# Allograft Rejection and the Role of the Immunoregulatory Cytokine Interleukin-12

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## SUMMARY

A major objective in transplant research is to achieve tolerance to alloantigen by modifying the host immune system at the time of grafting, thereby avoiding the need for exogenous immunosuppression. Much recent interest has focused on the cytokines produced by immunoregulatory cells, to determine whether manipulating secretion of such molecules may prove beneficial for allograft survival. These cytokines are a diverse group of proteins, produced during the effector phase of both the innate and the specific immune responses and they serve to mediate and regulate the immune response.

T helper cells are critical to the adaptive immune response and can be divided into functionally distinct subsets according to the repertoire of cytokines which they produce, Th1 cells producing Interleukin-2 (IL-2), Interferon-γ (IFN-γ) and Tumour Necrosis factor-β (TNF-β) and Th2 cells producing IL-4, IL-5 and IL-10, with Th0 cells being an undifferentiated common precursor. Th1 cells are responsible for cell-mediated immunity as they stimulate cytotoxic T cells and NK cells, activate macrophages and control antibody-dependent cell cytotoxicity. In contrast, Th2 cells provide optimal help for humoral immune responses by inducing production of IgE, IgG 1 and enhancing mucosal immunity.

Allograft rejection is primarily attributable to cell-mediated immunity and is associated with an increase in Th1 type cytokines. Immunological tolerance to allografts is usually associated with a decrease in Th1 cytokines and, in some, but not all situations, a preservation or increase in Th2 cytokines. Consequently,

induction of tolerance to allografts may be associated with the preferential activation of Th2 cells.

An important factor which determines the polarisation of undifferentiated Th0 cells into Th1 or Th2 cells is the cytokine environment present at the time of antigen presentation to the Th0 cell. IL-12 is a potent cytokine for induction of Th1 responses, while IL-4 promotes Th2 responses. These two cytokines have antagonistic effects on each other. IL-12 is, therefore, a key immunoregulatory cytokine: it induces Th0 cells to differentiate into Th1 cells; it promotes cytotoxic lymphocyte activity; it induces production of other Th1 cytokines such as IFN-γ, and it inhibits the generation of Th2 responses.

Antagonism of endogenous IL-12 can switch various pathological processes from a Th1 to a Th2 mediated disease and this can result in dramatic changes in the disease process (e.g. protozoan infections, acute allergic encephalomyelitis, experimental colitis). IL-12 is, therefore, an attractive target molecule in allograft rejection. By inhibiting IL-12, it may be possible to prevent the generation of the characteristic Th1 response to an allograft and, instead, induce a Th2 response. This, in turn, may lead to prolonged survival of the graft.

This aim of this study was to examine the effect of neutralising endogenous IL-12 on skin allograft survival in a mouse experimental model. The immune response following treatment was investigated to determine whether anti-IL-12 antibody could switch a Th1 to a Th2 response and whether this was associated with prolonged graft survival.

C57Bl/6 and DBA/2 mice were grafted with tail skin from semi-allogeneic (C57Bl/6 x DBA/2) $F_1$ ($BDF_1$) donors. Skin graft survival was compared between untreated mice and those treated with a neutralising goat anti-mouse IL-12 polyclonal antibody. In addition, mice were pre-treated with donor specific transfusion in the form of $BDF_1$ spleen cells injected intravenously prior to grafting, or with a combination of both anti-IL -12 antibody and donor cell pre-treatment.

Skin graft survival was not prolonged in animals which had been treated with anti-IL-12 antibody or donor cell injection alone compared to unmodified controls. Allograft survival was, however, significantly prolonged in animals pre-treated with both anti-IL-12 antibody plus donor cell injection. Interestingly, when a fully allogeneic mismatch was used, this combined treatment did not prolong graft survival.

The immunological processes involved with the rejection response were investigated. The spleens of treated and untreated mice were removed at various time points after grafting and single cell suspensions prepared and maintained in vitro. When re-stimulated with irradiated $BDF_1$ cells in a MLR, cells from unmodified C57Bl/6 graft recipients proliferated well. However, where animals had been pre-treated with donor cells, either with or without anti-IL-12 antibody, proliferation was markedly reduced, although such a reduction was not associated with prolonged graft survival. Pre-treatment with anti-IL-12 antibody alone did not reduce proliferation in vitro. Supernatants from cultures of spleen cells from treated animals re-stimulated with donor-specific antigen were analysed for their cytokine content by ELISA. Unmodified graft rejection was associated with

increased production of the Th1 cytokines, IL-2 and IFN-γ. Treatment with donor cells prior to grafting abolished this Th1 response in vitro, with cells producing little Th1 or Th2 cytokines, although grafts were still rejected at the normal time. Treatment with anti-IL-12 antibody alone induced cells to produce less IFN-γ in vitro and also slightly increased production of the Th2 cytokines, IL-5 and IL-10, although this did not alter allograft rejection. Pre-treatment with both anti-IL-12 antibody and donor cells abolished a Th1 response and significantly increased Th2 cytokine production and this was associated with prolonged allograft survival. In this model, therefore, prolonged skin graft survival appeared to be associated with an increased Th-2 response and a down-regulated Th-1 response when cells from treated animals were re-stimulated in vitro.

Specific cytotoxicity, as measured by CTL activity in spleen cell preparations was not reduced by neutralising endogenous IL-12, but NK cell activity was reduced. NK cells are responsible for the elimination of injected foreign cells, and persistence of such injected cells within the host has been shown by others to prolong allograft survival. However, using FACS analysis, it was not possible to demonstrate that reduced NK cell activity directly led to increased survival of injected $BDF_1$ cells in treated animals in this model.

Thus inhibition of endogenous IL-12, together with donor cell pre-treatment, prolonged skin graft survival and this was associated with an up-regulated Th2 response and a down-regulated Th1 response. This indicates that effecting a switch to a Th2 response can prove beneficial for allograft survival.

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## PRESENTATIONS OF THIS WORK

Parts of this work have been presented at various meetings.

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"Anti-IL-12 antibody together with donor leukocyte pre-treatment prolongs skin allograft survival."

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## CONTENTS

# CHAPTER 1

# INTRODUCTION AND REVIEW OF THE LITERATURE

## CHAPTER 2

## MATERIALS AND METHODS

## CHAPTER 3

## SKIN GRAFT SURVIVAL

## CHAPTER 4

## PROLIFERATION AND CYTOKINE PRODUCTION IN VITRO

## CHAPTER 5

## CYTOTOXICITY ASSAYS

## LIST OF FIGURES

## ABBREVIATIONS

| | |
|---|---|
| APC | antigen presenting cell |
| conA | concanavalin-A |
| cpm | counts per minute |
| CTL | cytotoxic T lymphocyte |
| DST | donor-specific transfusion |
| DTH | delayed-type hypersensitivity |
| EAE | experimental allergic encephalomyelitis |
| ELISA | enzyme-linked immunosorbent assay |
| FACS | fluorescence activated cell sorting |
| GVHD | graft versus host disease |
| IFN | interferon |
| IL | interleukin |
| ip | intraperitoneal |
| iv | intravenous |
| mAb | monoclonal antibody |
| MBP | myelin basic protein |
| MLR | mixed lymphocyte reaction |
| MST | mean survival time |
| NK | natural killer cell |
| PBS | phosphate buffered saline |
| PLP | proteolipid protein |
| Tc | Cytotoxic T cell |
| Th | T helper cell |

# CHAPTER 1

# INTRODUCTION AND REVIEW OF THE LITERATURE

## 1.1 The Immune System

The immune system of man and other mammals has evolved into a sophisticated and closely regulated series of mechanisms which serve to protect the individual against the threat of infection and colonisation. It has the ability to recognise foreign antigens, differentiate them from host or self constituents and to institute effector mechanisms to eliminate or destroy such antigens. In addition, it retains an immunological memory in order that, on re-exposure to such antigens, recognition and elimination proceed rapidly.

The mammalian immune system has conventionally been divided into 2 parts, the innate and the adaptive systems. It must be appreciated, however, that functionally no division exists, with both systems working in a co-ordinated fashion to provide immune responses to any antigen (reviewed in Austyn and Wood, 1993).

### 1.1.1 The Innate Immune System

The innate immune system includes all non-specific resistance mechanisms and can recognise and eliminate antigen in a variety of ways. A fundamental feature of this arm of the immune response is the speed with which such recognition and effector mechanisms can be instituted. The innate system does not, however, possess specificity of recognition or elimination and the mechanisms of destruction usually work indiscriminately. This involves the inflammatory response which starts with activation of both cellular and humoral components by antigen (Austyn and Wood, 1993).

Immune responses are produced primarily by leucocytes, of which there are several different types. One important group is the phagocytic cells such as monocytes, macrophages and polymorphonuclear neutrophils. They bind to antigen, internalise it and destroy it, using primarily non-specific recognition systems and thus mediate the innate response.

A wide variety of soluble molecules are also involved in the development of immune responses. The complement system is a group of serum proteins which interact with one another in a cascade which can opsonize bacteria, act as chemoattractants and cause damage to cell membranes leading to cell lysis. Cytokines (dealt with more fully below) form a large group of molecules involved in signalling between cells during an immune response. These components all serve to co-ordinate and direct the innate arm of the immune response, but can also interact with components of the adaptive response.

### 1.1.2 The Adaptive Immune System

The adaptive immune response is highly specific for any particular antigen and this response improves with each successive encounter with that antigen. Lymphocytes are the cell type central to the adaptive response since they specifically recognise individual pathogens either inside host cells or in tissue fluids or blood (Austyn and Wood, 1993). Lymphocytes can be characterised in many ways, but essentially fall into 2 groups, T and B lymphocytes. T cells recognise specific antigens via a specific receptor, the T cell antigen receptor (TCR). However, for this to occur, antigen must be presented in association with specific cell surface molecules, termed major histocompatibility antigens,

expressed on host cell surfaces (eg Shoskes and Wood, 1994, Larsen and Pearson, 1997). T cells have also been characterised by the surface molecules which they express: $CD4^+$ T cells express the CD4 molecule and are often, but not exclusively, T helper (Th) cells while $CD8^+$ cells express the CD8 molecule and are usually cytotoxic T (Tc) cells (Zamoyska, 1998).

During an immune response there is a considerable degree of interaction between lymphocytes and phagocytes. For example, phagocytic cells, such as dendritic cells, will take up antigen, process it and present it on their surface in a form which can be recognised by T cells. Such antigen presenting cells (APCs) play a vital role in presenting antigenic material to the adaptive immune system (eg VanBuskirk et al, 1997, Grewal and Flavell,1998). Some cells such as dendritic cells and Langerhans cells are "professional" APCs, whilst other cell types such as endothelial, epithelial and B cells can act as APCs when stimulated with cytokines. T cells also release soluble mediators in the form of various cytokines which can activate phagocytic cells and also influence other T and B cells. Thus many immune responses are made up of a variety of innate and adaptive components.

### 1.2 Cytokines

The cytokines are families of molecules such as interleukins, lymphokines, growth factors, interferons and chemokines which act as mediators of short range signals between cells (Sanchez-Cuenca et al, 1999). These signals are transduced via specific receptors on target cells (Taga and Kishimoto, 1990). They produce a wide array of biological effects which frequently overlap with the

effects of other cytokines, giving redundancy within these systems (Paul, 1989). In vivo, the effects of most cytokines are usually restricted to those cells in the immediate vicinity of release. Cytokine secretion is itself a brief, self-limited event as these molecules are not stored as preformed molecules, their synthesis being initiated by transient gene transcription. In addition, individual cytokines often influence the synthesis of other cytokines, leading to regulatory positive or negative feedback mechanisms and so they can amplify or antagonise each others actions (Gately et al, 1998).

The complete characterisation of many of the cytokines has made it possible to classify them according to their structure, biological activity and the structure of their specific receptor (Bazan, 1990, a). Interleukins were defined as the molecules which transmit signals between leukocytes and, structurally, they can be divided into 2 groups: those with 4-α-helical short chain structures or those with β-sheets. Most of the cytokines with a 4-α-helical structure bind to a receptor with a haemopoietin ligand binding domain (Bazan, 1990, b). The receptors for these cytokines are heterodimers with a unique, ligand binding chain and a common intracellular chain bearing domains necessary for signal transduction. Thus IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-13 and IL-15 all share a similar structure in the form of a 4-α-helical short chain while IL-1α and IL-1β have a β sheet structure. IL-2, IL-4, IL-7, IL-9 and IL-13 receptors all have a component specific for that cytokine, but this is linked to a common intracellular signalling component in the form of a γ chain. These receptors are all part of the haemopoeitin receptor family. Similarly, the receptors for IL-3 and IL-5 are also derived from the haemopoietin receptor family but share a common β chain, while

IL-6 has a gp 130 chain in its receptor which is essential for signal transduction (Taga and Kishimoto, 1997). The interferons (IFN) bind to a IFN receptor domain. These common structures suggest that cytokines and their receptors may have diversified together in the evolution of increasingly specialized effector functions and the shared use of signal transducers in the multichain receptor complexes has been used to explain the functional redundancy of many of the cytokines (Taga and Kishimoto, 1997). Individual cytokines are produced by different cell types and can have more than one cell type as a target. This is summarised in Figure 1.1. The functions of the cytokines relevant to this work are discussed later.

### 1.3 Transplantation

Transplantation of allogeneic organs from one individual to another has become increasingly common in recent years as improvements in surgical technique and post-operative care have been made. The major limitation in this field is, however, the problem of graft rejection. The transplantation of allogeneic tissue leads to a vigorous immune response as the host immune system recognises the grafted tissue as foreign and attempts to destroy it (Wood and Morris, 1995). Despite advances in immunosuppressive therapy, allograft rejection remains a significant problem, as does long term treatment with immunosuppressive agents which are associated with significant side-effects. Much attention has, therefore, been directed at looking for ways in which to modulate the immune system at the time of transplantation to prevent allograft rejection and so avoid the need for ongoing immunosuppression. In order to examine this, one must first have some

understanding of how the host immune system recognises and responds to the allogeneic graft.

### 1.4 Allograft Rejection

The immunological destruction of an allogeneic graft is a complex process and involves different effector mechanisms. In the clinical setting, 3 types of graft rejection are recognised. Hyperacute rejection is mediated by preformed antibody which destroys the blood supply of vascularised organs (Williams et al, 1968). Binding of preformed antibody to donor antigen on endothelium initiates a cascade of events leading to vascular thrombosis. Complement fixation and activation of bradykinins and other vasoactive substances are involved in the destructive process (Jooste et al, 1981, VanBuskirk et al, 1997) and this occurs within minutes of restoring the circulation to the grafted organ. In a clinical setting, however, hyperacute rejection can usually be avoided by the use of cross-match testing prior to grafting to indicate whether a recipient will reject a particular graft in the immediate post-operative period (Scornik et al, 1992, Chapman et al, 1986).

Rejection episodes occurring in the first few weeks after grafting are termed acute rejection and this is usually attributed to cell mediated immunity, provoking a delayed type hypersensitivity (DTH) reaction (Hall, 1991), although alloantibody may also be involved (Costa et al, 1997, Tweedle et al, 1996). Acute rejection is characterised by the presence of donor-reactive T cells and such cells are required for rejection to proceed (Rosenberg and Singer, 1992). The essential role of T cells in this process has been demonstrated by adoptive transfer studies

in T cell-deficient graft recipients. Thus athymic nude mice will accept tissue grafts from allogeneic sources (Manning et al, 1973), but rejection can be brought about by reconstituting such mice with purified syngeneic T cells (Rosenberg et al, 1986, Bolton et al, 1989). It is thought that T cells recognise alloantigen on APCs, and this leads to T cell activation. Cytokine production and other cellular interactions take place as Th cells recruit and activate other cells, including Tc and macrophages, and this leads to tissue destruction (VanBuskirk et al, 1997).

Most organs which undergo acute rejection do so within the first three months after transplantation. However, some organs are lost after this period and this has been termed chronic rejection. In the clinic, acute rejection can often be controlled pharmacologically and so chronic rejection has become a significant problem, occurring in around half of all transplant patients. Chronic rejection is characterised by the slow development of interstitial fibrosis and neointimal proliferation within larger blood vessels and this process is unresponsive to current immunosuppressive therapies (Orosz et al, 1997, Hayry et al, 1993). In the past, acute and chronic rejection have been viewed as separate entities, but more recent evidence has shown that the number of acute rejection episodes has a strong influence on the development of chronic rejection (Matas et al, 1994), suggesting that the 2 processes may be connected. Chronic rejection results from the progressive development of sclerosis in larger arteries in the graft, which decreases blood flow, inducing tissue ischaemia and fibrosis, leading to graft failure. T cells have been shown to contribute significantly to neointimal proliferation within the graft vessels (Cramer and Shirwan, 1998). In addition, however, circulating alloantibody is present in chronic rejection and can itself

cause neointimal proliferation (Russell et al, 1994, Cramer and Shirwan, 1998). Thus chronic rejection is a complex phenomenon where both cell-mediated and humoral mechanisms play a role but, as yet, a full understanding of their significance remains elusive.

### 1.4.1 Cell Types Involved in Rejection

Despite widespread agreement that T cells play a central role in allograft rejection (Bolton et al, 1989, Rosenberg and Singer, 1992), controversy remains regarding the actual mechanisms involved. While T cells invade grafts, other cell types are also to be found within the graft during rejection.

Progressive infiltration of the graft by host mononuclear cells is characteristic of acute rejection of allogeneic tissues (Tilney et al, 1994). Soon after the circulation to the allograft is restored there is invasion by host lymphocytes, initially into the perivascular areas and then throughout the graft. Over the following days the inflammatory cell infiltrate increases, with a large increase in the number of macrophages. Inflammatory changes within the interstitial tissues increase and this leads to tissue necrosis. At the same time, lymphocytes, both mature and blastic, rise in numbers within the graft (Orosz et al, 1997). These include T and B lymphocytes and NK cells.

Both $CD4^+$ and $CD8^+$ T cells are present within rejecting grafts, whether the graft cells express class I, class II or whole MHC differences (Mayer et al, 1988). However, the actual numbers of T cells within a graft do not appear to related to the speed with which a graft is rejected and this suggests that only a small proportion of the cells present may actually have specificity for allogeneic

antigens. Macrophages within the graft act both as APCs that initiate immune responses and as aggressive cells which contribute to graft destruction (Nathan and Murray, 1980, Yamamoto et al, 1998). When activated, macrophages produce cytokines which in turn activate $CD4^+$ lymphocytes which release cytokines themselves. Although NK cells infiltrate allografts rapidly, they have not been shown to be critical in allograft rejection. They are unable to bring about organ destruction without the participation of T cells (Strom et al, 1977) and thus the role of these cells is unclear.

While other cell types may be important in the later, effector stages of allograft rejection, it is the antigen-specific action of T cells which initiates rejection (Rosenberg and Singer, 1992). The role of T cells in skin graft rejection has been extensively studied. Antigen-specific cytotoxic T cells appear to be the major effector cells mediating skin allograft rejection, but the generation of cytotoxic T cells (Tc) from resting T cell precursors requires lymphokines secreted by activated Th cells (Keene and Forman, 1982). Both $CD4^+$ and $CD8^+$ T cell populations contain both Th and Tc cells, albeit in different proportions (Swain, 1983), and Rosenberg et al (1987) demonstrated that immunoincompetent nude mice could only reject skin grafts if adoptively-transferred cells reactive against the donor skin antigens contained both Th and Tc cells, although these cell types could be of identical or distinct phenotypes.

Targeting the CD4 or CD8 molecule with monoclonal antibodies (mAb) to eliminate or inactivate $CD4^+$ or $CD8^+$ T cells has been used in many different experiments. Depleting anti-CD4 antibodies induce long term survival of pancreatic islets and vascularised heart allografts but only delay skin graft

rejection in rodents. Anti-CD8 mAb treatment fails to induce permanent survival of heart grafts or pancreatic islets in the rat, and fails to prolongs skin graft survival in mice (reviewed in Krieger et al, 1996). Using CD4 and CD8 knockout mice, Krieger et al demonstrated that rejection of vascularised heart and skin grafts can be initiated in the absence of $CD8^+$ T cells, but that $CD4^+$ T cells are required in allorecognition. Waldmann has demonstrated that non-depleting CD4 and CD8-specific monoclonal antibodies can induce tolerance to minor mismatched skin grafts in mice and that such tolerance is antigen specific (Davies et al, 1996). This induction of tolerance using anti-$CD4^+$ and anti-$CD8^+$ antibodies alone is, however, strain dependent (Davies et al, 1997) as some strain combinations are resistant. It can be concluded, however, that allograft rejection is T cell dependent and requires activation of cells with helper function as well as activation of cells with cytotoxic function. In many cases, these cell types are two distinct populations of T cells which interact with one another (Rosenberg et al, 1988, Hall, 1991).

### 1.4.2 The Major Histocompatibility Complex

In all mammalian species there is a single genetic locus which encodes antigens which are central to immune recognition and this is termed the major histocompatibility complex (MHC). There is great genetic polymorphism within the MHC leading to vast differences in the antigens expressed within a population (Wood and Morris, 1995). The essential role of MHC antigens is to present foreign antigen to responding host T cells. T cell responses are extremely potent against allogeneic MHC antigens since approximately 2% of T cells can respond

to a particular MHC, whereas only around 1 per ten thousand T cells can respond to common environmental pathogens (Fischer-Lindahl and Wilson, 1977). Thus there is a huge increase in T cell precursor frequency against MHC antigens.

MHC molecules are divided into 3 classes: class I (in humans HLA-A, -B, -C, -E, -F, -G and -H genes), class II (HLA-DR, -DP and -DQ genes), and class III which includes genes coding for complement and tissue necrosis factor (Moss and Khanna, 1999). Both class I and class II molecules have many common structural features. They form stable complexes with peptide antigens, presenting them on the cell surface of antigen presenting cells for recognition by T cells. MHC molecules are transmembrane glycoprotein heterodimers, formed, in the case of class I molecules, by a membrane-bound polymorphic heavy chain (with extracellular domains $\alpha 1$, $\alpha 2$ and $\alpha 3$) and a soluble, non-polymorphic $\beta 2$ microglobulin chain (Bjorkman & Parham, 1990). Class II molecules are composed of membrane-bound polymorphic $\alpha$ and $\beta$ chains, containing extracellular domains $\alpha 1$ and $\alpha 2$, and $\beta 1$ and $\beta 2$ respectively. Both class I and class II molecules thus have 4 extracellular domains, the 2 membrane-proximal domains being immunoglobulin-like structures and the membrane-distal domains having a unique structure related to their function.

The three-dimensional structure of the extracellular domains of class I and class II molecules has now been determined using X-ray crystallography (Madden, 1995, Fremont et al, 1996). The first structure determined for an MHC molecule was that of the class I HLA-A2, where the $\alpha 1$ and $\alpha 2$ domains form an intrachain dimer, with each domain also contributing to a $\beta$ sheet (Saper et al, 1991). Two long, interrupted helices, one from the $\alpha 1$ and one from the $\alpha 2$

domain pack against the β sheet, but are separated by an extended groove approximately 30A long and 12A wide in the middle, which acts as the peptide binding groove. Highly variable residues within the α1 and α2 domains point into this groove, conferring unique peptide and TCR binding properties on each MHC molecule. Thus the MHC class I allele binds to a spectrum of peptides 8-10 amino acids long with high affinity (Jones, 1997). With class II molecules, the peptide binding groove is formed as an interchain dimer of the α1 and β1 domains. Natural ligands for class II molecules are longer than for class I, ranging from 12 to 25 amino acids in length (Falk et al, 1994).

Despite their similarities, differences between the structures of class I and class II peptide-binding grooves have important functional consequences. The grooves of class I MHC molecules are blocked at both ends, while those of class II molecules are open. Thus the ends of peptides presented on class II MHC molecules protrude out of the binding groove (Rammensee, 1995), while those on class I do not. From a structural point of view, however, an antigenic peptide acts as a keystone in the peptide binding domain, placing the peptide at the centre of the T cell binding surface.

The tissue distribution of class I and class II MHC antigens differs considerably. Class I MHC molecules are found on most nucleated somatic cells although levels of expression vary widely between tissues (Harris and Gill, 1986). Class II expression is, by contrast, much more limited, with constitutive expression being found predominantly on B lymphocytes, monocytes, macrophages and dendritic cells, all of which can act as antigen presenting cells

for T cells (Daar et al, 1984). In humans, but not in rodents, class II MHC molecules are found on vascular endothelium.

The primary function of MHC molecules is to scavenge peptides and express them on the surface of cells for surveillance by T cells. In general , MHC class I molecules deal with peptides derived from proteins in the intracellular environment while MHC class II molecules deal with those from the extracellular environment (Rammensee et al, 1993). Expression of MHC antigens can be augmented or induced by certain cytokines or by stimuli which activate cells. For example, IFN-γ can induce class II expression on fibroblasts, T cells and endothelium (Klareskog and Forsum, 1986). TNFα also increases class II expression on monocytes and macrophages and has a synergistic effect with IFN-γ (Arenzana-Seisdedos et al, 1988).

Different functional subsets of T cells are restricted to the MHC antigens against which they react. $CD4^+$ T cells respond to antigen presented in association with MHC class II molecules, while $CD8^+$ T cells respond to antigen associated with class I molecules (Davis and Bjorkmann, 1988, Krensky, 1997). CD4 and CD8 molecules have been shown to be co-receptors for MHC molecules, with the binding sites for both CD4 and CD8 mapping to structurally similar regions of the constant domains of MHC class II and class I molecules respectively (Konig et al, 1992, Salter et al, 1990). The fact that the binding sites for CD4 and CD8 on MHC molecules are separate from the peptide-binding domain of MHC molecules and, therefore, from the site of interaction with the TCR, shows that a single MHC molecule can be bound by both TCR and CD4 or CD8 simultaneously, and this increases the overall avidity of the interaction (Zomoyska, 1998).

### 1.4.3 T Cell Activation

T cells cannot be stimulated by soluble foreign antigens but rather require that antigen is presented to them on the surface of specialised APCs (Grewal and Flavell, 1998). This involves the breakdown of foreign proteins by APCs into peptides which are then associated with host MHC molecules and presented on the surface of the APC. T cells, via the T cell receptor (TCR), can then interact with these cells (Krensky et al, 1990). APCs such as dendritic cells, macrophages and B cells all express MHC class II antigens and so can stimulate $CD4^+$ T cells when antigen is presented associated to class II molecules. In addition, APCs communicate with T cells via other cell surface co-stimulatory molecules which are necessary for T cell activation (Grewal and Flavell, 1998, Lenschow et al, 1996).

For T cell activation to occur, there must be 2 signals: signal 1 involves TCR engagement with the MHC antigens on the APC while signal 2 involves the interaction of co-stimulatory molecules on the surface of both the T cell and the APC (Janeway and Bottomly, 1994, Jenkins, 1994). On activation of the T cell, there is a cascade of intracellular second messengers culminating in the transcription of genes responsible for mediating the effector functions of the T cell.

One such important co-stimulatory molecule is the CD28/B7 complex with the CD28 molecule on the T cell and the B7 complex on the APC (June et al, 1994, Chambers and Allison, 1997). Two ligands, B7-1 and B7-2, have been identified for CD28, B7-1 being expressed on activated B cells, dendritic cells and monocytes, while B7-2 is expressed on resting monocytes. Blockade of this

pathway prevents T cell activation (Harding et al, 1992), inducing a state of unresponsiveness or anergy, preventing differentiation and proliferation of T cells. CD40, another co-stimulatory molecule has also been shown to play a role in T cell activation, as well as in activation of APCs (Grewal and Flavell, 1998, Larsen and Pearson, 1997). CD40 is expressed on B cells and many other APCs including dendritic cells, macrophages and endothelial cells and CD154, the ligand for CD40 is expressed on T cells. Activated T cells rapidly express CD154 in response to TCR ligation (Roy et al, 1993). Recent evidence suggests that by antagonising the CD40/CD154 interaction, T cell priming and clonal expansion is prevented (Grewal and Flavell, 1998), suggesting that such co-stimulatory molecules are important in T cell activation. In addition, interaction between CD40 on B cells and CD40 ligand on activated T cells up-regulates B7 expression on B cells, thereby increasing T cell co-stimulation (Chamber and Allison, 1997). Thus, by preventing the interaction of these co-stimulatory molecules, T cell activation can be prevented, and this has been employed as a strategy to alter T cell responses in allograft rejection (e.g. Larsen and Pearson, 1997, Forster et al, 1999).

### 1.4.4 Presentation of Antigen

As already stated, T cells recognise antigens as processed peptides presented in the context of self MHC molecules and this is termed MHC restriction. APCs internalise foreign antigen and break it down into peptide fragments. These are then presented in the peptide-binding grooves of MHC molecules on the surface of the APC for recognition by host T cells (Wood and

Morris, 1995). Presentation of such peptide in a meaningful manner is only accomplished by a few specialised APCs, including dendritic cells, macrophages, B cells and endothelial cells (Germain, 1993). However, in transplanted tissue there is the unique situation where there are 2 sources of APC, that is either host derived which can process donor antigen and present it with self MHC, or donor derived which will present donor MHC. Host T cells can engage and respond to the high levels of donor MHC antigens expressed on donor APCs and these cells also provide the appropriate co-stimulatory signals to the host T cells. This is termed direct presentation (Cramer and Shirwan, 1998). Thus alloreactive T cells recognise peptides, either exogenous or endogenous, bound to a particular allo-MHC molecule or recognise the allo-MHC molecule itself. This may explain why there is such a high precursor frequency to allogeneic MHC (Heber-Katz et al, 1982, Lui et al, 1993). Direct recognition therefore induces a rapid and vigorous immune response which results in acute allograft rejection (Cramer and Shirwan, 1998, Benichou et al, 1999).

Allogeneic MHC molecules can also be processed by host APCs and thus presented as antigen bound to host MHC molecules, as in a normal immune response. Host T cells can recognise these allo-MHC-derived peptides presented with host MHC and this is termed indirect presentation. There is now increasing evidence that indirect presentation can play a role in allograft rejection (Bradley et al, 1992, Shoskes and Wood, 1994, Bradley, 1996, Valujskikh et al, 1998).

Sensitisation of host T cells may occur within the graft itself or else in the draining lymph node. Barker and Billingham (1967) demonstrated that donor APCs will migrate out of the graft to the regional lymph node where they will

encounter a large number of host T cells. Such migrating APCs have been termed "passenger leukocytes" and play an important role in the presentation of donor antigen to the host immune system.

### 1.5 T-helper Cells and Production of Cytokines

T cells play a central role in allograft rejection and they have been divided into a number of different types, being characterised in a number of different ways. Mosmann et al (1986) were, however, the first to characterise such cells on the basis of the different cytokines which they produce, attributing specific functions to the different cell types. According to the dominant cytokines which they produced, mouse $CD4^+$ T cell clones were divided into 3 groups: T helper 0 cells (Th0), T helper 1 cells (Th1) and T helper 2 cells (Th2).

Mouse Th1 cells produce Interleukin 2 (IL-2), Interferon-γ (IFN-γ) and Tumour Necrosis Factor-β (TNF-β), while Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Mosmann and Sad, 1996). Similar patterns have been observed in human T-cells, although the synthesis of IL-2 , IL-6 and IL-10 may not be as strictly limited to Th1 and Th2 cells as in the mouse (Del Prete et al, 1991). Th1 and Th2 cytokine secretion patterns represent activated effector cell phenotypes which have been generated during an immune response. Th0 cells produce a variety of cytokines common to both Th1 and Th2 cells, namely IL-2, IL-4 and IFN-γ (Mosmann and Sad, 1996).

### 1.5.1 Differentiation of T-helper Cells

When first stimulated by antigen presented on an antigen presenting cell, naive T cells produce IL-2 and then differentiate into cells which produce other cytokines (Mosmann and Sad, 1996, Swain, 1999). This is summarised in figure 1.2. The differentiation of Th0 cells appears to be dependent on the cytokines present at the time of antigen presentation. For example, by blocking differentiation of naive T cells using TGF-β and anti-IFN-γ antibody, $CD4^+$ T cells are kept in a proliferating state (Sad and Mosmann, 1994). When these cells are subsequently re-stimulated with specific cytokines they will then differentiate: the presence of IL-4 results in the generation of Th2 cytokine production while the presence of IFN-γ induces Th1 cytokine production. Thus Th1 and Th2 cells can be derived from a common precursor cell (Mosmann and Sad, 1996) with specific cytokines promoting the differentiation into a Th1 phenotype and others promoting a Th2 phenotype. Thus the pattern of cytokines present during the initial stimulation of naïve Th cells dictates whether Th0 cells will develop into Th1 or Th2 cells.

IFN-γ, and the subsequent down regulation of IL-4 promotes the development of Th1 cells (Hsieh et al, 1993, Gajewski et al, 1989). Another cytokine, IL-12 can promote Th1 differentiation independently, and also increases IFN-γ production by NK cells and T-cells, which further encourages a Th1 phenotype (Trinchieri, 1993). Positive feedback mechanisms therefore exist to amplify cytokine production, further polarising the Th1 response. An additional cytokine, interferon γ-inducing factor (IGIF), also known as IL-18, has recently been identified and it appears to synergise with IL-12 in inducing IFN-γ

production by both differentiating and committed Th1 cells (Robinson et al, 1997, Okamura et al, 1998). It also induces IFN-γ production by NK cells (Ushio et al, 1996), indicating that both IL-12 and IL-18 are required for maximal expression of the Th1 phenotype. Conversely, the generation of Th2 cells is critically dependent on the presence of IL-4 (Seder and Paul, 1994, Swain et al, 1990). This induces further IL-4 production by T cells in an analogous feedback loop.

