attempts were made however to demonstrate donor-specific hyporesponsiveness by MLR in a similar manner to DA strain rats treated with the OX34 protocol.

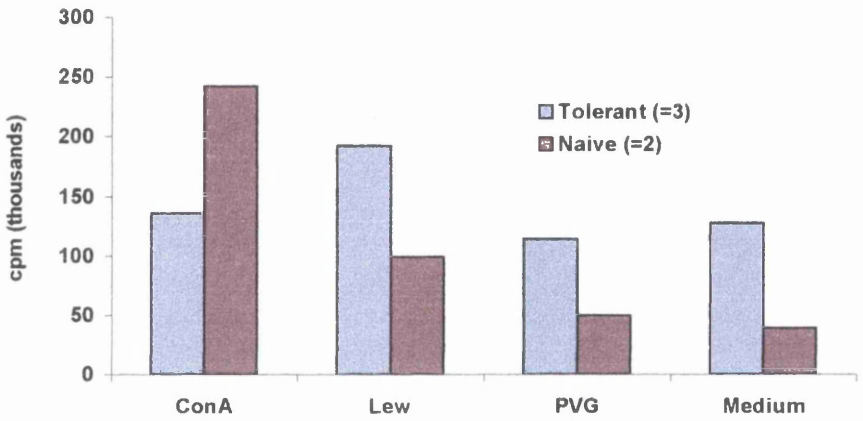

Figure 6.5 Proliferation of cells from naïve and tolerant DA rats after therapy with $CTLA_4Ig$ on *in vitro* stimulation with donor antigen

Mean thymidine incorporation from a single experiment of naive DA LNCs and LNCs from DA rats transplanted with Lewis allografts after post-operative therapy with $CTLA_4Ig$ (2.5mg/kg) Stimulator cells are Lewis and third-party PVG strain splenocytes. Control wells contain responder cells plus ConA or responder cells alone.

As can be seen in figure 6.5 donor-specific hyporesponsiveness is not demonstrated in DA rats treated with $CTLA_4Ig$. In contrast to the findings with DA rats treated with the OX34 protocol (figure 6.3) both tolerant and naïve rats respond to a greater degree to Lewis than to PVG strain stimulators.

6.4.4 Proliferative responses of tolerant Lewis rats treated with OX34 and OX38.

Of the two models of permanent engraftment developed using high responder Lewis rats transplant tolerance has been demonstrated in Lewis rats treated with OX34 and OX38 only. In a similar manner to above the ability of LNCs from tolerant rats to respond to donor and third party strain splenocytes was assessed in MLR experiments. Three sets of experiments were performed using LNCs from a total of seven tolerant Lewis rats, six naïve rats and three unmodified Lewis rats undergoing acute rejection of DA strain allografts. The proliferation indices were calculated for each group after three days of culture to allow comparisons across experiments and the results are demonstrated in figure 6.6.

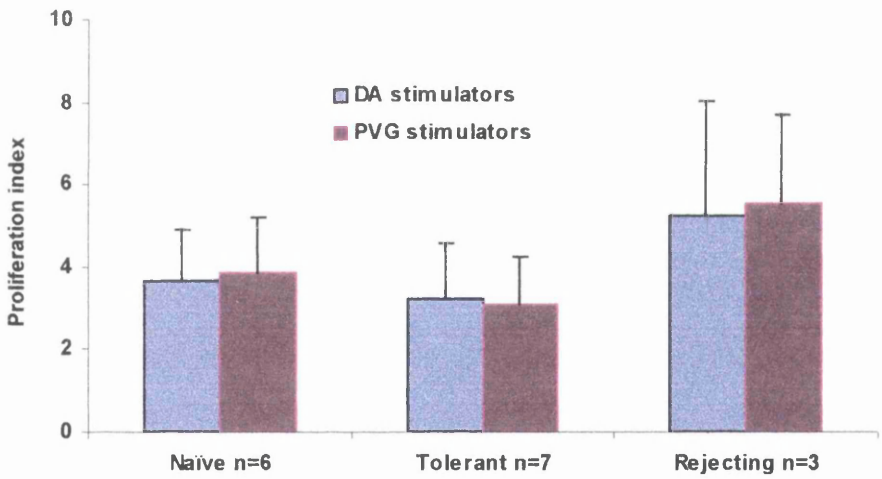

Figure 6.6 Proliferation of cells from tolerant Lewis rats after therapy with OX34 and OX38 on *in vitro* stimulation with donor antigen

Mean proliferation indices from three MLR experiments using LNCs from Lewis rats tolerant to DA allografts after therapy with OX34 (2x5mg/kg) and OX38 (16mg/kg) and naive and rejecting control Lewis rats. Proliferation Index = (specific proliferation/proliferation in medium).

These results demonstrate that lymphocytes from tolerant Lewis rats treated with OX34 and OX38 are able to proliferate normally to DA splenocytes in the

MLR. Again lymphocytes from rats undergoing acute allograft rejection demonstrate more dynamic proliferative responses to both stimuli.

6.4.5 Cytokine production by lymphocytes from tolerant Lewis rats treated with OX34 and OX38.

Supernatants from the above reactions were retained to assess the production of the cytokines IL2 and γIFN by lymphocytes from tolerant, naïve and rejecting rats. Figure 6.7 shows the concentration of IL2 in supernatants from the MLR experiments shown in figure 6.6 plus those of a further experiment (values for PI could not be calculated in this experiment as cells containing medium alone were not included). Supernatants from these four experiments were retained and used in IL2 bioassays in two groups, the means of which are demonstrated. The experiment was repeated using the same supernatants, with identical results.

