CYTOKINES IN THE IMMUNOPATHOGENESIS OF MURINE GRAFT-VERSUS-HOST DISEASE

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SUMMARY

Murine models of graft-versus-host disease (GvHD) provide important information relevant to clinical bone marrow transplantation (BMT), as well as to other types of T cell-mediated pathology. The nature of the GvHD which develops in (C57BL/6 x DBA/2)F_1 (BDF_1) mice injected with parental lymphocytes is dependent on whether C57Bl/6 (B6) or DBA/2 parental donor cells are used. BDF_1 mice injected with B6 donor cells (B6 ⇒ BDF_1) develop an acute GvHD with early lymphoid hyperplasia and NK cell activation, followed by immunosuppression, activation of anti-host cytotoxic T lymphocytes (CTL), weight loss and early death. In contrast, BDF_1 mice given DBA/2 donor cells (DBA/2 ⇒ BDF_1) exhibit a chronic, stimulatory GvHD, characterised by B cell hyperreactivity, autoantibody production and immune complex-mediated glomerulonephritis (ICGN).

Previous studies have shown that the distinct forms of GvHD in BDF_1 recipient mice are associated with different patterns of cytokine production. Whereas acute GvHD is characterised by production of high levels of Th1 cytokines, chronic GvHD is associated with a preferential Th2 response. Therefore, it was suggested that the two forms of GvHD may reflect differential activation of distinct subsets of $CD4^+$ T helper (Th) cells. However, when and why such T cell polarisation should occur has remained unclear. A number of recent studies have demonstrated that cytokines produced by cells of the non-specific immune system during the early phase of an immune response can strongly influence the type of specific response which develops subsequently. The main aim of this thesis was to explore the role of these early immune mediators in determining the outcome of the GvHD in BDF_1 mice.

The initial series of experiments described in Chapter 3 detailed the kinetics of acute and chronic GvHD in order to determine at what point the cytokine response becomes polarised towards a preferential Th1 or Th2 phenotype. I found that acute GvHD in B6 ⇒ BDF_1 mice is a biphasic disease, in which Th1 cytokines and intense

lymphoid hyperreactivity progress into a destructive disease characterised by death and immunesuppression. In contrast, chronic GvHD is an initailly milder, but ultimately more persistent disorder, which appears to involve a mainly Th2-type immune response. These results confirmed previous reports that differential cytokine production is associated with acute and chronic GvHD and extended them by showing that the responses elicited by B6 and DBA/2 parental donor cells diverged very rapidly.

IL-12, a heterodimeric cytokine produced by APCs, plays a key role in the early polarisation of Th cell responses in a variety of disease models by selectively promoting the growth and differentiation of Th1 cells. In Chapters 4-6, I therefore examined directly the role of IL-12 in determining the outcome of the GvHD in BDF_1 mice, by investigating the effect of neutralising IL-12 *in vivo*. A single injection of neutralising anti-IL-12 antibody abrogated many of the early proliferative features of acute GvHD, including splenomegaly and NK cell activation, but these effects were transient. Neutralising endogenous IL-12 in B6 $\Rightarrow$ BDF_1 mice for a longer period had a more dramatic effect on the development of acute GvHD, as repeated administration of anti-IL-12 during the first 8 days of the disease prevented all subsequent immunosuppression, weight loss and mortality. Anti-IL-12 not only conferred long-term protection from the disease, but also permitted full repopulation with donor B6 lymphocytes. The cytokine response of anti-IL-12 treated surviving mice was permanently polarised towards a Th2 phenotype similar to that observed in DBA/2 $\Rightarrow$ BDF_1 mice. However, long-term survivors did not develop the autoimmune pathology associated with the chronic form of disease. These results show that early production of IL-12 plays a critical role in acute, but not chronic GvHD. In contrast, neutralising IL-12 had no effect on the chronic GvHD in DBA/2 $\Rightarrow$ BDF_1 mice.

To explore further the influence of IL-12 in acute vs chronic GvHD, in Chapter 7, I examined the effects of giving rm IL-12 to both B6 $\Rightarrow$ BDF_1 and DBA/2

$\Rightarrow$ BDF_1 mice. Administration of IL-12 converted chronic GvHD into a more acute type of disease, with a characteristic pattern of early hyperplasia followed by late immunosuppression and destruction. Exogenous IL-12 also provoked anti-host CTL activity, weight loss and mortality in DBA/2 $\Rightarrow$ BDF_1 mice and converted the cytokine profile towards the Th1 pattern usually seen during acute GvHD. IL-12 also exacerbated the systemic acute GvHD in B6 $\Rightarrow$ BDF_1 mice. Although these results confirmed the important role of IL-12 in acute GvHD, enhanced levels of IL-12 were produced by spleen cells from both B6 $\Rightarrow$ BDF_1 and DBA/2 $\Rightarrow$ BDF_1 mice in response to LPS *in vitro*. However, B6 $\Rightarrow$ BDF_1 cells produced higher amounts of IL-12 over a longer period.

The principal immunomodulatory role of IL-12 is to induce IFN-γ production by $CD4^+$ and $CD8^+$ T cells and NK cells. In Chapter 8, I therefore examined directly the role of IFN-γ in acute GvHD, both in unmanipulated B6 $\Rightarrow$ BDF_1 mice and in DBA/2 $\Rightarrow$ BDF_1 mice given IL-12. Depletion of IFN-γ ameliorated many of the destructive features of both diseases, including weight loss, mortality and suppression of lymphoid responses, as well as reducing splenomegaly and increasing Th2 cytokine production. In contrast, IFN-γ was not required for CTL activation, induction of IFN-γ production and inhibition of B cell activity.

The source of the different cytokines produced in GvHD were examined in Chapters 9 and 10. $CD4^+$ T cells were the principal source of IL-2, IFN-γ, IL-5 and IL-10 in both acute and chronic GvHD, although $CD8^+$ T cells and other cells also contributed to some of the cytokine production. In particular, during acute GvHD, $CD4^+$, $CD8^+$ and non-$CD4^+CD8^+$ populations all appear to contribute to the high levels of IFN-γ produced by spleen cells from B6 $\Rightarrow$ BDF_1 mice. However, the exact source of IFN-γ appeared to vary at different times and I found evidence that several populations can produce or regulate the production of many of the critical cytokines involved in GvHD.

Finally, since NK cells are important during other models of GvHD and are a source of IFN-γ in various models of T cell-mediated immunity. In Chapter 10 I examined the involvement of NK cells in polarising immune effector responses early in acute GvHD. Depletion of NK cells with a single injection of anti-ASGM-1 antibody had no consistent effect on either the progression of the disease or the cytokines produced during its course. Thus, NK cells may not be involved in the initial polarisation of acute vs chronic GvHD.

My results thus extend previous findings on the distinct forms of immunopathology which develop in BDF_1 mice with acute and chronic GvHD. In addition, my studies have demonstrated the importance of individual cytokines in polarising the allogeneic T cell response and determining its pathological outcome. In particular, they emphasise the role of IL-12 in Th1 cell activation. These studies of the cellular and molecular interactions involved in murine GvHD have implications for understanding the pathogenesis of clinical GvHD and the development of specific therapy following BMT. In addition, they provide an important insight into the regulation of immune responses during other immunologically-mediated diseases.

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INDEX

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 5

CHAPTER 8

CHAPTER 9

CHAPTER 10

DECLARATION

These studies represent original work carried out by the author, and have not been submitted in any form to any other University. Where use has been made of material provided by others, due acknowledgement has been made.

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PUBLICATIONS

Parts of this thesis have been included in the following publications:

1. Williamson, E., Garside, P., Bradley, J. A. and Mowat, A. McI. 1996. Interleukin-12 is a central mediator of acute graft-versus-host disease in mice. Journal of Immunology, 157: 689-699.