One important factor in the Th1/Th2 paradigm is that once a specific direction is determined early in the immune response, it remains stable unless major changes take place in the balance of cytokine production (Mosmann and Sad, 1996). This is due to the fact that cytokines exert negative feedback on the cells which produce antagonistic cytokines. IL-12 and IFN-γ inhibit the differentiation and effector functions of Th2 cells and prevent production of IL-4 (Liblau et al, 1995). IL-4, IL-10 and IL-13 inhibit Th1 cell proliferation and oppose the effects of IFN-γ on macrophages (D'Andrea et al, 1993). Thus cross-regulatory interactions occur which further polarise the immune response with IL-12 and IFN-γ being the most important cytokines for inhibition of a Th2 phenotype and IL-4 the most important for inhibition of Th1 responses (Trinchieri, 1995).

In the primary response, antigen-specific T-cells are present at low concentrations and so other cell types probably secrete the cytokines which influence T-cell differentiation. IL-12 is synthesised by many cell types including macrophages and B cells (Gately et al, 1998). Natural killer (NK) cells synthesise IFN-γ in response to IL-12 (Trinchieri, 1993) or in response to antigen-mediated cross linking of antibody bound to Fc receptors (Trinchieri, 1989). In mice, early

production of IL-4 may be due to mast cells, basophils and some T-cells (Yoshimoto and Paul, 1994). Thus, the cytokines which determine the polarisation of the immune response into Th1 or Th2 are produced by the cells of the innate immune system early after exposure to antigen (Bogen et al, 1993).

The type of antigen presenting cell involved appears to be less important in Th1 versus Th2 differentiation than the types of cytokine present as dendritic cells, macrophages and B cells are capable of inducing Th1 or Th2 phenotypes in the presence of the appropriate cytokines. However, co-stimulatory molecules on APCs may also influence differentiation (Thomson, 1995, Abbas et al, 1996, O'Garra, 1998).

### 1.5.2 Functions of T-helper Cells

The in vivo relevance of the functional division of Th cells into different subsets has been extensively studied. Th1 cells are predominantly involved with cell-mediated immune responses. Delayed type hypersensitivity (DTH) is an inflammatory reaction mediated by the products of T cells (Hall, 1991). In an experimental system in which T cells plus antigen were injected into the footpads of mice, only Th1 clones were able to elicit antigen-specific swelling suggesting that Th1 cells are responsible for the generation of DTH (Cher and Mosmann, 1987). The DTH reaction may involve 2 phases with an initial activation signal resulting in recruitment of effector T cells into the site and these cells then recruit monocytes and granulocytes which mediate the subsequent stages of the reaction (Van Loveren et al, 1984). Th1 cytokines activate macrophages and NK cells (Romagnani, 1992) and activate cytotoxic T lymphocytes which, in turn, can

synthesise further Th1-type cytokines, amplifying the response (Mosmann and Coffman, 1989). Thus Th1 responses promote cell-mediated events and these have been proposed as central in allograft rejection (Piccotti et al, 1997, a).

As well as generation of cell mediated responses, Th1 cells have a role in B cell help. Th1 clones at low antigen concentrations can activate B cells to produce specific IgG isotypes. Th1 clones induce the production of IgG 2a (Coffman et al, 1988). IFN-γ, a Th1 cytokine, appears to be important for high levels of IgG 2a production, but other factors may also be involved. IFN-γ induces increased expression of Fc receptors for IgG 2a (Warren and Vogel, 1985) leading to increased antibody dependent macrophage cytotoxicity. In addition, IgG 2a can kill target cells by complement lysis.

Conversely, Th2 cells provide optimal help for humoral immune responses (Mosmann and Sad, 1996). Th2 clones induce proliferation and Ig secretion by B cells and activate populations of small resting B cells. Growth and differentiation of B cells requires direct contact with Th2 cells, while optimum proliferation and differentiation require both IL-4 and IL-5 (Mosmann and Coffman, 1989). IL-4 encourages IgE production as well as increased expression of IgE Fc receptors on B cells, while IL-5 causes proliferation of eosinophils. In addition, Th2 cells promote mucosal immunity through mast cell proliferation and facilitation of IgA synthesis. Thus Th2 clones induce IgM, IgG, IgA and IgE synthesis by B cells and have no cytolytic potential (Romagnani, 1994) while Th1 clones result in delayed type hypersensitivity with little antibody production.

## 1.6 Transplantation Tolerance

A large amount of research has been carried attempting to influence the recipient immune system by altering T cell function, and so render the host unresponsive, or tolerant, to an allograft. Tolerance can be defined as the indefinite survival of a graft in the absence of ongoing immunosuppression in the presence of an otherwise intact immune system and normal host defences (Charlton et al, 1994). Numerous strategies have been developed and successfully applied to achieve allograft tolerance in rodent models. Various mechanisms have been proposed to attempt to explain transplantation tolerance, and the predominance of one particular mechanism may be a result of many variables. Such mechanisms have been classified as central i.e. operate within the thymus, or peripheral i.e. operate outside and independently of the thymus.

Intrathymic events are of great importance in the development and maintenance of self-tolerance. The stromal elements of the thymus, including the bone marrow-derived and epithelial constituents, are capable of influencing the repertoire of emigrating, mature T cells. Through deletion due to apoptosis, and silencing by functional inactivation, T cells with anti-self reactivities are prevented from leaving the thymus (Kappler et al, 1988). However, important as central mechanisms are for self tolerance, long term graft acceptance can be achieved independently of thymic involvement either by treatments which alter the recipients immune system or by pre-treatment of the donor tissue (Lafferty et al, 1986, Waldmann et al, 1993). In such cases peripheral mechanisms of tolerance are responsible for controlling the rejection response. Several distinct mechanisms for peripheral tolerance have been proposed.

### 1.6.1 T Cell Ignorance

As already stated, T cells are activated by cross-linking of their TCR following the recognition of specific target antigen / MHC complexes presented by APCs. T cells are able to respond if antigen is presented by "professional" APCs. However, if a graft is devoid of such APCs, antigen may be presented by "non-professional" APCs which fail to stimulate any response from T cells with the appropriate TCR, leading to T cell ignorance and graft acceptance (Miller et al, 1990). Such a situation arises when tissue grafts such as thyroid or pancreatic islets are maintained in vitro prior to grafting (Lacy et al, 1979, Lafferty et al, 1986). The period in culture is thought to rid the graft of professional APCs (or passenger leukocytes) which migrate out of the graft and thus the allograft provides no stimulus to activate recipient T cells. However, while this technique is effective in tissue culture, it is more difficult to achieve the same results with whole organ grafting.

### 1.6.2 Deletion

As already stated, T cells able to recognise antigenic self-MHC in the thymus can be programmed to die through apoptosis and this is central to the maintenance of self-tolerance. Apoptotic deletion of mature T cells can, however, also occur in the periphery. This has been identified as the mechanism responsible for the induction of tolerance to superantigens following infusion of leukocytes into thymectomised adult mice (Webb et al, 1990). Despite this, while deletion of reactive T cells has been described after transplantation when therapeutic agents were employed, it has not been described as an ongoing mechanism of graft

tolerance and its role in transplantation tolerance does not appear to be significant (Charlton et al, 1994).

### 1.6.3 Anergy

Anergy in T cells was first described by Lamb et al (1983) who demonstrated that T cell clones exposed to antigen expressed on other T cells in culture failed to proliferate in response to subsequent re-exposure to the antigen presented by normal APCs. This functional unresponsiveness, termed T cell anergy, can result from antigen receptor stimulation in the absence of a second, co-stimulatory signal (Chambers and Allison, 1997). As already stated, T cell activation requires 2 separate signals: signal 1 involves TCR engagement with the APC while signal 2 requires the interaction of co-stimulatory molecules on the surface of the APC and the T cell.

Much attention has recently focused on the CD28/ B7 interaction. The B7 ligand has 2 forms, B7.1 and B7.2. B.71 is expressed on dendritic cells and at low levels on resting APCs and lymphocytes while B7.2 is expressed at low levels on resting APCs and moderate levels on resting T cells. Both ligands are up-regulated upon activation (June et al, 1994). CD28 is present on virtually all T cells and its expression is up-regulated during activation. Stimulation through CD28 induces IL-2 production, proliferation of T cells and enhances cell-mediated cytotoxicity (Lenschow et al, 1996), while blockade of the B7/ CD28 interaction inhibits proliferative responses to alloantigen (Tan et al, 1993). In vivo blockade of the B7/CD28 interaction has been achieved by the use of CTLA 4-Ig, a soluble form of CTLA 4, an alternative B7-binding T cell molecule. Thus T cells receive signal

1 but not signal 2, and are therefore inactivated This has been shown to be effective in vivo as treatment with CTLA 4-Ig prevents cardiac allograft rejection (Turka et al, 1992) and produces tolerance to xenogeneic islet grafts in mice (Lenschow et al, 1992).

Other measures are also capable of inducing anergy in vivo. Administration of cell types which do not provide second signals is a well established technique. Such non-professional APCs present the antigenic peptide-MHC complex, but lack the co-stimulatory cell surface molecules to induce T cell activation. Pre-treatment of graft recipients with donor antigen in the form of a blood transfusion uses this mechanism (Dallman et al, 1991, a). Other workers have used gene transfection experiments to induce unresponsiveness (e.g. Madsen et al, 1988) and a number of adhesion molecule-blocking antibodies are also effective in inducing anergy, such as those directed at CD 4, LFA-1 and ICAM-1 (Wood and Morris, 1995).

Anergy is thus defined as a proliferative unresponsiveness and the cytokine environment may also play a role in influencing this. It has been suggested that the Th2 cytokines IL-4 and IL-10 may be associated with unresponsiveness (Takeuchi et al, 1992), or that low or defective production of Th1 cytokines might also be responsible (Dallman et al, 1991, a). Many studies have shown that there is a difference in cytokine expression in rejecting and tolerant recipients (e.g. Bugeon et al, 1992) but, as yet, there is only indirect evidence that development of a Th2 like cytokine environment is critical for the induction of tolerance to alloantigen (see below).

### 1.6.4 Suppression

The term suppression has been used to describe a situation whereby potentially allo-reactive T cells are present but are prevented from responding by other cell populations. Antigen specific suppressors can be defined as cells which inhibit effector responses by recognition of the same antigen. These cells can be adoptively transferred from animals bearing long term allografts to a naive, syngeneic recipient and modify the rejection response to a fresh graft (Hutchison, 1986). For example, adoptive transfer of $CD4^+$ T cells from rats tolerant to kidney allografts render naive rats tolerant (Hall et al, 1984).

Evidence for active regulatory cells maintaining peripheral tolerance has come from adoptive transfer studies where injection of lymphocytes from an animal tolerised to a specific allograft into a T cell-depleted host can prevent injected T cells from an untreated animal rejecting an identical graft. $CD4^+$ T cells were found to be the active suppressor cells (Qin et al, 1993, Davis et al, 1996). Chai et al (1999) demonstrated that anergic T cells adoptively transferred into mice can induce prolongation of skin graft survival , indicating that anergic T cells can act as suppressor cells. Interestingly, they found that this suppression was not mediated by increased Th2-type cytokines, but other workers have found that the cytokine environment is important in suppression. Davis et al (1996) found that in a model of allogeneic skin grafting in mice, the presence of IL-4 was at least partially responsible for the suppressive effect. Similarly, Yang, L. et al (1998) demonstrated that, in a model of tolerance to an allogeneic skin graft induced by donor-specific transfusion in mice, the mechanism of tolerance was the generation of a population of suppressor cells. In this situation, tolerant animal were found to

have significantly elevated levels of IL-4 in their serum compared to controls. In another model of tolerance induced by donor-specific transfusion and anti-CD4 mAb treatment (Roelen et al, 1998), tolerance was mediated by a population of $CD4^+$ T cells acting as suppressor cells. The authors suggested that $CD4^+$ T cells from such animals produce increased amounts of IL-10 on re-stimulation, indicating that the Th1/Th2 paradigm may be relevant in the suppression phenomenon.

### 1.6.5 Donor-specific transfusion

Donor specific blood transfusion (DST) or donor antigen pre-treatment has been used extensively as a method for prolonging allograft survival in experimental models. In addition, it has been observed that patients who received multiple blood transfusions prior to renal transplantation have improved rates of graft survival compared to those who have not (Dossetor et al, 1967, Opelz et al, 1973, Persign et al, 1979 and Chavers et al, 1997). It has also been demonstrated that transfusion of patients with donor blood prior to renal transplantation increases allograft survival (Singal et al, 1985, Salvatierra et al, 1980). Others have shown that DST reduces the frequency of acute rejection episodes in the early period after transplantation (Bayle et al,1995, Opelz et al, 1997) and that $CD4^+$ T cells are involved in this transfusion effect (Van Rood and Claas, 1990). However, some studies have demonstrated that DST may actually sensitise patients, inducing the production of alloantibody, and this has deleterious effects on allograft survival (Opelz, 1985, Burlingham et al, 1989). In an effort to avoid sensitising patients to donor antigen at the time of DST, other workers have pre-

treated patients with DST plus immunosuppressive agents (Alexander et al, 1992, Alexander et al, 1993) and have demonstrated improved graft survival. More recently, Reinsmoen et al (1999) reported that patients treated with DST around the time of renal transplantation showed donor antigen-specific hyporeactivity in vitro compared to controls which had not received DST.

Cells derived from a variety of lymphoid tissues, as well as whole blood transfusion have been used to induce specific unresponsiveness to alloantigen in adult rodent models (e.g. Takeuchi et al, 1992, Cranston et al, 1986). In rodents, peripheral blood lymphocytes, erythrocytes and platelets have all been shown to be capable of inducing unresponsiveness in vivo (Wood and Morris, 1985), although studies using different lymphocyte subpopulations have produced conflicting results. Cranston et al (1986) found that B cells and $CD4^+$ T cells were effective, whereas more recently Gorczynski et al (1996, b) have demonstrated that purified dendritic cells are effective. The critical factors for the induction of unresponsiveness rather than sensitisation following pre-treatment with alloantigen are the route and timing of antigen administration relative to grafting (Bradley, 1991). Intravenous delivery of antigen often results in unresponsiveness and, if antigen alone is used, a finite time after antigen administration is required before a beneficial effect is seen.

In spite of much effort attempting to elucidate the mechanism responsible for the transfusion effect, clear evidence for a single underlying mechanism has not been found. This is partly explained by the great variety of model systems examined, the variable factors in different allografts and the complexity of the allograft rejection response. Some workers have suggested that pre-treatment with

donor antigen promotes a protective Th2 alloimmune response and, at grafting, this Th2 response is strengthened and so rejection is prevented. In support of this, Babcock and Alexander (1996) demonstrated that spleen cells harvested from transfused animals produce increased amounts of IL-4 and IL-10, and reduced levels of IL-2 when re-stimulated with mitogens in vitro. Others have demonstrated a decrease in Th1 cytokine production by re-stimulated splenocytes from animals treated with donor-specific transfusion (Dallman et al, 1991, a). Following blood transfusion, macrophages produce increased amounts of IL-10 and prostaglandin $E_2$ (Takeuchi et al, 1992) and this may promote activation of Th2 clones and inhibit the generation of Th1 cytokine producing cells. Josien et al (1995) demonstrated that, following DST which tolerises the recipient to an allogeneic heart graft, graft infiltrating cells are unable to produce a variety of cytokines including IL-2 and IFN-γ, compared to unmodified graft recipients where such cells exhibit strong expression of such cytokines.

Other workers have demonstrated that DST results in the generation of immunoregulatory T cells. Yang, L. et al (1998) described how DST in a TCR-transgenic mouse skin graft model resulted in permanent allograft survival. They suggested that DST promotes the induction of suppresser cells, dependent on the presence of IL-4, and these inhibit the activation of donor-reactive T cells. In addition, DST promotes IL-4 production in recipient animals, stimulating these suppresser cells. Roelen et al (1998) suggested that DST, in combination with anti-CD4 mAb, a regime which tolerises mice to an allogeneic heart graft, results in the generation of immunoregulatory $CD4^+$ T cells which inhibit allospecific responses. Interestingly, DST is successful in prolonging allograft survival when

the effector stage of rejection is a cell mediated event, but not when it is an antibody mediated event (Yang, C.P. et al, 1998). This is supported by the finding that tolerance to a heart allograft in a rat model induced by DST can be abolished by the administration of recombinant IFN-γ, but that this is associated not with an increase in aTh1-type cytokine response but by alloantibody production (Josien et al, 1999).

The persistence of donor antigen within the graft recipient may also be critical for the induction of allospecific tolerance. While an allograft survives within the recipient, donor antigens are continually shed into the recipient and here they may inactivate newly emerging T cells from the recipients thymus (Wood and Morris, 1995). This has been supported by the observation that, after removal of a graft, if the recipient is rechallenged with the same antigen some time later, tolerance is lost (Shizuru et al, 1990) indicating the ongoing need for the presence of donor antigen within the recipient.

It has been proposed that the development of microchimerism within the host is important for the induction and maintenance of tolerance (Bushell et al, 1995). The authors addressed this issue by examining donor-specific transfusion in a mouse cardiac allograft model where peripheral tolerance could be induced by a combination of injection of viable donor-specific cells plus anti-CD4 mAb. They found that when donor cells are irradiated prior to injection, graft survival is not prolonged. They concluded that when unmodified donor cells plus anti-CD4 mAb are given together, donor reactive T cells still encounter antigenic cells but are unable to react due to blocking of the second signal by anti-CD4 mAb and this leads to T cell unresponsiveness and the graft is not rejected. However, when

irradiated cells are injected, they fail to survive for long enough for these abortive T cell encounters to occur, and so, when a graft is added, normal T cell responses occur and the graft is rejected. When repeated injections of irradiated donor cells are given around the time of anti-CD4 antibody treatment, tolerance is induced, indicating that non-viable cells can induce tolerance as long as they persist within the host for sufficient time.

### 1.7 Th1/ Th2 Paradigm in Transplantation

The hypothesis has been proposed that therapies which induce immune deviation from a Th1 to a Th2 response early during the allograft response will result in graft tolerance or prolonged survival of the graft (Piccotti et al,1997, a). This arose from the observation that allograft rejection is often associated with a Th1 response, while tolerance is often associated with a down regulated Th1 response and an increased Th2 response. The Th1 cytokines, IL-2 and IFN-γ, as well as the CTL-specific marker granzyme B have all been detected in hosts undergoing unmodified allograft rejection (Dallman et al, 1991, b, Takeuchi et al, 1992). Takeuchi et al demonstrated that, by treating mice with donor specific transfusion, cyclosporin or anti-CD4 monoclonal antibodies, they could be tolerised to accept an allogeneic heart. When the grafted hearts and recipient spleens were analysed, there was greatly reduced RNA message for IL-2 and IFN-γ, and enhanced levels of message for IL-4 and IL-10. It was concluded that peripheral tolerance is linked to the differential activation of Th2 cells. In addition, adoptive transfer of cells from the spleens of tolerant animals prolonged heart graft survival in naive recipients and these cells were of the Th2 phenotype.

Dallman et al (1991, a) demonstrated that administration of IL-2 prevents long term graft survival when given to animals which have received a normally tolerising regime of anti-CD4 antibody plus donor specific transfusion. Peripheral tolerance in a rat model was also seen to be associated with reduced levels of IL-2 and IFN-γ production (Bugeon et al, 1992).

Rats pre-treated with a single injection of CTLA 4-Ig are tolerised to a renal allograft in 70% of cases (Sayegh et al, 1995). In tolerant animals, immunohistological analysis reveals that IFN-γ expression is reduced while IL-4 expression is increased compared to controls. Treatment with IL-2 following the tolerising therapy partially prevents long term engraftment.

Gorczynski and Wojcik (1994) demonstrated that pre-treatment of mice with irradiated donor specific splenocytes, injected via the portal vein, prolongs skin graft survival. When lymphocytes are removed from such animals and stimulated in vitro they produce increased levels of IL-4 and IL-10 with decreased IL-2 and IFN-γ. Cells from control grafted animals re-stimulated in vitro produce high levels of Th1 cytokines and low levels of Th2 cytokines, suggesting that preferential activation of Th2 cells is linked with prolonged allograft survival. In addition, in adoptive transfer studies, Th2-cytokine producing cells prolong skin graft survival when they are injected into mice pre-treated with donor cells via the portal vein (Gorczynski et al, 1996, a). More recently, Saggi et al (1999) demonstrated that tolerance to a renal allograft in a rat model induced by rapamycin and cyclosporine was associated with up-regulated IL-4 and IL-10 production, and that IFN-γ was only detectable in grafts showing evidence of rejection.

While these results lend support to the idea that Th2 activation equates with tolerance, other studies have failed to confirm this. In another model of tolerance to heart grafts in rats induced by donor specific transfusion, low levels of transcripts for both Th1 and Th2 cytokines are detected, thus failing to demonstrate any up-regulation of a Th2 response (Josien et al, 1995). Other studies have even demonstrated that a Th2 response may be associated with rejection, albeit somewhat delayed (Chan et al, 1995). In a study of pig islet xenografting into mouse recipients, rejection is associated with activation of Th2 responses, while tolerance is associated with reduced IL-4 expression. In addition, tolerance induced by anti-CD4 mAb therapy could be reversed and this is associated with an increase in IL-4 expression (Morris et al, 1995). Other workers have also demonstrated that Th2 cells alone are capable of mediating allograft rejection. VanBuskirk et al (1996) demonstrated that transfer of either alloreactive Th1 or Th2 cells could effect allograft rejection in athymic SCID mice. They demonstrated that both cell types can bring about rapid, acute rejection of grafts and that the transferred cells are found in the rejecting graft.

### 1.7.1 Treatment with Cytokines

Various workers have attempted to manipulate the cytokine environment directly at the time of alloantigen exposure in order to influence the fate of an organ or tissue allograft. Levy and Alexander (1995) assessed the effects of intragraft infusion of recombinant IL-4 or IL-10 using an osmotic minipump in a rat heart allograft model which had been pre-treated with cyclosporin. They found

that when rats are treated with IL-10 there is no effect on graft survival, but in the animals treated with IL-4 there is a significant prolongation in graft survival.

Gorczynski et al (1995) found that in their model of prolonged skin graft survival in mice where recipient animals are pre-treated with donor-strain splenocytes injected via the portal vein prior to grafting, increased graft survival is associated with an up-regulated Th2 response and a down regulated Th1 response, with increased IL-4 and IL-10 production and decreased IFN-γ and IL-2 production by immunocompetent cells from treated animals re-stimulated in vitro by donor antigen. However, prolongation of graft survival can be prevented by treatment with anti-IL-10 antibody plus recombinant IL-12. In addition, this induces a switch from a Th2 to a Th1 cytokine production pattern when cells are re-stimulated in vitro, with increased IL-2 and IFN-γ and reduced IL-4 and IL-10 production. Qin et al (1996) described a gene transfer model in mice, in which treatment with a retrovirus producing IL-10 significantly prolongs survival of a cardiac allograft. They demonstrated that retro-viral mediated gene transfer of viral IL-10 prolongs graft survival in a non-vascularised neonatal cardiac transplant model. Interestingly, this method has also been applied to a vascularised cardiac transplant model in which a plasmid encoding viral IL-10 is perfused through the graft. This viral IL-10 gene transfer significantly prolongs heart graft survival and is associated with a decrease in Th, Tc and alloantibody responses (Piccotti et al, 1997, a).

Other workers have been less successful when using cytokine therapy. IL-10 treatment of mice grafted with allogeneic hearts produces a slight increase in graft survival (Lowry et al, 1995). However, others have found that treatment with

IL-10, modified to prolong its half-life within the host, does not prolong survival of murine pancreatic islets (Zheng et al, 1995).

In an attempt to elucidate the precise role of cytokines in transplantation, the ability of mice bearing disruptions to the genes encoding for specific cytokines to reject allografts has been examined. Mice deficient in IL-2 production reject pancreatic islet grafts, although rejection is delayed compared to wild type controls (Steiger et al, 1995). However, IFN-γ is strongly expressed in these grafts, suggesting that a switch to a Th2 response has not occurred. IFN-γ or IL-4 knockout mice are both able to reject cardiac allografts (Strom et al, 1996). IL-12 knockout mice have also been examined (Piccotti et al, 1998). Cardiac allografts are rejected more rapidly in IL-12 knockout mice than in wild-type controls. Interestingly, however, these mice are still capable of producing both IFN-γ and Th1 cells, indicating that IL-12 is not essential for the generation of a Th1 response. These results are, however, difficult to interpret as the immune systems of such mice may not be directly comparable to wild type controls.

**1.8 Interleukin-12**

As manipulation of certain cytokines has not demonstrated conclusive support for a Th2 switch being required for allograft survival, attention has turned to other cytokines. IL-12, with its key role in the differentiation and maintenance of Th1 cells and its inhibition of Th2 cells (Gately et al, 1998), is an obvious choice as a target molecule to assess the influence of the Th1/Th2 paradigm in allograft rejection.

IL-12 is a heterodimer, composed of two covalently linked glycosylated chains, one of 40 kDa and the other of 35 kDa which are encoded by separate genes (Wolf et al, 1991). The p35 light chain has limited homology with IL-6 while the p40 heavy chain is not homologous with any other cytokine and most closely resembles the IL-6 receptor. It belongs to the haemopoietin receptor family and resembles the extracellular domain of the IL-6 receptor α-subunit. Thus it is possible that the covalently linked IL-12 heterodimer is derived from a primordial cytokine of the IL-6 family covalently linked to the soluble form of its own receptor (Trinchieri, 1993).

The IL-12 receptor chains are expressed mainly by activated T cells and NK cells. Two IL-12 receptor chains have so far been identified, termed β1 and β2 because they share the general features of β type cytokine receptors, each belonging to the gp 130 subgroup of the cytokine receptor superfamily (Wang et al, 1999). Each chain alone binds IL-12 with low affinity, but when both chains are expressed together, they bind IL-12 with high affinity (Piccotti et al, 1999). Th1 and Th2 cells both express the IL-12 β1 receptor chain, but only Th1 cells express the β2 chain and this accounts for the inability of Th2 cells to respond to

IL-12 (Lamont and Adorini, 1996). In addition, expression of the IL-12 receptor β-2 chain appears to be regulated by cytokines such as IL-10 and TGF-β (Wu et al, 1997). Thus control of this receptor chain expression may constitute a pivotal mechanism for regulating IL-12 responsiveness.

### 1.8.1 Production of Interleukin-12

IL-12 is produced mainly by dendritic cells, macrophages and neutrophils and production can be induced by their interaction with activated T cells which provide co-stimulatory signals (Shu et al, 1995, Maruo et al, 1997). Keratinocytes and Langerhans cells are capable of low levels of IL-12 production (Trinchieri, 1993). Thus IL-12 is produced primarily by antigen presenting cells and exerts immunregulatory effects on both T and NK cells, inducing production of large amounts of IFN-γ (Gately et al, 1998).

### 1.8.2 Effects of Interleukin-12

IL-12 acts at different stages in the immune response, being involved in both its induction and maintenance by regulating the balance between Th1 and Th2 cells. In vitro, it has stimulatory effects on NK cells and T cells and these include induction of transcription and secretion of cytokines. IL-12 stimulates both NK and T cells to produce increased levels of IFN-γ (Chan et al, 1991). This induction of IFN-γ leads to an antagonistic effect on the production of IL-4 (Heinzel et al, 1993) and so IL-12 promotes Th1 cytokine production while inhibiting the generation of Th2 cytokines. In addition, IL-12 itself may promote the differentiation of naive T helper cells into Th1 cells. When highly purified

$CD4^+$ T cells are cultured with immobilised anti-CD 3, without APCs, IL-12 enhances priming for IFN-γ production implying that IL-12 can act directly on the naive T-helper cell (Hsieh et al, 1993). In addition, neutralising IFN-γ using anti-IFN-γ antibodies does not diminish IL-12-mediated priming for IFN-γ production, indicating that IL-12 does act directly on the naive T-helper cell to induce differentiation into a Th1 cell (Seder and Paul, 1994).

As well as inducing cytokine production, IL-12 enhances NK cell cytotoxic activity and activates macrophages (Chan et al, 1991). Allospecific $CD8^+$ cytotoxic T lymphocytes proliferate in response to IL-12 (Bloom and Horvath, 1994), indicating that IL-12 plays a key role in the regulation of cell-mediated cytotoxicity. McKnight et al (1994) investigated the effects of IL-12 in vivo using a hapten-protein conjugate as an immunising system. They found that systemic IL-12 strongly inhibits the development of IL-4-producing T cells. It also promotes the production of IFN-γ by antigen-responsive T cells. They concluded that, in vivo, IL-12 blocks the development of Th2 cells and stimulates differentiation towards the Th1 pathway. This may be as a result of direct effects on activated T cells or from stimulation of NK cells leading to increased secretion of IFN-γ.

### 1.9 Th1 to Th2 Switching

While the Th cell phenotype appears stable after differentiation in normal conditions, various experimental systems have demonstrated that, by adding or blocking the cytokines necessary for differentiation, Th cell phenotype can be altered. IL-10 inhibits antigen-stimulated proliferation by Th1 clones (Fiorentino

et al, 1989), while Th2 clones cultured in the presence of IL-12 produce increased IFN-γ and reduced IL-4, in keeping with a Th1 phenotype. When Th1 cell lines are cultured in the presence of anti-IL-12 antibodies during antigenic stimulation, they produce elevated levels of IL-4 (Manetti et al, 1993).

The direction of the immune response in vitro can therefore be altered by either blocking or adding the key regulatory mediators of differentiation at antigen presentation. The question has, therefore, been asked as to whether this is possible in an in vivo setting and whether this would alter immune-mediated disease processes such as allograft rejection.

### 1.10 Effects of Interleukin-12 in vivo

As IL-12 has been demonstrated to play a central role in the differentiation of T-helper cells and in vitro work has suggested that neutralising the effects of IL-12 on immunocompetent cells can encourage the generation of a Th2 phenotype, considerable attention has focused on the role of IL-12 in animal models of disease.

#### 1.10.1 Parasitic Infection

In the context of the Th1/Th2 paradigm, one of the most closely studied models has been that of parasite infection in mice. Studies have demonstrated that the ability of a host to eradicate an invading organism effectively depends on the type of specific immune response generated and this is influenced by the genetic control of the host. Leishmania major is an intracellular protozoan parasite which evokes different responses in different strains of mice. C57Bl/6 mice respond to

Leishmania major infection with a strong Th1 response, characterised by strong DTH. Antibody levels are low, with no elevation of IgE. There are high levels of IFN-γ and low levels of IL-4 expression. This results in a localised infection which is ultimately cleared by the host. In marked contrast, Balb/c mice produce a strong Th2 response with high antibody levels. High levels of IL-4 production are seen and IFN-γ expression is low and there is no DTH response. This leads to a severe and progressive generalised disease which ultimately proves fatal (Heinzel et al, 1991).

IFN-γ appears to be required for the generation of a protective Th1 response and a single injection of anti-IFN-γ monoclonal antibody to neutralise endogenous IFN-γ converts a normally resistant mouse into a susceptible mouse which develops the generalised disease (Scott et al, 1989). In addition, treatment with recombinant IL-12 provides protection to normally susceptible mice infected with Leishmania. In contrast, treatment of resistant C57Bl/6 mice with a neutralising IL-12 antibody abolishes their protective immunity and exacerbates the infection. This is associated with a decrease in Th1 cytokine production by lymphocytes re-stimulated in vitro with specific antigen, suggesting an abrogation of the Th1 response (Sypek et al, 1993). Protection against Leishmania major infection is dependent on the production of IL-12 by macrophages (Doherty and Coffman, 1999). To confirm that these results are directly attributable to Th1 and Th2 phenomena, Th1 and Th2 cell lines have been prepared which are specific for Leishmania antigens. When these cells are injected back into infected mice, the Th1 line completely cures the infection while the Th2 line exacerbates the course of the disease (Scott et al, 1988).

The relative contribution of Th1 and Th2 cells to the immune response against other infective agents has also been examined. Listeria monocytogenes is a facultative intracellular bacterium and production of IFN-γ is required to clear the infection in mice. Immunocompetent CB-17 mice usually clear Listeria infection by the generation of Th1 cells. However, when animals are treated with polyclonal anti-IL-12 antibodies mice are rendered more susceptible to infection and die in response to a normally sublethal dose of Listeria (Tripp et al, 1994). These findings suggest that IL-12 is necessary for the generation of an effective Th1 response, and when this is blocked, the disease process is radically altered.

Trypanosoma cruzi is another intracellular parasite in which a Th1 response is protective, while a Th2 response leads to disease progression. Susceptible mice can, however, be rendered immunocompetent by injection of Th1 cells specific for the antigen (Nickell et al, 1993).