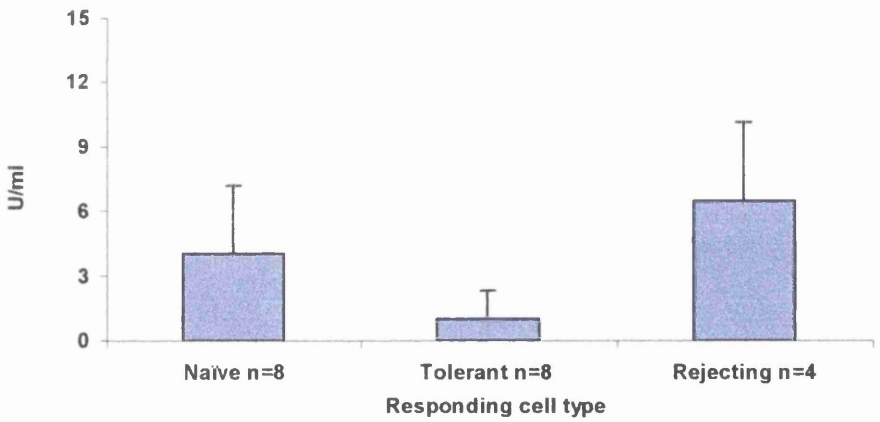

Figure 6.7 Production of IL2 by cells from tolerant Lewis rats after therapy with OX34 and OX38 on *in vitro* stimulation with donor antigen

IL2 concentration in supernatants of MLRs using cells from naïve rats, Lewis rats tolerant to DA allografts after therapy with OX34 (2x5mg/kg) and OX38 (16mg/kg) and Lewis rats rejecting a DA strain allograft. DA strain splenocytes used as stimulators. Supernatants taken from four separate experiments (tolerant v naïve p=0.015, t test for unpaired data).

This experiment demonstrates that lymphocytes from tolerant Lewis rats produce less IL2 on *in vitro* stimulation with donor-type antigen than do naïve rats, despite showing normal levels of proliferation. As demonstrated in proliferation assays cells from rats undergoing an acute rejection process show an enhanced IL2 response after *in vitro* stimulation with donor antigen. To assess if this paralysis of IL2 production by cells from transplanted animals is specific to stimulation with donor antigen, supernatants were harvested from one MLR, in which cells from tolerant Lewis rats were stimulated with donor-type DA cells and third party PVG cells, and assayed for IL2 in the same manner.

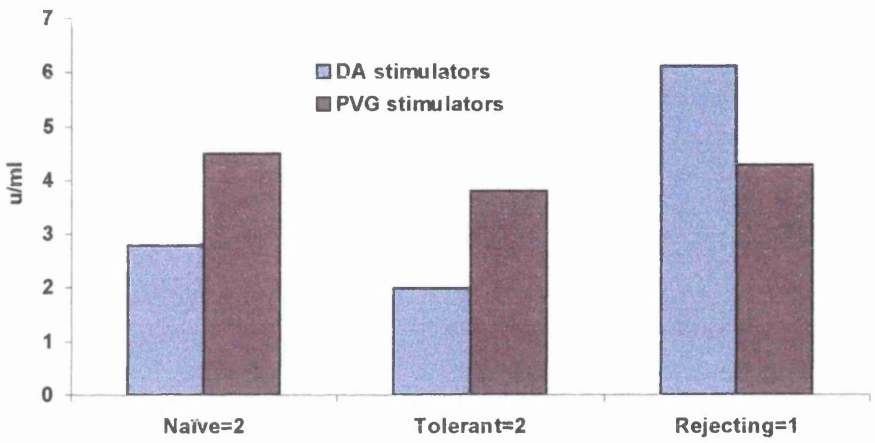

Figure 6.8 Production of IL2 by cells from tolerant Lewis rats after therapy with OX34 and OX38 on *in vitro* stimulation with donor antigen and third party antigen

Mean IL2 concentration in supernatant of single MLR using LNCs from tolerant Lewis rats after therapy with OX34 (2x5mg/kg) and OX38 (16mg/kg) on *in vitro* stimulation with donor type DA splenocytes or third party PVG splenocytes.

Figure 6.8 demonstrates that in a sample of the animals assayed in figure 6.7 IL2 production by cells from tolerant animals demonstrate diminished IL2 production on stimulation by both donor type and third party antigen.

In a similar manner the ability of cells from tolerant animals to produce γIFN on *in vitro* stimulation was measured. The reduced IL2 elaboration by cells from tolerant Lewis rats was not reproduced for γIFN, the production of which was similar

by cells from all sources measured (γIFN production was not measured for cells from animals rejecting their allografts) (figure 6.9).

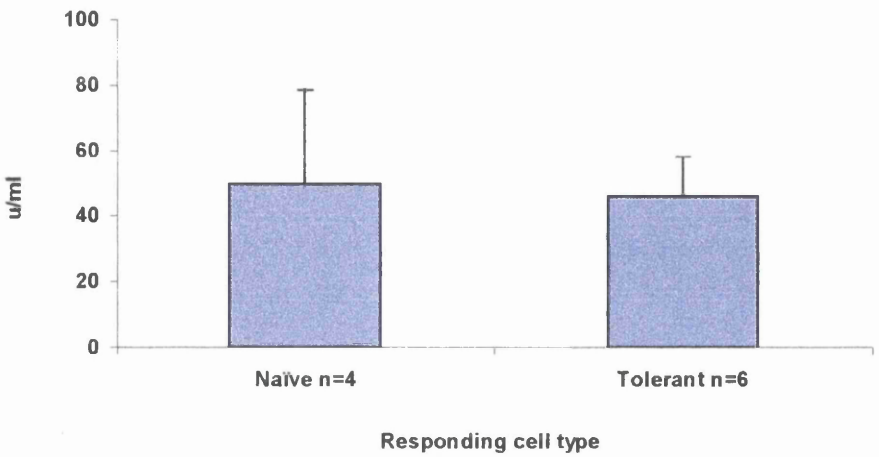

Figure 6.9 Production of γIFN by cells from tolerant Lewis rats on *in vitro* stimulation with donor antigen

Mean γIFN concentration in supernatants of two MLR experiments using LNCs from tolerant Lewis rats after therapy with OX34 (2x5mg/kg) and OX38 (16mg/kg) on *in vitro* stimulation with donor type DA splenocytes.