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ABBREVIATIONS

APC	Antigen Presenting Cell(s)
ASGM-1	Asialo GM-1
BBS	Borate Buffered Saline
BSA	Bovine Serum Albumin
BMT	Bone Marrow Transplantation
Con A	Concanavalin A
CTL	Cytotoxic T Lymphocyte(s)
DC	Dendritic Cell(s)
ddH_2O	Deionised Distilled H_2O
ds-DNA	Double-Stranded DNA
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscopy
FACS	Fluorescence-Activated Cell Scanner
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GvHD	Graft-versus-Host Disease
GvHR	Graft-versus-Host Response
GBM	Glomerular Basement Membrane
^3H	Tritium
H & E	Haematoxylin and Eosin Staining
IC	Immune Complex(es)
ICGN	Immune Complex-Mediated Glomerulonephritis
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin

LM	Light Microscopy
LPS	Lipopolysaccharide
Mϕ	Macrophage(s)
mAb	Monoclonal Antibody
2-ME	2-mercaptoethanol
MHC	Major Histocompatability Complex
$Na_2{}^{51}CrO_4$	Sodium ^{51}Chromate
NCS	Newborn Calf Serum
NK cell	Natural Killer Cell
NO	Nitric Oxide
O.D.	Optical Density
PBS	Phosphate Buffered Saline
pCTL	Precursor Cytotoxic T Lymphocyte(s)
PE	Phycoerythrin
RID	Radial Immunodiffusion
rm	Recombinant Murine
SA	Streptavidin
SB	Staining Buffer
SEM	Standard Error of the Mean
SD	Standard Deviation
TcR	T Cell Receptor
TGF-β	Transforming Growth Factor-β
Th cell	T helper Cell
Th1	T helper 1
Th2	T helper 2
TMB	3,3'5,5'-tetramethylbenzidine
TNF-α	Tumour Necrosis Factor-α

CHAPTER 1: INTRODUCTION

Graft-versus-host reactions (GvHR) are initiated when mature T lymphocytes are transferred into allogeneic recipient animals, thereby provoking the development of a multi-organ inflammatory syndrome termed graft-versus-host disease (GvHD). Experimental GvHD was first described by Billingham, Brent & Medawar [1, 2], who found that newborn mice inoculated with allogeneic lymphocytes developed a "runt disease", associated with malabsorption, growth retardation, and damage to the liver, skin, intestinal tract and lymphoid organs. Although initially described as a disorder of experimental animals [1-3], GvHD was subsequently found to be a common and often fatal, complication of human allogeneic bone marrow transplantation (BMT) [4-6]. Human acute GvHD closely resembles acute GvHD in mice, and similar pathological changes in the skin, gut and liver are observed [3, 4, 7-9]. Clinical chronic GvHD can evolve directly from primary acute disease or develop "de novo" [10]. In either case, the clinical manifestations of chronic GvHD are similar to those of collagen vascular diseases, such as systemic lupus erythematosous (SLE) or scleroderma [11, 12].

Since BMT remains the treatment of choice for a variety of diseases, including aplastic anaemia, severe combined immunodeficiency (SCID) and leukaemia [5, 6, 13-15], GvHD presents an obstacle in the therapeutic use of BMT. Despite the introduction of immunosuppressive agents such as Cyclosporine A and more recently, FK506 and rapamycin, as GvHD prophylaxis [10, 16], clinically significant GvHD develops in up to 60% of patients receiving an HLA-matched sibling BMT [9, 13]. Conversely, the more selective approach of depleting T cells from the donor BM to prevent GvHD [17-19] leads to failure of engraftment and causes leukaemic relapse [20]. Alternative GvHD preventative strategies are clearly needed and a better understanding of the complex interactions involved in GvHD will assist their development. Several animal models have therefore provided experimental systems to explore the pathogenesis of clinical

GvHD and other forms of T cell-mediated pathology in the relevant tissues [8, 21-23].

EXPERIMENTAL MODELS OF GvHD

Murine models of GvHD allow experimental manipulation of effector mechanisms in a broad range of donor/host strain combinations of distinct genetic backgrounds. Furthermore, the pathological alterations of acute murine GvHD correlate well with those in the human disease and has provided important insights into its pathogenesis. The essential requirements for induction of GvHD in experimental animals are as follows:- 1) The donor inoculum must contain immunocompetent T lymphocytes, since their depletion from allogeneic bone marrow grafts prevents GvHD initiation [17]. 2) The donor and host must be genetically incompatible i.e. the host must express minor or major histocompatibility (MHC) antigens which are lacking in the donor inoculum and so are recognised by the donor T cells as foreign. 3) The host mice must be unresponsive to the injected donor cells, either because they are immunocompromised, or because they are genetically tolerant to the donor.

There are two principal models of experimental GvHD:-

1). Irradiated model:- the immune system of recipient mice is deliberately destroyed by irradiation (or chemotherapy). The subsequent injection of bone marrow and low numbers of donor T cells initiate a very severe, often lethal GvHD.

2). Parent $\Rightarrow$ F_1 model:- occurs when unirradiated, immune competent F_1 hybrid mice are given parental donor T cells. In this case, donor lymphocytes can respond to the non-shared host MHC antigen(s), whereas host T cells are genetically tolerant to the injected cells. The severity of this form of GvHD is dependent on factors such as the nature of the donor and host MHC disparity [21, 24-29], the phenotype of the cell population used to induce the GvHR [30, 31], and the microbiological status of the host [32-35]. This was the model of GvHD

chosen for the studies contained in this thesis, which focuses on the events involved in the induction and development of GvHD in unirradiated (C57Bl/6 x DBA/2)F_1 (BDF_1) recipient mice injected with either C57Bl/6 (B6) or DBA/2 parental donor spleen cells.

The BDF_1 model of GvHD is particularly useful because the nature of the disease can be modified by using either C57Bl/6 (B6) or DBA/2 parental donor cells. B6 (H-2^b) donor cells provoke an acute GvHD, with early lymphoid hyperplasia, followed by immunosuppression, lymphoid atrophy, activation of anti-host cytotoxic T lymphocytes (CTL), weight loss and early death [21, 24, 27, 28, 30, 31]. In contrast, DBA/2 (H-2^d) donor cells elicit a chronic, stimulatory GvHD, characterised by B cell hyper-reactivity, autoantibody production and Immune Complex mediated Glomerulonephritis (ICGN) [23, 24, 36-40]. Previous studies have shown that the distinct forms of GvHD which develop in BDF_1 host mice are associated with different patterns of cytokine production. Acute GvHD is characterised by production of high levels of IFN-γ and IL-2 [41-45], while chronic GvHD is associated with production of the Th2-type cytokines, IL-4, IL-5 and IL-10 [42, 46-48]. Thus, it has been suggested that the two forms of GvHD reflect differential activation of $CD4^+$ T helper (Th) cell subsets [42, 46]. However, the reason for this polarisation has remained unclear.
The spectrum of pathological alterations observed in acute and chronic GvHD is illustrated in Fig. 1.1.

IMMUNOPATHOGENESIS OF ACUTE GvHD

The acute GvHD which develops in B6 ⇒ BDF_1 mice is characterised by an initial period of immune stimulation, during which donor T cells expand in the host lymphoid organs [27, 29, 45] and enhanced NK cell activity is observed [49]. However, the early hyperplasia is rapidly replaced by suppressive manifestations, including loss of B and T cell function, hypogammaglobulinaemia and destructive pathology in the skin, liver, small intestine and lymphoid tissue [7,

21, 24, 27, 28]. The host lymphohaemopoietic system is destroyed, severe weight loss is evident and the majority of animals die from an increased susceptibility to secondary infection and/or shock [21]. However, a small number of mice survive acute GvHD and because the initial splenic donor inoculum contains a high frequency of stem cells [50], these survivors are eventually repopulated with donor-derived lymphohaemopoeitic cells [33, 51, 52].

The cellular requirements for initiation of acute GvHD have been studied extensively. Optimal induction of the disease requires the presence of both $CD4^+$ and $CD8^+$ T cells in the donor inoculum [30] and incompatibility in the F_1 recipient at both MHC class I and class II loci [25, 26, 29]. The immunosuppression and lethality can be inhibited by depleting either $CD4^+$ or $CD8^+$ T cells from the inoculum, [26, 30, 31] and CD8 depletion converts the acute disease into a chronic, stimulatory GvHD, similar to that found in unmodified DBA/2 $\Rightarrow$ BDF_1 mice [31]. The use of F_1 mutant mouse strains which differ from donor mice solely at class I and/or class II MHC loci also shows that acute GvHD is characterised by sequential activation of class II-specific donor $CD4^+$ T cells, followed by class I-specific $CD8^+$ T cells [21, 29].