Th2 responses are required to deal adequately with other infectious agents such as the multicellular helminths. Trichuris muris infection induces a Th2 response in resistant mice. In susceptible strains, however, a Th1 response is generated and they fail to clear the infection. When susceptible mice are pre-treated with neutralising IFN-γ antibody, they develop resistance. Similarly, when they are treated with IL-4 they also clear the infection whilst, when animals are pre-treated with neutralising IL-4 antibody, the condition is exacerbated (Else et al, 1994). With another helminth, Nippostrongylus brasiliensis, a Th2 response is generated by immunocompetent Balb/c mice with stimulation of IL-3, IL-4, IL-5 and IL-9 production which induces IgE, eosinophil and mast cell responses and results in clearance of the infection. Treatment of animals with IL-12 reduces

protection, with increased egg production by parasites and suppression of adult worm expulsion. IL-12 also inhibits IgE production and eosinophil activity as well as the generation of the Th2 cytokines (Finkelmann et al, 1994).

Thus treatment with IL-12 can inhibit the production of Th2 cytokines and stimulate Th1 cytokine production in vivo, even for antigens which would normally evoke Th2 responses. It is therefore possible, in models of murine infection, to polarise the immune system in a specific direction by exposing the early immune response to key immunoregulatory cytokines. Other workers have, therefore, questioned whether this would be possible in other immune-mediated diseases, and, as IL-12 has been demonstrated to play a definitive role in the generation of T-helper responses, work has concentrated on using neutralising IL-12 antibodies to block IL-12 effects in a variety of models.

### 1.10.2 Experimental Colitis

Neurath et al (1995) described a murine model of chronic intestinal inflammation induced by colonic installation of the chemical hapten 2,4,6-trinitrobenzene. This leads to the development of chronic, transmural colitis with weight loss and diarrhoea. The disease observed is similar to that of Crohn's disease in humans. The inflamed murine colon contains a heavy $CD4^+$ T cell infiltrate. When infiltrating cells are isolated and re-stimulated in vitro, they produce increased IFN-γ and IL-2 and decreased IL-4 when compared to cells from untreated controls, indicating that this is a Th1 type disease. Neutralising endogenous IL-12 by administration of a monoclonal anti-IL-12 antibody during the disease leads to a striking improvement in the clinical and histological picture

and is often curative. In addition, T cells from colitic animals treated with anti-IL-12 antibody do not produce IFN-γ when re-stimulated in vitro, indicating that by neutralising IL-12 one can abrogate the Th-1 response and significantly alter clinical outcome.

### 1.10.3 Contact Hypersensitivity

Contact hypersensitivity is a delayed type immune reaction which, in mice, is mediated by a Th1 type response. When a hapten reagent which induces contact hypersensitivity is applied to the skin, mRNA for IL-12 is increased in regional lymph nodes. However, when mice are pre-treated with monoclonal anti-IL-12 antibody prior to sensitisation there is suppression of the clinical hypersensitivity reaction in a dose-dependent manner. Injection of anti-IL-12 antibody into pre-sensitised mice significantly suppresses the hypersensitivity reaction, indicating that by neutralising IL-12, both the induction of the Th1 response and the effector phase of the response can be abolished (Riemann et al, 1996).

### 1.10.4 Experimental Allergic Encephalomyelitis

Experimental allergic encephalomyelitis (EAE) is an inflammatory autoimmune disease of the central nervous system and has been widely used as an animal model for multiple sclerosis. The experimental disease is characterised by the onset of a progressive paralysis which usually recovers spontaneously. Histologically, there is perivascular infiltration of the CNS by mononuclear cells. The disease can be induced in a number of animal species by immunisation with

myelin basic protein (MBP) or proteolipid protein (PLP) or by the adoptive transfer of MBP or PLP-sensitised $CD4^+$ T cells (Liblau et al, 1995). EAE appears to be a Th1 mediated disease: the inflammatory lesions seen in the CNS are similar to a DTH reaction, and immunohistochemical studies of CNS lesions reveal the presence of IL-2, TNF and IFN-γ, but little IL-4 in lesions during the acute disease. However, during the recovery phase, IFN-γ is low or undetectable while IL-4, IL-10 and TGF-β are elevated. In mice injected with $CD4^+$ T cells which have been stimulated in vitro with PLP in the presence of IL-12, the resulting disease is more severe and prolonged. Interestingly, when mice are treated with a polyclonal neutralising IL-12 antibody injected intraperitoneally after $CD4^+$ T cell transfer, paralysis is, to a large extent, prevented, with only 40% of animals developing mild symptoms of the disease. IL-12 therefore plays a pivotal role in the development of EAE and, by neutralising endogenous IL-12, the disease can be prevented and the Th1 response abolished (Leonard et al, 1995, Gately et al, 1998).

### 1.10.5 Graft versus Host Disease

Several studies have analysed the relative contribution of Th1 and Th2 responses to the development of graft versus host disease (GVHD) in murine models (Via et al, 1994, Williamson et al, 1996). The injection of DBA/2 spleen cells into immunocompetent (C57Bl/6 x DBA/2) $F_1$ mice ($BDF_1$) induces a chronic GVHD in which the donor $CD4^+$ T cells become activated and produce Th2 type cytokines which stimulate autoreactive B cells to differentiate into antibody secreting cells. Serum Ig levels of all isotypes, including IgE are

increased. Conversely, CTL function is reduced and donor antihost CTL activity is barely detectable. An autoimmune disease resembling human systemic lupus erythromatosis develops in this strain combination. In marked contrast, injection of C57Bl/6 spleen cells into $BDF_1$ strain mice results in an acute GVHD in which donor CTLs become activated and cause the death of the host by destruction of the host immune and haemopoetic systems. The acute disease is associated with increased IFN-γ production (Via et al, 1994). In the chronic GVHD model, when $BDF_1$ mice are treated with IL-12 at the time of DBA/2 cell transfer, mice develop an acute rather than chronic GVHD. Specifically, treatment with IL-12 blocks autoantibody production, induces antihost CTL activity and leads to the elimination of most host T and B lymphocytes. However, when mice with established chronic GVHD are treated with IL-12, there is no decrease in autoantibody responses and there is no induction of anti-host CTL suggesting that treatment with IL-12 may be most effective if given early during the immune response.

In the same experimental model, when mice are pre-treated with neutralising IL-12 antibody, C57Bl/6 spleen cell injection fails to provoke the acute form of GVHD (Williamson et al, 1996). Associated with this is reduced production of IFN-γ with increased production of IL-10, indicating that, by blocking the effects of IL-12 in vivo, an anticipated Th1 response can be polarised to a Th2 response and this can alter the clinical disease.

### 1.11 Interleukin-12 in Transplantation

In view of the central role of IL-12 in the differentiation and maintenance of Th1 cells and its ability to inhibit the development of Th2 cells, IL-12 is an obvious choice as a target molecule for attempts to manipulate the rejection response.

Gorczynski et al (1996, b) assessed the effect of a neutralising IL-12 antibody on skin graft survival in a minor mismatch model as described earlier. Portal vein injection of donor strain cells prolongs skin graft survival in this murine model. Skin graft survival is further prolonged when graft recipients are treated with both a neutralising anti-IL-12 antibody plus portal vein injection of donor strain cells. When cells from such treated animals are re-stimulated in vitro with donor antigen Th2 cytokine production is enhanced compared to that by cells from unmodified graft recipients and from those treated with portal vein injection of donor strain cells alone. Furthermore, treatment with a combination of anti-IL-12 antibody plus recombinant IL-13 (a Th2 cytokine) further prolongs graft survival.

Conversely, in a mouse cardiac allograft model, treatment with anti-IL-12 antibody actually increased allograft rejection (Piccotti et al, 1996). Unmodified rejection in this model is characterised by a Th1 response within the graft, while treatment with anti-IL-12 induces some Th2 cytokine expression with enhanced expression of IL-4 and IL-10, but not IL-5 mRNA within the graft. However, IFN-$\gamma$ production is not inhibited by antagonism of IL-12. This failure of blockade of endogenous IL-12 to prevent allograft rejection has been further investigated (Piccotti et al, 1997, b). The Th1 cells generated by alloantigen exposure in the

presence of an IL-12 antagonist are predominantly $CD8^+$ T cells. Interestingly, prolonged cardiac allograft survival is observed when mice are treated with a combination of IL-12 $(p40)_2$, an IL-12 antagonist, and in vivo depletion of $CD8^+$ T cells, although neither of these treatments alone prolong allograft survival. The authors suggested that blockade of endogenous IL-12 alone may not be sufficient to prevent Th cells from differentiating into Th1 cells. However, when the generation of Th1 cells is prevented, allograft survival may be prolonged. Treatment of mice with recombinant IL-12 significantly increases the Th1 response generated to an allogeneic heart graft (Piccotti et al, 1999), with increased levels of IFN-γ produced by cells from treated animals when they are re-stimulated in vitro. Interestingly, however, this up-regulated Th-1 response is not associated with accelerated graft rejection, as grafts are rejected at the same time as untreated controls.

Li et al (1998) demonstrated that treatment with anti-IL-12 antibody injected intra-peritoneally around the time of allogeneic pancreatic islet grafting in a mouse model is able to polarise the normal Th1 rejection response to a Th2 response, with high expression of IL-4 and IL-10 within the grafts. Thus, by neutralising endogenous IL-12, a Th1 to Th2 shift is produced. Interestingly, this Th2 shift was not associated with prolonged graft survival in MHC-mismatched strain combinations, but was associated with increased graft survival in minor antigen mismatch combinations.

Controversy does, therefore exist as to the relevance of the Th1/Th2 paradigm in transplantation. While there is strong evidence that unmodified rejection is associated with a Th1 response, there is conflicting evidence as to

whether tolerance is associated with a Th2 response. In experimental models where the cytokine response has been manipulated, rejection can certainly still occur in the presence of a Th2 response (e.g. Chan et al, 1995, Piccotti et al, 1996). Further work is, therefore, required to analyse the cytokine responses in transplantation to determine the role of Th2 activation in tolerance.

### 1.12 Aims of This Study

Interleukin-12 is a key immunoregulatory cytokine involved in the regulation of Th1/Th2 balance both in vitro and in vivo. It promotes a Th1 response by inducing IFN-γ production by both Th1 cells and NK cells and by directly stimulating Th1 cells. In addition, it has inhibitory effects on Th2 responses. IL-12 is also able to direct the polarisation of the immune response away from an anticipated Th2 response to that of a Th1 response in certain experimental models and this can have profound effects on the progression of pathological conditions. In addition, by neutralising endogenous IL-12, an anticipated Th1 response can be abrogated, and a Th2 response generated instead. Th1 responses initiate allograft rejection by promoting both CTL and DTH responses which serve as the final effector mechanisms by which allograft rejection occurs. IL-12 is, therefore, a suitable target molecule in experiments investigating allograft rejection and the generation of tolerance.

The central aim of this study was to investigate the role of IL-12 in the rejection of allogeneic tissue, and, in particular, to determine the relevance of the Th1 / Th2 paradigm in transplantation. Endogenous IL-12 was neutralised using a polyclonal anti-IL-12 antibody in a mouse host which was then transplanted with

allogeneic tail skin and graft survival assessed. This experimental model was used to determine whether anti-IL-12 antibody could effect a switch from the normal Th-1 driven rejection response to a potentially less harmful Th-2 response, and whether this would equate with prolonged graft survival.

The immune response in both control and treated animals was analysed to determine the mechanisms involved. This was done using a variety of in vitro techniques including mixed lymphocyte reactions, cytokine analysis by ELISA and cytotoxicity assays.

| Cytokine | Mol. Wt. (kD) | Source | Target cell | Receptor |
|---|---|---|---|---|
| IL-1α | 17 | macrophages | T and B cells, macrophages | immunoglobulin superfamily |
| IL-1β | 17 | epithelial cells | T and B cells, macrophages | immunoglobulin superfamily |
| IL-2 | 15.5 | T cells | T and B cells, NK cells | haemopoietin receptor family common γ chain |
| IL-3 | 20-32 | T cells | mast cells, macrophages, basophils & eosinophils | haemopoietin receptor family common β chain |
| IL-4 | 18-20 | T cells, basophils & mast cells | T and B cells, mast cells & monocytes | haemopoietin receptor family common γ chain |
| IL-5 | 12<br>24 dimer | T cells<br>mast cells | eosinophils, basophils & B cells | haemopoietin receptor family common β chain |
| IL-6 | 21-26 | macrophages, fibroblasts, T cells & mast cells | T and B cells, hepatocytes & osteoclasts | haemopoietin receptor family gp 130 |
| IL-7 | 25 | stromal cells | T and B cells, monocytes | haemopoietin receptor family common γ chain |
| IL-8 | 8-10 | monocytes, macrophages, T cells, fibroblasts, neutrophils, endothelial & NK cells | T and B cells, neutrophils, basophils, monocytes and endothelial cells | chemokine receptor family |
| IL-9 | 14 | T cells | T cells, macrophages | haemopoietin receptor family common γ chain |
| IL-10 | 17-21 | T cells, monocytes | T and B cells, mast cells | |
| IL-12 | p35<br>p40 | B cells, monocytes, macrophages | T cells, NK cells | haemopoietin receptor family gp 130 |
| IL-13 | 9-17 | T cells | monocytes, macrophages and B cells | haemopoietin receptor family common γ chain |
| IL-14 | 50-60 | T and B cells | B cells | |
| Il-15 | 14-15 | monocytes, epithelial cells | T cells, LAK cells | haemopoietin receptor family common γ chain |
| IFN-γ | 20-25 | T cells, NK cells | many cell types | IFN-γ receptor |

**Figure 1.1**: a summary of some of the different cytokines which play a role in T cell function.

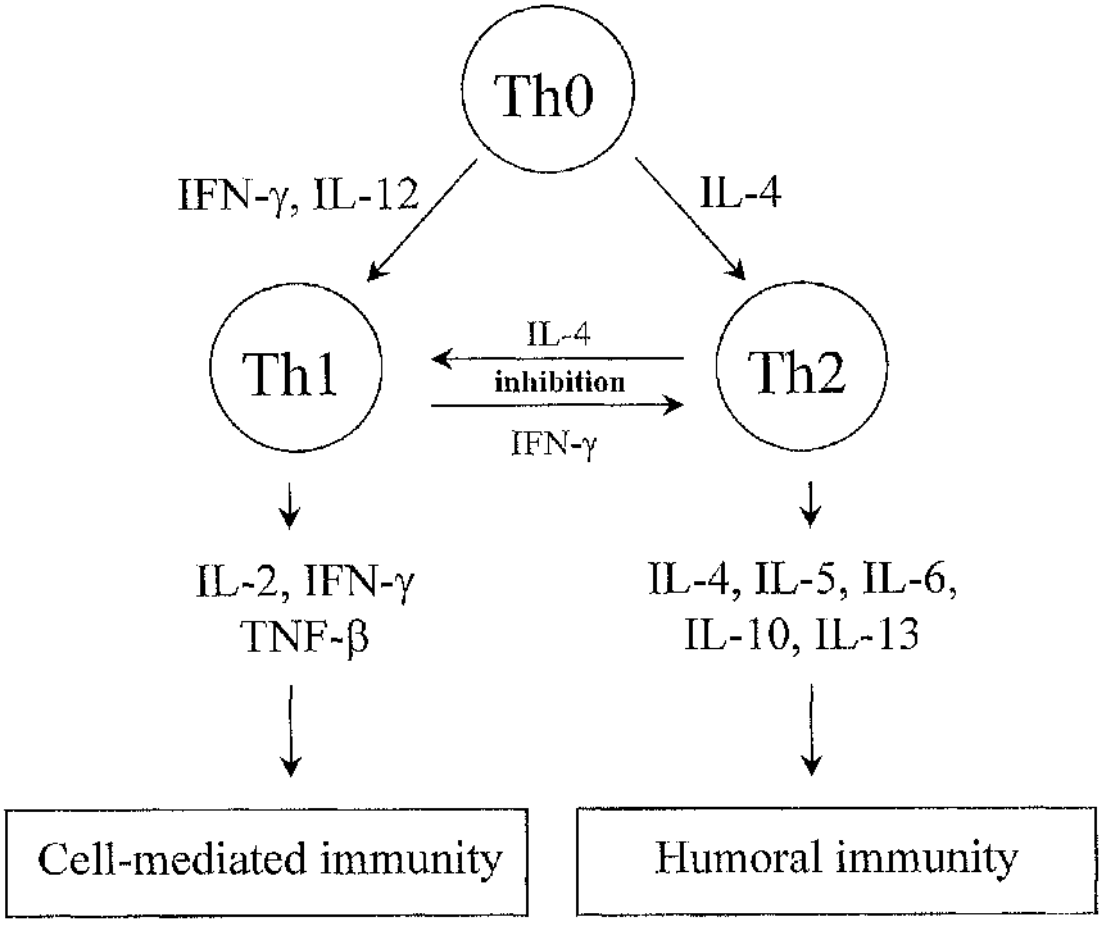



**Figure 1.2: Differentiation of CD4$^+$ T cells.** T helper cell subsets differentiate from a common undifferentiated precursor Th0 cell, the differentiation being influenced by the cytokines present at the time of antigen presentation. IL-12 promotes differentiation into Th1 cells whereas IL-4 promotes differentiation into Th2 cells.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Animals

Skin graft survival was assessed in various in-bred strains of mice. C57Bl/6 ($H2^b$) and DBA/2 ($H2^d$) mice were used as recipients of tail skin grafts from semi-allogeneic (C57Bl/6 x DBA/2) $F_1$ ($BDF_1$) donors ($H2^{bd}$). These mouse strain combinations were chosen because, in previous studies, treatment with IL-12 and anti-IL-12 antibody has been shown to polarise the GVH alloimmune responses to Th1 and Th2 responses respectively (Williamson et al, 1996). In some experiments, fully allogeneic skin grafts were performed between the two parental strains.

Animals were housed in the Central Research Facility of the University of Glasgow and were given food and water ad libitum.

## 2.2 Skin Grafting

Mice aged between 6 and 10 weeks were used for skin grafting experiments. Tail skin grafts were carried out using a modification of the method described by Billingham and Madawar (1951). Donor animals were sacrificed by cervical dislocation and their tail skin removed and cut into pieces approximately 1cm. by $^1/_2$ cm. Recipient animals were anaesthetised using halothane and oxygen inhalation supplied through a nose cone from a vaporiser in a standard anaesthetic machine. The thorax was shaved with clippers and the recipient site on the left hemithorax prepared using an operating microscope. Thin slivers of full thickness trunk skin were removed to form the edges of a square slightly larger than the tail skin graft and the central portion of the square then gently removed, taking care not to damage the fine, vascularised layer of the panniculus carnosus which acted

as the graft bed and from which recipient blood vessels grew into the graft to give it a blood supply. This layer could be clearly visualised with the operating microscope. The graft was then laid onto the recipient site and held in place using "spot welds" of Histoacryl tissue glue (Braun, Germany) to attach each corner of the graft to the recipient skin edge.

A small square of paraffin gauze dressing (Smith and Nephew, U.K.) was then placed over the graft and a circumferential bandage of sleek wrapped around the thorax of the recipient, taking care not to cause respiratory impairment. Animals were allowed to recover from the anaesthetic under close observation and were then housed individually to prevent disruption of the bandages or the grafts. Bandages were removed 7 days after grafting and the following day scoring of grafts was started.

### 2.2.1 Assessment of Skin Graft Survival

Skin grafts which had not been accepted when the bandages were removed were classed as technical failures and discounted. Grafts were assessed daily and given a score of 1 to 4 with 4 being a healthy, well vascularised graft with no reduction in the original size of the skin graft, 3 being a decrease in size of the graft of up to 20%, 2 being a reduction in size of up to 50% and 1 being loss of greater than 80 % of the grafted tissue. A score of 1 was taken as the point at which rejection was deemed to have occurred (Azuma et al, 1989).

**2.3 In Vivo Neutralisation of Interleukin-12**

Mice were treated with a polyclonal goat anti-mouse IL-12 IgG antibody. This was a generous gift from Dr. M. Gately (Hoffman-La Roche, New Jersey, U.S.A.). Animals were treated with the anti-IL-12 antibody (0.5 mg. in 0.2 mls. of phosphate-buffered saline (PBS)) i.p. on days -1, 2, 5 and 8 with grafting on day 0. Additional animals were treated with a combination of anti-IL-12 antibody and donor-specific spleen cells (see below). These mice were treated with the anti-IL-12 antibody either on days -1, 2, 5 and day 8 with donor spleen cell injection on day -7 or on days -8, -5, -2 and day 1 again with spleen cell injection on day -7.

Preparation of the antibody used in this study has been described (Tripp et al, 1994). Briefly, a goat was immunised with 200μg of recombinant mouse IL-12 in complete Freund's adjuvant injected subcutaneously. This was boosted with further recombinant IL-12, 100μg on day 28, 50 μg on day 42 and monthly injections of 10 μg of recombinant IL-12 thereafter. Serum was collected fortnightly beginning on day 42. The IgG fraction of the antiserum was purified by affinity chromatography on protein-G Sepharose. IgG-enriched fractions were pooled, dialysed overnight at 4°C against PBS and stored at -20°C. At 0.2 μg/ml, the antibody completely neutralised 200 pg of IL-12 (M. Gately, personal communication). In addition, 450 μg of the antibody brought about significant changes in the Listeria monocytogenes murine infection model (Tripp et al, 1994). Thus, 2 mg, the dose with which mice were treated in this study, represents a fourfold increase in a dose which was seen to produce biological effects in vivo.

As a negative control, purified goat IgG (Sigma, St. Louis, Missouri, U.S.A.) was injected ip. as 0.5 mg. in 0.2 mls. PBS at the same time points as anti-IL-12 antibody.

**2.4 Donor Spleen Cell Treatment**

Groups of mice were treated with donor-strain spleen cells injected intravenously (iv.) 7 days prior to skin grafting. Spleens were harvested from donor-strain animals and cells freed by gentle rubbing over a sterile stainless steel wire mesh. Cells were washed twice in wash medium (see below) and then passed through a 100 μm nylon mesh to remove any cell clumps or debris. Cells were resuspended in RPMI-1640 medium plus 25 mM Hepes (both Life Technologies Ltd., Paisley, U.K.), counted and made up to a final concentration of $2.5 \times 10^8$ cells per ml.

Recipient mice were gently warmed in a light box to dilate their tail veins and then 0.2 mls. of the cell suspension injected intravenously, giving a dose of $5 \times 10^7$ cells per animal.

**2.5 In vitro Assays**

To investigate the immunological mechanisms associated with the allogeneic graft response, spleens were removed from grafted animals and splenocytes analysed in vitro. For this part of the study, C57Bl/6 mice were used as graft recipients and $BDF_1$ mice as donors.

### 2.5.1 Washing Medium

Cells were washed in Hanks Balanced Salt Solution (Life Technologies Ltd.) with 2 % fetal calf serum (FCS) (Sigma, St. Louis, Missouri, U.S.A.) and 100 u/ml of penicillin and 100 μg/ml of streptomycin (both Life Technologies Ltd.).

### 2.5.2 Standard Medium

Cells were cultured in standard medium made up of RPMI-1640 containing 5 x $10^{-2}$ M mercaptoethanol, 2mM L-glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin (all Life Technologies Ltd.) with 5 % fetal calf serum (Sigma).

## 2.6 Mixed Lymphocyte Reaction

Spleens were removed from animals at various times after grafting and spleen cells used in mixed lymphocyte reactions to assess T cell proliferation in response to donor alloantigen. Single cell suspensions of spleen cells were obtained by passing spleens through a sterile fine wire mesh and then washing the cells 4 times in washing medium. Spleen cells were used as responders and were resuspended in standard medium at a final concentration of 2 x $10^6$ cells/ml.

Stimulator cells were harvested from the spleens of $BDF_1$ mice, rubbed through a wire mesh, washed 3 times and then resuspended in standard medium. Stimulator cells were irradiated with 2000R, counted and resuspended at a final concentration of 4 x $10^6$ cells/ml in standard medium.

Cells were plated out in triplicate wells in 96 U-bottomed well sterile culture plates (Nunclon, Kamstrup, Denmark) with 2 x $10^5$ responder cells per well and 4 x $10^5$ stimulator cells per well, with a final volume of 200 µl/well. Negative controls with responder cells plus 100 µl of standard medium and positive controls with responder cells plus 100 µl of medium containing 10 µg/ml concanavalin-A (conA) (Life Technologies Ltd.) were also prepared. Cultures were incubated in a humidified atmosphere at 37°C in 5 % $CO_2$ and harvested at various time points.

Eighteen hours prior to harvesting, plates were pulsed with 1 µCi/well of $^3$H-thymidine, and then harvested using a Skatron semiautomatic cell harvester (Skatron Instruments, As, Norway). Filter papers were allowed to dry and then analysed on a Betaplate Beta scintillation counter (Wallac, Finland) and expressed as a mean of counts per minute.

**2.7 Cytokine Assays**

Supernatants from MLRs were analysed to determine the cytokines produced by responder cells when re-stimulated in vitro by donor antigen. Cultures were prepared as for MLRs and supernatants were harvested at various time points. After harvesting, supernatants were centrifuged at 1200 rpm to remove any cellular debris and then stored in aliquots at -70 °C until used for cytokine analysis.

For cytokine analysis, samples were thawed and the cytokine content quantified using sandwich ELISAs to detect IL-2, IL-4, IL-5, IL-10 and IFN-γ. All analyses were performed using capture and detection monoclonal antibodies from

Pharmingen (San Diego, U.S.A.) and compared against known concentrations of recombinant mouse cytokines used as standard concentrations (Pharmingen).

Flat bottomed microtitre plates (Immulon 4, Dynatech Laboratories, Chantilly, Virginia, U.S.A.) were coated with capture antibody at the concentrations shown in table 2.1 made up to 50 µl in a coating buffer of 0.1M $NaHCO_3$ at pH 8.2 and incubated overnight at 4 °C. Plates were washed twice with phosphate-buffered saline (PBS) plus Tween 20 (0.05%) and then blocked with 200 µl of PBS / 10 % FCS solution and incubated for one hour at 37 °C. Plates were washed twice and then standards and samples added: 100 µl of standard cytokine solution were added, in triplicate wells, starting at the concentration shown in table 2.2 and then diluted in doubling dilutions with PBS / 10 % FCS.

Undiluted culture supernatant, 100 µl of each test sample, was added, again in triplicate, and plates incubated at 37 °C for 3 hours. Plates were washed 4 times and then biotinylated anti-cytokine detecting monoclonal antibody, diluted in PBS / 10 % FCS , added at the concentrations shown in table 2.1 and plates incubated for a further hour.

Plates were washed 6 times, extravidin-peroxidase 2 µg/ml (Sigma) in PBS/ 10 % FCS added and plates incubated for one hour. Thereafter, plates were washed 8 times and 100 µl of 3,3,5,5 tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, U.S.A.) added and the colour allowed to develop. The reaction was stopped with 100µl of 0.2M $H_2SO_4$ per well, after colour changes had developed, and then plates read on a Dynatech MR 5000 ELISA plate reader using Biolinx software programme

(Dynatech Laboratories) for analysis of results. Plates were read at a wavelength of 450 nm. Cytokine concentrations were calculated with reference to standard curves constructed using recombinant cytokines (Pharmingen). The results of triplicates of test samples were quantified and expressed as a mean.

### 2.8 Assays for Cytotoxicity

Spleen cells from treated animals were assessed for their ability to effect cell-mediated cytotoxicity of donor lymphoblasts and NK-susceptible target cells.

### 2.9 Specific Cell-Mediated Cytotoxicity

Specific host anti-donor CTL activity was quantified using chromium release cytotoxicity assays. Two different target cell types were used.

#### 2.9.1 Preparation of Cells

The mouse mastocytoma cell line P815, which expresses $H2^d$ was used as the allospecific target cell. Cells were maintained in vitro and used when required. In addition, DBA/2 conA blasts were also used as allospecific targets. Spleens were harvested from DBA/2 mice and a single cell suspension prepared as before. Cells were cultured for 72 hours in standard medium which had 10 % FCS and 5 μg/ml conA added. The cultured cells were then harvested, washed and resuspended in standard medium without FCS. Target cells were then incubated for 1 hour at 37°C with 50 μCi of $^{51}Cr$ per $2 \times 10^7$ cells.

Spleen cells from experimental animals were used as responder cells to assess specific CTL activity. Spleens were removed from grafted animals and

single cell suspensions prepared as described before, with cells used at a final concentration of 1.3 x $10^7$ cells/ml in standard medium.

### 2.9.2 Cytotoxic T Lymphocyte Assays

Responder cells were cultured in 96 well V-bottomed microtiter plates (Titertek, Flow Laboratories, Richmansworth, U.K.), with 75 µl of 1.3 x $10^7$ cells/ml. in each well and then in doubling dilutions for each sample. Triplicates of each sample were cultured. 75 µl of target cells at a concentration of 1.3 x $10^5$ cells/ml. were added to each well to achieve effector to target cell ratios of 100:1, 50:1, 25:1, 12.5:1, 6.25:1 and 3.125:1 for each sample. Negative controls of target cells with no responders, plus positive controls of target cells with 75 µl of Triton X (Sigma) were also set up.

Culture plates were centifuged briefly (1000 rpm for 1 minute) and then incubated for 4 hours at 37 °C. Supernatants were harvested, taking care not to disturb the cell pellet at the bottom of the culture well. Samples were analysed for chromium release with a 1282 Compugamma Universal Gamma Counter (Wallac). The percentage cytotoxicity was calculated using the formula:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

### 2.10 Natural Cytotoxicity

NK cell activity was measured in experimental animals using a chromiun release cytotoxicity assay. YAC-1 cells were used as specific targets for NK cells (Williamson et al, 1996). YAC-1 cells were suspended in standard medium

without FCS and incubated for 1 hour at 37 °C with 50 μCi of $^{51}Cr$ per 2 x $10^7$ cells.

Effector cells were prepared from the spleens of treated animals as described before and resuspended in standard medium at a final concentration of 1.3 x $10^7$ cells/ml. 75 μl of effector cells were plated out onto microtiter plates in triplicate and then in doubling dilutions to give final effector to target cell ratios as before. YAC-1 target cells were added (75 μl of 1.3 x $10^5$ cells/ml) and the plates spun for 1 minute at 1000 rpm. Controls for maximum and spontaneous release were also prepared and plates were then incubated for 4 hours at 37 °C. Supernatants were harvested and analysed and % cytotoxicity calculated as before.

**2.11 Assays for Microchimerism**

The persistence of injected donor cells in treated animals was assessed by flow cytometric fluorescence activated cell sorting (FACS) analysis. C57Bl/6 animals were injected with 5 x $10^7$ donor $F_1$ splenocytes, and sacrificed 24 hours later. Other animals were treated with either 0.5 mg. anti-IL-12 ip. or 0.5 mg. goat IgG ip. 24 hours prior to spleen cell injection, and again animals were sacrificed 24 and 72 hours after donor cell treatment.

Spleens and lymph nodes were removed from animals and single cell suspensions prepared. Cells were resuspended in PBS /2% FCS at a final concentration of 2 x $10^7$ cells/ml. 50 ml aliquots of each sample were incubated with antibody directed against donor MHC. Thus, 1 μl of biotinylated anti-$H2D^d$ (Pharmingen) was incubated with samples at 4 °C for 40 minutes in the dark to label any persisting $BDF_1$ cells ($H2^{bd}$) in C57Bl/6 recipients ($H2^b$). Positive

controls, labelling with 4 µl of biotinylated anti-$H2D^b$ (Pharmingen) were also prepared. Further samples were incubated with anti-CD 3 antibody (Pharmingen) in order to differentiate the lymphocyte population from any red blood cells.

Following incubation with antibodies, samples were washed twice with PBS / 2% Bovine Serum Albumin (Sigma) and then 10 µl of Streptavidin R. Phycoerythrin (Sigma) or FITC (Vector Laboritories, Peterborough, UK) added to each tube and again incubated at 4 $^{o}$C for 40 minutes. Samples were washed in PBS / BSA twice and then resuspended in PBS.

Analysis of samples was carried out using a Coulter Epics-XL flow cytometer (Hialileah, Florida, U.S.A.).

**2.12 Statistics**

Survival of skin grafts in each of the experimental groups was compared using a Mann-Whitney U-test. This generated a p value for the comparison of 2 groups and differences were deemed to be significant when $p<0.05$.

For in vitro assays, results were taken as the mean of three different experiments. The standard deviation of these results was calculated and results expressed as the mean +/- one standard deviation. Values for cytokine production in vitro by cells from treated animals were compared at the various time points. Cytokine levels in the treatment groups were compared using the Wilcoxon matched pairs test and differences were taken to be significant when $p<0.05$.

**Table 2.1**

| | Capture mAb µg/ml | Antibody Isotype (clone) | Detection mAb µg/ml | Antibody isotype (clone) |
|---|---|---|---|---|
| IL-2 | 2 | IgG2a (JES6-1A12) | 1 | IgG2b (JES6-5H4) |
| IL-4 | 2 | IgG2b (BVD4-1D11) | 1 | IgG1 (BVD6-2462) |
| IL-5 | 4 | IgG1 (TRFK5) | 4 | IgG2a (TRFK4) |
| IL-10 | 4 | IgG1 (JES5-2A5) | 2 | IgM (SXC-1) |
| IFN-γ | 2 | IgG1 (R4-6A2) | 1 | IgG1 (XMG1.2) |

Concentrations of capture and detection antibodies used in ELISAs. All were rat anti-mouse monoclonal antibodies and detection antibodies were all biotinylated. The cell clones used to produce the antibodies are shown in brackets.

**Table 2.2**

| Cytokine | Standard Samples |
|---|---|
| IL-2 | 200 u/ml |
| IL-4 | 200 u/ml |
| IL-5 | 200 u/ml |
| IL-10 | 40 ng/ml |
| IFN-γ | 200 u/ml |

Concentrations of recombinant mouse cytokines used in ELISAs for the generation of standard curves.