6.4.6 Proliferative responses of tolerant Lewis rats treated with OX34 and $CTLA_4Ig$

Long-term survival of DA allografts in Lewis rats was obtained by using a combination of OX34 and $CTLA_4Ig$ at varying doses. True transplant tolerance was not demonstrated however in this model by performing donor specific second allografts but investigations were performed on cells from rats with long surviving grafts to investigate features of the immune environment in these animals. By using standard MLR experiments the ability of LNCs from rats with long surviving allografts to respond to donor and third party strain splenocytes was assessed. Two separate experiments were performed using LNCs from a total of five engrafted Lewis rats, two naïve rats and two unmodified Lewis rats undergoing acute rejection

of DA strain allografts. The proliferation indices were calculated for each group to allow comparisons across experiments and the results are demonstrated in figure 6.10.

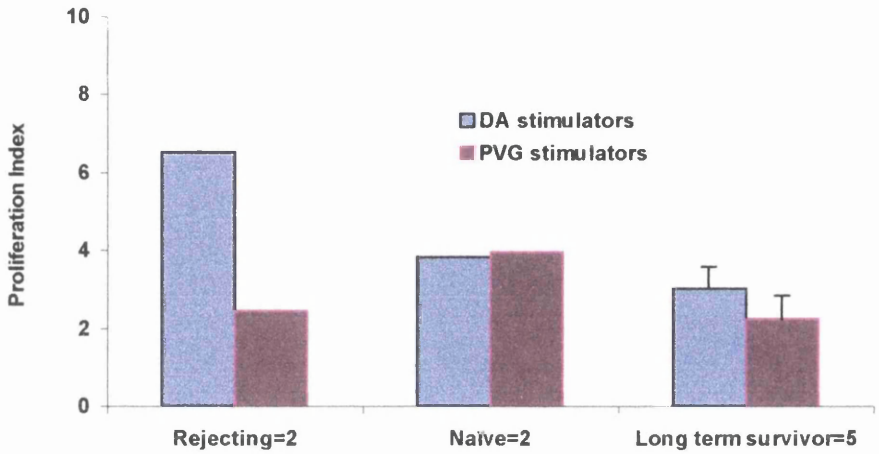

Figure 6.10 Proliferation of cells from Lewis rats with long surviving grafts after therapy with OX34 and $CTLA_4Ig$ on *in vitro* stimulation with donor antigen

Mean relative proliferation of two experiments using LNCs from naive Lewis rats, Lewis rats undergoing rejection of a DA allograft and permanently engrafted Lewis rats after treatment with OX34 (10 or 20 mg/kg) and $CTLA_4Ig$ (2.5mg/kg) on stimulation with donor-specific and third party splenocytes.

This graph demonstrates that lymphocytes from engrafted Lewis rats treated with OX34 and $CTLA_4Ig$ proliferate to a broadly similar degree as those of naïve Lewis rats after stimulation by DA splenocytes in the MLR, in a similar fashion to the other models of allograft survival assessed in this project. These results demonstrate that an alloreactive T cell repertoire persists in all experimental animals examined in this project, excluding clonal deletion as a mechanism of tolerance.

Supernatants from a single MLR in which cells from transplanted rats treated with OX34 and $CTLA_4Ig$ were stimulated with donor type splenocytes were assessed for IL2 production. Very low levels of IL2 were noted in these supernatants that did not allow discrimination between samples.

6.5 Analysis of allo-specific effector mechanisms in tolerant Lewis rats after therapy with OX34 and OX38.

The presence of alloreactive T cells in tolerant Lewis rats transplanted with DA allografts has been established by proliferation assay. As has been outlined in the introduction this assay is not a precise replica of transplant alloimmunity as the stimulating cell (DA splenocytes in these experiments) differs from the stimulating cell in the transplant model (DA myocytes). We therefore wished to establish if allo-sensitised cells are present in tolerant animals.

6.5.1 Assessment of allospecific CTL in tolerant Lewis rats.

Firstly we assessed the ability of tolerant Lewis rats treated with this regime to generate specific anti-DA cytotoxic lymphocytes. Splenocytes were prepared from six tolerant Lewis rats >100 days post transplant treated with OX34 and OX38 and four Lewis rats 14 days after the onset of acute allograft rejection of an unmodified DA allograft. Both groups of cells were used as effectors as described in materials and methods. Con A treated DA and PVG strain splenocytes were used as target cells. As can be seen in figure 6.11 tolerant Lewis rats generate specific cellular cytotoxicity to DA strain targets, but to a lesser degree than rejecting rats. As expected neither group of rats show specific cytotoxicity to third party PVG strain targets.