Although both $CD4^+$ and $CD8^+$ T cells are required for acute GvHD progression, the relative role of each of these populations in disease pathology is still not fully understood and remains very controversial. The main effector function of $CD4^+$ T cells is to secrete cytokines and they can be divided into two subsets depending on the pattern of these mediators they secrete [53]. T helper type-1 (Th1) cells are responsible for cell-mediated immunity, while T helper type-2 (Th2) cells mediate humoral responses. Recent studies have suggested that during acute GvHD, activated Th cells appear to differentiate preferentially towards a Th1 phenotype [42-44, 54]. Using semi-quantitative polymerase chain reaction (PCR) to detect cytokine mRNA, Allen et al. [42] demonstrated that spleen cells from acute GvHD mice produced much higher levels of IFN-γ mRNA than did cells from either chronic GvHD or control mice. Furthermore, Troutt et

al. [54] observed that up to 70% of spleen cells in B6 ⇒ BDF_1 mice were producing IFN-γ mRNA compared with < 1% of normal cells. Several studies have also suggested that acute GvHD is critically dependent on the high levels of Th1 cytokines produced during its development [55-57]. Administration of anti-IL-2 monoclonal antibody (mAb) to B6 ⇒ BDF_1 mice inhibits anti-host CTL cytotoxicity and prevents the destruction of host B cells usually observed [57]. The intestinal pathology associated with this model can also be prevented by neutralising IFN-γ *in vivo* [55].

Th1 cytokines could account for several features of acute GvHD. Firstly, IFN-γ activates macrophages (mϕs) [58] and induces the release of inflammatory mediators such as IL-1, IL-6, TNF-α and nitric oxide (NO) [59, 60]. Cells from mice with acute GvHD show an enhanced capacity to produce IL-1, IL-6 and TNF-α when stimulated with LPS *in vitro* [41, 43, 61] and inhibition of IL-1 [62, 63] or TNF-α [64, 65] *in vivo* reduces the severity of the disease. Similarily, injection of the NO synthesis inhibitor L^G-monomethyl-arginine (L-NMMA) to mice with GvHD prevents enteropathy [66]. Further potential functions of Th1 cytokines in acute GvHD include activating NK cells, providing help for CTL responses and contributing to immunosuppression. IL-2 and IFN-γ act synergistically to trigger NK cells to secrete cytokines and develop into activated killer cells [67, 68], while IL-2 is required for $CD8^+$ cells to differentiate into anti-host CTL during acute GvHD [57]. The final way in which IFN-γ may be important in the acute disease is by suppressing lymphoid cell responses. Several mechanisms have been implicated in GvHD-asociated immunosuppression, including the destruction of lymphoid tissue by CTL [23, 31] and deficient lymphopoiesis of new B and T cell populations secondary to GvHD-attack on the thymus and bone marrow [50, 69, 70]. However, active suppression of lymphoid responses, demonstrated by the ability of GvHD cells to reduce B or T cell responses of cocultured normal host or donor cells, also occurs [27, 28, 30] and has been linked consistently to the production of IFN-γ [71-73]. IFN-γ has

cytostatic properties *in vitro* [74] and anti-IFN-γ has been shown to prevent much of the suppression by GvHD cells *in vitro* [71, 73]. IFN-γ may act by inducing release of additional immunosuppressive agents such as transforming growth factor-β (TGF-β) [71] or NO [75] which then modulate immune responses. Although it is not clear why IFN-γ production predominates in acute GvHD, IL-12 has been shown to be central to its induction in other systems [76-82].

$CD8^+$ T cells are also critical for the development of acute GvHD, although their exact role is unknown. The disease can be prevented by depleting $CD8^+$ cells from the donor inoculum [30, 31] and $CD8^+$ T lymphocytes from mice with acute GvHD can lyse host alloantigen bearing target cells *in vitro* [24, 27, 31]. The kinetics of this CTL response correlate with destruction of host lymphoid cells *in vivo* [31], while chronic GvHD in DBA/2 ⇒ BDF_1 mice is not associated with either tissue destruction or detectable CTL activity [23, 24, 27, 31]. However, the presence of CTL does not always correlate with tissue damage and lethality [24, 27, 83]. An alternative possibility is that allospecific $CD8^+$ T cells act by producing mediators which inhibit immune function and/or cause tissue damage. These cells were first described as "allosuppressor" T cells in both murine and clinical GvHD, based on their ability to inhibit the function of other lymphocyte populations *in vitro*. [27, 30, 84]. Recent studies in other systems have also suggested that $CD8^+$ T cells produce IFN-γ [85-88] and TGF-β [89], both with known inhibitory properties. Finally, it should be noted that under certain conditions, $CD8^+$ cells can also produce a variety of other cytokines, including IL-4, IL-5 and IL-10 [90-94] and it therefore remains important to define the role(s) of $CD8^+$ T cells directly.

A widely described feature of murine and clinical GvHD is NK cell activation [7, 49, 95-99], although their precise role in GvHD is unclear. NK cells are classified as large granular lymphocytes (LGL) and like $CD8^+$ CTL, can be directly cytotoxic [100]. However, in contrast to specific CTL-mediated cytotoxicity, the susceptibility of target cells to NK cell-mediated lysis is inversely

proportional to their expression of class I MHC antigens [101]. Increased NK cell activity is observed early during several models of GvHD [7, 49, 95-98] and involves both donor and host NK cell populations [95, 102, 103]. NK cell depleted donor populations induce a less severe acute GvHD [102, 104, 105] and depletion of NK cells in recipient mice by *in vivo* injection of anti-ASGM-1 antiserum [105, 106] also reduces lethal disease. NK cells are found in areas of epithelial cell damage [102, 107-109] and therefore may contribute to disease by cytotoxicity. NK cells are thought to mediate rejection of certain parental bone marrow grafts by F_1 hybrid mice (hybrid resistance) [110, 111]. However NK cells may also contribute to acute GvHD by releasing pathogenic cytokines such as IFN-γ. This possibility is consistent with current ideas that, by providing an early source of IFN-γ, NK cells are an important component of innate defence against pathogens, by activating phagocytic cells [112-114] and promoting Th1 cell differentiation [115-117]. NK cells are recruited and activated by IL-12 [118-121], a proinflammatory cytokine released by mϕs and other APC [76, 122, 123]. IL-12 therefore provides a link between innate resistance and T cell-dependent responses. The role of NK cells in polarising GvHD has not been examined previously, nor has the relationship between IL-12 and NK cells been explored.

IMMUNOPATHOGENESIS OF CHRONIC GvHD

DBA/2 ⇒ BDF_1 mice develop a chronic GvHD, characterised by persistent immune stimulation of mainly host B lymphocytes. This leads to increased levels of serum IgG and IgE [37-39, 48] and production of autoantibodies against nuclear antigens, double-stranded DNA (ds-DNA), erythrocytes and thymocytes [21, 23, 38, 39]. Unlike the acute GvHD, tissue destruction and early death are not features of the chronic disease. However, after several months, DBA/2 ⇒ BDF_1 mice develop immune complex-mediated glomerulonephritis (ICGN), as evidenced by proteinuria, ascites and deposits of

immune-complexes in renal glomeruli and usually die from kidney failure, or lymphoma [21, 36-39].

Chronic GvHD appears to be mediated entirely by $CD4^+$ T cells [26, 30], which are activated by allogeneic class II MHC antigens expressed on host B cells and then release cytokines which activate the B cells to proliferate and produce autoantobodies [39, 124]. No expansion of donor $CD8^+$ T cells is observed and anti-host CTL activity is not detectable [27, 28, 31]. As a result, host lymphohaemopoietic cells are not eliminated and instead of repopulation by the donor cells, mixed chimerism develops [21, 23]. Chronic GvHD can also be induced by the injection of purified B6 $CD4^+$ T cells [30, 125], while depletion of $CD8^+$ T cells prevents the ability of B6 cells to induce acute GvHD and promotes development of chronic GvHD [30, 31]. Similarly, chronic GvHD is induced by B6 cells when the host expresses an isolated MHC class II disparity [25, 26, 29]. Previous studies suggest that the key-factor in chronic GvHD is the continued stimulation of host reactive donor $CD4^+$ T cells by host MHC class II-expressing B cells [39, 124, 126, 127] and that this occurs because host B cells are not eliminated by the inflammatory processes which underlie acute GvHD [24, 27, 31]. Potentially autoreactive B cells exist even in normal mice [21] and when adequate Th cell help is provided (in this case by alloreactive donor $CD4^+$ T cells), these can be triggered to secrete high affinity autoantibodies [21, 39, 124].

Recent work has suggested that chronic GvHD may be associated with a Th2-type cytokine phenotype [42, 46-48, 128]. This is consistent with the fact that Th2 cytokines drive B cell responses [53] and recent studies have shown elevated levels of mRNA for IL-4 and IL-10, but not for IFN-γ, in unstimulated cultures of chronic GvHD spleen cells [42, 46, 47]. Furthermore, the majority of antibodies produced during chronic GvHD are of the IgG_1 and IgE subclasses [48], both of which are highly dependent on IL-4 production [129] and are therefore preferentially associated with Th2 responses. Anti-IL-4 treatment *in vivo*

has also been shown to prevent the elevated serum immunoglobulin and proteinuria associated with the disease, in addition to reducing mortality [48, 128].