# CHAPTER 3

# EFFECTS OF ANTI-IL-12 ANTIBODY ON SKIN GRAFT SURVIVAL

## 3.1 Introduction

As already described, IL-12 is a key immunoregulatory cytokine, playing a central role in the differentiation of Th0 cells into committed Th1 cells (Trinchieri, 1995). The Th1 response is associated with cell mediated immunity and a DTH response, and this is characteristic of the response generated during allograft rejection (Hall, 1991). Neutralising endogenous IL-12 has been shown to reduce or prevent the Th1 response generated in GVH disease, and in other models of immune-mediated disease, and to have significant effects on the subsequent pathological process (Williamson et al, 1996, Gately et al, 1998).

The effect of neutralising endogenous IL-12 on allograft survival was, therefore, investigated. Allograft survival in a semi-allogeneic mouse skin graft model was assessed to determine whether antagonism of endogenous IL-12 would produce effects on allograft survival, and to determine whether this was associated with a down-regulated Th1 response or the generation of a Th2 response.

## 3.2 Effects of Anti-IL-12 Antibody Alone on Skin Graft Survival

Tail skin was taken from $BDF_1$ animals and grafted on to the thorax of either C57Bl/6 or DBA/2 recipients as described in materials and methods. After removal of bandages, skin graft survival was assessed daily and rejection defined as loss of greater than 80% of the grafted tissue (Azuma et al, 1989). $BDF_1$ skin grafts were rejected rapidly by unmodified C57Bl/6 recipients, with a mean survival time (MST) of 11.8 days (n=11). $BDF_1$ donor skin grafted onto DBA/2 recipients was rejected less rapidly with a MST of 13.2 days (n=6). Syngeneic

grafts onto either parental strain survived indefinitely (n=6). These results are shown in Fig. 3.1 and Fig. 3.2.

C57Bl/6 and DBA/2 mice were treated with anti-IL-12 antibody prior to skin grafting to assess the effects of neutralising endogenous IL-12 on skin allograft survival. Mice were treated with 0.5 mg. of a goat anti-mouse IL-12 polyclonal antibody on day -1, 2, 5 and 8 with grafting on day 0 (Figs 3.1 and 3.2). There was no prolongation of graft survival in either of the groups treated with the neutralising antibody. Skin grafts in the C57Bl/6 group had a MST of 11.25 days (n=4) and those in the DBA/2 group had a MST of 12.8 days (n=5). When these results are compared statistically using the Mann-Whitney U test there is no significant difference between unmodified graft rejection and rejection in recipients treated with anti-IL-12 antibody in either strain combination. From these results, it can be concluded that treatment with a neutralising polyclonal anti-IL-12 antibody on its own is not sufficient to prolong allograft survival in this model.

### 3.3 Pre-treatment with Donor Spleen Cells

Donor-specific blood transfusion or donor antigen pre-treatment has been used extensively as a method for prolonging allograft survival in experimental models (e.g Takeuchi et al, 1992, Gorczynski et al, 1996, b, Wood and Morris, 1995). This finding does have some clinical relevance as it has been observed that patients who received multiple blood transfusions prior to renal transplantation have greater graft survival rates compared to those who have not (Dossetor et al, 1967, Chavers et al, 1997). In addition, it has been demonstrated that transfusion

of patients with donor blood prior to renal transplantation increases allograft survival (Singal et al, 1985).

In experimental murine models using minor MHC mismatched strains, intravenous injection of unaltered lymphoid cell populations leads to prolonged survival of allogeneic skin grafts (e.g. Heeg and Wagner, 1990). In this study mice were treated with 5 x $10^7$ donor-strain spleen cells injected intravenously 7 days prior to skin grafting. This method has been shown to prolong skin graft survival in a minor mismatch model on a C57Bl/6 background (Hori et al, 1992). Results from pre-treatment of C57Bl/6 mice with donor spleen cells prior to grafting with $BDF_1$ skin are shown in Fig. 3.3. The MST of grafts was 9.9 days (n=9) and this is not significantly different from unmodified graft recipients. Similarly, with DBA/2 mice pre-treated with $BDF_1$ spleen cells and then grafted, MST was 11.2 days (n=11) and again this is not significantly different from unmodified DBA/2 graft recipients (Fig. 3.4). Thus, in this experimental model, pre-treatment with donor-specific antigen 7 days prior to grafting did not significantly alter graft rejection.

### 3.4 Timing of Donor Spleen Cell Pre-treatment

The timing of pre-treatment with donor antigen has been shown to be important in the outcome after transplantation (Wood and Morris, 1995). To investigate the lack of effect from donor cell pre-treatment 7 days prior to grafting, other C57Bl/6 mice were pre-treated with 5 x $10^7$ $BDF_1$ cells injected intravenously 4 days prior to grafting with $BDF_1$ tail skin. Allograft survival in this group was 12.5 days (n=6) and this is not significantly different from graft

survival in unmodified recipients or in the group pre-treated with donor cells 7 days prior to grafting.

### 3.5 Combined Treatment with Anti-IL-12 Antibody and Donor-Specific Spleen Cells

Since neither treatment with anti-IL-12 antibody nor donor-specific spleen cells significantly altered skin allograft survival when given alone, mice were treated with a combination of the two therapies to determine whether they could prolong allograft survival together. Other workers have shown that combined treatment with donor alloantigen plus some other immunoregulatory therapy can prolong allograft survival. For example, murine heart grafts across a fully allogeneic mismatch survive indefinitely when mice are pre-treated with donor-specific transfusion plus anti-CD4 mAb (Bushell et al, 1995). In the model which they described, the timing of the donor antigen and the mAb therapy is critical for tolerance induction. Similarly, in a rat vascularised heart model, treatment with donor-specific transfusion plus intragraft continuous IL-4 therapy along with cyclosporin significantly prolongs graft survival (Levy and Alexander, 1995).

Mice of both C57Bl/6 and DBA/2 strains were therefore treated with donor antigen in the form of $5 \times 10^7$ $BDF_1$ spleen cells injected intravenously one week prior to skin grafting. Anti-IL-12 antibody was given as 0.5 mg. ip. on day -8, -5, -2 and 1 with skin grafting on day 0. These results are shown in Fig. 3.5 and 3.6. In the C57Bl/6 group, skin graft survival was prolonged, with a MST of 17.0 days (n=9). These results, when compared with those for unmodified skin graft recipients, showed that this prolonged graft survival was statistically significant

(Mann-Whitney U test, $p<0.002$). As a control for the anti-IL-12 antibody, C57Bl/6 mice were treated with $BDF_1$ donor cells plus a purified goat IgG (Sigma). This was given, like the anti-IL-12 antibody, as 0.5 mg. on day -8, -5, -2 and 1. Skin graft survival in this group was not prolonged with a MST of 11.8 days (n=7). When compared statistically, this was not significantly different from allograft rejection in unmodified graft recipients, but was significantly reduced compared to treatment with donor spleen cells plus anti-IL-12 antibody ($p<0.002$).

DBA/2 mice treated with both $BDF_1$ spleen cells and anti-IL-12 antibody also showed prolonged skin graft survival, with the MST being 17.7 days (n=6). When compared with unmodified DBA/2 graft recipients, the treated group had significantly prolonged survival ($p<0.002$).

Thus treatment with a combination of anti-IL-12 antibody and donor spleen cells significantly prolongs skin graft survival in both of the parental strains.

### 3.6 Timing of Anti-IL-12 Antibody Treatment

Many other studies have demonstrated that the timing of potentially tolerising therapies is critical to their success (e.g. Pearson et al, 1992). The importance of the timing of anti-IL-12 antibody treatment, relative to donor splenocyte injection, was therefore assessed. C57Bl/6 mice were given $5 \times 10^7$ $BDF_1$ splenocytes iv. 7 days prior to grafting. They were then treated with 0.5 mg. anti-IL-12 antibody ip. on days -1, 2, 5 and 8, that is starting one week after the time which had already been shown to prolong allograft survival (Fig. 3.7). Mean survival time of grafts in animals treated with the later anti-IL-12 antibody

injections was 13.8 days (n=6). When compared with graft survival in the group treated with donor cells plus the earlier anti-IL-12 antibody injections, there is a difference, with the earlier injection group surviving significantly longer than those given the anti-IL-12 antibody later ($p<0.05$). In addition, when graft survival in the group treated with donor cells plus later anti-IL-12 antibody injections is compared to that of unmodified graft recipients there is no significant difference, indicating that this treatment confers no benefit to allograft survival.

### 3.7 Treatment with Additional Donor Antigen

The effect of giving an additional injection of donor spleen cells was next investigated. Some workers have indicated that persistence of donor antigen may be important for allograft survival, and this may be one mechanism whereby donor-specific transfusion has its effect (e.g. Gorczynski et al, 1996, b, Bushell et al, 1995). Thus, giving an additional treatment of donor spleen cells during the pre-treatment protocol may be anticipated to have a beneficial effect on allograft survival.

C57Bl/6 mice were pre-treated with the same regime which had been shown to significantly prolong allograft survival, namely 0.5 mg. anti-IL-12 antibody on day -8, -5, -2 and 1 with $5 \times 10^7$ $BDF_1$ spleen cells on day -7. In addition, mice were treated with a further iv. injection of donor spleen cells on the day of grafting, some 7 days after the first spleen cell injection. Results are shown in Fig. 3.8. Skin graft survival in this group was significantly prolonged compared to unmodified graft recipients, with a MST of 17.0 days ($n=5$, $p<0.05$). However,

this was exactly the same as in the group treated with a single injection of donor cells at day -7, plus anti-IL-12 antibody.

An additional treatment with donor-specific antigen on the day of grafting did not, therefore, significantly enhance graft survival compared to the group treated with a single spleen cell injection when both groups also received anti-IL-12 antibody.

### 3.8 Grafting Across a Fully Allogeneic Mismatch

Skin graft survival across a full MHC mismatch was also assessed by grafting skin from one parental strain onto the other parental strain (Figs. 3.9 and 3.10). DBA/2 tail skin was grafted onto C57Bl/6 recipients and the MST was 10.0 days (n=10). When this was compared to $BDF_1$ tail skin grafted onto C57Bl/6 recipients (MST 11.8 days), there was no significant difference. When C57Bl/6 skin was grafted onto DBA/2 recipients the MST was 11.8 days (n=10) and this too was not significantly different from $BDF_1$ grafts to DBA/2 recipients (MST 13.2 days).

The effect of antigen pre-treatment, either with or without anti-IL-12 antibody was also assessed (Fig 3.11). C57Bl/6 mice pre-treated with $5 \times 10^7$ DBA/2 spleen cells 7 days prior to grafting were transplanted with DBA/2 skin grafts. The MST was 9.75 days (n=4) and this was not significantly different from unmodified graft recipients. Mice were also treated with donor specific spleen cells plus anti-IL-12 antibody on day -8, -5, -2 and 1 and graft survival assessed. MST in this group was 11.8 days (n=5) and this also was not significantly different from unmodified graft recipients. This was, however, significantly

reduced compared to the semi-allogeneic $BDF_1$ graft onto C57Bl/6 animals pre-treated with donor-specific spleen cells plus anti-IL-12 antibody ($p<0.05$). Thus pre-treatment with donor strain spleen cells plus anti-IL-12 antibody was not able to prolong fully allogeneic skin graft survival in this strain combination.

### 3.9 Discussion

As discussed earlier, Th1 cells are the principal mediators of allograft rejection as they promote the cell mediated immune response, particularly CTL, and these serve as the terminal effector cell types in rejection (Hall, 1991). The hypothesis that polarisation of a Th1 response to a Th2 response may favour allograft survival has been suggested (Takeuchi et al, 1992, Dallman et al, 1991, Gorczynski et al, 1996, a). Reciprocal regulation of this response exists, whereby an up-regulated Th2 response leads to a down-regulated Th1 response (Mosmann and Sad, 1996). It may be anticipated, therefore, that, by preferential activation of an antigen-specific Th2 response, the destructive Th1 response will be inhibited and allograft tolerance induced. Specific therapies which promote the polarisation of the immune system to a Th2 response are therefore being investigated for their role in allograft survival. IL-12, as a key regulator of the Th1 response, is a suitable target molecule around which therapeutic strategies can be manipulated.

In the studies described here using a model of semi-allogeneic tail skin grafts in a murine host, neutralising endogenous IL-12 did not prolong allograft survival. There is a number of possibilities as to why neutralising endogenous IL-12 by itself failed. The amount of anti-IL-12 antibody used in this study may not have been sufficient to neutralise all endogenous IL-12. Against this, however, is

the fact that this same polyclonal goat anti-mouse IL-12 antibody has been used in another experimental mouse model and was effective at inhibiting a Th1 response and generating a Th2 response (Tripp et al, 1994). In addition, the dose used here was 4 times that which was effective in these experiments. Furthermore, as discussed in later chapters, while anti-IL-12 antibody alone did not prolong allograft survival, it was effective in deviating the cytokine response away from Th1 towards Th2. Thus, while it was not formally shown that all endogenous IL-12 was neutralised by this treatment, strong supportive evidence exists to suggest that it was.

Despite neutralisation of endogenous IL-12, skin allograft survival was not prolonged. These findings are in agreement with those of Piccotti et al (1996) who used the same polyclonal goat anti-mouse IL-12 antibody from Dr. M. Gately, as well as IL-12 p40 homodimer in a murine vascularised cardiac allograft model. The IL-12 p40 homodimer is a soluble form of the p40 chain of IL-12 which competes with the cell surface IL-12 receptor, blocking the effects of IL-12. Thus they used 2 different methods for blocking the action of IL-12 in a heart allograft model, and found that inhibition of IL-12 around the time of grafting fails to prolong survival. In fact, neutralising IL-12 appears to cause a modest acceleration in rejection. Similarly, Li et al (1998) demonstrated that pre-treatment of recipients of pancreatic islet cell grafts with a neutralising anti-IL-12 antibody was unable to prolong allograft survival in full MHC-mismatch mouse models. Interestingly, however, such treatment was capable of prolonging graft survival in minor mismatch combinations. Thus neutralising endogenous IL-12 alone does not prolong allograft survival in MHC-mismatch strain combinations.

Piccotti et al (1996) also investigated the effect of anti-IL-12 antibody on cytokine gene expression within the rejecting cardiac allografts and found that in unmodified mice, cardiac allograft rejection is characterised by a Th1 response, demonstrated by detectable levels of IFN-γ, with little expression of Th2 cytokine message. Conversely, in animals treated with anti-IL-12 antibody, IL-4 and IL-10 expression is detectable, indicating an increase in Th2 cytokine message. IFN-γ expression is not, however, inhibited. Thus, while treatment with anti-IL-12 antibody induces Th2 cytokine expression, it does not completely prevent the generation of Th1 cytokines. Recently, IFN-γ inducing factor (IGIF or also named IL-18) has been identified (Okamura et al, 1995, Okamura et al, 1998). This cytokine augments NK cell activity and is produced by activated macrophages. IGIF may, therefore, mediate a second pathway for inducing IFN-γ production by Th1 cells (Okamura et al, 1998). Neutralising IL-12 alone may not, therefore, be sufficient to completely inhibit the generation of IFN-γ in an allograft model as there appears to be some redundancy within cytokine pathways. Thus a pathway for Th1 cytokine generation would still exist and this may be sufficient to effect allograft rejection.

The ability of IL-12 p40 knockout mice to reject an allograft has also been studied (Piccotti et al, 1996). When grafted with a vascularised allogeneic heart, IL-12 knockout mice reject their grafts at the same time as wild type animals treated with anti-IL-12 antibody, that is slightly earlier than unmodified controls. In addition, lymphocytes taken from IL-12 knockout grafted mice and re-stimulated in vitro with donor antigen are able to produce significant amounts of IFN-γ, indicating that IL-12 is not required for the generation of Th1 responses in

an allograft model. Cytokine data from the work done in this study is presented and discussed in later chapters. Thus there appears to be considerable complexity within cytokine responses, and blocking one specific cytokine may not be effective in completely abrogating a specific T-helper cell response, as other pathways may be activated which ultimately stimulate the same effector cells.

In this study donor spleen cell injection was not sufficient to prolong allograft survival in either semi- or fully allogeneic skin grafts. When donor-specific cells were given, no other form of immunomodulation was administered, and so host T cells, on encountering such antigen, would be able to react normally. In both C57Bl/6 and DBA/2 recipients pre-treated with $BDF_1$ spleen cells and then skin grafted, rejection occurred more quickly than in the unmodified grafted groups (MST 9.9 days in C57Bl/6 mice treated with spleen cells and 11.8 days in unmodified animals, and MST 11.2 days in DBA/2 mice pre-treated with donor cells compared to 13.2 days in unmodified graft recipients), although in neither strain combination were these differences statistically significant. It may be, therefore, that in this strain combination, pre-treatment with donor spleen cells alone only serves to sensitise the recipient and so make it reject the graft more quickly.

While the administration of donor antigen alone is, in certain experimental models, an effective strategy for the induction of peripheral tolerance to alloantigen, it does not result in tolerance in every situation. Indefinite survival of skin allografts is very difficult to achieve, as here. Other workers have, therefore sought to combine the benefits of immunological specificity achieved by using donor antigen pre-treatment with low levels of less specific immunosuppressive

therapies. For example, donor-specific transfusion plus cyclosporin treatment prolongs cardiac allograft survival in a rat model (Brunson et al, 1991). In the work of Wood's group, donor-specific transfusion combined with anti-CD4 mAb results in antigen-specific peripheral tolerance. They observed that the anti-CD4 mAb therapy induces short-term immunosuppression which is transient. If donor cells are given at the same time, however, while immunocompetence to other antigens recovers, as demonstrated by the ability to reject a third party heart graft, specific unresponsiveness to the donor antigen remains, and this results in peripheral tolerance to that alloantigen (Pearson et al, 1992). They have suggested that this specific unresponsiveness is due to the short-term persistence of donor-specific antigen in the host (Bushell et al, 1995).

In this study, combining 2 treatments, neither of which prolonged allograft survival alone, produced significant prolongation of skin graft survival. This prolongation of skin graft survival was found when $BDF_1$ skin was transplanted onto either the C57Bl/6 or DBA/2 parental strain when recipients were pre-treated with $BDF_1$ spleen cells plus anti-IL-12 antibody. Unlike the other combination methods described above, however, neither of these therapies are non-specifically immunosuppressive and therefore other mechanisms must be involved in the prolongation of graft survival.

Anti-IL-12 antibody has been shown to induce polarisation to a Th2 response in a number of experimental models, including murine infections and graft versus host disease. However, in a murine vascularised cardiac allograft model, while expression of Th2 cytokines was up-regulated, allograft survival was not increased by treatment with anti-IL-12 antibody alone (Piccotti et al, 1996). As

discussed earlier, pre-treatment with donor cells may also polarise the immune response in a Th2 direction (Babcock and Alexander, 1996). It may be, therefore that these 2 therapies are additive and, when given together, they polarise the immune response more strongly in a Th2 direction, and this is sufficient to prolong allograft survival. Thus, treatment with one therapy alone may not be sufficient to polarise all alloreactive T cells to a Th2 response, while both given together are. This is supported by the findings of Gorczynski et al (1996, b) who found that when mice were treated with donor strain dendritic cells injected into the portal vein, allogeneic skin graft survival was prolonged. When immunocompetent cells were removed from such animals and re-stimulated in vitro, they produced reduced levels of Th1 cytokines and increased levels of Th2 cytokines. When mice were treated with portal vein injection of dendritic cells plus an anti-IL-12 antibody and recombinant IL-13, skin graft survival was further prolonged. Cells removed from these animals and re-stimulated in vitro produced even less of the Th1 cytokines and even greater levels of IL-4 and IL-10 compared to those from animals given portal vein injections alone. Piccotti et al (1997, b) demonstrated that pre-treatment with a combination of IL-12 p40 homodimer, an IL-12 antagonist, plus a depleting anti-CD8 mAb in a mouse cardiac allograft model can significantly prolong allograft survival. Treatment with either the p40 homodimer or anti-CD8 mAb alone does not prolong graft survival. They concluded that IL-12 antagonism inhibits the generation of $CD4^+$ Th1 cells. However, $CD8^+$ T cells are unaffected and these cells alone are capable of inducing allograft rejection in this model. Inhibiting $CD4^+$ Th1 cells by IL-12 antagonism, and depleting $CD8^+$ T cells together is, however, sufficient to prolong

heart graft survival. Thus there is evidence that different treatments can have additive effects on the polarisation of the allograft rejection response and this can increase allograft survival.

The finding that delaying anti-IL-12 antibody treatment in this model did not prolong skin graft survival is, however, at variance with this. When mice were treated with donor spleen cell injection on day -7 and anti-IL-12 antibody treatment delayed until day -1, 2, 5 and 8 (with grafting on day 0), skin allograft survival was not significantly prolonged compared to that in unmodified graft recipients. If both treatments were additive and act by polarising the immune response in the Th2 direction, one would anticipate that survival would also be increased by late treatment with anti-IL-12 antibody. The potential exists, therefore, that polarisation of the immune response is not ultimately responsible for graft prolongation and that other mechanisms may be involved.

Allogeneic cells, when injected into full histocompatibility mismatched hosts are rapidly eliminated. Sheng-Tanner and Miller (1992) demonstrated that injection of DBA/2 lymphoid cells into C57Bl/6 recipient mice leads to rapid destruction of the donor cells. By labelling donor cells with FITC, the fate of such cells can be followed and it was found that at 3 days following injection, no donor cells can be detected within the circulating lymphocyte population. They concluded that the rapid elimination of fully allogeneic cells is due to NK cell killing because when NK cell activity is inhibited by treatment of recipient mice with antiasialo-GM1 mAb in a C57Bl/6 host, fully allogeneic Balb/c cells were detectable 3 days after injection. Interestingly, when they injected $BDF_1$ cells into C57Bl/6 recipients they could still detect $BDF_1$ cells at day 3. It was also

demonstrated that in this model, iv. injection of $BDF_1$ lymphoid cells leads to a prolongation of $BDF_1$ skin graft survival in DBA/2 recipients and that this was associated with donor cells persisting in the host for 3 days. They concluded that for donor cell injection to prolong allograft survival, donor cells must be able to survive within the host for at least 3 days. Sheng-Tanner and Miller also suggested that elimination of $BDF_1$ cells is also attributable to NK cell destruction. It was demonstrated that when recipient mice are treated with polymerised inosinc cytidine, a procedure which boosts host NK cell activity (Djeu et al, 1979), $BDF_1$ donor cells cannot be detected at day 3 and skin graft survival is not prolonged.

These results are obviously at variance with the graft survival data presented in this study. Sheng-Tanner and Miller found that $BDF_1$ cell infusion indefinitely prolonged $BDF_1$ tail skin survival in DBA/2 recipients. In this study, $BDF_1$ cell injection did not prolong allograft survival and, if anything, increased rejection rates. These differences are somewhat difficult to reconcile. In the experiments of Sheng-Tanner and Miller, however, donor cells were injected 4 days prior to grafting and, in addition, a combination of both spleen cells plus lymph node cells were injected after removal of red blood cells. It may be, therefore, that the differences which they observed are due to differences in the cell populations injected as in this study, a population of spleen cells, without removal of any red cells, were injected.

In addition, in an attempt to investigate their results, C57Bl/6 mice were injected with $BDF_1$ spleen cells 4 days prior to skin grafting and it was found that this did not prolong skin graft survival in this study. It must be assumed, therefore, that the cell population injected in the 2 different protocols was responsible for the

differences in graft survival observed. Hori et al (1989) found that after injection of an unpurified donor-specific spleen cell population 7 days prior to grafting a MHC class II disparate skin graft in a mouse model, allograft survival is not prolonged. However, when the donor cell population is passed over Sephadex G-10 columns to remove APCs, skin graft survival is significantly prolonged. Thus, the nature of the cell populations injected can have significant effects on allograft survival, and this may explain the differences observed between this work and that of Sheng-Tanner and Miller.

Other groups have highlighted the significance of persistence of donor cells in the host for induction of tolerance. Bushell et al (1995) determined that tolerance to allogeneic heart grafts in mice is due to prolonged survival of donor cells following anti-CD4 mAb therapy. When irradiated donor cells are injected, tolerance does not follow. However, when repeated doses of irradiated cells are given, tolerance ensues. They concluded that a single dose of irradiated cells does not remain within the host for sufficient time to generate donor-specific tolerance.

Persistence of donor cells may also be important in the model described in this work. There is some evidence to suggest that $BDF_1$ cells are eliminated by NK cells in C57Bl/6 mice (Sheng-Tanner and Miller, 1994). By inhibiting NK cell-mediated destruction of donor cells, the ability of injected donor cells to increase allograft survival may be enhanced. IL-12 is a potent activator of NK cells and was originally called NK cell stimulatory factor (Kobayashi et al, 1989). Blockade of endogenous IL-12 would, therefore, be expected to inhibit NK cell activity and possibly reduced NK cell killing of donor cells. This may allow

persistence of donor antigenic cells for long enough to allow prolongation of allograft survival.

In this model, the effect of donor-specific spleen cell injection plus anti-IL-12 antibody may not, therefore, be related to polarisation of immune response to Th2, but may be a consequence of the inhibitory effect of anti-IL-12 antibody on NK cells, allowing persistence of donor antigen. These possibilities have been analysed using in vitro methods and the results are presented in the following chapters.

Interestingly, in a fully allogeneic mismatch, treatment with donor-specific cells plus anti-IL-12 antibody did not prolong allograft survival. This may be due to the rapid destruction of donor cells by NK cells despite anti-IL-12 antibody treatment. Thus blocking of endogenous IL-12 activity may not be sufficient to prevent NK cell killing across a fully allogeneic mismatch. Similar results were found by Li et al (1998) in a murine pancreatic islet allograft model. They found that pre-treatment of graft recipients with anti-IL-12 antibody was able to induce a Th-1 to Th-2 shift within the graft but that this was not associated with prolonged allograft survival in a full MHC-mismatch model. However, such treatment in minor mismatch models, again inducing a Th1 to Th2 shift, was associated with prolonged allograft survival.

### 3.10 Summary of Results of Chapter 3

**1.** Pre-treatment of recipient mice with either a neutralising anti-IL-12 antibody or an intravenous injection of donor-strain spleen cells alone does not prolong skin graft survival in this semi-allogeneic mismatch model.

**2.** Pre-treatment of recipient mice with a combination of anti-IL-12 antibody and donor strain spleen cells does significantly prolong skin graft survival in this model.

**3.** The timing of treatment with anti-IL-12 antibody relative to donor spleen cell injection was important. Pre-treatment of mice with anti-IL-12 antibody on day -8, -5, -2 and 1 and donor spleen cell injection on day -7 prolonged skin graft survival, while delaying treatment with the anti-IL-12 antibody did not prolong graft survival.

**4.** Giving an additional donor spleen cell injection on the day of grafting after pre-treatment with anti-IL-12 antibody and donor spleen cell injection as before did not further increase skin graft survival.

**5.** Pre-treatment with a combination of anti-IL-12 antibody and donor spleen cell injection did not prolong skin graft survival in a fully allogeneic mismatch.

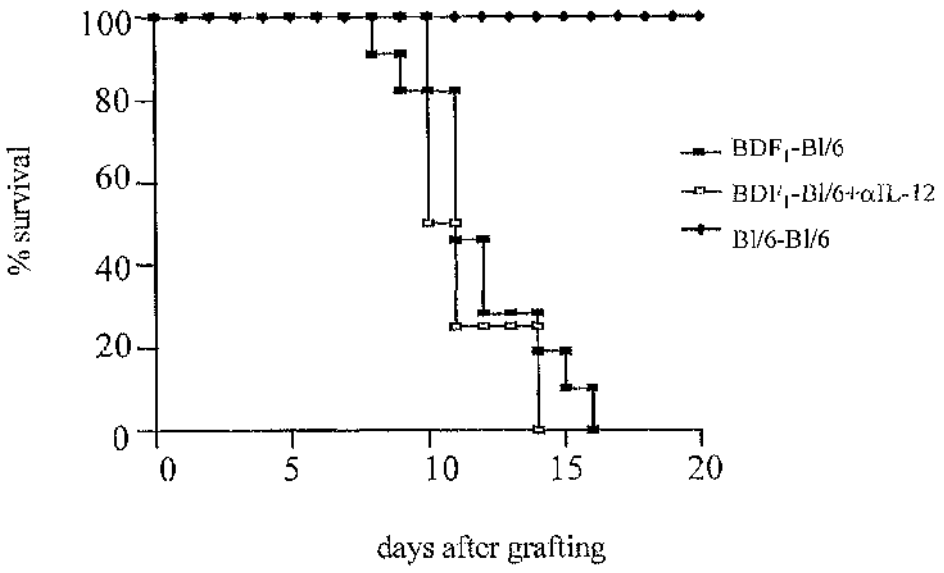



**Figure 3.1 Skin graft survival after treatment with anti-IL-12 antibody.** C57Bl/6 mice were grafted with tail skin from either $BDF_1$ donors (BDF1-Bl/6) or syngeneic animals (Bl/6-Bl/6). One group was treated with 0.5mg anti-IL-12 antibody on days -1, 2, 5 and 8 with grafting of BDF1 skin on day 0 (BDF1-Bl/6 + αIL-12).

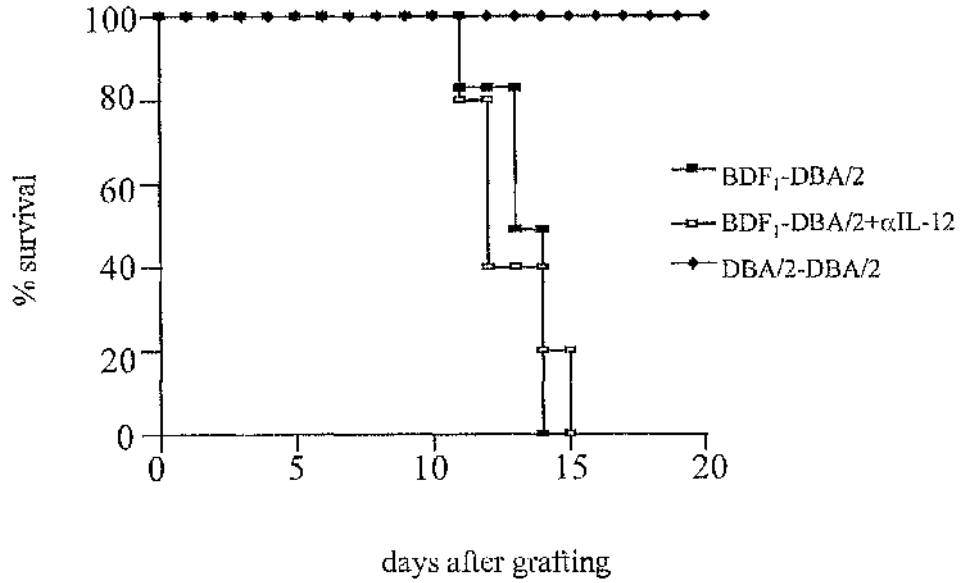



**Figure 3.2 Skin graft survial in DBA/2 mice treated with anti-IL-12 antibody.** DBA/2 mice were treated with anti-IL-12 antibody as in Figure 3.1.

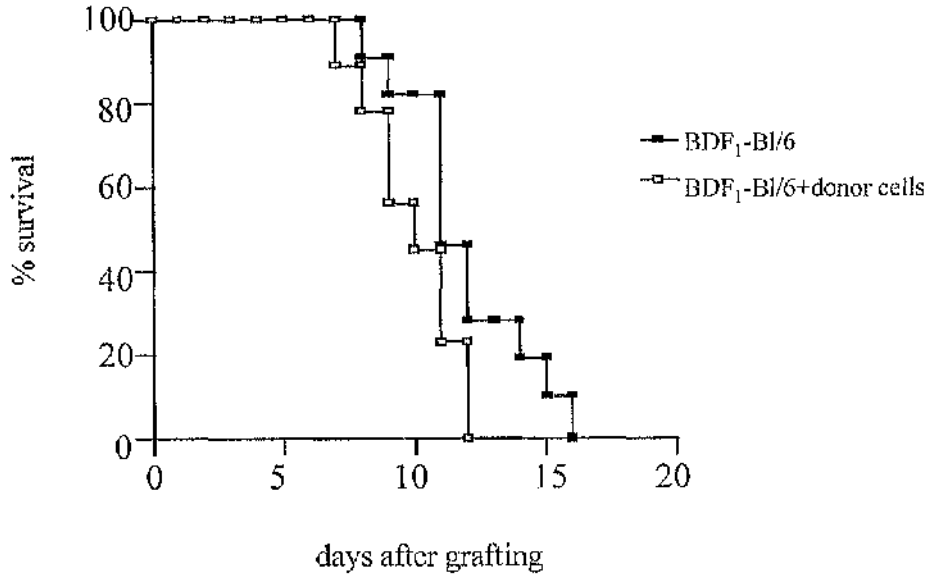



**Figure 3.3 Survival of skin grafts on Bl/6 recipients after treatment with donor antigen.** Mice were injected with 5 x $10^7$ $BDF_1$ splenocytes 7 days prior to skin grafting with $BDF_1$ skin ($BDF_1$-Bl/6 + donor cells).