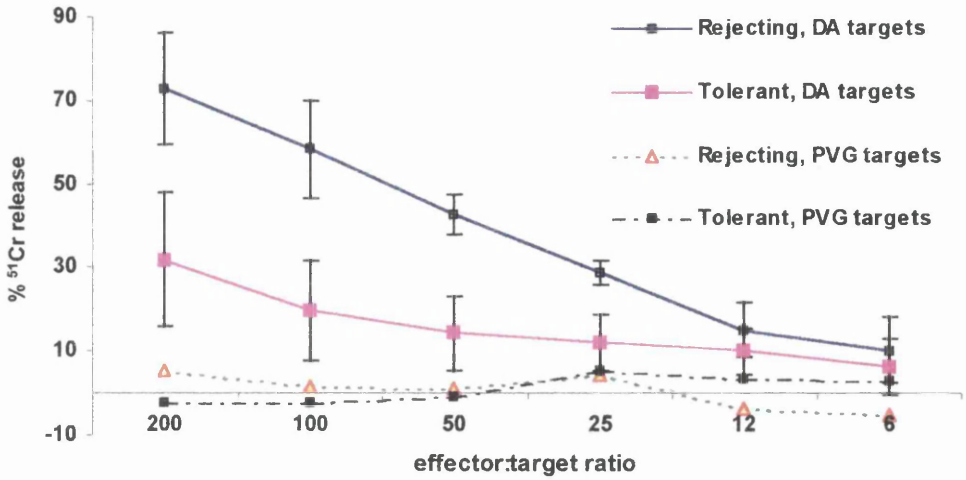

Figure 6.11 Generation of donor-specific allo-CTL by tolerant Lewis rats treated with OX34 and OX38

Mean % specific cytotoxicity (DA targets) and non-specific cytotoxicity (PVG targets) of splenocytes from unmodified Lewis rats 10 days after transplant of a DA allograft (n=4) and Lewis rats tolerant to DA allografts after pre-operative therapy with OX34 (2x5mg/kg) and OX38 (16mg/kg)(n=6).

6.5.2 Assessment of alloantibody production by tolerant Lewis rats after therapy with OX34 and OX38.

In a similar manner and using the same rats as a source of serum we assessed the production of specific cytotoxic antibody in tolerant and rejecting rats. Naïve Lewis rats were also used as a source of unmodified serum for use as a negative control. Serial dilutions of serum were then incubated with ^{51}Cr labelled DA strain blasts and guinea pig serum used as a source of complement as described in materials and methods. As can be seen in figure 6.12 the six tolerant Lewis rats tested in this way produce no demonstrable cytotoxic antibody.

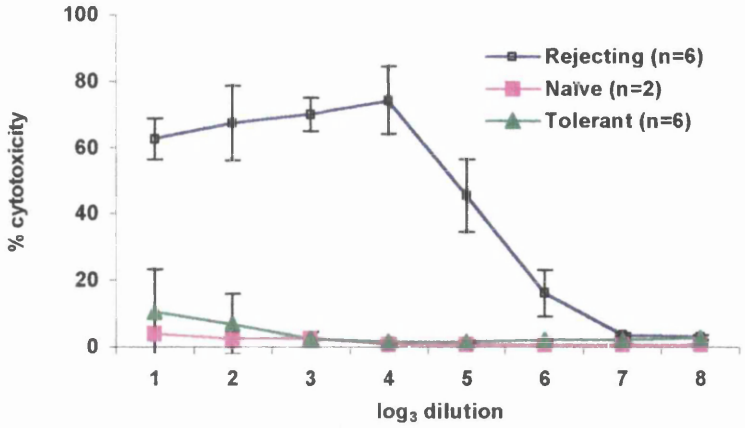

Figure 6.12 Generation of donor-specific cytotoxic alloantibody by tolerant Lewis rats treated with OX34 and OX38

Mean % specific cytotoxicity (DA targets) of serum from unmodified Lewis rats 10 days after transplant of a DA allograft (n=4), from naïve Lewis rats and from Lewis rats tolerant to DA allografts after pre-operative therapy with OX34 (2x5mg/kg) and OX38 (16mg/kg)(n=6).

The absence of specific antibody mediated cytotoxicity can be explained by either the absence of specific antibody or by peripheral mechanisms of immune regulation involving competing antibody isotypes. We therefore investigated the presence, in addition to the function, of specific alloantibody by a flow cytometric method in rejecting and tolerant Lewis rats. Serum was taken from four unmodified Lewis rats seven days after receiving a DA strain allograft and compared to serum from tolerant Lewis rats 100 days post-transplant treated with OX34 and OX38 preoperatively. Serial dilutions of serum were incubated with DA strain LNCs before labelling with specific mouse anti-rat IgG and IgM and analysis by FACS. Figures 6.13 and 6.14 show that tolerant Lewis rats fail to produce anti-DA strain antibody of IgM or IgG class.

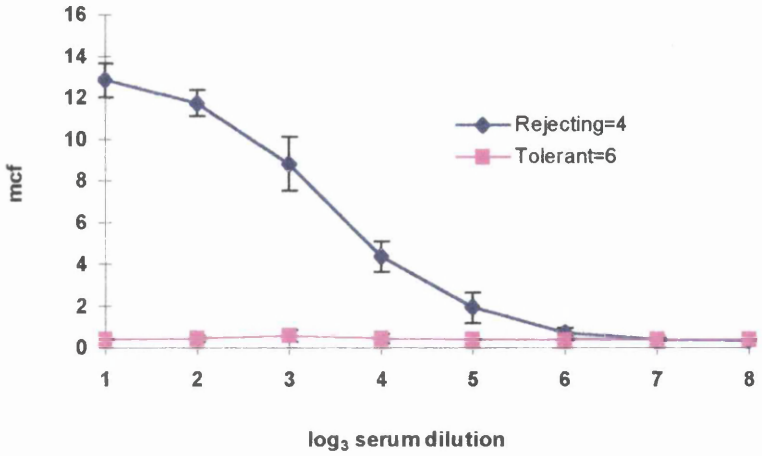

Figure 6.13 Generation of donor-specific alloantibody of IgM subclass by tolerant Lewis rats treated with OX34 and OX38

Flow cytometric analysis of serum from Lewis rats tolerant to DA strain allografts (>100 days post transplant) after preoperative treatment with OX34 (10mg/kg total) and OX38 (16mg/kg total) and serum from unmodified Lewis rats seven days after receiving a DA cardiac allograft. DA target cells were labelled with FITC-conjugated mouse anti-rat IgM antibody after incubation with test serum.