The reasons why the same class I and class II MHC antigens cause different pathological outcomes depending on the donor cell origin are unclear. Using limiting dilution analysis, it has been shown that DBA/2J mice have a nine-fold lower anti-BDF_1 precursor CTL (pCTL) frequency than B6 mice [31] and thus are unable to generate a comparable $CD8^+$-mediated cytotoxic attack on BDF_1 recipients. However, when spleen cells from congenic DBA/2HaSmm mice are used to induce GvHD in BDF_1 mice, instead of the DBA/2J cells normally used in this model, a classic acute GvHD with anti-host CTL activity and immunosuppression develops [130]. As DBA/2HaSmm mice are also H-2^d and have a similarily low anti-H-2^b pCTL frequency as that of DBA/2J mice [130, 131], it would appear that a low pCTL frequency does not in itself prevent the development of acute GvHD. Furthermore, these experiments show that chronic GvHD does not simply result from injecting H-2^d donor cells into BDF_1 mice and cells from other H-2^d strains, including C57Bl congenic B10.D2 mice elicit acute GvHD normally [21]. Thus, the inherent differences in the T cell responses of B6 and DBA/2 mice responsible for modulating GvHD remain unidentified.

Taken together, it appears that Th1 cytokine production, together with $CD8^+$ donor T cell engraftment provokes acute GvHD, while a preferential Th2 cytokine response and a lack of donor $CD8^+$ T cell engraftment, leads to chronic GvHD. Determining why these different T cell responses were elicited by B6 and DBA/2 donor cells was the main focus of my work and it is important to understand what factors are responsible for polarising the specific immune response in other systems.

Th1 and Th2 CELL DIFFERENTIATION

The cytokine profile produced by activated $CD4^+$ T helper (Th) cells determines the specialised effector function of the cell [53, 132]. Th1 cells secrete

cytokines which provide help for cell mediated immune responses and are functionally defined as cells which produce IFN-γ, while Th2 cells secrete cytokines which induce antibody production by B cells and are defined on the basis of their ability to produce IL-4, IL-5, IL-6, IL-10 and IL-13 [53]. The reason(s) why a naive or precursor $CD4^+$ T cell (pTh) becomes polarised to differentiate into a Th1 or a Th2-type cell are not fully understood and are the focus of many current studies. Recent use of TcR transgenic (TcR Tg) mice, in which the majority of $CD4^+$ T cells express a TcR specific for known antigen/class II MHC molecule complexes, has strongly suggested that a single precursor cell may differentiate to either a Th1 or Th2 phenotype, depending on the conditions present during its initial contact with specific antigen [76, 78, 133-135]. These studies have also been instrumental in showing that many factors can play a role in aquisition of T helper cell phenotype, including the type of APC involved [132, 136] antigen dose [137, 138] and genetic background [139, 140]. However, the most potent influence on Th phenotype acquisition appears to be exerted by cytokines themselves [76-78, 133-135, 141]. The majority of studies agree that differentiation towards a Th2 response is critically dependent on the presence of IL-4 during priming [134, 135, 141] and that mast cells, basophils and/or $CD4^+$ $NK1.1^+$ cells may be important IL-4-producing cell types during the early non-specific immune response [116, 142-146]. In addition, much interest has focused on the role of the recently described APC-derived cytokine, IL-12, in determining the outcome of $CD4^+$ T cell-mediated immune responses [76-78, 147].

INTERLEUKIN-12

IL-12 (originally known as Natural Killer Cell Stimulatory Factor (NKSF)) is a heterodimeric cytokine consisting of a 35kD and a 40kD chain [123]. It is produced by phagocytic cells, particularly mφs, in response to microbial constitutents [76, 122], or when CD40 on the mφ surface is ligated by CD40 ligand (CD40L) on activated T cells [148, 149]. Originally cloned from

EBV-transformed B cell lines, IL-12 was first functionally described as a potent inducer of IFN-γ in T and NK cells and an enhancer of cytotoxic activity in $CD8^+$ T cells and NK cells [118, 120, 150, 151]. It is now known that IL-12 acts at several different levels during T cell-mediated immune responses, as illustrated in Figure 1.2.

Early during the response, APC-derived IL-12 stimulates NK and T cells to produce IFN-γ. Both resting and activated NK cells produce IFN-γ in response to IL-12 [121, 123], alone, or in synergy with IL-1 and TNF-α [119, 152]. This early, T cell-independent pathway of IFN-γ production may therefore be extremely important in determining the outcome of the specific adaptive response [112-114, 117, 152, 153]. IL-12 also induces IFN-γ production by $CD4^+$ and $CD8^+$ T cells [92, 120, 121, 151], but maximal production of IFN-γ by T cells also requires TcR/CD3 engagement and costimulatory signals delivered via the CD28 molecule on the T cell surface by B7 on APC [154, 155]. In addition to eliciting IFN-γ from NK cells and T cells, IL-12 also enhances their cytotoxic responses [118, 120, 121]. IL-12 augments both NK cell-mediated and CTL-mediated cytotoxicity and promotes the expansion of activated NK and $CD8^+$ T cells [123, 156, 157].

B cells and mϕs can both produce IL-12 [76-78, 120, 122, 152, 158, 159], but mϕs appear to be the major producers of IL-12 under physiological conditions, as LPS induces similar levels of IL-12 in normal and B cell deficient SCID mice [152]. Although stimulation with bacteria or bacterial products (e.g. LPS), or signalling via the CD40 antigen can activate mϕs to release IL-12, mϕs also usually require priming with IFN-γ for optimal IL-12 production [148, 160]. Very recently, it has been suggested that dendritic cells (DCs) can also produce IL-12, albeit at much lower levels than are produced by activated Mϕs [123]. The signals required for IL-12 production by DCs are not yet fully characterised, however the ability of DCs to produce IL-12 while acting as APCs may be important in influencing Th cell differentiation.

IL-12 is critically required for the differentiation of pTh into effector Th1 cells [76-78, 159]. Hsieh et al. [76] were the first to demonstrate this by showing that the addition of heat killed Listeria monocytogenes (HKLM) to unprimed cultures of TcR Tg T cells augmented their subsequent IFN-γ production. This effect was only observed if mϕs were present in the original culture and was found even if the mϕs were not recognised by T cells in a cognate fashion. This was prevented by the addition of anti-IL-12 mAb to the cultures. IL-12 appears to act directly on pTh to promote Th1 differentiation, as it also enhances priming for IFN-γ in an APC-indendent system [78], but it may also increase Th1 responses by inhibiting priming for IL-4 production, thereby preventing Th2 cell differentiation.

Whether IFN-γ is necessary for Th1 responses induced by IL-12 is, as yet, unresolved [77, 78, 159, 161]. Studies in some systems, suggest that in the presence of neutralising anti-IFN-γ antibodies, IL-12 can still promote differentiation of pTh towards a Th1 phenotype [77, 78]. However, it is thought that the ability of IL-12 to inhibit Th2 differentiation is mediated by IFN-γ. This hypothesis is supported by a number of *in vivo* studies [162-164], and is particularly well illustrated in mice treated *in vivo* with anti-IgD antibody to induce a Th2 response [163]. In such mice, recombinant IL-12 reduced IL-4 mRNA levels in an IFN-γ dependent manner, while augmenting IFN-γ production in an IFN-γ independent fashion.

Taken together, it appears that IL-12 provides an important link between the early innate immune response, mediated by phagocytes and NK cells, and the subsequent cell-mediated response, dominated by Th1 cells and CTL. It would therefore seem likely that IL-12 was responsible for polarising the T cell response during BDF_1 GvHD. This had not been examined before and was the principal aim of my thesis.

AIMS OF THIS STUDY

Distinct pathological syndromes are elicited by the injection of B6 or DBA/2 parental cells into unirradiated BDF_1 recipient mice, although the factors responsible for this polarisation are poorly defined. Dissecting the cellular and molecular interactions involved in the two disease processes would lead to a better understanding of GvHD and other immunologically-mediated diseases and is essential for the development of specific therapies following BMT. The primary aim of this study was to examine whether differing patterns of cytokine production were associated with acute and chronic GvHD and to examine what factors may be responsible for this polarisation. Therefore, in the experiments described in Chapter 3, I firstly characterised the kinetics of acute and chronic GvHD using *in vitro* and *in vivo* indices of disease progression and subsequently examined a time course of cytokine production during the early period of the two diseases.