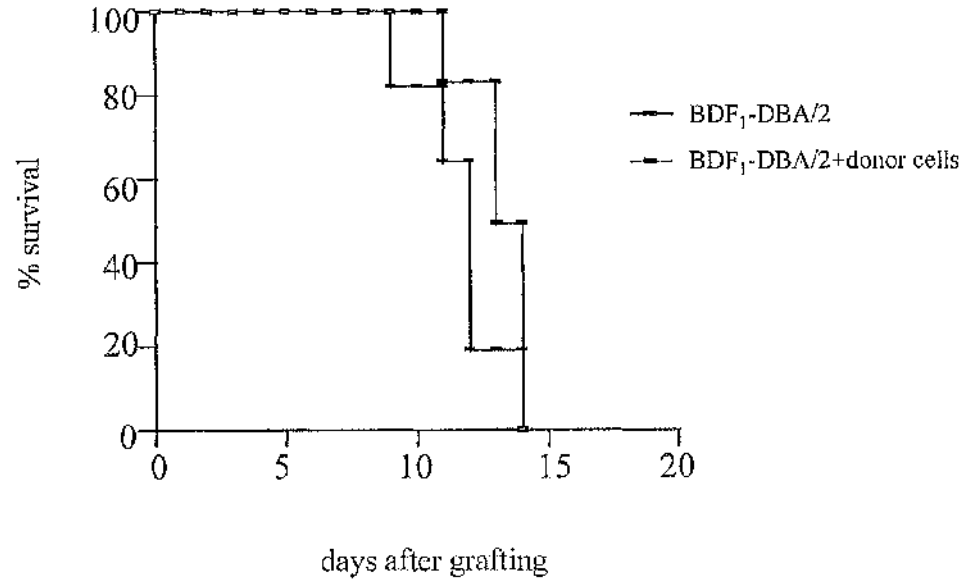



**Figure 3.4 Survival of skin grafts on DBA/2 recipients after treatment with donor antigen.** DBA/2 mice were treated as above.

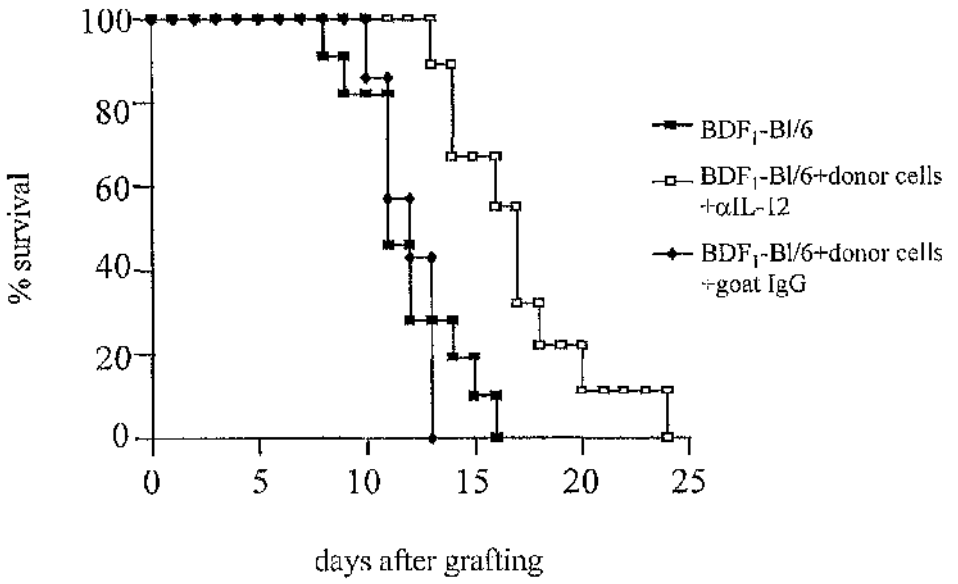



**Figure 3.5 Skin graft survival in C57Bl/6 mice treated with a combination of donor antigen plus anti-IL-12 antibody.** Mice were treated with donor spleen cells 7 days prior to grafting plus 0.5 mg. anti-IL-12 antibody on day -8, -5, -2 and 1 ($BDF_1$-Bl/6 + donor cells + αIL-12). Another group was treated with purified goat IgG plus donor spleen cells as a negative control ($BDF_1$-Bl/6 + donor cells + goat IgG).

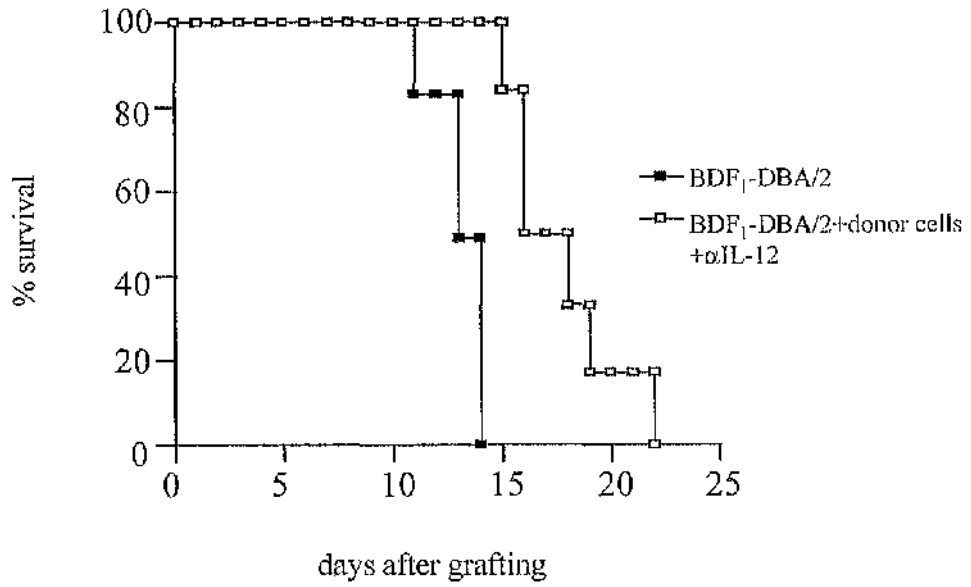



**Figure 3.6 Skin graft survival in DBA/2 mice treated with both donor antigen plus anti-IL-12 antibody.** The treatment regime was identical to that in Figure 3.5.

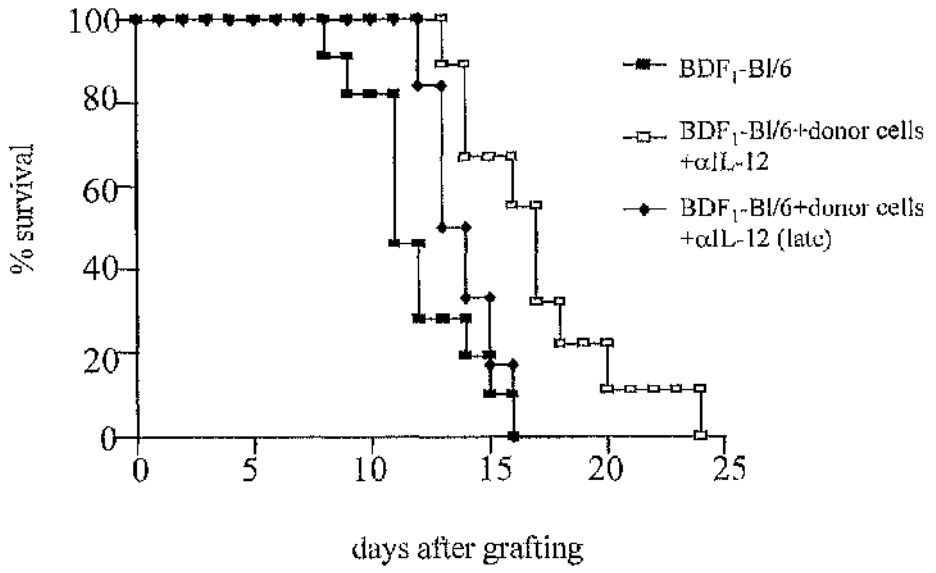



**Figure 3.7 Timing of treatment with anti-IL-12 antibody.** C57Bl/6 mice were grafted with $BDF_1$ tail skin after treatment with donor spleen cells on day -7 as before, plus anti-IL-12 antibody. This was given as the same dose as before, but either on days -8, -5, -2 and 1 or on days -1, 2, 5 and 8 (late group).

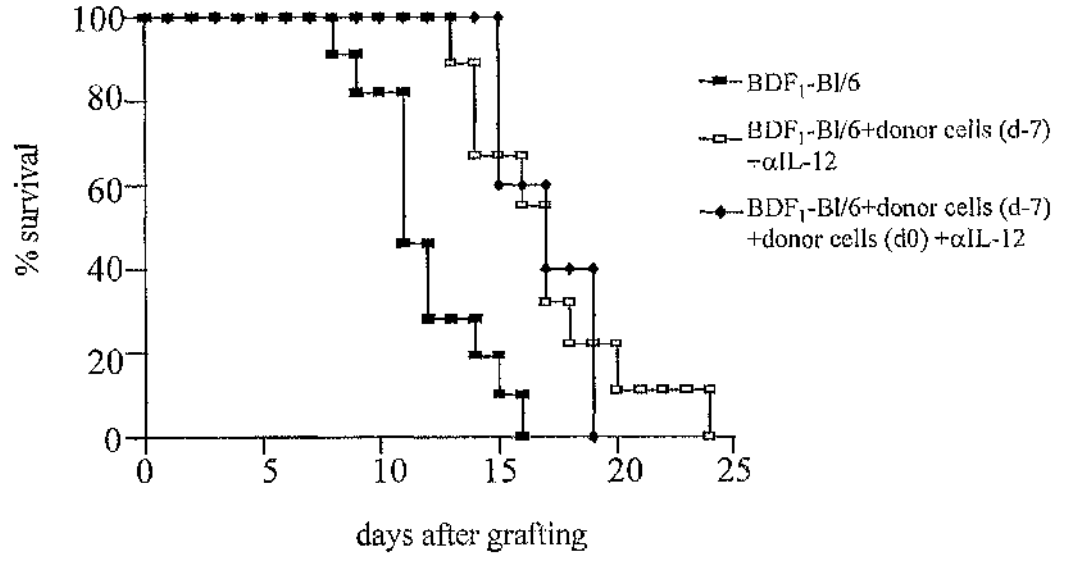
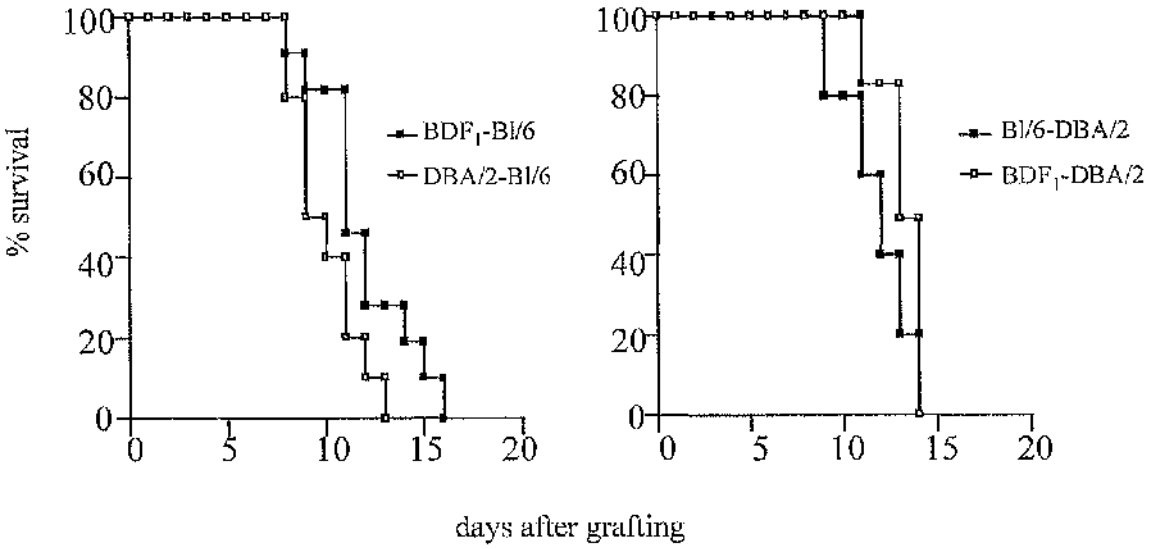



**Figure 3.8 Additional treatment with donor cells.** C57Bl/6 mice were grafted with $BDF_1$ tail skin and one group also treated with donor cells and anti-IL-12 antibody as before. A third group was given donor cells on day -7 and anti-IL-12 antibody plus a further injection of donor spleen cells on day 0 (donor cells d-7 & 0).




**Figures 3.9 and 3.10 Survival of fully allogeneic skin grafts.** C57Bl/6 mice were grafted with DBA/2 tail skin and vice versa. Results are compared with grafting of $BDF_1$ skin.

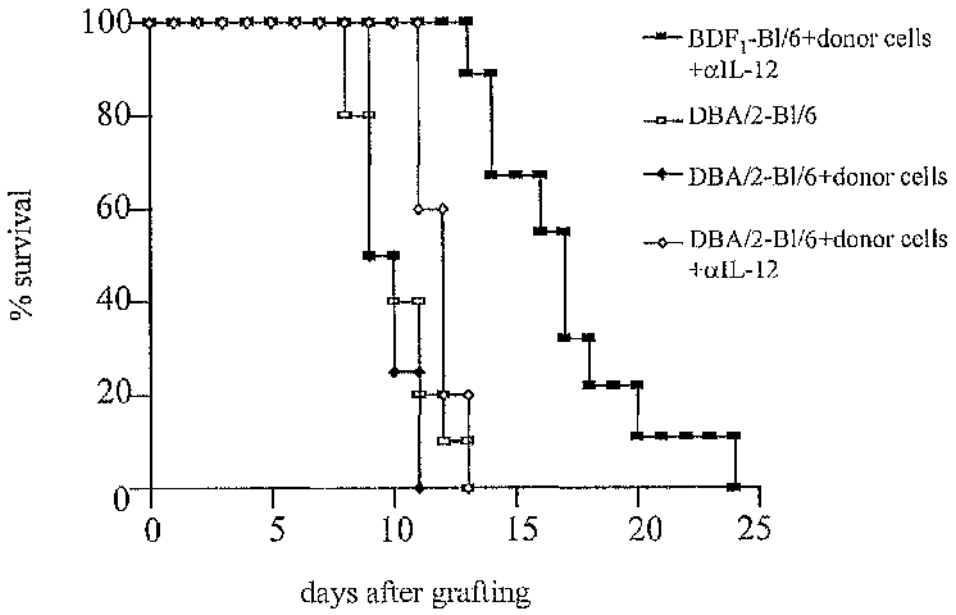



**Figure 3.11 Survival of fully allogeneic grafts after treatment of recipients.** C57Bl/6 mice were treated in the same manner as for assessment of semi-allogeneic graft survial.

# CHAPTER 4

# PROLIFERATION AND CYTOKINE PRODUCTION IN VITRO

## 4.1 Introduction

In order to analyse the immune mechanisms underlying prolonged graft survival compared to unmodified graft rejection, spleen cell cultures were prepared from C57Bl/6 mice which had received $BDF_1$ skin grafts. Cells from animals in the different treatment regimes were used in a variety of in vitro experiments.

In a mixed lymphocyte reaction (MLR), single cell suspensions of responder T cells are cultured with allogeneic stimulator lymphocytes. The activating stimulus is the foreign histocompatibility antigens on the stimulator cells and, in mice, both $CD4^+$ and $CD8^+$ T cells will proliferate, although $CD4^+$ T cells will proliferate to a greater extent (Kitagawa et al, 1991). For such proliferation to occur, a source of APCs is also required. In addition to producing a measure of proliferative responses to alloantigen, MLRs are also useful for the assessment of cytokine production by the proliferating responder cells. For this, supernatants can be harvested from cultures and analysed for specific cytokines.

## 4.1 Determination of Optimal Conditions for MLR

Spleens were removed from C57Bl/6 mice which had received no therapeutic intervention or else had received a $BDF_1$ skin graft 3 days previously, and a single cell suspension prepared as described earlier. Cells were cultured in 96 well culture plates at a concentration of $2 \times 10^6$ cells/ml. Stimulator spleen cells were removed from $BDF_1$ mice, irradiated with 20 Gy and added at a concentration of $4 \times 10^6$ cells/ml. Cultures were maintained for different lengths of time before $^3$H-thymidine, 1 μCi per well, was added. After a further 18 hours, cultures were analysed using incorporation of thymidine as a measure of proliferation.

With cells taken from naive, ungrafted animals, there were moderate levels of proliferation at the 5 time points assessed, namely 24, 48, 72, 96 and 120 hours in vitro. These results are shown in figure 4.1. With cells taken from animals which had been sensitised to donor antigen by means of a skin graft, proliferation was more pronounced. The counts obtained from these groups were compared with the counts obtained from wells containing responder cells from animals which had received a skin graft with no stimulators added and which thus acted as a negative control. At 24 hours, proliferation in the group which had received a skin graft was slightly greater than in the naive group and this in turn was slightly greater than in the unstimulated group, but actual counts were low at this early time point. At 48 hours in vitro, proliferation in the skin grafted group had increased to 3 fold greater than in the naive group and 5 fold greater than in the unstimulated group whilst at 72 hours proliferation in the skin grafted group had increased considerably to 5 fold greater than the naive group and 10 fold greater than in the unstimulated group. At 96 hours in vitro, the proliferation pattern in the 3 groups was similar to that at 72 hours and it was at these 2 time points that proliferation in the skin grafted group was at its greatest, with counts of 27 128 counts per minute (cpm) at 72 hours and 30 245 cpm at 96 hours. At 120 hours in vitro, proliferation in all groups had reduced considerably, although the pattern was the same as at the other time points. Values were calculated as the mean of 3 separate MLRs with 6 repetitions in each MLR.

As in vitro proliferative responses were most pronounced at 72 and 96 hours in vitro, it was decided in subsequent experiments to determine proliferative responses in each of the treatment groups at these 2 time points.

### 4.3 MLR with cells taken from pre-treated animals

Both untreated and pre-treated C57Bl/6 mice were grafted with $BDF_1$ tail skin and their spleens removed at various times after grafting. Mice were pre-treated with anti-IL-12 antibody alone, donor spleen cell injection alone or a combination of the 2 treatments, as detailed below. Single cell suspensions were prepared from the spleens of grafted animals and cultured with irradiated $BDF_1$ cells. In vitro proliferative responses were assessed at 72 and 96 hours. The various treatment groups which all received $BDF_1$ skin grafts were: (i) unmodified recipients of a $BDF_1$ graft; (ii) mice pre-treated with $5 \times 10^7$ $BDF_1$ spleen cells injected intravenously 1 week prior to skin grafting; (iii) mice pre-treated with donor spleen cells at day -7 and also given purified goat IgG, given as 0.5 mg. ip. on day -8, -5, -2 and 1 with grafting on day 0; (iv) animals pre-treated with donor cells on day -7 plus 0.5 mg. anti-IL-12 antibody ip. on day -8, -5, -2 and 1, and grafted on day 0; (v) mice treated with anti-IL-12 antibody alone on day -1, 2, 5 and 8, and grafted on day 0. The spleens were removed from animals 3, 7, 10 and 15 days after grafting and MLRs prepared as above. Results were calculated as the mean of 3 separate MLRs as before. The values expressed are the counts in each well minus the background counts from cultures of responder cells in medium alone, with no stimulation.

#### 4.3.1 Results of MLRs with cells taken 3 days after grafting

Animals from each treatment group were sacrificed 3 days after grafting and MLRs prepared. At 3 days, results from such MLRs at 72 and 96 hours followed a similar pattern, shown in figure 4.2. With cells taken from naive animals, there were moderate levels of proliferation. With cells from unmodified graft recipients high

counts were detected at both time points with counts averaging approximately 30 000 cpm at both time points, a 3 to 4-fold increase in proliferation compared to cells from naive animals. Counts were calculated as described before. Interestingly, cells taken from animals which had been pre-treated with donor cells 1 week prior to grafting showed little proliferation at either time point with counts averaging around 3 000 cpm. This was the case with cells from grafted animals treated with donor spleen cells alone, or with those pre-treated with donor cells plus either anti-IL-12 antibody or purified goat IgG. Thus pre-treatment with donor spleen cells appeared to reduce the proliferative response in vitro to donor antigen, irrespective of additional treatments. In addition, there were no real differences between the treatment groups which had received donor cells in the pre-treatment protocol.

### 4.3.2 Results of MLRs with cells taken 7 days after grafting

Cells from unmodified animals grafted 7 days previously proliferated well at both time points and these results are shown in figure 4.3. As with cells taken from animals 3 days after grafting, cells from mice pre-treated with donor antigen either with or without additional treatment of anti-IL-12 antibody or goat IgG showed reduced proliferation compared to cells from unmodified graft recipients, with counts being reduced by half. These results were similar at both time points. Interestingly, cells taken from animals treated with anti-IL-12 antibody alone showed increased proliferation with counts comparable to those from unmodified graft recipients. Thus at day 7 after grafting, cells from animals pre-treated with donor antigen continued to show reduced proliferation, but treatment with anti-IL-12 antibody alone did not affect proliferation in vitro.

### 4.3.3 Results of MLRs from cells taken 10 days after grafting

Cells from unmodified graft recipients 10 days after grafting continued to show good proliferation, while those pre-treated with donor antigen, either with or without further antibody treatment, showed reduced proliferation, with counts being reduced some 2 to 3-fold (figure 4.4). As with day 7 after grafting, cells from graft recipients treated with anti-IL-12 antibody alone proliferated to a comparable degree to those from unmodified grafted animals.

### 4.3.4 Results of MLRs from cells taken 15 days after grafting

By day 15 after grafting, unmodified graft recipients and those pre-treated with donor spleen cells would both have rejected their grafts, while those pre-treated with donor cells plus anti-IL-12 antibody would only be starting to reject. At day 15 after grafting, differences in proliferation between the various treatment groups was less marked. This was most obvious after 96 hours in vitro and these results are shown in figure 4.5. At this time, proliferation in all groups was similar, with cells from unmodified graft recipients achieving counts of 16 659 cpm, and those from grafted animals pre-treated with donor cells giving 13 669 cpm. Interestingly, the lowest counts achieved at this time was in the group pre-treated with donor cells plus anti-IL-12 antibody, with an average of 10 187 cpm. By day 15, therefore, the large differences seen in proliferation at earlier times with cells from unmodified graft recipients compared to those from animals pre-treated with donor cells had largely disappeared, although proliferation in the group treated with donor cells plus anti-IL-12 antibody was still lower.

It would appear, therefore, that injection of donor cells prior to grafting markedly reduces the proliferative response to donor antigen in vitro. This effect does, however, diminish with time. Such reduced proliferation in a MLR does not, however, correlate with prolonged graft survival as all treatment groups which received donor cell pre-treatment did not show prolonged graft survival, this only being exhibited in the group treated with both donor cells plus anti-IL-12 antibody.

**4.4 Discussion**

In this study, spleen cells cultivated from grafted mice pre-treated with donor antigen in the form of $5 \times 10^7$ donor spleen cells injected intravenously 1 week prior to skin grafting showed a reduced in vitro proliferative response on re-stimulation with donor strain spleen cells. This effect was not dependent on any additional treatment such as the neutralisation of endogenous IL-12. It is interesting to note that such reduced proliferation in vitro was not associated with a prolongation of skin graft survival.

These findings correlate well with previously published results. Josien et al (1999) have shown that, in a model of allograft tolerance induced by donor -specific transfusion in rats, such tolerance is characterised by inhibition of both T cell and macrophage activation. Such treatment profoundly inhibits proliferation of spleen cells from graft recipients re-stimulated with donor antigen in MLR. Kitagawa et al (1991, a) assessed proliferation in vitro in cells from mice which had been injected with MHC class I disparate spleen cells 1 week previously. They found that proliferation by cells from pre-treated animals re-stimulated with irradiated donor cells in vitro is comparable to background levels while proliferation is increased

some 10-fold in cultures of cells from animals which had not received donor cells. Increasing the number of cells given in the donor innoculum makes no difference. Most significantly, they reported that despite this reduction in proliferation in vitro, intravenous donor spleen cell injection does not prolong skin graft survival across a full MHC class I mismatch. They also determined that proliferation of both $CD4^+$ and $CD8^+$ T cells is inhibited by donor cell injection in their model. Despite a profound reduction in MLR responses, CTL activity is not affected by donor specific transfusion, and it is these cells which are responsible for allograft rejection in their model.

This group had previously described how donor spleen cell injection in a class I minor mismatch model does markedly reduce proliferation in a MLR and is also associated with significant prolongation of skin graft survival (Azuma et al, 1989). They concluded that suppression of MLR, even though it can be profound, is not always correlated with a reduction in the capacity to reject grafts in vivo.

Other workers have investigated proliferative responses in MLR when C57Bl/6 mice are injected with $BDF_1$ lymphoid cells (Kiziroglu and Miller, 1991). They reported that cells removed from C57Bl/6 mice pre-treated with iv. donor cells fail to proliferate when re-stimulated with irradiated donor cells. It was concluded that injection of cells leads to inactivation of Th cells, although the exact mechanism for this is unclear. This work is not, however, directly comparable to the work presented here because animals were not exposed to donor antigen in the form of a skin graft prior to removal of cells for MLR. It does, however broadly agree with the findings presented here, namely that pre-treatment with donor cells leads to reduced responsiveness in a MLR.

Donor-specific cell injection prior to skin grafting in minor MHC class I and II mismatches is associated with a reduction in proliferative responses when cells are re-stimulated in a MLR, a reduction in CTL activity and prolongation of skin graft survival (Hori et al, 1989). Conversely, when fully disparate MHC class I strains are used, while there is a reduced response in a MLR, this is not associated with prolongation of skin graft survival. The results presented for the semi-allogeneic mismatch model used here support these findings. While pre-treatment with donor cells profoundly inhibited proliferation in a MLR, it did not prolong allograft survival. In addition, treatment with anti-IL-12 antibody alone had no effect on proliferation in vitro.

**4.5 Cytokine production in vitro**

To investigate the production of cytokines in vitro, the supernatants of cultures of spleen cells from grafted C57Bl/6 mice re-stimulated with irradiated donor cells were harvested. Initially, cells were removed from animals which had received $BDF_1$ tail skin grafts 3 days earlier or from animals which had not received a graft and cultures prepared. Cells were stimulated either with irradiated $BDF_1$ donor cells or with medium containing 10 μg/ml conA and cells cultured for 48, 72 and 96 hours. These results are shown in figure 4.6. Supernatants were harvested at these time points and analysed for their content of IFN-γ and IL-4 using sandwich ELISAs as described earlier. When spleen cells from grafted animals were stimulated with conA, IFN-γ production was most marked at 72 hours, although levels of IFN-γ were high at all 3 time points examined. Similarly with cells removed from grafted animals re-stimulated with donor antigen, IFN-γ production was greatest at 72 hours in vitro, and, although levels were reduced compared to cells stimulated with conA, levels were still high. In cells from ungrafted animals stimulated with $BDF_1$ cells, production of IFN-γ was low at all 3 time points.

When spleen cells from grafted animals were stimulated with conA they produced moderate amounts of IL-4 at all 3 time points examined, while spleen cells from graft recipients re-stimulated with donor cells produced smaller, although detectable levels of IL-4 at all 3 time periods, with no great differences between them. Spleen cells from ungrafted animals stimulated with $BDF_1$ cells failed to produce much IL-4 at any time point.

As production of both cytokines appeared to be maximal after 72 hours of culture, supernatants were harvested from cultures of cells in the various treatment

groups after 72 hours and sandwich ELISAs were carried out to quantify cytokine production. Spleens were removed from treated C57Bl/6 mice at 3, 7, 10 and 15 days after grafting and MLRs prepared as before. The Th1 cytokines IL-2 and IFN-γ and the Th2 cytokines IL-4, IL-5 and IL-10 were quantified. Results were calculated using the mean of 3 separate ELISA assays from different cultures. Analyses were performed on C57Bl/6 mice in each of the following treatment groups: (i) unmodified $BDF_1$ graft recipients; (ii) $BDF_1$ graft recipients pre-treated with $5 \times 10^7$ donor spleen cells iv. 1 week prior to grafting; (iii) $BDF_1$ graft recipients pre-treated with donor cells as before and 0.5 mg. anti-IL-12 antibody on day -8, -5, -2 and 1; (iv) $BDF_1$ graft recipients given iv. donor cells as before plus 0.5 mg. goat IgG on day -8, -5, -2 and 1 as a negative control; (v) $BDF_1$ grafted animals treated with 0.5 mg. anti-IL-12 antibody on day -1, 2, 5 and 8.

Spleen cells prepared from animals in each of the treatment groups were cultured with irradiated $BDF_1$ splenocytes which acted as antigen-specific stimulators. Negative control cultures, where cells from grafted animals were cultured in standard medium alone, and positive control cultures, where splenocytes from animals in each of the treatment groups were cultured with standard medium containing 10 μg/ml conA were also prepared. The results of cytokine production in these cultures are shown in tables 4.1 to 4.5. At each time point, and in each experimental group, production of the various cytokines tested was not detectable in the cultures of cells from grafted animals cultured with standard medium alone. This indicates that there was no spontaneous production of cytokines by unstimulated cells. Splenocytes from animals in each of the treatment groups at all time points tested were, however, capable of producing detectable levels of cytokines when

stimulated with the mitogen conA. These levels were similar in each of the treatment groups, indicating that splenocytes in each of the treatment groups were responsive to mitogens and that none of the treatments used induced non-specific suppressive effects. In the groups re-stimulated with irradiated donor strain cells there were, however, significant differences between the treatment groups and at the different time points examined. These differences are discussed in the following sections.

### 4.5.1 Production of IL-2 in vitro

With cells taken from animals 3 days after grafting and re-stimulated in vitro, IL-2 production was greatest in spleen cells obtained from unmodified graft recipients (figure 4.7). Spleen cells from this group produced twice the amount of IL-2 compared to cells from animals in any of the treatment groups and these differences were statistically significant ($p<0.05$) when compared using a Wilcoxon matched pairs test. Interestingly, all the other treatment groups assessed at day 3 after grafting, namely grafted animals pre-treated with donor cells, those pre-treated with donor cells plus anti-IL-12 antibody and those given donor cells plus goat IgG produced comparable, but reduced amounts of IL-2.

Production of IL-2 by cells removed 7 days after grafting was broadly similar in all the experimental groups, including those treated with anti-IL-12 antibody alone, with all groups producing around 15 u/ml and there were no significant differences between groups. At 10 days after grafting, IL-2 production was slightly elevated in the groups which had been pre-treated with donor spleen cells, either with or without antibody, with production around 10 u/ml. However, differences were small and standard deviations were relatively large and so such differences were not significant.

Interestingly, at day 15, production of IL-2 was also increased in all the treatment groups compared to unmodified grafted animals although again differences were small. These differences were, however, significant in the groups treated with donor cells alone or with donor cells plus goat IgG, but not in the groups treated with anti-IL-12 antibody, either with or without donor cells.

Thus IL-2 production was increased in cells from unmodified grafted animals compared to all the other treatment groups early on in the rejection process. However, as time went on, IL-2 production was similar in all groups and latterly was increased in groups which had received donor cells without anti-IL-12 antibody. It is of interest to note that pre-treatment with donor cells either with, or without anti-IL-12 antibody was capable of reducing IL-2 production early after grafting even though pre-treatment with donor cells alone did not correlate with prolonged skin graft survival. In addition, treatment with purified goat IgG plus donor cells had no effect over and above treatment with donor cells alone.

### 4.5.2 Production of IFN-γ in vitro

The cells from unmodified graft recipients taken 3 days after grafting produced considerably more IFN-γ than those from the other groups, that is those treated with donor cells either with or without anti-IL-12 antibody or goat IgG. Results are shown in figure 4.8. In the unmodified grafted group, IFN-γ production was some 20 fold greater compared to the other groups, all of which were very similar and these differences were statistically significant ($p<0.05$). Cells taken 7 days after grafting from treated animals all produced more IFN-γ than those from unmodified graft recipients with little differences between the groups treated with

donor cells either with or without additional antibody. This increase was, however, modest, being approximately twice that of unmodified graft recipients although the differences were significant. At this time the greatest production of IFN-γ was seen in cells taken from grafted animals treated with anti-IL-12 antibody alone. Despite this, levels of IFN-γ production by cells from mice 3 or 7 days after grafting are relatively low.

The most dramatic differences in IFN-γ production were seen at the later time points. At 10 days after grafting, cells from unmodified graft recipients produced large amounts of IFN-γ, averaging 51.7 u/ml. In addition, production of IFN-γ in the groups pre-treated with donor cells, either with or without additional antibody, remained similar to that seen at the earlier time points for the same groups. Thus, 10 days after grafting, IFN-γ production by cells from animals which had received pre-treatment with iv. donor spleen cells was 50 times less than in the unmodified grafted group, at around 1.0 u/ml. Cells from animals treated with anti-IL-12 antibody alone produced elevated amounts of IFN-γ compared to the other treatment groups, recording levels of 27.0 u/ml.

At day 15 after grafting, production of IFN-γ was similar to that seen at day 10 in the various treatment groups. Cells from unmodified graft recipients produced large amounts, cells from animals treated with anti-IL-12 antibody alone produced moderate amounts and cells from animals pre-treated with donor spleen cells either with or without further antibody treatment produced small amounts.