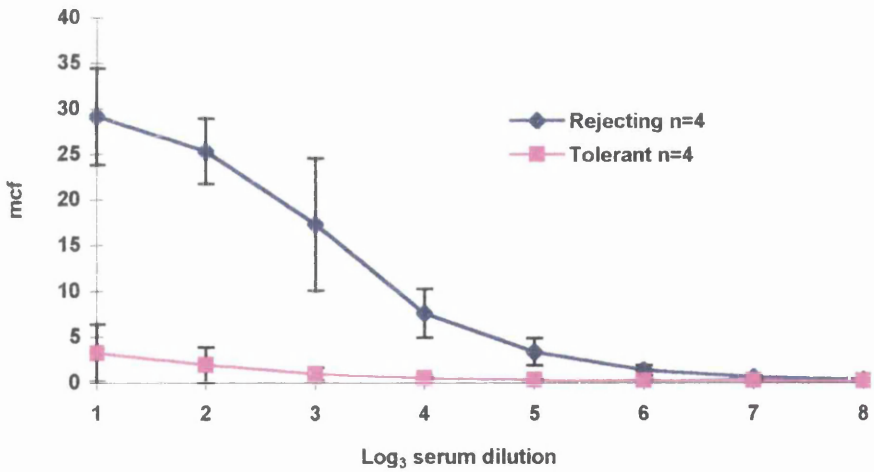

Figure 6.14 Generation of donor-specific alloantibody of IgG subclass by tolerant Lewis rats treated with OX34 and OX38

Flow cytometric analysis of serum from Lewis rats tolerant to DA strain allografts (>100 days post transplant) after preoperative treatment with OX34 (10mg/kg total) and OX38 (16mg/kg total) and serum from unmodified Lewis rats seven days after receiving a DA cardiac allograft. DA target cells were labelled with FITC-conjugated mouse anti-rat IgG antibody after incubation with test serum

Specific immunological tolerance in this model is therefore associated with abrogation of the alloantibody response to the graft in long-term recipients. Experiments were also performed with serum from transplanted Lewis rats treated as above using neat serum harvested seven and forty days post transplant, to ensure detection of an early IgM or IgG peak. Very low levels of specific anti-DA antibody are demonstrable at all time points in tolerant rats (figure 6.15).

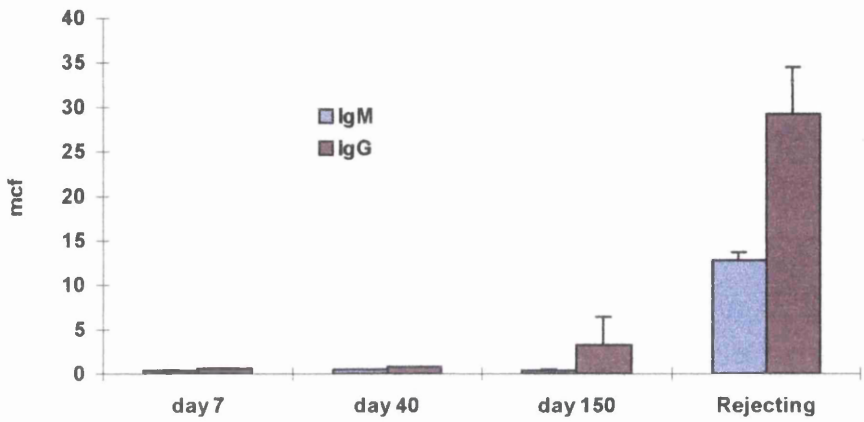

Figure 6.15 Generation of donor-specific alloantibody of IgG and IgM subclass by tolerant Lewis rats treated with OX34 and OX38 during the induction of transplant tolerance

Mean channel fluorescence of DA target cells incubated with serum from Lewis rats transplanted with DA allografts and treated with OX34 and OX38 taken at 7 (n=3), 40 (n=3) and 150 (n=6) days post-transplant compared to unmodified Lewis rats (n=4) 14 days post-transplant. Target cells are labelled with FITC-conjugated mouse anti-rat IgM and IgG antibody.

6.6 Conclusions and discussion of results

6.6.1 Demonstration of transplant tolerance

Tolerance to donor tissue has been demonstrated for the DA to Lewis model treated pre-operatively with OX34 (2x5mg) and OX38 (10,2,2,2mg/kg). In the Lewis to DA model treated with OX34 alone (2x5mg/kg pre-operatively) three of four

retransplanted animals accepted a second donor type allograft whilst all rejected a third party allograft; the logrank test for these data is very close to significance. Transplant tolerance in Lewis rats treated with OX34 and OX38 however can be concluded by:

1) The persistence of a strong, palpable heart beat

2) The microscopic appearance of minimal tissue damage

3) The absence of detectable alloantibody in transplanted Lewis rats

The observation that tolerant rats of both strains reject third-party allografts at a slower tempo than naïve rats suggests the presence of continued immunosuppression in treated rats. This observation is supported by the continued depletion of CD4 T cells more than 100 days after therapy with OX34 in DA strain rats. Furthermore MLR experiments reported in this section suggest a depressed response of LNCs from tolerant Lewis rats treated with OX34 and OX38 against both donor specific and third-party alloantigen in terms of proliferation and cytokine production, although the small number of animals used does not allow the demonstration of statistical significance. As noted in the text tolerance in Lewis rats treated with OX34 and $CTLA_4Ig$ was not successfully demonstrated due to technical difficulties with the procedure of cervical transplantation.