The second important aim of my research was to assess the role played by the early non-specific immune response in determining the outcome of parental cell injection and the role of IL-12 was assessed in Chapters 4-7. From the results of these chapters, it was clear that IL-12 was instrumental in directing the specific response during GvHD and several findings suggested that the role of IL-12 was to induce the production of IFN-γ. In Chapter 8, I therefore examined directly the role of IFN-γ in acute GvHD, both in unmanipulated B6 $\Rightarrow$ BDF_1 mice and in DBA/2 $\Rightarrow$ BDF_1 mice given IL-12, using the parameters which I had characterised in previous chapters.

Finally, it was of interest to determine the cellular source of the cytokines produced during acute and chronic GvHD. In Chapter 9, I therefore explored how $CD4^+$ and $CD8^+$ T cells contributed to cytokine production in acute and chronic GvHD. In addition, since NK cells are important for the pathogenesis of GvHD and are implicated as an important early source of IFN-γ in other T-dependent

models of infection, I carried out a detailed study of their involvement in the IL-12 mediated acute form of GvHD. This is described in Chapter 10.

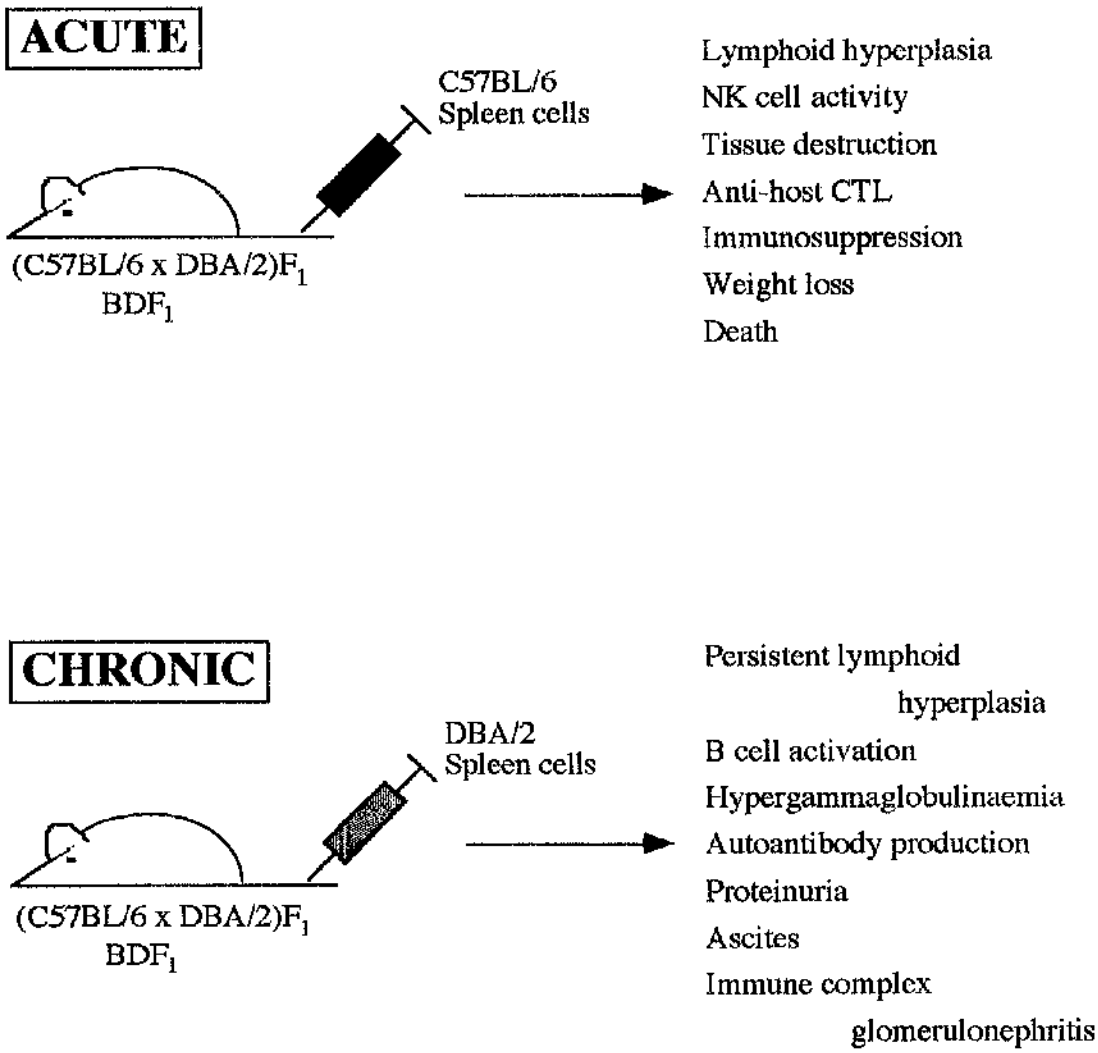


Fig. 1.1. The Spectrum of Pathological Alterations Associated with Acute and Chronic GvHD.

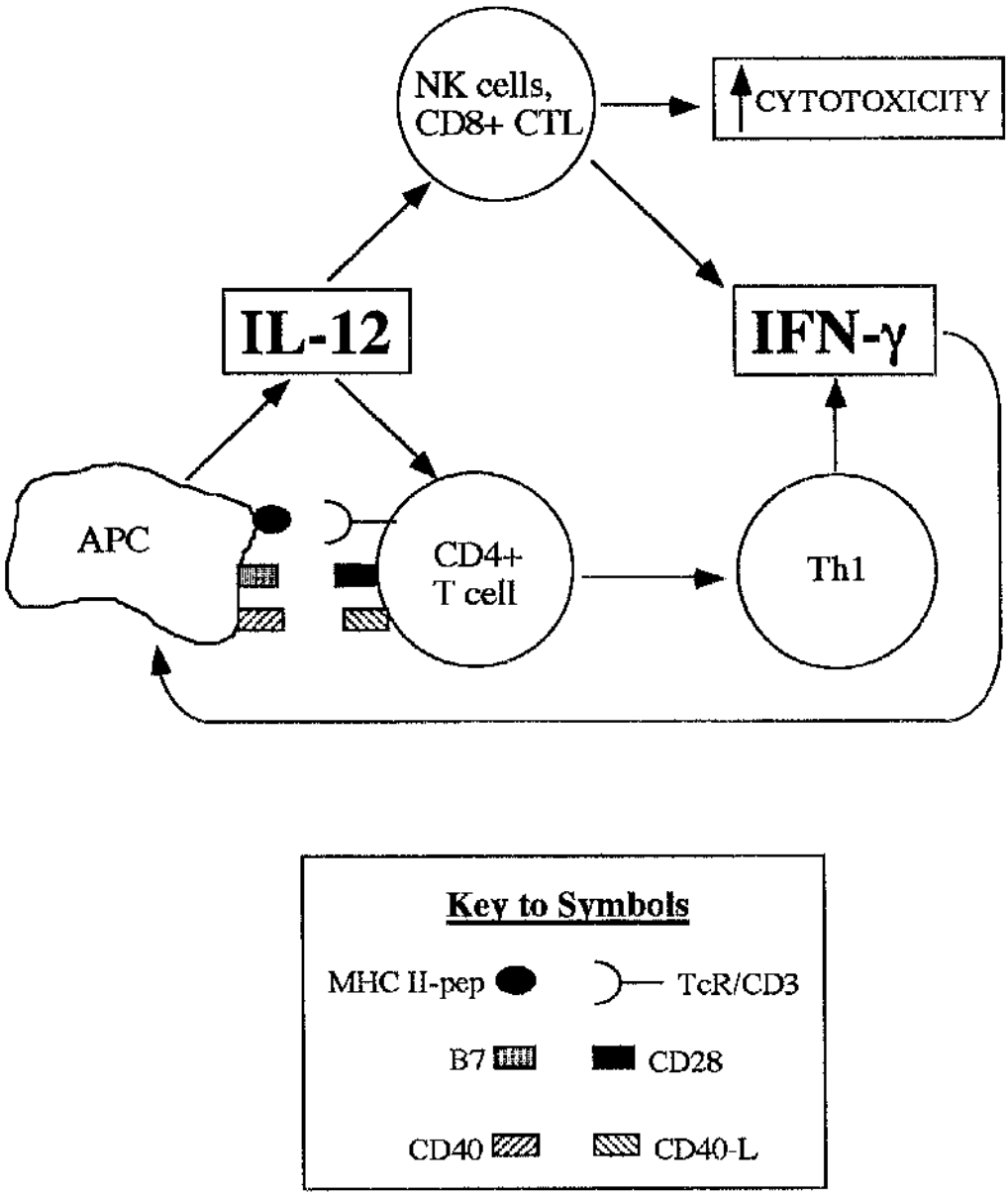


Fig. 1.2. The Role of IL-12 in Cell-mediated Immune Responses.