IFN-γ production in unmodified graft recipients was, therefore, high. However, when mice are pre-treated with iv. donor spleen cells, production was considerably reduced and this was statistically significant. This reduction appeared to

be dependent on donor cell injection rather than additional antibody treatment, either with anti-IL-12 antibody or goat IgG, as levels were equally reduced in all 3 groups. Treatment with anti-IL-12 antibody alone did reduce IFN-γ production to a moderate level, but this was always considerably higher than in the groups treated with donor cells. Thus treatment with anti-IL-12 antibody alone did not completely prevent production of IFN-γ in this model, while treatment with donor cells was more effective.

### 4.5.3 Production of IL-4 in vitro

Production of IL-4 in cultures showed small differences between the various treatment groups (figure 4.9). Cells removed from animals 3 days after grafting all produced small amounts of IL-4 upon re-stimulation, although a statistically significant reduction was seen in the groups treated with donor cells either with or without goat IgG, but not in the groups treated with anti-IL-12 antibody. At day 7, however, production of IL-4 was increased in cells from animals pre-treated with anti-IL-12 antibody, either with or without donor spleen cells. IL-4 production was 12.7 u/ml in the group treated with anti-IL-12 antibody plus donor spleen cells and 12.5 u/ml in the group treated with anti-IL-12 antibody alone compared to 6.4 u/ml in cells from unmodified graft recipients, although such differences were not significant. At 10 days after grafting, IL-4 production was greatest in cultures from unmodified graft recipients with this being twice the level detected in the other groups and this was statistically significant. At day 15, the pattern was similar to that seen at day 7, with groups which had received anti-IL-12 antibody pre-treatment,

either with or without donor cells, showing a slight increase in IL-4 production compared to other groups.

Thus pre-treatment with anti-IL-12 antibody tended to promote IL-4 production by re-stimulated cells compared to the groups which had not received it. The differences observed were, however, small, the maximum increase in IL-4 release being only 2 fold. In addition, there were no real differences in the production of IL-4 by cells from mice pre-treated with anti-IL-12 antibody alone compared to those treated with both anti-IL-12 antibody and donor spleen cells. Interestingly, treatment with donor cells alone appeared to reduce IL-4 production compared to other groups.

### 4.5.4 Production of IL-5 in vitro

At day 3 after grafting, only cells obtained from mice pre-treated with anti-IL-12 antibody plus donor spleen cells produced detectable levels of IL-5 on re-stimulation in vitro. These results are shown in figure 4.10. In unmodified grafted controls and in those treated with donor cells or donor cells plus goat IgG, IL-5 was undetectable. With cells removed 7 days after grafting, IL-5 production was confined to cells from animals which had been pre-treated with anti-IL-12 antibody, either with or without donor cells. In the other 3 groups, IL-5 was undetectable. Interestingly, at this time, production of IL-5 in the group treated with anti-IL-12 antibody alone was less than half that of the group treated with both anti-IL-12 and donor cells.

Cells from animals in all the treatment groups produced small amounts of IL-5 when cultured 10 days after grafting with no real differences between the groups.

However, 15 days after grafting, the group treated with both anti-IL-12 and donor cells produced large amounts of IL-5, with little or none being detected in the other groups.

Significant production of IL-5 was, therefore, confined to cultures of cells from mice which had been pre-treated with anti-IL-12 antibody. This was greatest in the group treated with both anti-IL-12 antibody and donor spleen cells, suggesting that both treatments together produced a stronger cytokine response. Treatment with donor cells alone, or no treatment at all, did not promote the generation of IL-5.

### 4.5.5 Production of IL-10 in vitro

In cultures of cells removed 3 days after grafting, IL-10 production was detectable in all of the treatment groups, but was most pronounced in the group treated with both donor spleen cells and anti-IL-12 antibody (figure 4.11). These differences were not, however, significant. Production was lowest in the group treated with donor cells, either with or without goat IgG control antibody, and these were 2 fold less than in the highest group. By day 7 after grafting, production of IL-10 was greatest again in the group treated with both donor cells plus anti-IL-12 antibody, with this being 3 times greater than that in the unmodified grafted group or in those pre-treated with donor cells alone and this difference was significant ($p<0.05$). In the group treated with anti-IL-12 antibody alone, IL-10 production was slightly increased compared to unmodified graft recipients.

IL-10 production by cells removed 10 days after grafting was considerably increased in the group pre-treated with donor cells plus anti-IL-12 antibody, with this being increased 5 fold compared to all other groups and again this was significantly

increased. Cells from animals which had been grafted 15 days earlier all produced detectable levels of IL-10 when re-stimulated in vitro. Interestingly, at this time levels were greatest in the groups treated with donor spleen cells, either with or without goat IgG. In the groups treated with anti-IL-12 antibody, with or without donor cells, IL-10 production at this time was reduced, although the differences between groups at this time were not statistically significant.

Thus, at the earlier time points after grafting, pre-treatment with anti-IL-12 antibody, either with or without donor spleen cell injection, promoted IL-10 production in vitro. This was considerably more pronounced when both treatments were combined. At the later time points, differences between the groups were much less obvious.

**4.6 Discussion**

In this experimental model of semi-allogeneic skin grafting in mice, unmodified rejection was associated with a strong Th1 cytokine response as determined by cytokine measurements in cultures of spleen cells from grafted animals. When cells from skin grafted animals were re-stimulated with donor-specific antigen in vitro there was an early increase in IL-2 production at 3 days after grafting, followed by a large increase in IFN-γ production as rejection became established. In addition, there was little Th2 cytokine production during rejection. Cytokine production was seen to be a dynamic process, with different cytokines being produced in greater or lesser amounts at different times after grafting. Such findings support the results of many other workers. Gorczynski and Kiziroglu (1994) found that cells removed from animals which had received a minor MHC

mismatched skin graft 7 days earlier and re-stimulated in vitro with donor antigen produce elevated levels of IL-2 and IFN-γ. Takeuchi et al (1992) demonstrated increased transcripts of mRNA for Th1 cytokines in their model of unmodified rejection of cardiac allografts in mice. They found that message for IFN-γ is greatest 5 days after grafting and declines thereafter. Equally, message for IL-2 is greatest 5 days after grafting and then decreases.

Levels of IFN-γ in this model were greatest around the time of rejection in unmodified grafted animals (day 10). However, the highest levels of IL-2 were found much earlier than this (day 3) and, by the time of rejection, levels of IL-2 were actually less than in other groups. Takeuchi et al (1992) also found that the peak IL-2 production preceded graft rejection in their model of a murine cardiac allograft where tolerance was induced by treating recipient mice with donor specific transfusion, cyclosporin or anti-CD4 mAb. O'Connell et al (1993) demonstrated that rejection of unmodified recipients of murine pancreatic islet cell grafts is preceded by the production of IL-2 within the graft as detected by polymerase chain reaction-assisted reverse transcription. In addition, production of IL-4 during unmodified islet cell rejection was not detected. More recently, it has been demonstrated that unmodified rejection of pancreatic islet cell grafts in mice is associated with increased expression of IL-2 and IFN-γ, with levels of IL-4 barely detectable ( Li et al, 1998). Thus these results, and the results presented here, demonstrate that unmodified rejection is characterised by a strong Th1 response although the levels of cytokine production at various times do differ.

Pre-treatment of graft recipients with iv. donor spleen cells 1 week prior to grafting altered the cytokine producing potential of cells re-stimulated in vitro. In

culture, such cells failed to produce large amounts of the Th1 cytokines at any time point examined. Most significantly, levels of IFN-γ were markedly reduced compared to those from unmodified grafted groups. Pre-treatment with donor cells also failed to induce any significant increase in the production of Th2 cytokines. Such a reduction in Th1 cytokine generation did not, however, correlate with any increase in allograft survival. Results for the negative control group where mice were treated with donor cells plus purified goat IgG were similar to those for cells from animals treated with donor cells alone. Cells from animals pre-treated with donor cells were, however, able to produce detectable levels of cytokines when stimulated with mitogens, indicating that this reduction in cytokine production was antigen specific. Other workers have reported similar findings. Kitagawa et al (1991, a) described how donor-specific cell injection prior to skin grafting reduces proliferative responses and IL-2 production in vitro when cells are re-stimulated with donor antigen. Graft rejection in their model is due to $CD8^+$ CTL which are resistant to donor cell pre-treatment, and so skin graft survival is not prolonged by injection of donor cells alone. However, when donor cell pre-treatment is combined with anti-CD8 mAb therapy, skin graft survival is significantly prolonged. It was concluded that pre-treatment with donor cells induces elimination, or functional inactivation of particular T cell subsets, but this does not necessarily equate with prolongation of graft survival. Only when donor cell injection is combined with treatments eliminating either $CD8^+$ CTL or $CD4^+$ Th cells is graft survival increased

Dallman et al (1991, a) demonstrated in a rat kidney allograft model that donor-specific transfusion produces long term graft survival and that the resulting transplantation tolerance is associated with reduced IL-2 production by tolerant cells

in vitro. They concluded that donor-specific transfusion induces T cell anergy by alloantigen stimulation in the absence of a second signal and that this is the mechanism for acceptance of kidney grafts. Bugeon et al (1992) extended this work and found that donor-specific transfusion prior to grafting leads to decreased levels of both IL-2 and IFN-γ mRNA in the cardiac allografts of tolerant animals.

In the model used in this thesis, pre-treatment with donor cells led to reduced levels of both Th1 and Th2 cytokine production. It did not, however, result in prolonged graft survival and so, while T cells may have been rendered unresponsive in terms of cytokine production, animals were still capable of effecting allograft rejection. The underlying mechanism behind the blood transfusion effect remains unresolved. However the results presented here suggest that the generation of a Th1 environment is not an absolute requirement for allograft rejection and that other mechanisms may also be involved in activating the cells which mediate allograft rejection in this model. This is supported by other workers findings. For example, VanBuskirk et al (1996) demonstrated that allograft rejection can proceed in the absence of Th1 cells.

Pre-treatment of skin graft recipients with anti-IL-12 antibody alone did not prolong allograft survival. However, when cells from such animals were removed and re-stimulated in vitro, diminished Th1 and increased Th2 cytokine responses were produced. Interestingly, while pre-treatment with anti-IL-12 antibody reduced production of IFN-γ, it did not abolish it, or reduce it to the levels produced by cells from animals pre-treated with donor spleen cells. Thus neutralising endogenous IL-12 did not prevent the generation of IFN-γ, indicating that IL-12 is not the only signal required for IFN-γ production. Treatment with anti-IL-12 antibody did, however push

cells towards a Th2 phenotype with increased levels of IL-5 and IL-10 being generated in vitro. Piccotti et al (1996) found similar effects in a murine cardiac allograft model. They found that neutralising endogenous IL-12 leads to increased mRNA for IL-4 and IL-10, although message for IFN-γ is still present. In addition, when spleen cells are removed from treated animals and re-stimulated in vitro, they can still produce IFN-γ. Thus while neutralising IL-12 induces a shift towards a Th2 response, the Th1 response is not completely abrogated. In addition, this shift towards a Th2 response does not prolong cardiac allograft survival. Similarly, Li et al (1998) found that, in a mouse pancreatic allograft model, while pre-treatment with anti-IL-12 antibody did induce a Th1 to Th2 cytokine shift, this was not associated with prolonged allograft survival in MHC-mismatch strain combinations.

In this model, despite treatment with anti-IL-12 antibody alone generating a Th2 response, allograft survival was not prolonged. This may be because not all Th1 cytokine production was prevented. IL-18, a recently identified cytokine, induces production of IFN-γ and thus an alternative pathway for the generation of Th1 responses exists (Okamura et al, 1998). Allograft rejection may proceed by activation of this alternative pathway. Equally, anti-IL-12 antibody treatment alone only generated a weak Th2 response compared to treatment with both anti-IL-12 antibody and donor spleen cell injection. Such a weak Th2 response may be insufficient to overcome the Th1 response which still exists.

Pre-treatment of graft recipients with both anti-IL-12 antibody and donor spleen cells significantly prolonged the survival of skin allografts. When immunocompetent cells were removed from such animals and re-stimulated in vitro, production of the Th1 cytokines was reduced and that of the Th2 cytokines increased.

Donor antigen pre-treatment appears, therefore, to have additive effects with anti-IL-12 antibody in terms of Th2 cytokine production because levels of Th2 cytokines in this group were greater than those in the group treated with anti-IL-12 antibody alone. Thus prolonged allograft survival was associated with a Th2 response. One explanation for this may be that, while pre-treatment with anti-IL-12 alone is insufficient to polarise the immune response strongly in a Th2 direction, once a strong Th2 response is generated allograft survival can be prolonged. However, in the group treated with anti-IL-12 antibody and donor spleen cells, while skin graft survival is prolonged, grafts are not accepted permanently. At day 15 after grafting, cells from animals pre-treated with both anti-IL-12 antibody and donor cells continue to produce elevated Th2 cytokines with very little IFN-γ, indicating that a Th2 response in itself does not necessarily equate with tolerance as skin grafts are rejected not long after this.

The assumption has been made that while a Th1 response is associated with allograft rejection, conversion to a Th2 response may be associated with tolerance. The results presented in this study do not support this. A weak Th2 response, generated by treatment with anti-IL-12 antibody alone, had no effect on allograft survival, while a strong Th2 response, generated by donor cell plus anti-IL-12 pre-treatment, did prolong allograft survival by a few days but, even in the absence of Th1 response, rejection did still occur. Similar findings have been reported. Chan et al (1995) depleted $CD8^+$ T cells in a murine cardiac allograft model and found that this had no effect on allograft survival compared to unmodified controls. They did, however, find that the rejection mechanisms were different: unmodified rejection was associated with a heavy CTL infiltrate and the generation of Th1 type cytokines;

rejection in $CD8^+$ T cell-depleted animals was associated with a reduced CTL infiltrate, a heavier eosinophil infiltrate and Th2 cytokine production. They concluded that while the ultimate effector mechanisms were different, allograft rejection could still occur in either a Th1 or a Th2 environment. They therefore questioned the relevance of the Th1/ Th2 paradigm in transplantation. More recently, Josien et al (1999) demonstrated that DST prior to grafting in a rat allogeneic heart graft model induced long term graft survival, and this was associated with down-regulated production of cytokines within the graft. Treatment with recombinant IFN-γ abolished the beneficial effect of DST, but this was not associated with any change in the cytokine profiles from treated animals. Rather, treatment with rIFN-γ enhanced anti-donor IgG alloantibody production and it was this which induced rejection, indicating that, even in the presence of a down-regulated cytokine response, humoral immunity can induce allograft rejection. Similarly, Yang, C.P. et al (1998) demonstrated that DST can iduce long-term graft survival when rejection is dependent on cell-mediated events, but not when it is mediated by humoral mechanisms.

The results presented here would also challenge the idea that a Th2 response is necessarily associated with tolerance. At day 15 after grafting, lymphoid cells from animals pre-treated with donor spleen cells and anti-IL-12 antibody continued to produce Th2 cytokines with little evidence of Th1 cytokine production, but the skin grafts rejected around day 17. It may be, therefore, that other mechanisms rather than Th1/ Th2 deviation account for the prolongation of allograft survival seen in these studies. Additional experiments performed to investigate this possibility are described in the following chapter. Alternatively, it must be appreciated that

achieving tolerance to skin grafts is extremely difficult and it is possible that treatment with both anti-IL-12 antibody and donor spleen cells, effecting a deviation in the immune response from Th1 to Th2, may prove to be effective in inducing transplantation tolerance in a less exacting experimental model such as allogeneic cardiac transplantation.

## 4.7 Summary of Results of Chapter 4

**1.** Preparations of cells from the spleens of treated animals proliferate in vitro when stimulated with irradiated donor cells. Pre-treatment of graft recipients with injected donor cells prior to skin grafting reduced the proliferation of such spleen cells irrespective of any other treatment which recipient animals received.

**2.** Cells prepared from the spleens of skin graft recipients pre-treated with anti-IL-12 antibody alone did not exhibit reduced proliferative responses.

**3.** Cells prepared from the spleens of unmodified skin graft recipients produce increased amounts of the Th1 cytokines IL-2 and IFN-γ when re-stimulated in vitro with donor alloantigen.

**4.** Cells from the spleens of skin graft recipients pre-treated with injected donor spleen cells prior to grafting produce reduced levels of both Th1 and Th2 cytokines when re-stimulated with donor alloantigen in vitro.

**5.** Spleen cells from skin graft recipients pre-treated with anti-IL-12 antibody alone produce reduced levels of Th1 cytokines and low levels of Th2 cytokines when re-stimulated in vitro.

**6.** Spleen cells from grafted animals pre-treated with both anti-IL-12 antibody and donor spleen cells prior to grafting produce low levels of Th1 cytokines and

increased levels of the Th2 cytokines, IL-5 and IL-10, and this treatment regime is associated with prolonged skin graft survival.

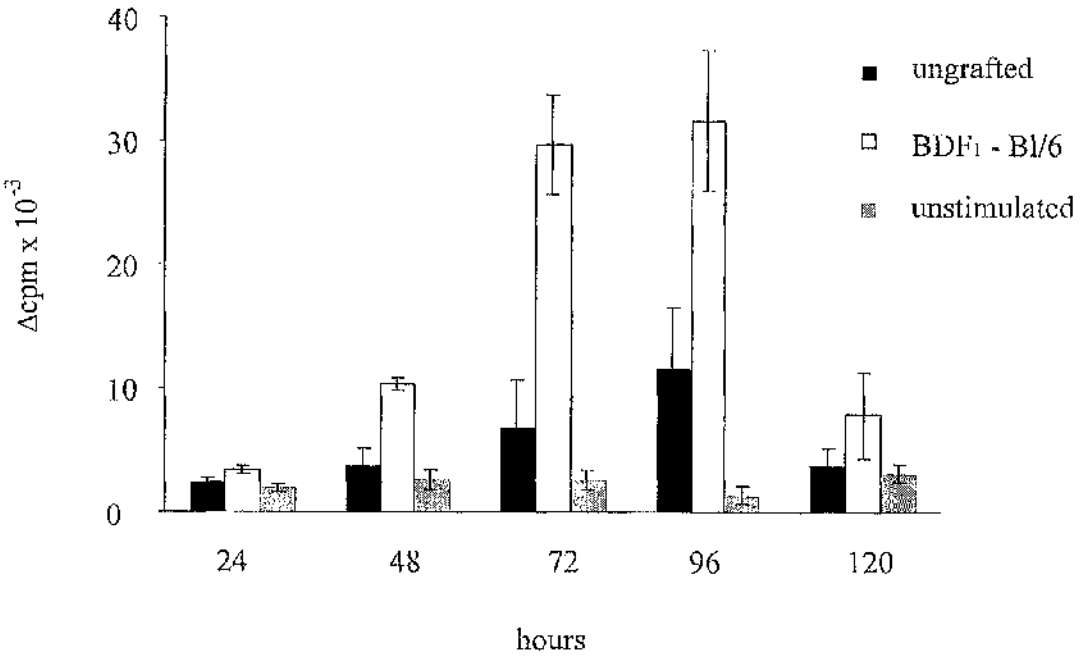



**Figure 4.1 Proliferation in a MLR at various time points.** Spleens were removed from C57Bl/6 mice and single cell suspensions prepared. Cells were re-stimulated in vitro with irradiated BDF1 cells and maintained in vitro for 24, 48, 72, 96 or 120 hours after which 3H thymidine was added and, 18 hours later, proliferation quantified. The responder cell types examined were: cells from naïve, ungrafted animals (ungrafted); cells from animals which had received a BDF1 tail skin graft 3 days earlier (BDF1 - Bl/6); cells from animals which had received a skin graft 3 days earlier but had no stimulator cells added to the MLR (unstimulated). Counts are the means of 3 experiments +/- 1 standard deviation.

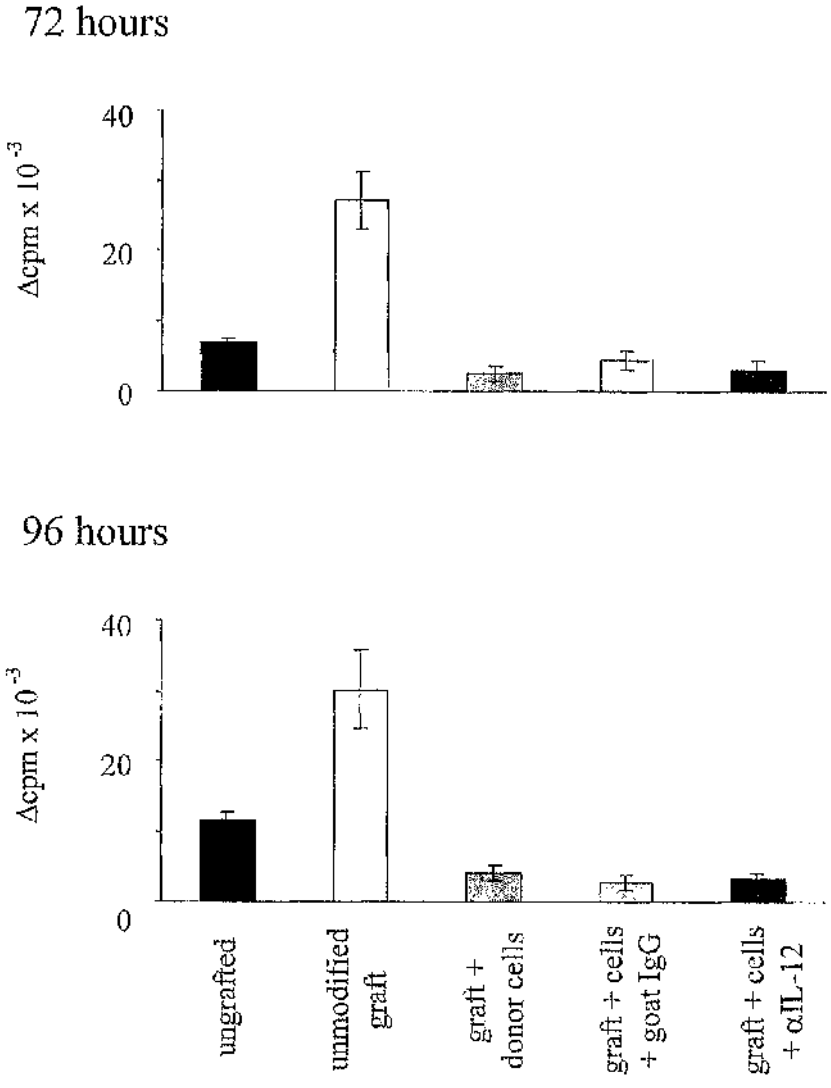



**Figure 4.2 Proliferation in vitro by cells 3 days after grafting.** Spleen cells were removed from C57Bl/6 mice and re-stimulated in vitro with irradiated $BDF_1$ cells. Cultures were harvested at 72 and 96 hours in vitro. Proliferation was quantified in the following groups: (i) ungrafted - cells removed from naïve mice; (ii) unmodified graft - cells removed from mice which had received a $BDF_1$ skin graft 3 days previously; (iii) graft + donor cells - cells removed from animals which had been pre-treated with iv donor cells 7 days prior to receiving a skin graft; (iv) graft + cells + goat IgG - cells from animals pre-treated with both donor cells on day -7 and 0.5 mg goat IgG ip on days -8, -5, -2 and 1; (v) graft + cells + αIL-12 - cells from mice pre-treated with donor cells on day -7 and 0.5 mg anti-IL-12 antibody on days -8, -5, -2 and 1. Counts are the means of 3 separate experiments +/- 1 standard deviation.

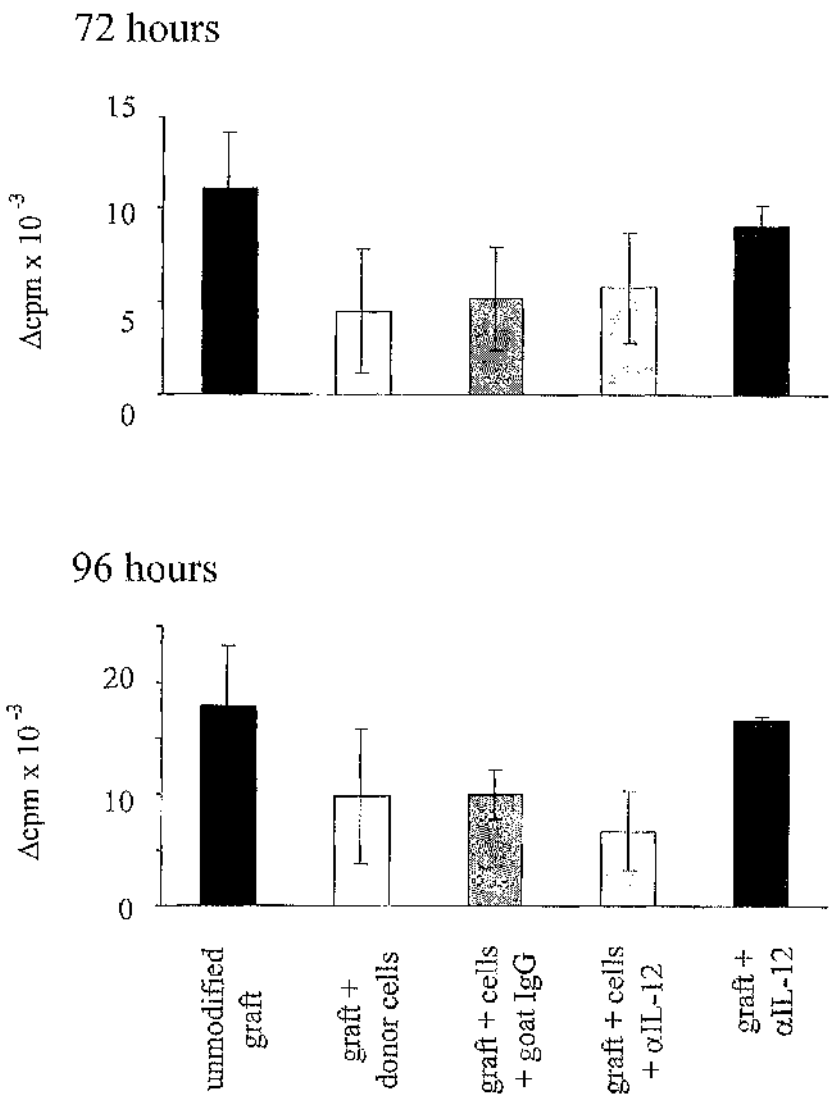



**Figure 4.3 Proliferation in vitro of cells removed 7 days after grafting.** MLRs were prepared as before from cells removed from C57Bl/6 mice 7 days after grafting and maintained in vitro for 72 or 96 hours. Experimental groups are as for Figure 4.2 except the group graft + αIL-12 where mice were treated with 0.5 mg anti-IL-12 antibody on day -1, 2, 5 and 8 with grafting on day 0.

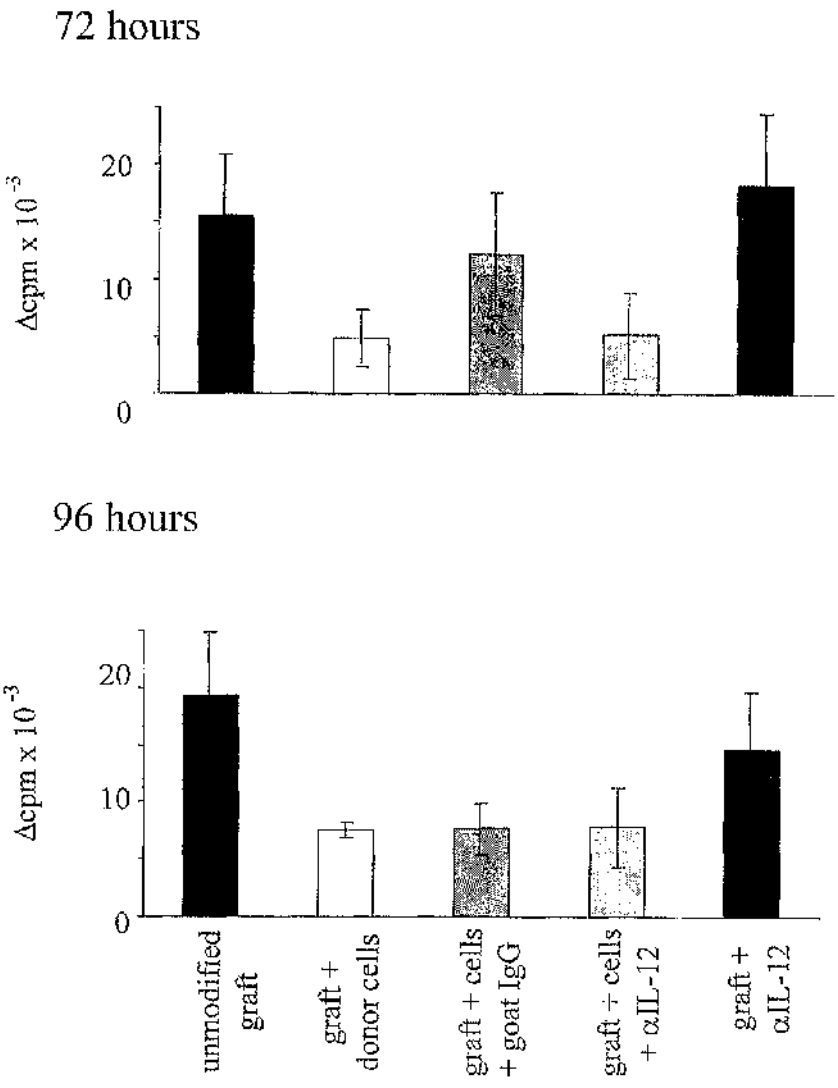



**Figure 4.4 Proliferation in vitro of cells removed 10 days after grafting.** MLRs were prepared as before with cells removed from C57Bl/6 mice 10 days after grafting. Experimental groups are as for Figure 4.3.

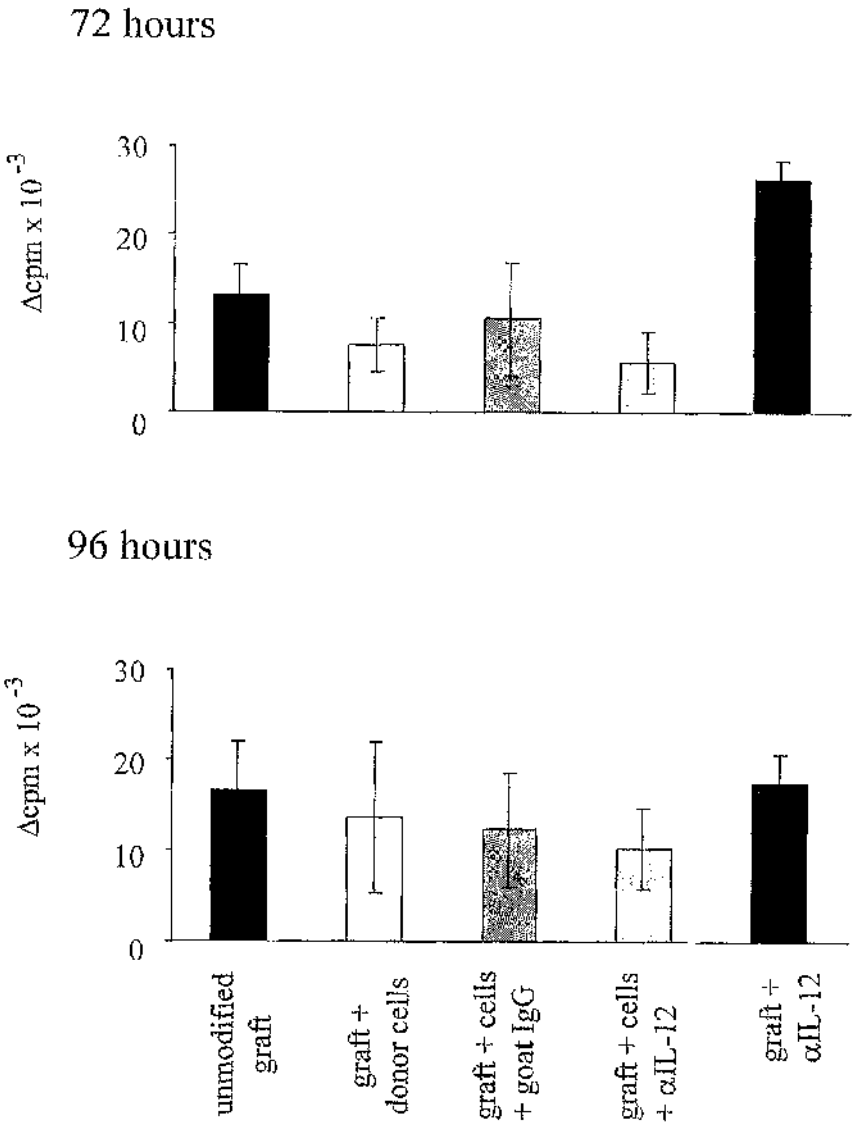



**Figure 4.5 Proliferation in vitro of cells removed 15 days after grafting.** Cells were removed from C57Bl/6 mice 15 days after grafting with BDF1 tail skin. Treatment groups were as for Figure 4.3.