6.6.2 Analysis of proliferative responses in transplanted rats.

In contrast to the response of DA and Lewis rats to second donor and third party allografts, donor type hyporesponsiveness is not demonstrated in MLR experiments for any of the models under study, since LNCs from successfully transplanted rats are consistently able to proliferate on stimulation with donor type cells. Estimates of relative proliferation between LNCs from naïve and transplanted animals against donor and third party splenocytes are difficult to draw from MLR

experiments as the pattern of proliferation differs between experiments performed under identical conditions. A safe conclusion from these experiments however is that cells from tolerant animals are able to respond to donor strain lymphocytes *in vitro* ('split tolerance'). The only other consistent observation from the MLR experiments performed is that cells from sensitised animals (undergoing acute allograft rejection) produce greater degrees of proliferation on *in vitro* stimulation with donor type antigen than cells from either naïve or tolerant animals.

6.6.3 Analysis of cytokine production in MLR experiments

IL2 and γIFN production by cells from OX34 treated DA strain rats with long surviving Lewis allografts on stimulation with Lewis splenocytes was measured in the supernatant of one MLR. No difference was noted in cytokine production between cells from naïve and transplanted animals, in keeping with their similar degree of proliferation. In contrast a striking difference was noted in IL2 production between cells from naïve Lewis rats and Lewis rats tolerant to DA allografts after therapy with OX34 and OX38 on stimulation with DA splenocytes. This was not matched by a similar reduction in γIFN production. This difference in IL2 production by tolerant cells may be either stimulator cell-type specific or may represent a generalised reduction in IL2 production by cells from animals treated with OX34 and OX38. To address this question supernatants from a single MLR only in which the cells from two naïve Lewis rats and two tolerant rats were stimulated by PVG splenocytes was also assessed for IL2 production (figure 6.8). Unfortunately this MLR produced supernatant with the highest IL2 content on stimulation with both strains of splenocyte stimulator cell. Although tolerant Lewis LNCs produced less IL2 on stimulation by both types of stimulator cell, adequate numbers of experiments

were not performed to reliably discriminate between donor-specific hyporesponsiveness and a persistent global suppression of T cell function.

No reliable comment can be made regarding cytokine concentration in MLRs using cells from Lewis rats treated with OX34 and $CTLA_4Ig$.

6.6.4 Analysis of effector mechanisms in tolerant Lewis rats after therapy with OX34 and OX38

The results of experiments assessing effector mechanisms in tolerant Lewis rats show a dichotomy between humoral and cellular alloresponses. Tolerant rats are clearly able to generate cytotoxic T cells directed against alloantigen, although to a lesser degree than rats undergoing acute allograft rejection. The controls used in this experiment (figure 6.11) are unmodified Lewis rats 10 days post-transplant, a time point where cellular responses may be expected to be very high. The lower level of cytotoxicity found in cells from tolerant Lewis rats may represent either a lower titre of allo-reactive CTL or impaired lysis by CTL.

Very low levels of antibody-mediated cytotoxicity are generated by these Lewis rats, in contrast to the high levels of cytotoxic alloantibody found in serum from rejecting animals. Analysis of alloantibody class in tolerant Lewis rats reveals that no IgM is present, although very low levels of T-dependent IgG directed at DA antigen are present. This antibody is however only detectable during the maintenance phase of immune-tolerance (>100 days) and is absent during the induction phase (measured at days 7 and 40). The inability of Lewis rats treated with this combination of mAb to generate either an IgG or IgM antibody response despite the presence of large numbers of B cells suggests that in Lewis rats both classes of antibody are T cell dependent, and also that a degree of B cell tolerance has developed in Lewis rats during the prolonged period of T cell depletion.

7 Summary

The starting point of this discussion is the observation made in Chapter 3 on the efficacy of anti-CD2 mAb in transplant models either when used alone in low responder strain rats or in combination with other modalities in high responder strain rats. The subsequent two chapters contain the bulk of the experimental work of this project which aims to investigate the mechanism by which anti-CD2 mAb acts as an immunosuppressive agent in rat experiments. These investigations therefore concentrate on the mechanisms of induction, rather than maintenace, of transplant tolerance and seek to compare these mechanisms to those of the established immunosuppressive agents anti-CD4 mAb and $CTLA_4Ig$. The final chapter begins to explore the mechanisms of the maintenance of transplant tolerance in DA and Lewis rats, although the investigation of tolerance mechanisms is a secondary aim of this project. This chapter will bring together findings from each of the sections of the thesis to give an overview of the biological effects and mechanisms of anti-CD2 mAb.

7.1 The *in vivo* properties of anti-CD2 mAb

The ability of OX34 to induce permanent allograft survival in DA strain rats but not in Lewis rats is described, and the related anti-CD2 mAb OX55 is noted to be less potent than OX34. An analysis of timing and dose of OX34 administration reveals that post-operative therapy is ineffective at reversing graft rejection and that a 'threshold' dose of OX34 (2x5mg/kg) is required to prevent acute allograft rejection, with lower doses having minimal impact on allograft survival. After intravenous administration OX34 induces a marked depletion of CD4 T cells to similar levels in

both Lewis and DA strain rats, so that differential levels of depletion do not explain the difference in graft survival seen in the two strains. The depletion of CD4 T cells by OX34 is greater than that induced by the depleting anti-CD4 antibody OX38, whilst little effect is detectable on CD8 T cells, despite equal expression of CD2 by the two subsets. The anti-CD2 mAb OX55 induces a similar overall level of depletion of T cells but depletes CD4 and CD8 T cells equally.