CHAPTER 2

MATERIALS AND METHODS

Animals

Female C57Bl/6J (H-2^b), DBA/2J (H-2^d) and (C57Bl/6J x DBA/2) F_1 (BDF_1) (H-2^{bxd}) mice were obtained from Harlan Olac Ltd (Bicester, Oxon). Animals were specified pathogen-free (SPF) and were maintained under standard animal house conditions either in the Joint Animal Facility, or the Central Research Facility at the University of Glasgow. Mice were normally used at 6-8 weeks of age.

Collection of Blood Samples

Under light anaesthesis using 5% Halothane BP (Rhone Mericux Ltd., Harlow, Essex), a maximum of 200µl of blood was removed from the retro-orbital plexus using heparinised capilliary tubes (Hawksley & Sons LtD., Lancing, Sussex). Serum was separated by centrifuging capillary tubes for 10 mins at 2000 rpm and stored at -20°C until use.

Histopathological Analysis of Kidney Sections

Histopathological analysis was carried out by Dr. Robin Reid (Dept. Pathology, Western Infirmary, Glasgow). Mice were sacrificed by cervical dislocation and kidneys removed by dissection. Kidneys were then fixed in 10% formalin solution and embedded in paraffin wax before 4µm sections were cut and stained with either Haematoxylin and Eosin (H and E) or Periodic-Acid Schiff (PAS) reagents. Sections were analysed by light microscopy (Nikon Labophot x 40 objective).

Electron Microscopy of Kidney Sections

Electron microscopy was performed by Mrs Jane Hare (Dept. of Pathology, Western Infirmary, Glasgow). Kidneys were removed as above and fixed in 5% paraformaldehyde solution. Sections were then negatively stained with 3% phosphotungstic acid (pH6.6) and analysed on a Philips CM10 transmission electron microscope (TEM).

Preparation of Lymphoid Cells

Mice were sacrificed by asphyxiation in CO_2. Spleens and thymi were removed immediately and placed in a petri dish containing 10ml RPMI 1640 medium (Gibco BRL. Paisley, Scotland). Single-cell suspensions were prepared by gently rubbing the tissues through a stainless steel mesh with the plunger of a 5ml syringe (Becton Dickinson, Cowley, Oxford) and the resultant suspension passed through Nitex mesh (Cadisch & Sons, London) to remove clumps and debris. Cells were then washed twice in RPMI by centrifuging for 7 mins at 450g and resuspending the cell pellet in 10ml fresh RPMI between washes. Viable cells were counted by phase contrast microscopy (Nikon Labophot x 40 objective) using an Improved Neubauer haemocytometer (Weber Scientific International Ltd., Teddington, Middlesex). The final cell pellets were resuspended to a concentration of 5 x 10^8 cells/ml in RPMI.

Induction of GvHR

The GvHR was induced by intravenous (i.v.) injection of 10^8 viable C57Bl/6J or DBA/2J donor spleen cells, in a volume of 0.2-0.4 ml RPMI, into the tail vein of BDF_1 recipients. Control mice received 0.2ml of RPMI only.

Assessment of Systemic GvHR

The intensity of the systemic GvHR in BDF_1 mice was assessed by regular weighing of mice to gauge weight loss, noting the onset of mortality and determining splenomegaly in individual animals.

Calculation of Splenomegaly

BDF_1 mice were sacrificed, their spleens removed and weighed and their body weight noted. The relative spleen weight was expressed in mg/10g body weight and the Spleen Index calculated for each GvHD mouse using the formula :-

$$\frac{\text{Relative Spleen Weight of GvHD Mouse}}{\text{Mean Relative Spleen Weight of Control Mice}}$$

A Spleen Index of greater than 1.3 was considered to be indicative of significant GvHD in all experiments [3].

Measurement of Proliferative Responses *In Vitro*

Spleen cell suspensions of spleens pooled from GvHD or control mice were prepared in RPMI as described above. After washing twice in RPMI by centrifuging for 7 min at 450g, viable cells were counted and the cell pellet was resuspended at a final concentration of 10^6 cells/ml in RPMI medium containing 10% heat-inactivated foetal calf serum (FCS), 100U/ml penicillin, 100µg/ml streptomycin, 1.25µg/ml fungizone and 2mM l-glutamine, (all Gibco BRL) and 5 x 10^{-5}M 2-mercaptoethanol (2-ME, Sigma-Aldrich Company Ltd., Poole, Dorset) (complete RPMI). The cells were plated in quadruplicate in 96 well flat-bottomed microtitre plates (Costar, Northumbria Biologicals, Cramlington, Northumberland) at 2 x 10^5 cells/ well in a total volume of 200µl complete RPMI medium, either alone, or with 10µg/ml of either Concanavalin A (Con A) or

Lipopolysaccharide derived from *Salmonella Enteritidis* (LPS; both Sigma-Aldrich). Plates were sealed with ICN Titertek non-toxic platesealers (ICN Biomedicals, Flow Laboratories, Irvine, Scotland) and placed in a humidified 37^0C incubator with a 5% CO_2 atmosphere. Either immediately, or after various culture periods, individual wells were pulsed with 1μCi ^{3}H-thymidine (West of Scotland Radionucleotide Dispensary, Western Infirmary, Glasgow) for 4 hours (spontaneous '*ex-vivo*' proliferation) or 24 hours before being harvested onto glass fibre filters using a 1295 Betaplate 96 well harvester. DNA-^{3}H-thymidine incorporation was assessed by liquid scintillation using a 1205 Betaplate scintillation counter (all LKB Wallac, Turku, Finland).

Induction and Measurement of Cytokine Production *in vitro*

Single spleen cell suspensions were resuspended at a final concentration of 4 x 10^6 cells/ml and cultured in 1ml aliquots in 24 well tissue culture plates (Costar) in complete RPMI. Cells were cultured in medium alone (to assess spontaneous production of cytokines) or with 10μg/ml Con A or LPS. Supernatants were harvested after 1-4 days of culture, centrifuged in a Microcentaur Microfuge (Scotlab) at 13000 rpm for 5 minutes to remove cellular debris and stored at -20°C until assayed.

Production of IL-2, IFN-γ, IL-4, IL-5 and IL-10 were quantified using sandwich Enzyme-Linked Immunosorbent Assays (ELISA). 96-well ELISA plates (Immunlon 4, Dynatech Laboratories Ltd., Billingshurst, West Sussex) were coated with 50μl per well of monoclonal anti-cytokine capture antibody (mAb) at the predetermined optimal concentration (Table 1) in carbonate buffer (Appendix 1) and then incubated overnight in a sealed container at 4^0C. The contents of the wells were emptied and the plates washed twice by filling the wells with phosphate buffered saline (PBS; Appendix 1) containing 0.05% Tween 20 (BDH, Poole, Dorset) (PBS/Tween), leaving to stand for 1 min and emptying the contents again. Non-specific protein binding sites were blocked by incubation

with 200µl of PBS containing 10% FCS (PBS/FCS) for 1hr at 37^{0}C. After blocking, the plates were washed twice with PBS/Tween and culture supernatants or standard recombinant murine cytokines at predetermined concentrations (Table 2) were added to individual wells in a volume of 50µl. Standard recombinant cytokines were then serially diluted to provide the range of concentrations shown in Table 2 and the plates were incubated at 37^{0}C for 3hrs. Plates were then washed 4 times with PBS/Tween and 50µl of biotinylated anti-cytokine detecting antibody, diluted in PBS/10% FCS, was added at the concentrations shown in Table 1. After incubation for 1hr at 37^{0}C, the plates were washed 6 times with PBS/Tween and 100µl of extravidin-peroxidase, (Sigma), prediluted to 2µg/ml in PBS/10% FCS, was added to each well. Following a final incubation for 1hr at 37^{0}C, the plates were washed 8 times with PBS/Tween, before 100µl 3,3'5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Dynatech) was added to each well. The plates were then read at 630nm using an MR5000 automatic microplate reader (Dynatech) using Mikrofit version 4.5 software (Mikrotek Laborsysteme GmbH) and the cytokine concentration in each test supernatant calculated with reference to a standard curve constructed using serial dilutions of recombinant cytokines.