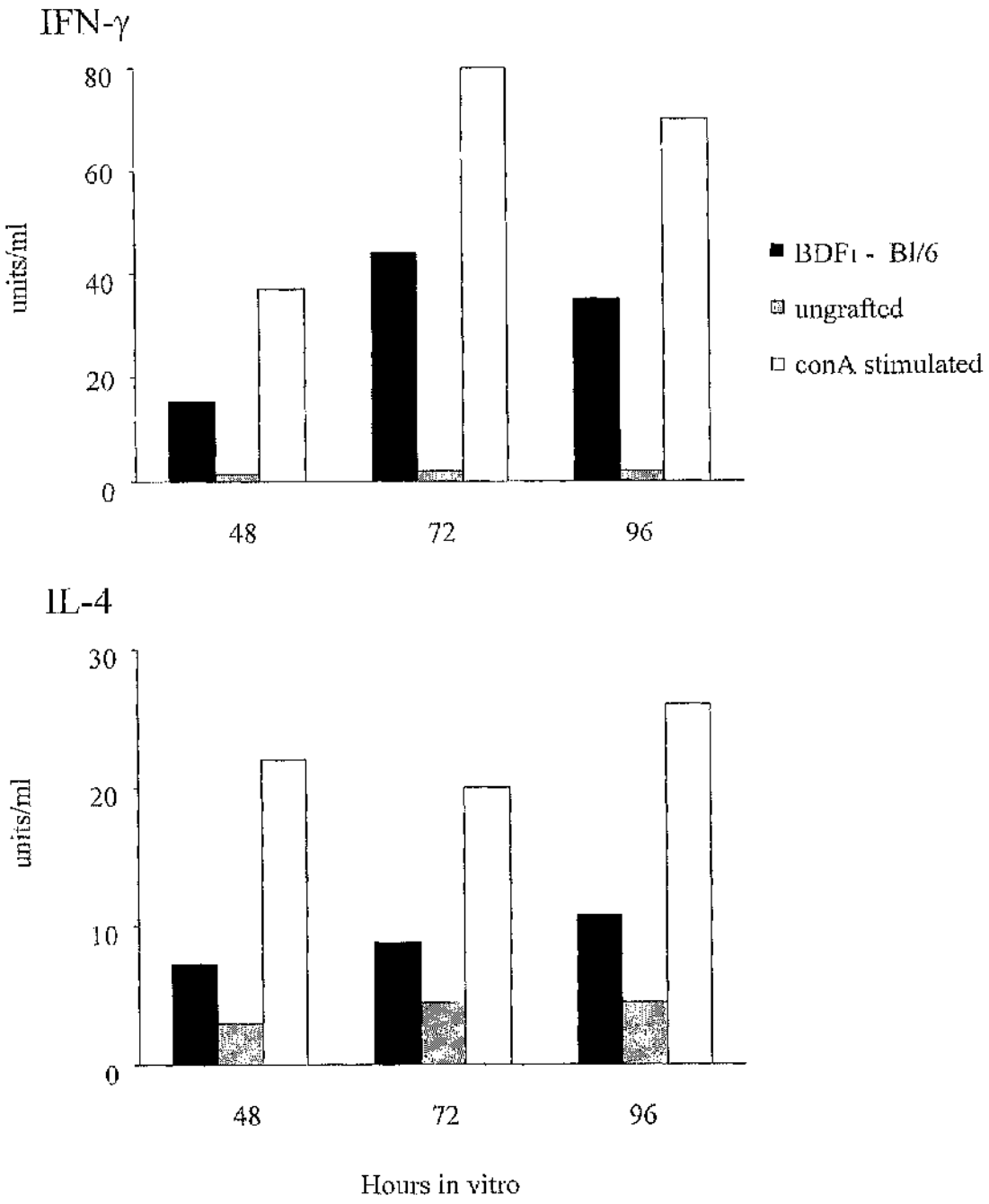



**Figure 4.6 Quantification of cytokines produced in vitro.** Cells were removed from C57Bl/6 mice which had received a BDF1 skin graft 3 days earlier or which had received no treatment and cultures prepared. Cells from grafted animals were restimulated with irradiated donor cells (BDF1 - Bl/6) or with medium containing 10 μl/ml conA (conA). Cells from animals which had not received a graft were stimulated with irradiated BDF1 cells (ungrafted). Supernatants were harvested at 48, 72 and 96 hours and cytokine content measured by ELISA. Values are expressed as the mean of 3 experiments.

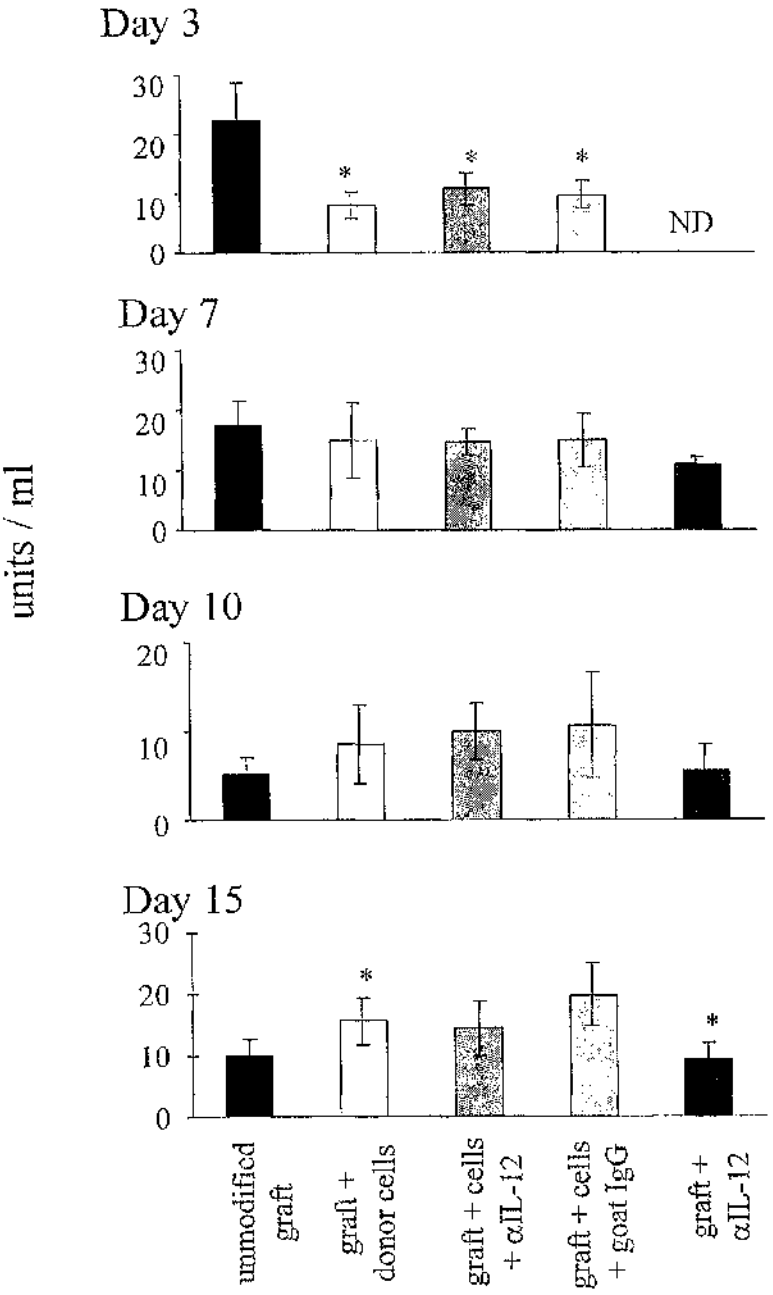



**Figure 4.7 Production of IL-2 in vitro.** Spleens were removed from treated C57Bl/6 mice which had received a $BDF_1$ skin graft at different time points after grafting (day 3, 7, 10 and 15), and single cell suspensions prepared. These cells were re-stimulated in vitro with irradiated $BDF_1$ cells. Supernatants were harvested after 72 hours and analysed for IL-2 content by ELISA.
Treatment groups are as for Figure 4.2. and 4.3.
ND = not done. Values are expressed as the mean of 3 experiments +/- 1 standard deviation (SD), as are all further cytokine experiments. * denotes statistical significance compared to results for unmodified graft recipients ($p<0.05$) using Wilcoxon matched pairs test.

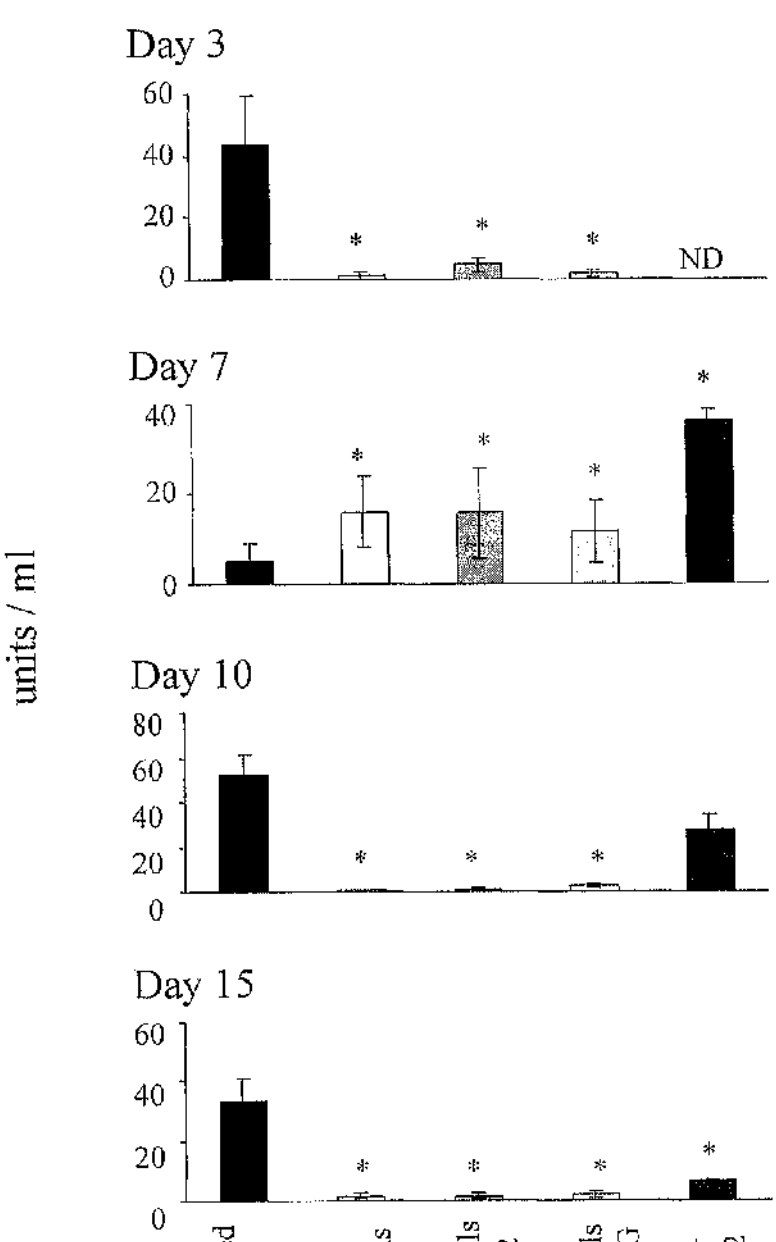



**Figure 4.8 Production of IFN-γ in vitro.** Results are expressed as mean +/- SD. * denotes statistical significance compared to results for unmodified graft recipients ($p<0.05$). ND = not done.

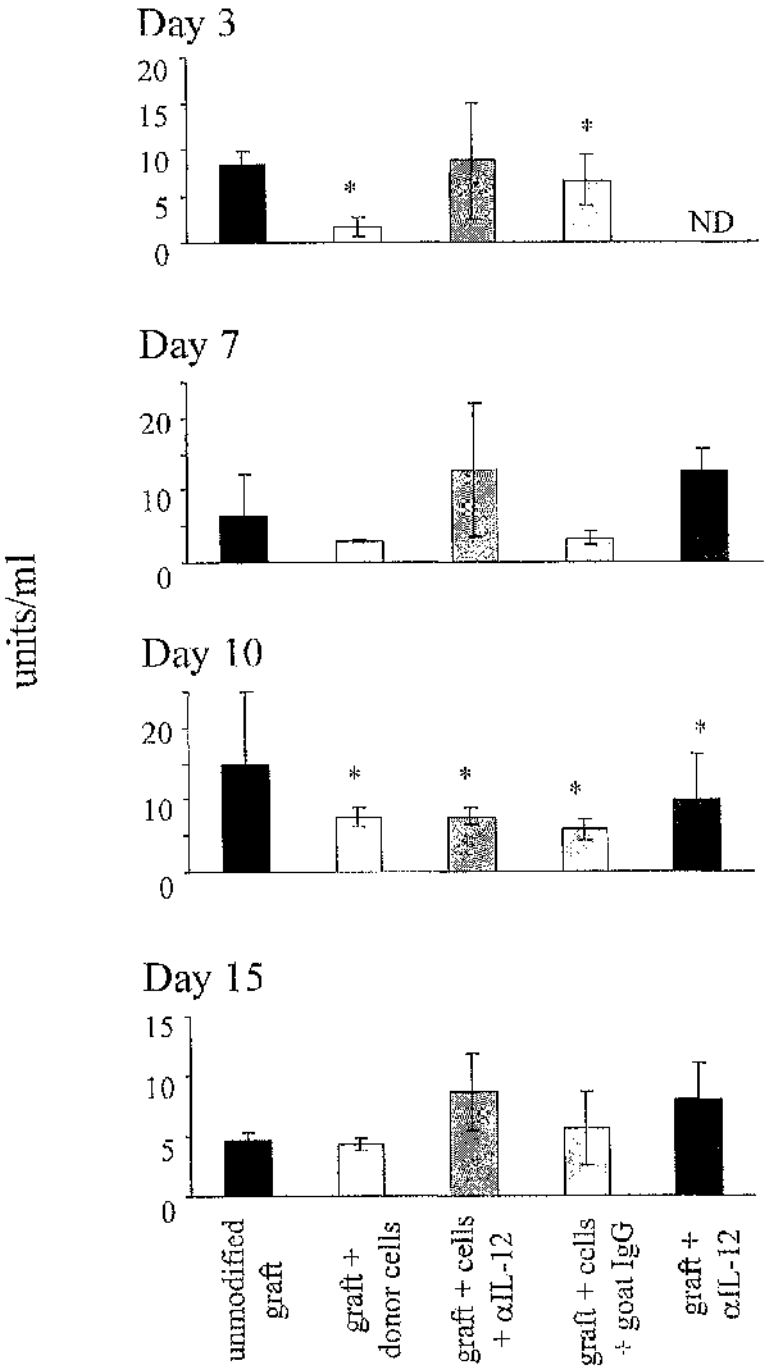



**Figure 4.9 Production of IL-4 in vitro.** Results are expressed as mean +/- SD. * denotes statistical significance compared to results for unmodified graft recipients ($p<0.05$). ND = not done

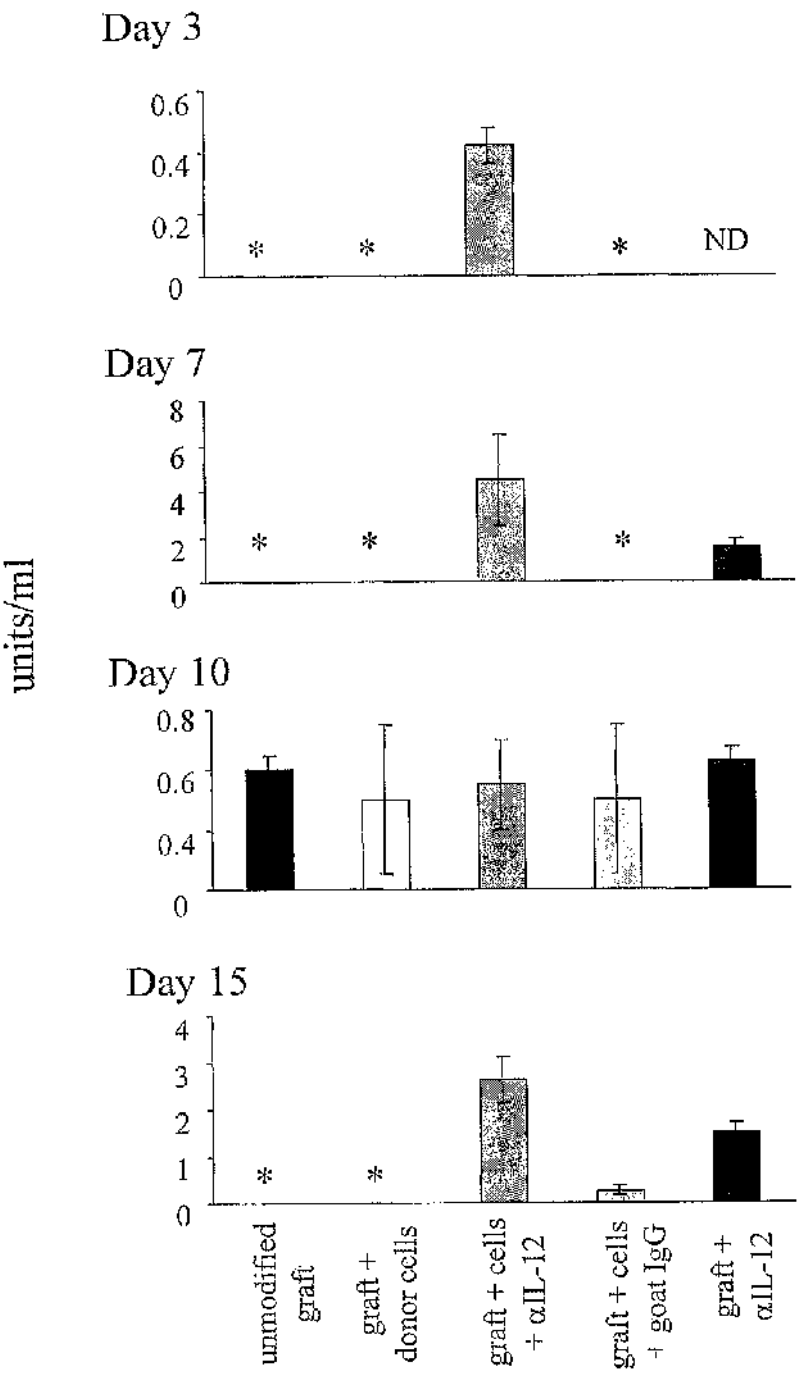



**Figure 4.10 Production of IL-5 in vitro.** Results are expressed as mean +/- SD. * = not detected. ND = not done.

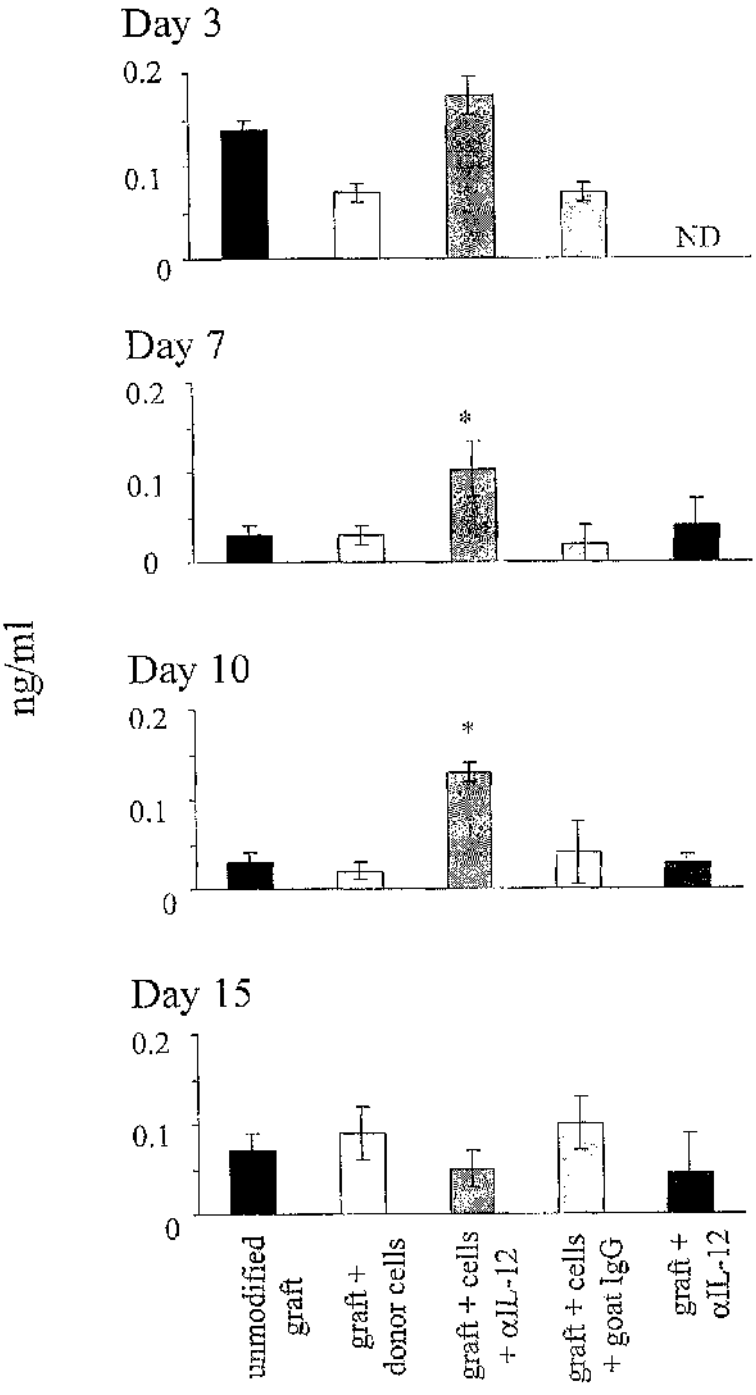



**Figure 4.11 Production of IL-10 in vitro.** Results are expressed as mean +/- SD. * denotes statistical significance compared to results for unmodified graft recipients ($p<0.05$). ND = not done.

**Table 4.1: Production of IL-2 (units/ml)**

| **DAY 3** | graft | graft + donor cells | graft +donor cells + αIL-12 | graft + donor cells + goat IgG |
|---|---|---|---|---|
| stimulators | 22.3 +/- 6.4 | 8.1 +/- 2.2 | 10.7 +/- 2.7 | 9.7 +/- 2.3 |
| unstimulated | <0.2 | <0.2 | <0.2 | <0.2 |
| conA | 40.1 +/- 5.2 | 33.6 +/- 4.6 | 32.1 +/- 6.3 | 30.7 +/- 3.6 |

| **DAY 7** | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 17.7 +/- 4.0 | 14.9 +/- 6.4 | 14.6 +/- 2.3 | 14.9 +/- 4.5 | 11.0 +/- 0.1 |
| unstimulated | <0.2 | <0.2 | <0.2 | <0.2 | <0.2 |
| conA | 51.6 +/- 8.1 | 38.0 +/- 7.9 | 45.5 +/- 3.8 | 41.9 +/- 6.3 | 48.1 +/- 5.5 |

| **DAY 10** | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 5.2 +/- 1.8 | 8.5 +/- 4.5 | 10.0 +/- 3.2 | 10.6 +/- 6.0 | 5.6 +/- 3.0 |
| unstimulated | <0.2 | <0.2 | <0.2 | <0.2 | <0.2 |
| conA | 32.8 +/- 5.4 | 38.1 +/- 3.1 | 28.2 +/- 4.9 | 25.1 +/- 3.9 | 33.9 +/- 6.7 |

Production of IL-2 in vitro as measured by ELISA. Spleen cell suspensions were prepared from C57Bl/6 mice in each of the treatment groups at 3, 7 and 10 days after grafting (graft: recipients of $BDF_1$ skin graft; graft + donor cells: recipients of $BDF_1$ graft pre-treated with 5 x $10^6$ $BDF_1$ cells injected iv 7 days prior to grafting; graft + donor cells + αIL-12: $BDF_1$ graft recipients pre-treated with 5 x $10^6$ $BDF_1$ cells on day -7 plus 0.5 mg anti-IL-12 antibody ip on day -8, -5, -2 and 1; graft + donor cells + goat IgG: $BDF_1$ recipients pre-treated with 5 x $10^6$ $BDF_1$ cells iv on day -7 plus 0.5 mg goat IgG ip on day -8, -5, -2 and 1; graft +αIL-12: $BDF_1$ graft recipients pre-treated with 0.5 mg anti-IL-12 antibody on day -1, 2, 5 and 8). Cells were cultured with irradiated $BDF_1$ cells (stimulators), standard medium (unstimulated), and standard medium containing 10 µl/ml conA (conA). Supernatants were harvested 72 hours later and cytokine content quantified using sandwich ELISAs. Results are the means of 3 experiments +/- 1 standard deviation.

**Table 4.2: Production of IFN-γ (units/ml)**

| DAY 3 | graft | graft + donor cells | graft +donor cells + αIL-12 | graft + donor cells + goat IgG |
|---|---|---|---|---|
| stimulators | 43.7 +/- 15.0 | 1.3 +/- 0.36 | 4.7 +/- 2.7 | 1.7 +/- 0.4 |
| unstimulated | <0.06 | <0.06 | <0.06 | <0.06 |
| conA | 82.6 +/- 12.3 | 72.3 +/- 8.6 | 65.3 +/- 9.1 | 70.8 +/- 11.1 |

| DAY 7 | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 5.2 +/- 3.9 | 15.9 +/- 8.0 | 15.6 +/- 9.9 | 11.5 +/- 6.7 | 36.0 +/- 2.8 |
| unstimulated | <0.06 | <0.06 | <0.06 | <0.06 | <0.06 |
| conA | 45.1 +/- 4.3 | 55.2 +/- 7.3 | 52.6 +/- 6.4 | 61.3 +/- 9.7 | 58.0 +/- 8.1 |

| DAY 10 | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 51.7 +/- 3.9 | 0.65 +/- 0.27 | 1.1 +/- 0.2 | 2.3 +/- 1.0 | 27.0 +/- 7.0 |
| unstimulated | <0.06 | <0.06 | <0.06 | <0.06 | <0.06 |
| conA | 72.2 +/- 10.5 | 62.0 +/- 8.1 | 69.3 +/- 7.7 | 68.1 +/- 14.2 | 52.5 +/- 7.9 |

| DAY 15 | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 33.1 +/-7.9 | 1.57 +/- 1.0 | 1.44 +/- 0.9 | 1.99 +/- 1.4 | 6.2 +/- 1.0 |
| unstimulated | <0.06 | <0.06 | <0.06 | <0.06 | <0.06 |
| conA | 68.9 +/- 8.4 | 61.2 +/- 5.5 | 54.7 +/- 3.0 | 49.8 +/- 9.1 | 51.4 +/- 6.4 |

Production of IFN-γ in vitro as measured by ELISA. Experimental groups are as for Table 4.1.

**Table 4.3: Production of IL-4 (units/ml)**

| DAY 3 | graft | graft + donor cells | graft +donor cells + αIL-12 | graft + donor cells + goat IgG |
|---|---|---|---|---|
| stimulators | 8.3 +/- 1.5 | 1.7 +/- 1.9 | 8.7 +/- 6.7 | 6.7 +/- 2.7 |
| unstimulated | <0.1 | <0.1 | <0.1 | <0.1 |
| conA | 22.8 +/- 4.5 | 21.6 +/- 2.9 | 30.4 +/- 6.4 | 27.8 +/- 7.1 |

| DAY 7 | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 6.4 +/- 5.0 | 2.9 +/- 0.3 | 12.7 +/- 9.0 | 3.3 +/- 0.9 | 12.6 +/- 3.2 |
| unstimulated | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| conA | 19.8 +/- 3.1 | 24.2 +/- 6.8 | 21.6 +/- 5.2 | 18.0 +/- 2.1 | 21.2 +/- 3.4 |

| DAY 10 | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 14.9 +/-10.1 | 7.4 +/- 1.3 | 7.5 +/- 1.2 | 5.8 +/-1.5 | 9.9 +/- 6.2 |
| unstimulated | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| conA | 23.3 +/- 6.4 | 20.1 +/-3.8 | 20.8 +/- 4.5 | 22.4 +/- 5.1 | 26.1 +/- 3.0 |

| DAY 15 | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 4.7 +/- 0.5 | 4.3 +/- 0.5 | 8.6 +/- 3.2 | 5.6 +/- 2.6 | 8.0 +/- 3.0 |
| unstimulated | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| conA | 26.8 +/- 5.3 | 27.1 +/- 3.7 | 30.1 +/- 7.5 | 22.4 +/- 6.1 | 28.4 +/- 3.9 |

Production of IL-4 in vitro as assessed by ELISA. Groups are as for Table 4.1.

**Table 4.4: Production of IL-5 (units/ml)**

| **DAY 3** | graft | graft + donor cells | graft +donor cells + αIL-12 | graft + donor cells + goat IgG |
|---|---|---|---|---|
| stimulators | <0.01 | <0.01 | 0.42 +/- 0.06 | <0.01 |
| unstimulated | <0.01 | <0.01 | <0.01 | <0.01 |
| conA | 3.2 +/- 0.2 | 2.1 +/- 0.3 | 2.8 +/-0.55 | 2.7 +/- 0.27 |

| **DAY 7** | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | <0.01 | <0.01 | 4.5 +/- 2.0 | <0.01 | 1.54 +/- 0.3 |
| unstimulated | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| conA | 6.2 +/- 0.8 | 6.8 +/- 0.3 | 6.2 +/- 0.65 | 7.4+/- 0.7 | 6.8 +/- 1.1 |

| **DAY 10** | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 0.81 +/-0.1 | 0.6 +/-0.5 | 0.7 +/-0.3 | 0.6 +/- 0.5 | 0.85 +/- 0.1 |
| unstimulated | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| conA | 3.0 +/- 0.6 | 3.1 +/- 0.5 | 2.1 +/- 0.3 | 2.6 +/- 0.6 | 2.2 +/- 0.3 |

| **DAY 15** | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | <0.01 | <0.01 | 2.61 +/- 0.5 | 0.25 +/- 0.1 | 1.5 +/- 0.2 |
| unstimulated | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| conA | 4.8 +/- 0.4 | 4.0 +/- 0.8 | 3.9 +/- 0.2 | 4.4 +/- 0.6 | 3.8 +/- 0.6 |

Production of IL-5 in vitro as assessed by ELISA.

**Table 4.5: Production of IL-10 (ng/ml)**

| **DAY 3** | graft | graft + donor cells | graft +donor cells + αIL-12 | graft + donor cells + goat IgG |
|---|---|---|---|---|
| stimulators | 0.138 +/- 0.02 | 0.071 +/- 0.04 | 0.176 +/- 0.02 | 0.071 +/- 0.02 |
| unstimulated | <0.01 | <0.01 | <0.01 | <0.01 |
| conA | 1.0 +/- 0.25 | 0.8 +/- 0.16 | 0.6 +/- 0.07 | 0.8 +/- 0.04 |

| **DAY 7** | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 0.03 +/- 0.01 | 0.03 +/- 0.1 | 0.103 +/- 0.07 | 0.07 +/- 0.02 | 0.04 +/- 0.03 |
| unstimulated | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| conA | 0.53 +/- 0.03 | 0.76 +/- 0.1 | 0.61 +/- 0.09 | 0.52+/- 0.06 | 0.42 +/- 0.12 |

| **DAY 10** | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 0.03 +/- 0.01 | 0.02 +/- 0.01 | 0.13 +/- 0.01 | 0.075 +/- 0.035 | 0.028 +/- 0.01 |
| unstimulated | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| conA | 0.41 +/- 0.03 | 0.38 +/- 0.08 | 0.55 +/- 0.14 | 0.41 +/- 0.07 | 0.39 +/- 0.02 |

| **DAY 15** | graft | graft + donor cells | graft + donor cells + αIL-12 | graft + donor cells + goat IgG | graft + αIL-12 |
|---|---|---|---|---|---|
| stimulators | 0.07 +/- 0.02 | 0.09 +/- 0.03 | 0.05 +/- 0.02 | 0.1 +/- 0.03 | 0.045 +/- 0.05 |
| unstimulated | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| conA | 0.45 +/- 0.06 | 0.38 +/- 0.07 | 0.41 +/- 0.03 | 0.36 +/- 0.04 | 0.34 +/- 0.08 |

Production of Il-10 in vitro as assessed by ELISA. Groups are as for Table 4.1.

# CHAPTER 5

# CYTOTOXICITY ASSAYS

## 5.1 Introduction

Cytotoxic T cells recognise and kill target cells by direct cell-cell contact. CTL binding to an appropriate target stimulates a calcium-dependent degranulation process in the effector cell releasing perforin and granzyme from secretory granules into the local environment. This leads to the formation of pores in the target cell membrane and this is followed by fragmentation of the target cell DNA and cell lysis. A second, non-secretory pathway has also been proposed (reviewed in Berke, 1995) where the CTL induces apoptosis of the target cell via specific cell surface molecules such as Fas, and this may also be important in CTL killing. CTL are thought to play an important role in allograft rejection. Rosenberg et al (1987) demonstrated that skin allograft rejection in mice occurred only in animals which possess both T helper and cytotoxic T cells specific for alloantigens within the graft. Hori et al (1992) demonstrated that mouse skin graft rejection is associated with a potent induction of allospecific CTL responses. When such CTL responses are inhibited following donor-specific transfusion, skin graft survival is significantly prolonged.