Further examination of the cellular depletion induced by OX34 shows that the level of CD4 T cell depletion differs between the vascular and lymphatic compartments, suggesting that an external factor more accessible in the vascular compartment contributes to the depletion process. Furthermore the depletion of T cells is progressive over a period of weeks, during which time there is excess mAb in the circulation. This may be due to saturation of Fc receptors on fixed tissue macrophages in the liver and spleen limiting the depletion of antibody-coated cells.

The T cell population recovers slowly after administration of OX34 mAb and depletion of T cells continues for at least 100 days, whilst the mAb is eliminated from the circulation by day 21. The depletion of CD4 T cells induced by OX34 is accompanied by a relative preponderance of the B cell fraction, whilst the 'null' cell (OX12-/R73-) fraction remains constant in size. In keeping with this the lytic function of NK cells is not reduced by OX34 therapy.

Acute graft rejection in these models requires unbound CD2 molecules as OX34 therapy at the dose which induces permanent allograft survival saturates all OX34 binding sites 24 hours after administration, and induces modulation of the CD2 molecule from the surface of undepleted cells. The less potent mAb OX55 induces greater modulation of the CD2 molecule than OX34, suggesting that modulation is not a crucial aspect of the immunosuppressive effect of anti-CD2 mAb. Analysis of the effects of low dose OX34 therapy (2.5mg/kg) reveals that a

similar level of T cell depletion is obtained as by high dose therapy but without saturation of OX34 binding sites. This demonstrates that saturation of the CD2 molecule is not necessary to induce depletion of T cells. The ability of rats treated with low dose OX34 to mount an acute rejection response also suggests that the remaining unbound CD2 molecules are involved in allosensitisation. The CD2 molecule is therefore able to act as an accessory molecule in the alloimmune response to an organ graft, but can also become a gateway for transmission of a negative signal when labelled with OX34 mAb.

The differential effect of OX34 therapy on graft survival in Lewis and DA strain rats may relate to the fact that Lewis rats are able to generate an anti-globulin response to OX34 mAb leading to its early elimination from the circulation. Despite the preponderance of the B cell fraction noted in DA strain rats after OX34 therapy these animals eliminate OX34 by mechanisms not involving humoral immunity.

7.2 The *in vitro* properties of anti-CD2 mAb

Having demonstrated potent effects of OX34 mAb in rat experiments in terms of graft survival and cellular depletion, we then explored its effect in *in vitro* assays to define its mechanism of action. In contrast to anti-CD4 mAb and $CTLA_4Ig$, which have predictable and potent effects on *in vitro* correlates of alloimmunity, anti-CD2 mAb has only subtle effects, which make a stark contrast with the findings noted above.

In MLR experiments we have shown that both the anti-CD4 mAb OX38 and $CTLA_4Ig$ are able to inhibit proliferation whilst neither OX34 nor OX55 have a consistent effect. In proliferation experiments using anti-TCR antibody, where costimulatory signals are less crucial in activation, anti-CD4 mAb is partially able to inhibit T cell activation while $CTLA_4Ig$ and anti-CD2 mAb have no effect. Analysis

of the production of the cytokines IL2 and γIFN in these proliferation assays shows that both anti-CD4 mAb and $CTLA_4Ig$ are able to inhibit cytokine production in a similar manner to their inhibition of proliferation. Anti-CD2 mAb has minimal impact on cytokine production in these assays. Anti-CD2 mAb therefore does not inhibit T cell activation either by influencing signal 1 or signal 2 in the manner of anti-CD4 mAb and $CTLA_4Ig$. Furthermore lymphocytes can be cultured with OX34 *in vitro* without undergoing cell death on the scale seen after intravenous administration. The exception to this is when cells are cultured with OX34 fixed to the walls of a culture plate, which has a negative impact on cell survival. This suggests that physical anchoring of the OX34 mAb may be important in its effect. The greater depletion of OX34 in the vascular compartment may be a result of OX34 binding via Fc receptors to fixed tissue macrophages.

According to the above results CD2 modulation by anti-CD2 mAb is unable to prevent *in vitro* allosensitisation despite the role of the CD2 molecule in sensitisation to an organ graft in an intact animal. This suggests that undepleted cells have other accessory pathways which can augment T cell activation in the absence of CD2. Further experiments were then performed to examine if the effect of anti-CD2 mAb lies in the generation of effector functions in MLR experiments. OX34 was noted to inhibit the *in vitro* generation of allo-CTL, but to a lesser degree than OX38. In a similar manner OX34 is able to prevent the amplification of NK cells in MLR bulk cultures. Assessment of lysis by CTL and NK cells showed that OX34 is unable to inhibit the effector arm of cellular cytotoxicity. These observations show that despite the inability of OX34 therapy to prevent allosensitisation in terms of proliferation and Th_1 cytokine release it is still able to inhibit the provision of helper signals for CTL induction and NK cell amplification.

In an attempt to explain the depleting effect of mAb, *in vitro* experiments showed that neither OX34 nor OX38 are able to fix rat complement, excluding complement mediated lysis as a mechanism of depletion. The preferential depletion of CD4 T cells by OX34 is more marked within the vascular compartment suggesting that factors outside the cell are also important in T cell lysis. In support of a role for Fc-mediated functions is the observation that both OX55 (IgG1) and OX34 (IgG2a) can act as opsonins to promote the activity of NK cells in *in vitro* assays.

The difference in survival between cells exposed to OX34 *in vivo* and *in vitro* indicates that the proliferation assays described above do not accurately reproduce biological events within an intact animal and therefore limited conclusions can be drawn from these assays.