IL-12 p75 Heterodimer Specific ELISA

Production of IL-12 was also quantified using a sandwich ELISA. 96-well ELISA plates (Immunlon 4, Dynatech) were coated with 100µl per well of monoclonal anti-mouse p75 antibody (kindly provided by Dr. M. K. Gately, Hoffmann-La Roche, Nutley, NJ) diluted to 5µg/ml in IL-12 coating buffer (Appendix 1) and then incubated overnight in a sealed container at 4^{0}C. The plates were then washed three times with PBS/Tween before being blocked by the addition of 200µl per well of PBS containing 3% bovine serum albumin (BSA; PBS/BSA) for 1hr at 37^{0}C. After blocking, the plates were washed three times with PBS/Tween and culture supernatants were added to individual wells in a

volume of 100µl. Recombinant murine IL-12, diluted to 20ng/ml in IL-12 assay buffer (Appendix 1), was also added to individual wells in a volume of 100µl and serially diluted to construct a standard curve of IL-12 concentrations from 20ng/ml - 15.6pg/ml. After incubation for 3hrs at room temperature, the plates were washed 4 times with PBS/Tween and 100µl of horseradish peroxidase conjugated (HRPO) rat anti-mouse p40 detecting mAb (Dr. M. K. Gately) was added at 500ng/ml diluted in IL-12 assay buffer for 2hr at room temperature. The plates were washed a further 5 times before 100µl TMB peroxidase substrate was added to each well. The plates were then read as before using an MR5000 automatic microplate reader (Dynatech) and the IL-12 concentration in each test supernatant calculated with reference to the standard curve constructed using serial dilutions of recombinant IL-12.

Maintenance of Cell Lines *in vitro*

EL4 cells [derived from a thymoma of C57Bl/6 origin (H-2^b)], P815 cells [derived from a methylcholanthene-induced mastocytoma of DBA/2 origin (H-2^d)] and YAC-1 cells (derived from a thymoma of A strain origin) were maintained in RPMI containing 10% heat-inactivated FCS, 100U/ml penicillin, 100µg/ml streptomycin, 1.25µg/ml fungizone and 2mM l-glutamine in a humidified 37^{0}C incubator with a 5% CO_2 atmosphere. These cell lines were subcultured every 2-3 days, by adding 1-2ml of cell suspension (approximately 10^7 cells) to 10ml of fresh medium and were always subcultured 2 days before use, to ensure that the majority of cells would be in the log phase of growth.

Measurement of Specific and Non-Specific Cytotoxicity

EL4 and P815 target cells were used to detect anti-H-2^b and anti-H2^d allospecific CTL activity respectively, while YAC-1 cells were used to detect NK cell activity. Aliquots of 2.5 x 10^6 target cells were labelled with ^{51}Cr by incubation in 1ml RPMI 1640/5% newborn calf serum (NCS, Gibco BRL)

containing 2 MBq $Na_2{}^{51}CrO_4$ (West of Scotland Radionucleotide Dispensary) for 50 min at 37^{0}C. They were then washed 5 times in RPMI/5% NCS by centrifuging at 450g for 7 minutes and the number of viable cells counted before resuspending at a final concentration of 2 x 10^5cells/ml. 100µl of single cell suspensions of effector cells, resuspended at a final concentration of 2 x 10^7 cells/ml in RPMI/5% NCS were added in quadruplicate to 100µl aliquots of ^{51}Cr-labelled target cells in V-bottomed microtitre plates (Costar), giving final effector cell : target cell (E :T) ratios of 100:1, 50:1, 25:1 and 12.5:1. After 4hrs incubation in a humidified 37^{0}C incubator with a 5% CO_2 atmosphere, 100µl of supernatant was removed from each well to assess ^{51}Cr release in a 1282 Compugamma counter (LKB Wallac). The cytotoxic activity was then calculated by the following formula:

$$\% \text{ Specific Cytotoxicity} = \frac{(\text{Experimental cpm} - \text{Spontaneous cpm})}{(\text{Maximum cpm} - \text{Spontaneous cpm})} \times 100\%$$

In all assays, maximum release was obtained by adding 100µl 10% Triton-X 100 (Sigma) to wells containing 2 x 10^5 ^{51}Cr labelled target cells. In CTL assays, spontaneous release was calculated using spleen cells from control mice, while in NK cell assays, thymocytes from control mice were used.

Depletion of T Cell Subsets *in vitro*

CD4$^+$ and CD8$^+$ T cell subsets were depleted *in vitro* by complement-mediated lysis. Spleen cells were resuspended at 10^7 cells/ml in RPMI/5% FCS containing either 200µg/ml anti-CD4 mAb (YTS 191.1) or anti-CD8α mAb (YTS 169.4) (both kindly provided by Dr. Eleanor Bolton, Dept. Surgery, University of Glasgow) and incubated for 60 min on ice. After two washes in RPMI/5% FCS

by centrifuging for 7 min at 450g, the cells were resuspended at 5 x 10^6 cells/ml in RPMI/5% FCS containing 10% (v/v) rabbit complement (Low-Tox M, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and incubated for 60 min in a 37°C humidified incubator with a 5% CO_2 atmosphere. The cells were then washed twice in RPMI, counted and resuspended at 4 x 10^6 cells/ml in complete RPMI. Control cells were incubated in RPMI/5% FCS containing 10% rabbit complement alone.

The efficacy of the depleting antibodies was assessed by flow cytometry (as described below) and the percentage of residual $CD4^+$ or $CD8^+$ cells remaining after depletion was consistently ≤ 0.2%, as shown in Fig. 9.1.

Depletion of NK Cells *In Vivo*

NK cells were depleted *in vivo* by the i.v. injection of 30µl polyclonal anti-asialo-GM-1 rabbit antiserum (anti-ASGM-1; Wako Chemicals, Germany) diluted in 200µl PBS. Control mice received 0.2mls of normal rabbit serum (provided by CRF animal facility). The effect of anti-ASGM-1 treatment on splenic NK cell activity in GvHD and control mice is shown in Fig.10.2.

Phenotypic Analysis of Lymphocytes by Flow Cytometry

Aliquots of 10^6 spleen cells were resuspended in 50µl staining buffer (SB; Appendix 1) containing the appropriate concentration of primary antibody (Table 3). The samples were incubated for 30-40min on ice in the dark and then washed twice in SB for 7min at 450g. For two colour analysis, biotinylated anti-H-$2D^d$ and anti-H-$2D^b$ MHC Class I primary antibodies were then detected by incubating the cells in 50µl SB containing either 1/100 fluoroscein isothiocyanate-streptavidin (FITC-SAV) or 1/50 phycoerythrin-streptavidin (PE-SAV) (both from Vector Laboratories, Peterborough) for 30-40min on ice in the dark. The cells were then washed twice in SB and erythrocytes lysed by incubating the cells in 0.5ml of cold "Facslyse Solution" (Becton Dickinson) pre-diluted 1/10 in distilled water for 10

minutes at room temperature. The cells were again washed twice in SB and resuspended in 0.5ml "Facsflow" solution (Becton Dickinson) before being analysed using a FACscan IV flow cytometer (Becton Dickinson) with a 488nm argon laser. Lymphocytes were gated on the basis of their forward and side light scatter properties and the data analysed using Lysis II software (Becton Dickinson). In all experiments, negative control samples were cells incubated with FITC-SAV or PE-SAV in the absence of primary antibody.