CTL are usually $CD8^+$ T cells and thus respond to class I MHC alloantigen. However, some CTL are $CD4^+$ T cells and are thus reactive against MHC class II alloantigen (Rosenberg and Singer, 1992). IL-12 promotes specific CTL responses and can enhance the lytic activity of NK cells (Gately et al, 1998). Both CTL and NK cell activity was, therefore, assessed in the experimental model presented here. Single cell suspensions from the spleens of treated animals were used for in vitro cytotoxicity analysis. Specific cytotoxicity by CTL and non-

specific cytotoxicity by NK cells was quantified in each of the experimental groups

**5.2 Specific Cytoxicity 7 Days after Grafting**

Specific CTL activity in C57Bl/6 animals which had received a $BDF_1$ skin graft was assessed in spleen cell cultures prepared from spleens removed from animals 7 and 10 days after grafting. Two different target cell types were used, namely that of conA blasts prepared from DBA/2 mouse spleen cells ($H2^d$) and P815 cells. The latter cell line constitutively expresses $H2^d$. Thus if CTLs specifically directed against the allo-MHC of $BDF_1$ cells were present, lysis of both types of target cell would occur.

Cells prepared from spleens removed 7 days after grafting from unmodified graft recipients produced killing of DBA/2 conA blasts of 40 % at an effector:target cell ratio of 100:1 and this reduced as the ratio decreased. Results are shown in Figure 5.1. Cells from animals pre-treated with $5 \times 10^7$ $BDF_1$ spleen cells iv. 7 days prior to grafting also produced similar levels of target cell killing in vitro when targets were conA blasts. Cells from mice pre-treated with $5 \times 10^7$ donor cells on day -7 and 0.5 mg. anti-IL-12 antibody ip. on days -8, -5, -2 and 1 showed levels of target cell killing which were slightly reduced compared to the previous 2 groups. However, the differences between the groups are relatively small. The results shown are that of one experiment , but each experiment was repeated and produced similar results.

Spleen cells prepared from the spleens of mice 7 days after grafting were also tested against P815 target cells (figure 5.2). In this experiment, target cell

lysis was comparable in all 3 groups tested, that is cells from unmodified graft recipients, those from animals pre-treated with donor cells alone and those from animals treated with both anti-IL-12 antibody and donor cells. Thus, against P815 targets at day 7 after grafting, pre-treatment of animals did not appear to affect CTL activity.

### 5.3 Specific Cytotoxicity 10 Days after Skin Grafting

Cells were obtained from animals in each of the 3 groups 10 days after grafting and CTL activity assessed against both DBA/2 conA blasts and P815 cells. At day 10, the percentage cytotoxicity in each group was somewhat lower than in each group at day 7. As with day 7 after grafting, CTL activity was detectable in all groups but there were no real differences between percentage cytotoxicities when measured against DBA/2 conA blasts. These results are shown in Figure 5.3.

When CTL activity in cells removed 10 days after grafting was assessed using P815 cells as targets, percentage cytotoxicity was lower than at day 7 (figure 5.4). However, at this time, CTL activity was similar in cells from unmodified grafted animals and in those pre-treated with donor cells alone. By contrast, CTL activity in the group treated with both anti-IL-12 antibody and donor spleen cells was lower. Thus at day 10 after grafting there was little difference between the groups in CTL activity against DBA/2 conA blasts, whilst against P815 cells CTL activity was reduced in the group which exhibited prolonged graft survival. This difference is difficult to explain. Against P815 targets, however, levels of killing were generally low and so differences between groups are small.

These results must, however, be interpreted with some caution. While in some groups pre-treatment with both donor cells and anti-IL-12 antibody did appear to reduce CTL activity, this was, by no means, a consistent finding at both time points or against both target cell types.

**5.4 Non-specific Cytotoxicity**

$CD8^+$ CTL and NK cells represent 2 major populations of cytotoxic lymphocytes, both cell types being able to kill tumour cells, MHC-incompatible grafted cells and autologous cells infected with intracellular pathogens such as viruses (Austin and Wood, 1993). While CTL killing requires T cell help to generate effector cytotoxic cells, NK cells exist as pre-activated cytotoxic cells capable of mediating their effector function without the need for T cell help, making them an important component of the innate immune system (Kos and Engelman, 1996). IL-12 activates NK cells, increasing their cytotoxic activity and secretion of cytokines (Trinchieri, 1995) and so it could be assumed that, by inhibiting the activity of endogenous IL-12, NK cell activation could be prevented. NK cells have been proposed as the cell type responsible for the elimination of injected $BDF_1$ cells in parental strain recipients (Sheng-Tanner and Miller, 1992) and so, by inhibiting NK cell activation, destruction of injected donor-specific cells may be prevented, allowing persistence of such donor cells for longer within the host. Thus persistence of donor antigen after anti-IL-12 antibody treatment may potentially explain the prolonged skin graft survival in this group.

To investigate this, NK cell activity was assessed in vitro using a $^{51}Cr$ release cytotoxicity assay and the NK cell target cell, YAC-1 as described in

Williamson et al (1996). Spleens were removed from experimental animals, single cell suspensions were prepared and the cells were incubated with $^{51}$Cr-labelled YAC-1 cells. Spleen cells were tested from untreated naive animals, animals pre-treated with 5 x $10^7$ $BDF_1$ cells injected iv. 24 hours previously, and animals pre-treated with 5 x $10^7$ $BDF_1$ cells iv. 24 hours previously plus 0.5 mg. anti-IL-12 antibody ip. 24 hours prior to that. Results are those from 1 experiment but are representative of 2 further repetitions and are shown in figure 5.5.

The percentage cytotoxicity in the unmodified, naive group was 40 % for an effector: target cell ratio of 100:1 and steadily fell as the ratio of effector to target cells was reduced. In mice which had been pre-treated with $BDF_1$ cells , the percentage cytotoxicity was consistently increased compared to the naive group, indicating that, when animals are pre-treated with allogeneic cells iv., NK cell activity is increased. Interestingly, in the group treated with both donor cells and anti-IL-12 antibody, the percentage cytotoxicity was consistently reduced compared to naive controls. Thus treatment with anti-IL-12 antibody inhibits NK cell activity in this model.

**5.5 Persistence of Donor Antigen**

The persistence of injected donor strain cells within recipient mice was assessed using FACS analysis. Control C57Bl/6 mice were injected with 5 x $10^7$ $BDF_1$ cells intravenously and their spleens and lymph nodes harvested 24 and 72 hours later. Other C57Bl/6 mice were treated with anti-IL-12 antibody or goat IgG injected ip 24 hours prior to spleen cell injection as in the control group. Donor

cells were labelled with FITC and quantified as a percentage of the spleen or lymph node cell population of the recipient animals.

At 24 hours after spleen cell injection, only small numbers of donor $BDF_1$ cells were detectable in either of the treatment or control groups, and these levels were at the threshold of detection. There were no differences in the percentages detected in each of the groups. These results are shown in Table 5.1 and are expressed as the means of 3 repetitions of each experiment.

Similarly, at 72 hours after spleen cell injection, the percentage of $BDF_1$ cells detectable in the spleens and lymph nodes of C57Bl/6 mice in the control or treated groups was again very small, and no real differences were seen between the groups (Table 5.2).

Thus, in this experimental model using FACS analysis of spleen and lymph node cell populations, no differences were observed in the survival of donor cells in the group treated with anti-IL-12 antibody compared to unmodified controls.

**5.6 Discussion**

In this study, the results of specific cell mediated cytotoxicity were equivocal. While treatment with both donor-specific spleen cells plus anti-IL-12 antibody reduced CTL activity in some experiments, it was not a consistent finding at both time points against both target cell types. Pre-treatment with donor spleen cells alone did not reduce CTL activity. IL-12 is one factor responsible for the activation of CTL (Trinchieri, 1995) and thus, had reduced cytotoxicity been demonstrable in the groups treated with anti-IL-12 antibody, this may have

explained the mechanism whereby prolonged allograft survival was achieved in this model.

Other workers have demonstrated the importance of inhibiting CTL activity in allograft survival. Kitagawa et al (1991, a) showed that while donor cell injection prior to skin grafting generally inhibited MLR responses to alloantigen in vitro, allograft survival was prolonged only if CTL activity was also reduced. They concluded that iv. pre-treatment with donor cells resulted in the elimination of the proliferative and IL-2-producing capabilities of host $CD4^+$ and $CD8^+$ T cells without affecting the capacity of $CD8^+$ CTL precursors to reject the graft. Donor cell pre-treatment prior to skin grafting in a class I MHC mismatch was able to prolong allograft survival only when combined with anti-CD8 mAb therapy (Kitagawa et al, 1991, b). The results described in the present study correlate well with these. However, additional treatment with anti-IL-12 antibody did not consistently reduce CTL activity in vitro. It can be concluded, therefore, that IL-12 is not the only factor required for CTL activation. Thus, in this model of prolonged skin graft survival, inhibition of CTL activity did not appear to be the mechanism whereby graft survival was increased. Similar findings have been reported by Piccotti et al (1996), who found that CTL invasion of vascularised heart grafts is not influenced by antagonism of endogenous IL-12. Neutralising IL-12 did not prevent CTL invading allogeneic cardiac grafts, and rejection was not prevented by this treatment. IL-18, (IGIF) has recently been demonstrated to synergise with IL-12 in the generation of IFN-γ production and in Th1 phenotype generation (Okamura, et al, 1998) and it may be, therefore, that other cytokines such as IL-18 have a role in activation of CTL. This may explain why antagonism

of IL-12 alone is insufficient to inhibit CTL activity. In the current study, however, CTL activity was assessed in preparations of cells from the spleens of treated animals. Thus only one compartment of lymphoid tissue was assessed. It may be that CTL activity in draining lymph nodes, for example, was reduced by pre-treatment with anti-IL-12 antibody and this would be an interesting issue to address in the future.

Interestingly, while treatment with anti-IL-12 antibody did not reduce CTL activity, it did have a significant effect on non-specific cytotoxicity. Cells removed from animals pre-treated with both anti-IL-12 antibody and donor cells showed considerably less NK cell cytotoxic activity in vitro compared to cells from animals pre-treated with donor cells alone. The possibility that such reduced NK cell activity may be important in the prolongation of allograft survival by preventing the rapid destruction of injected donor cells was addressed.

Various authors have described how persistence of donor antigen within the host leads to hyporesponsiveness when the host is rechallenged by a graft (Bushell et al, 1995, Sheng-Tanner and Miller, 1994). NK cells are thought to be the cell type responsible for the rapid elimination of fully allogeneic cells injected intravenously, and probably also for the removal of semi-allogeneic cells (Sheng-Tanner and Miller, 1992). Thus, by inhibiting NK cell cytotoxicity, anti-IL-12 antibody may permit increased survival of injected donor splenocytes, allowing microchimerism to develop within the host and so induce hyporesponsiveness to a subsequent skin graft.

Against this, however, is the finding that, in this experimental model, pre-treatment with anti-IL-12 antibody did not increase survival of injected donor

strain spleen cells in host lymphoid tissue when assessed by FACS analysis. These findings differ from those of Sheng-Tanner and Miller (1994), who found that $BDF_1$ cells, previously labelled with FITC and then injected intravenously into C57Bl/6 mice, could be detected in host lymphoid tissue 3 days after injection. They concluded that persistence of these injected cells leads to a state of microchimerism and this is necessary for the prolongation of graft survival. In their experiments, as already discussed, injection of $BDF_1$ cells alone led to prolonged skin graft survival in C57Bl/6 recipients. The finding in this work that $BDF_1$ cells were not present at 3 days after injection either in unmodified mice or in those pre-treated with anti-IL-12 antibody is obviously at variance with their findings. However, in the work presented here, as no prolongation of graft survival was found in the group treated with donor cells alone, but was seen in the group treated with both donor cells and anti-IL-12 antibody, this indicates either that a state of microchimerism is not important for allograft survival in this model, or else detection of donor cells by FACS analysis labelling cells after injection into this host is not effective. Differences in the injected cell population in Sheng-Tanner's work, or differences in the methods used to detect persisting donor cells may, therefore, be of relevance. It would be of interest, therefore, to investigate this in the future by labelling donor cells with FITC prior to injection and then attempting to quantify persistence of donor cells.

In the work presented here, pre-treatment with anti-IL-12 antibody prior to donor cell treatment did not produce a detectable state of microchimerism within host animals. This finding is supported by the work of Woods' group (Bushell et al, 1995) who found that, in their model of prolonged heart graft survival in mice

treated with donor-specific transfusion plus anti-CD4 antibody, the generation of a state of microchimerism is not required for tolerance induction. Tolerance could be achieved by injecting irradiated donor cells with anti-CD4 antibody, indicating that persistence of viable cells is not required in that model.

Another possibility is that NK cells, via production of IFN-γ in response to IL-12, are directly responsible for the polarisation of T-helper cells, with IFN-γ and IL-12 inducing a Th1 phenotype. Romagnani (1992) proposed that the innate immune response, including NK cell activity, determines the subsequent specific immune response of $CD4^+$ T cells. Antigenic challenge of undifferentiated $CD4^+$ T cells in association with activation of NK cells produces a cascade of events resulting in the development of a Th1 response or the deviation of an expected Th2 response to a Th0 or Th1 phenotype. Manetti et al (1993) showed that the ability of IL-12 to promote the development and differentiation of Th1-type cells in vitro was, at least in part, due to its stimulatory effects on NK cells. They proposed that the generation of Th1 responses was due to the components of the innate immune response providing optimum conditions for the development of Th1 cells. Thus, in this model, allograft survival may be a consequence of anti-IL-12 antibody's inhibitory action on NK cell activation, which, in turn, fails to create the optimum conditions for Th1 cell generation and, instead, leads to Th2 cell generation. The presence of donor antigen at this time may mean that when the host immune response is re-exposed to that antigen in the form of a skin graft, a Th2 response is generated.

## 5.7 Summary of Results of Chapter 5

**1.** Spleen cells prepared from skin graft recipients pre-treated with anti-IL-12 antibody and donor spleen cell injection show similar levels of CTL activity as cells from unmodified graft recipients when assessed in chromium release cytotoxicity assays.

**2.** NK cell activity is, however, reduced in cultures of spleen cells from mice pre-treated with anti-IL-12 antibody compared to those from unmodified animals.

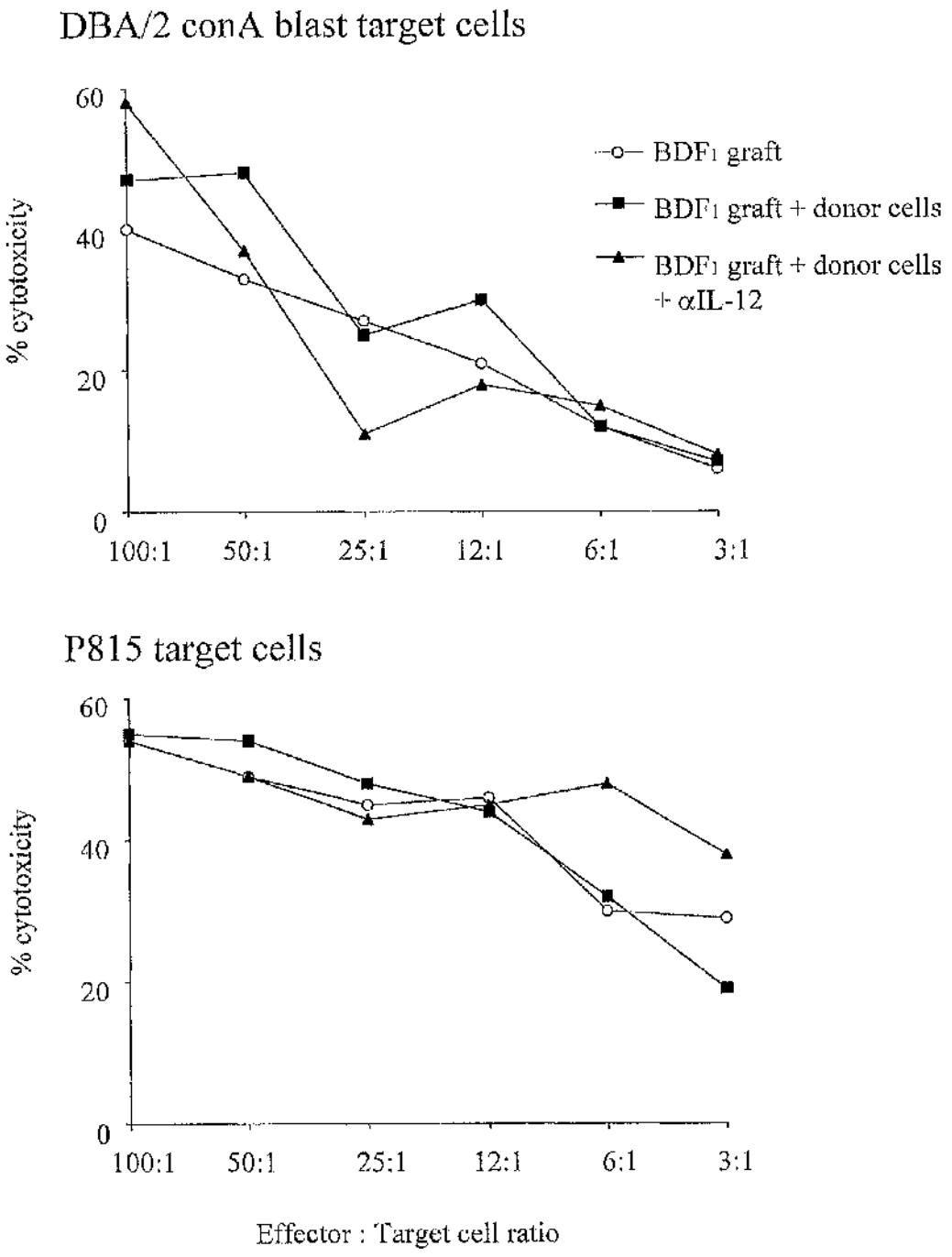
**3.** Pre-treatment of mice with anti-IL-12 antibody is not associated with increased survival of injected donor strain spleen cells within the host, indicating that such treatment does not encourage a state of microchimerism to develop.

| | spleen (%) | lymph node (%) |
|---|---|---|
| donor cells | 1.8 +/-0.3 | 0.9 +/-0.1 |
| donor cells + goat IgG | 1.9 +/-0.2 | 0.9 +/-0.2 |
| donor cells + αIL-12 | 1.4 +/-0.4 | 0.7 +/-0.1 |

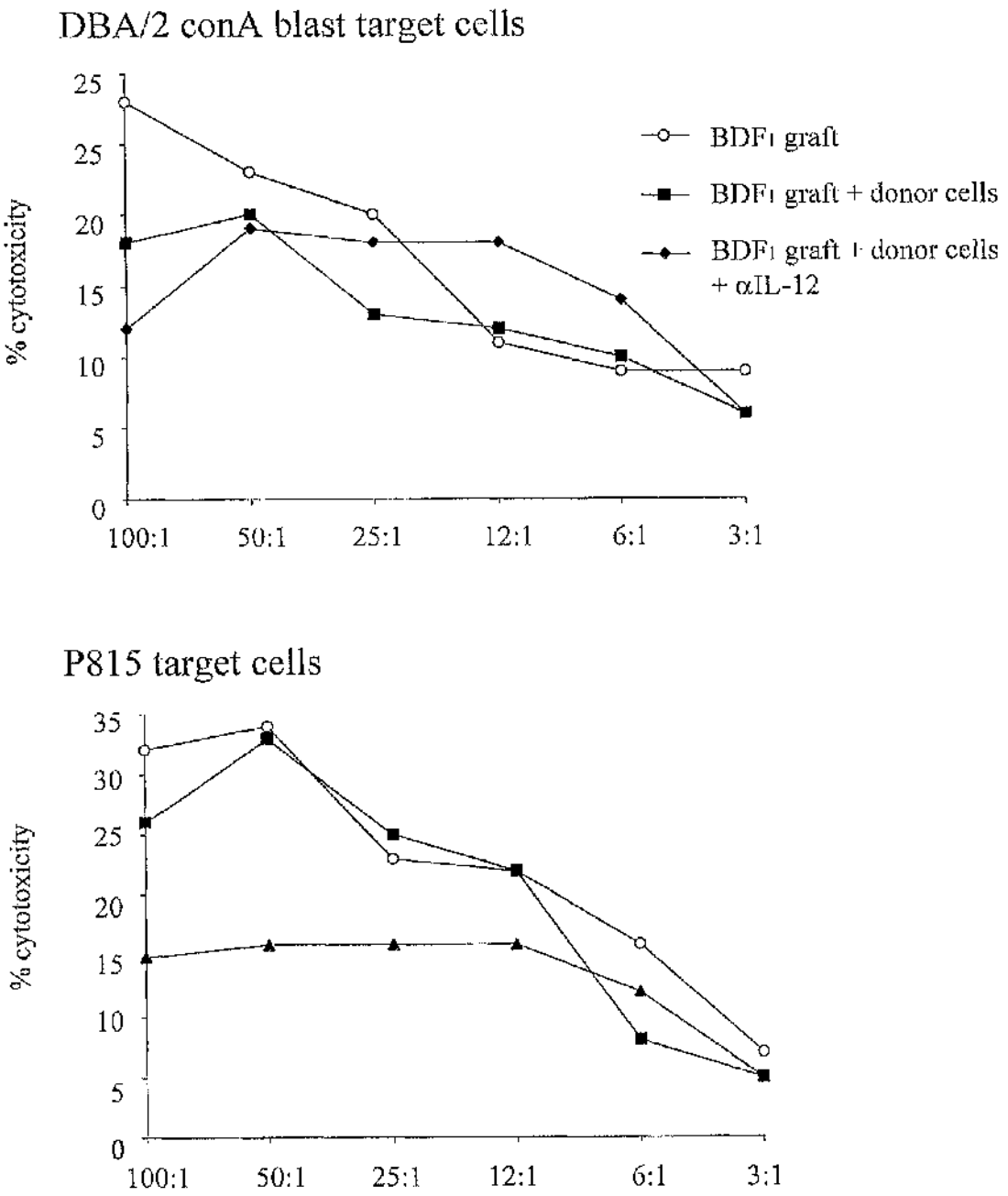
**Table 5.1 Persistence of injected $BDF_1$ cells at 24 hours in the spleen and lymph nodes of recipient animals.** 5 x $10^7$ $BDF_1$ spleen cells were injected iv into C57Bl/6 mice and cells harvested from the spleens and lymph nodes of recipient animals 24 hours later. The percentage of injected donor cells +/- 1 standard deviation, as detected by FACS analysis, is shown for animals injected with spleen cells alone (donor cells), animals pre-treated with 0.5 mg goat IgG injected ip 24 hours prior to $BDF_1$ spleen cell injection (donor cells + goat IgG) and animals pre-treated with 0.5 mg anti-IL-12 antibody injected ip 24 hours prior to $BDF_1$ spleen cell injection (donor cells + αIL-12). Percentages are the means of 3 experiments.

| | spleen (%) | lymph node (%) |
|---|---|---|
| donor cells | 1.1 +/-0.1 | 4.2 +/-2 |
| donor cells + goat IgG | 0.7 +/-0.1 | 1.4 +/-0.3 |
| donor cells + αIL-12 | 0.6 +/-0.1 | 1.7 +/-0.4 |

**Table 5.2 Persistence of injected $BDF_1$ cells at 72 hours in the spleen and lymph nodes of recipient animals.** 5 x $10^7$ $BDF_1$ spleen cells were injected iv into C57Bl/6 animals in each of the groups as above. Spleens and lymph nodes were harvested at 72 hours after donor cell injection and the percentages of donor cells detected are shown.




**Figures 5.1 and 5.2 Percentage cytotoxicity 7 days after grafting.**
Spleen cells were removed from C57Bl/6 mice which had received BDF1 skin grafts 7 days earlier and used in chromium release cytotoxicity assays with either DBA/2 conA blasts or P815 cells as targets. Cells were taken from unmodified graft recipients (BDF1 graft), animals which had been pre-treated with 5 x $10^7$ BDF1 donor cells iv. 7 days prior to grafting (BDF1 graft + donor cells) and animals pre-treated with both donor cells and 0.5 mg anti-IL-12 antibody on days -8, -5, -2 and 1 (BDF1 graft + donor cells + αIL-12).




**Figures 5.3 and 5.4 Percentage cytotoxicity 10 days after grafting.**
Cells were removed from grafted C57Bl/6 mice in the same treatment groups as in Figures 5.1 and 5.2 and cytotoxicity assessed against either DBA/2 conA blasts or P815 target cells in a chromium release assay.

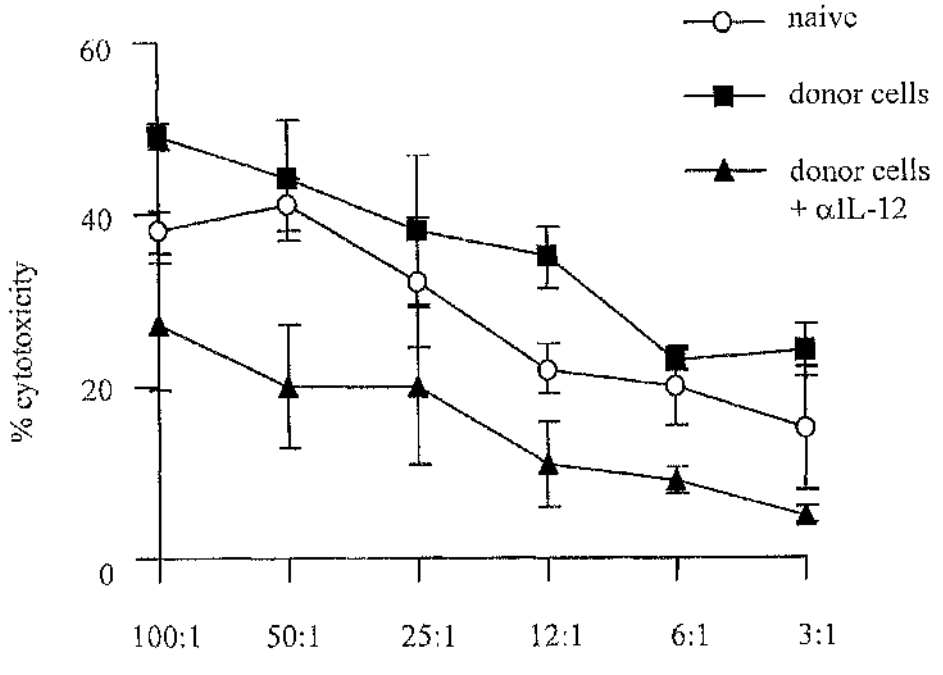



**Figure 5.5 NK cell activity in vitro.** Spleen cells were removed from C57Bl/6 mice and tested in a chromium release assay against YAC-1 target cells. Cells were removed from naïve animals (naïve), animals which had received 5 x $10^7$ $BDF_1$ cells iv. 24 hours previously (donor cells) and animals which had received the same donor cell pre-treatment plus 0.5 mg anti-IL-12 antibody ip. 24 hours prior to that (donor cells + αIL-12). Results are the calculated percentage cytotoxicity for each effector cell concentration +/- 1 standard deviation from 1 experiment which was representative of 3.

# CHAPTER 6

# FINAL DISCUSSION

Interleukin-12 is a key immunoregulatory cytokine, playing a central role in the differentiation of T helper cells. It stimulates both NK cells and T cells to produce IFN-γ, amplifying the Th1 response, and induces the differentiation of Th0 into Th1 cells. Allograft rejection is usually associated with an up-regulated Th1 response, and prolonged graft survival associated with a reduced Th1 response and an up-regulated Th2 response. The aim of this work was, therefore, to investigate whether, by neutralising endogenous IL-12 around the time of skin grafting, allograft survival would be prolonged and whether this would be associated with an up-regulated Th2 response.

Pre-treatment with a polyclonal anti-IL-12 antibody prior to skin grafting did not prolong allograft survival in a semi-allogeneic murine model with $BDF_1$ tail skin grafted onto either C57Bl/6 or DBA/2 recipient mice. Intravenous injection of donor strain spleen cells 7 days prior to grafting also failed to prolong graft survival. However, when these two therapies were combined, namely pre-treatment with both anti-IL-12 antibody plus donor strain spleen cell injection, allograft survival was significantly prolonged. Interestingly, the timing of each treatment was significant, because spleen cell injection just prior to skin grafting in mice which had been pre-treated with anti-IL-12 antibody as before failed to prolong allograft survival. The combination of donor cell injection plus anti-IL-12 antibody pre-treatment was not able, however, to prolong skin graft survival in a fully allogeneic mismatch, where C57Bl/6 skin was grafted onto DBA/2 recipients or vice versa.

Unmodified skin graft rejection was characterised by an up-regulated Th1 response when cells prepared from the spleens of grafted animals were re-

stimulated in vitro with donor antigen. Pre-treatment of graft recipients with anti-IL-12 antibody alone reduced this Th1 response, with less IFN-γ and IL-2 being produced by spleen cells in vitro, but did not abolish it. Antagonism of endogenous IL-12 alone also led to a weak Th2 response when cells were re-stimulated in vitro. This indicates that antagonism of IL-12 alone is not sufficient to prevent the generation of a Th1 response. This finding supports recent evidence that another cytokine, Interferon-γ-Inducing Factor, or IL-18, is capable of inducing IFN-γ production independently of IL-12 and so generating a Th1 response.

When cells from grafted animals pre-treated with a combination of donor spleen cells injected intravenously and anti-IL-12 antibody were re-stimulated in vitro, the Th1 response was markedly down regulated and a Th2 response was generated. This treatment regime was associated with prolonged allograft survival. In this model, while the generation of Th2 response was associated with prolonged allograft survival, the levels of the Th2 cytokines produced in vitro were still low. Thus, while a Th2 response was detectable, it may not be the actual cause of the prolonged graft survival. Interestingly, pre-treatment with donor spleen cells alone was associated with a down-regulated Th1 response when cells from treated animals were re-stimulated in vitro, but no increase in the Th2 response. Despite these changes, no increase in skin graft survival was seen in this group. This would indicate that allograft rejection can occur in the absence of a Th1 response and rejection takes place at the same speed as with a normal Th1 response. However, in this group, there was no up-regulation of the Th2 response and it may be that this is the significant factor for prolonging allograft survival.

Pre-treatment with anti-IL-12 antibody after donor cell injection did not affect CTL activity as assessed by an in vitro chromium-release cytotoxicity assay, indicating that the prolongation of skin graft survival in this group was not attributable to the inhibition of CTL activity. Similar findings have been reported in a cardiac allograft model, where antagonism of endogenous IL-12 alone did not prolong graft survival, and inhibition of IL-12 did not significantly affect in vivo CTL responses (Piccotti et al , 1997, b).

Pre-treatment with anti-IL-12 antibody did, however, reduce NK cell activity compared to unmodified controls. This result would be anticipated as IL-12 is known to act directly on NK cells, stimulating them to produce IFN-γ. The significance of this finding in the context of allograft rejection is, however, less clear. Despite this reduction in NK cell activity, a state of microchimerism in animals pre-treated with both donor spleen cells and anti-IL-12 antibody, where donor strain cells survive indefinitely within the graft recipient, could not be demonstrated. Thus the mechanism for prolonged graft survival in this model could not be demonstrated to be due to prolonged survival of injected donor cells due to a reduction in NK cell destruction of such cells. This finding, however, merits further investigation.

Prolonged skin graft survival was associated with a down-regulated Th1 response and an up-regulated Th2 response. This may be due to the effects of anti-IL-12 antibody and injected donor cells directly on Th cells, or it may also be due to effects on NK cells. By inhibiting NK cell function, the optimal environment for the differentiation of Th0 cells into Th1 cells may have been prevented, and a Th2 response generated instead.

These cytokine responses were, however, seen in single cell suspensions from the spleens of grafted animals. Obviously, such in vitro findings may not accurately reflect the local cytokine environment within the rejecting graft. In addition, in many of the experiments, only spleen cells were assessed and it may be that differences in responses could have been detected if cells from different lymphoid compartments such as draining lymph nodes had been assessed. However, spleen cell preparations did show consistent and reproducible differences between the treatment groups.

This work has demonstrated that the Th1/Th2 paradigm does have some relevance in allograft rejection. Antagonism of endogenous IL-12, with its central role in the differentiation of Th cells, can have some influence on the immune response to a graft but, on its own, is not sufficient to prolong allograft survival. However, when inhibition of IL-12 is combined with some other immunomodulating treatment, in this case the injection of donor strain spleen cells, prior to grafting, a strong Th2 response can be induced and allograft survival prolonged.

Prolonged graft survival in a skin graft model is often difficult to achieve and therefore these results indicate that the prolongation in allograft survival seen in this model is significant. It may be that by using a similar treatment protocol in a less demanding model, such as a vascularised heart graft, further increases in survival may be achieved. In addition, as evidence accumulates on the role of other cytokines such as IL-18 on Th1 cell generation, it would be interesting to attempt to neutralise the effects of both IL-12 and IL-18 in this model.

This work has demonstrated that, by effecting a shift from a Th1 to a Th2 response, allograft survival can be prolonged and this may have some implications for clinical transplantation. Treatment of transplant recipients with mAb has been used before and so antagonism of immunoregulatory cytokines at the time of transplantation by such means may prove to be beneficial. Clinical trials using recombinant IL-12 in a variety of conditions such as AIDS have started (Gately et al, 1998) but, as yet, an IL-12 antagonist suitable for clinical testing has not yet been identified. Were such an antagonist found it may prove to have a role in the prevention of transplant rejection.

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