7.3 Combination therapy in high responder Lewis strain rats

Survival data show that OX34, OX38 and $CTLA_4Ig$ are only able to prolong allograft survival in Lewis rats, but a combination of OX34 with either of the other two reagents allows the induction of permanent allograft survival. This interaction is probably synergistic because high doses of OX34 and OX38 in other treatment regimes do not show an improved effect over low dose therapy. An exploration of the mechanisms involved in the induction of tolerance in these regimes shows that a combination of treatment modalities causes more profound CD4 T cell depletion than OX34 does alone. Anti-CD4 mAb has also been shown to transmit negative signals to T cells and the proportion of cells which are resistant to cell death induced by a single mAb may be susceptible to a combination of effects. The increased depletion seen in animals treated with OX34 and $CTLA_4Ig$ may be due to blockade of the B7

molecule by $CTLA_4Ig$, which has been shown to promote cell death by apoptosis. Another effect of $CTLA_4Ig$ in the Lewis model is to prevent the generation of an anti-globulin response, thereby allowing prolonged circulation of OX34 and cellular depletion. The CD2 molecule remains modulated from the cell surface of the few remaining CD4 T cells for the duration of circulation of OX34 in Lewis rats treated with either regime.

An anlysis of the mechanisms involved in the maintenance of transplant tolerance in these regimes shows no evidence for the induction of anergy. Cells removed from both DA and Lewis rats with long surviving allografts retain the ability to proliferate in *in vitro* culture when stimulated by donor cells. It is noteable however that cells from a tolerant Lewis rat treated with OX34 and OX38 show deficient IL2 production when stimulated *in vitro*. This may be a long-term effect of mAb-induced immunosuppression. Estimation of effector functions in tolerant Lewis rats treated with OX34 and OX38 shows an almost complete abrogation of the humoral response to graft antigens and a reduction in cytotoxic T cell activity compared to that of a rejecting, unmodified control rat.

7.4 Final synopsis

Previous publications which have reported experience with anti-CD2 mAb in the mouse suggest a mechanism of action which involves the modulation of the CD2 molecule without cellular depletion (292, 304), the inhibition of costimulatory signals in T cell activation (291) and the induction of a Th_2 phenotype amongst alloreactive cells (289). More recent studies in the rat (300, 301, 303) have advanced a similar mechanism for the effect of mAb to the rat CD2 molecule. However the mouse CD2 molecule shows fundamental differences to the rat CD2 molecule in terms of:

1) Its distribution on B as well as T cells (352)

2) The presence of allotypic variants (287)

These differences suggest that the molecule may also function in a different manner and respond differently to anti-CD2 mAb. In keeping with this is our observation that whilst anti-CD2 mAb is ineffective when used as monotherapy in the mouse and has a synergistic action with anti-CD3 mAb (296), anti-CD2 mAb has a reverse pattern of effects in the rat, as it is effective in a low responder strain when used as monotherapy and shows no synergy of action with anti-CD3 mAb. The results reported in this thesis therefore do not support a construction based on the inhibition of costimulation but rather suggest that the principle mechanism of action of OX34 mAb is the transmission of a negative signal via the CD48-binding domain of the CD2 molecule on CD4 T cells using an Fc-mediated interaction with a host cell. This results in death of the mAb-bound cell and consequent disabling of immune responses. The following novel observations presented in this thesis support this proposition:

1) Intravenous OX34 therapy causes profound and long lasting depletion of CD4 T cells (greater than that caused by OX38)
2) Depletion is not caused by complement fixation
3) OX34 labels CD4 and CD8 T cells equally yet only depletes CD4 T cells
4) OX34-mediated death of CD4 T cells occurs more readily within the vascular than the lymphatic compartment, despite saturation of binding sites in both compartments
5) Depletion of T cells is progressive in the presence of excess OX34 in the serum of treated rats
6) OX34 mAb in solution in *in vitro* lymphocyte cultures causes more marked modulation of the CD2 molecule than after intravenous

administration in an intact animal yet has minimal impact on T cell activation and cytokine release in the MLR or after stimulation with anti-TCR mAb

7) Rat Fc-receptors recognise mouse Fc, and OX34 acts as an opsonin on NK target cells
8) OX55 mAb causes more profound modulation of the CD2 molecule than OX34 yet is less effective in transplant models
9) OX55 mAb causes less cellular depletion and does not discriminate between CD4 and CD8 T cells
10) Coadministration of $CTLA_4Ig$ with OX34 exaggerates the depletion of CD4 T cells (blockade of B7 is known to promote apoptosis)
11) Coadministration of OX38 with OX34 also exaggerates the depletion of CD4 T cells (anti-CD4 mAb can also act by delivery of a negative signal)
12) The more profound and prolonged depletion of CD4 T cells induced by these combination regimes is associated with permanent allograft survival in high responder rats.

In contrast results presented in this thesis provide little evidence that OX34 mAb acts by inducing hyporesponsiveness through blockade of costimulatory signals in the classic manner described by Bretscher and Cohn (90).

7.4.1 Future pospects

Two broad areas of research arise from this thesis which are worthy of further study. The potent ability of OX34 to deliver negative signals and cause T cell depletion could be corroborated by analysis of apoptosis both *in vivo* and *in vitro* by assays of DNA fragmentation. The nature of the host agent responsible for cooperating with CD2-mediated apoptosis could be investigated by using radio-

labelled OX34 to determine the site of T cell lysis. Attempts could also be made to saturate host Fc receptors with Fc fragments to assess their involvement in this process.

The findings reported in the tolerant Lewis rats after therapy with OX34 and OX38 are unusual and merit further study. Particularly interesting are the related findings of abnormal IL2 production by cells from tolerant animals and the failure of upregulation of B7 expression by these cells. These findings suggest that tolerance in these animals may be due to an abnormality of the IL2 pathway, a finding which has been noted before in the context of tolerance induced by DST (277). This finding could be pursued further by a more extensive analysis of B7 expression in these animals and by assessment of isolated graft infiltrating cells.

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