Measurement of Total Serum Immunoglobulin Levels

i) Total IgG Levels

Serum levels of total IgG were measured by radial-immunodiffusion (RID) using agarose gel RID kits, according to the manufacturers' instructions (Bind A RID NL RN272, The Binding Site, Birmingham). Test serum samples were diluted 1/10 and 5µl of each sample was applied to individual wells in the agarose gel (which contains antibodies to mouse IgG). On each RID plate, 3 wells were also filled with 5µl of one of three standard samples containing known concentrations of IgG. The plates were then resealed, wrapped in damp tissue paper and incubated at 4^0C for 72 hrs to allow diffusion of IgG through the agarose gel. After incubation, the lids were removed and the diameter of the precipitin ring formed for each sample was measued using a pocket focusing magnifier with measuring scale (Philip Harris Scientific, Clydebank, Scotland). A linear calibration curve was constructed by plotting the square of the diameter of the precipitin rings formed by the three standard samples against their specific protein concentration and the concentration of IgG in each test sample was then read off the calibration curve.

ii) Total IgE Levels

Serum IgE levels were measured using sandwich ELISA techniques. 50µl purified rat IgG anti-mouse IgE monoclonal antibody (Clone R35-72, Pharmingen) was added to the wells of flat bottomed microtiter plates (Corning Easy Wash, Bibby Sterilin Ltd., Newport, Gwent) at 1µg/ml in carbonate buffer. The plates were incubated overnight at 4^{0}C and then washed twice with PBS/Tween before blocking by incubation with 100 µl per well PBS/15% FCS for 2 hours at room temperature. 100µl of each serum sample was added in duplicate to individual wells, either neat or diluted 1/50 in PBS/15% FCS. Purified murine IgE standard (Clone IgE-3, Pharmingen) diluted to a starting concentration of 200ng/ml in PBS/15% FCS was added to additional wells in 100µl and serial dilutions made to construct a standard curve. The samples and standards were incubated on the plates overnight at 4^{0}C, after which the plates were washed 4 times in PBS/Tween before addition of 50µl per well of biotinylated rat anti-mouse IgE detecting antibody (Clone R35-92, Pharmingen) at 4µg/ml in PBS/15% FCS. After incubation for 1 hour at 37^{0}C, the plates were washed 6 times in PBS/Tween and 100µl streptavidin peroxidase (Sigma), diluted 1/1000 in PBS/15% FCS was added to each well. Following a final incubation for 1 hour at room temperature, the plates were washed 8 times in PBS/Tween prior to addition of 100µl/well TMB peroxidase substrate (Dynatech). The plates were then read at 630nm using an MR5000 automatic microplate reader (Dynatech).

iii) Measurement of Anti-Double-Stranded DNA Antibodies

Serum levels of anti-double stranded DNA (ds DNA) antibodies were assessed using sandwich ELISAs. Flat-bottomed microtitre plates (Immulon 2, Dynatech) were coated with 100µl per well of poly-l-lysine (Sigma) diluted to 50µg/ml in borate buffered saline (BBS; Appendix 1) for 1hr at 37^{0}C, then overnight at 4^{0}C. The plates were washed three times with BBS containing 0.05% Tween 20 (BBS/Tween) and then coated with 50µl per well of ds DNA at

10µg/ml for 1hr at 37^{0}C. After a further three washes in BBS/Tween, non-specific protein binding sites were blocked by the addition of 100µl per well of BBS containing 1% BSA, (BSA/BBS) for 1hr at room temperature. Following blocking, the plates were washed twice in BBS/Tween and 50µl of test serum samples diluted 1/100, 1/500 and 1/1000 in BSA/BBS containing 0.05% Tween 20 (BSA/BBS/Tween) were then added to individual wells for 2hr at room temperature. After four more washes with BBS/Tween, 50µl of alkaline phosphatase conjugated anti-mouse IgG (Sigma) diluted 1/10000 in BSA/BBS/Tween was added to the wells for 2hr at room temperature. Finally, the plates were washed 6 times in BBS/Tween before the addition of 100µl per well of p-nitrophenyl phosphate-disodium substrate (Dynatech) in 1% Diethanolamine solution (BDH) and 0.01% MgCl2. The colour was allowed to develop for 30-60min and then the plates were read at 405nm using an MR5000 automatic microplate reader (Dynatech).

Neutralisation of IL-12 *In Vivo*

Endogenous IL-12 was neutralised by the i.p. injection of 0.5mg purified goat anti-mouse IL-12 polyclonal IgG (provided by Dr. Maurice K. Gately, Hoffmann-La Roche Inc., Nutley, New Jersey, USA), diluted in 0.2ml PBS. Control mice received 0.5mg of purified normal goat IgG (Sigma; Reagent Grade) diluted in PBS.

Administration of Exogenous IL-12 *In Vivo*

IL-12 was administered by the i.p. injection of 100ng purified recombinant murine IL-12 (rm IL-12; Dr Maurice K. Gately, Hoffmann-La Roche) in a total volume of 0.2ml PBS containing 1% homologous mouse serum. Control mice received 0.2ml PBS containing 1% homologous mouse serum.

Neutralisation of IFN-γ *In Vivo*

Endogenous IFN-γ was neutralised by the i.p. injection of 0.5mg purified hamster anti-mouse IFN-γ IgG mAb H22 (provided by Dr. Adrienne Morgan, Celltech Ltd., Slough, Berkshire). Control mice received 0.5 mg of isotype matched hamster control antibody (also provided by Dr. Adrienne Morgan).

Statistics

Unless otherwise stated, results were expressed as means +/- standard deviations (SD) and were compared by Student's t-test.

TABLE 2.1

Monoclonal Antibodies used in Cytokine Sandwich ELISAs

i) Purified Capture Antibodies

Specificity	Clone	Isotype	Concentration (μg/ml)
IL-2	JES6-1A12	RAT IgG2a	2
IFN-γ	R4-6A2	RAT IgG1	2
IL-10	JES5-2A5	RAT IgG1	2
IL-4	BVD4-1D11	RAT IgG2b	2
IL-5	TRFK5	RAT IgG1	4

ii) Biotinylated Detecting Antibodies

Specificity	Clone	Isotype	Concentration (μg/ml)
IL-2	JES6-5H4	RAT IgG2b	1
IFN-γ	XMG1.2	RAT IgG1	1
IL-10	SXC-1	RAT IgM	2
IL-4	BVD6-24G2	RAT IgG1	1
IL-5	TRFK4	RAT IgG2a	4

The optimal concentration of capture and detecting antibodies for use in cytokine ELISAs was determined by chequerboard titrations.

All of the above monoclonal antibodies were purchased from Pharmingen, San Diego, U.S.A.

TABLE 2.2

Recombinant Murine Cytokine Standards used in Sandwich ELISAs

Cytokine	Concentration Range	Source
IL-2	200U/ml - 1.56U/ml	Pharmingen
IFN-γ	40ng/ml - 1.25ng/ml	Pharmingen
IL-10	40ng/ml - 1.25ng/ml	Genzyme (West Malling, Kent)
IL-4	200U/ml - 1.56U/ml	Genzyme
IL-5	200U/ml - 1.56U/ml	Pharmingen

TABLE 2.3

Antibodies used in Flow Cytometric Analysis

i) Monoclonal Antibodies

Specificity	Clone	Isotype	Source	Dilution
PE-anti-CD4	GK1.5	Rat IgG2b	Becton Dickinson	1:16
FITC-anti-CD8α	53-6.7	Rat IgG2a	Becton Dickinson	1:16
Bio-anti-H-2Db	KH95	Balb/c IgG2b,κ	Pharmingen	1:12
Bio-anti-H-2Dd	34-2-12	C3H IgG2a, κ	Pharmingen	1:50

KEY:

Bio -: Antibodies conjugated to biotin.

PE -: Antibodies conjugated to phycoerythrin.

FITC -: Antibodies conjugated to fluorescein isothiocyanate.

ii) Polyclonal Antibodies

Specificity	Nature & Source	Dilution
Bio-anti-mouse Ig	Rabbit F(ab')2 fragments Dako Ltd., High Wycombe, Bucks	1:25

APPENDIX 1

Reagents and Solutions

Complete RPMI

RPMI medium containing-:

10% heat-inactivated foetal calf serum (FCS)

100U/ml penicillin

100µg/ml streptomycin

1.25µg/ml fungizone

2mM l-glutamine

5×10^{-5}M 2-mercaptoethanol

Cell Culture Medium (for P815, EL4, YAC-1 cell lines)

RPMI medium containing-:

10% heat-inactivated foetal calf serum (FCS)

100U/ml penicillin

100µg/ml streptomycin

1.25µg/ml fungizone

2mM l-glutamine

Carbonate Buffer

0.1M $NaHCO_3$ in dcionised, distilled water (dd H_2O)

Phosphate Buffered Saline (PBS)

80g NaCl

11.6g Na_2HPO_4

2g KH_2PO_4

2g KCL

dissolved in 10 litres of dd H_2O

PBS/Tween

PBS containing 0.05% (v/v) Tween-20

IL-12 ELISA Coating Buffer

1M $NaHCO_3$

1M Na_2CO_3 in dd H_20 and Ph adjusted to 9.6

IL-12 ELISA Assay Buffer

1% w/v BSA

0.5M NaCl

0.02M Na2HPO4

0.05% (v/v) Tween-20

0.01% (w/v) thimerosal

dissolved in dd H_20 and Ph adjusted to 6.5

Facs Staining Buffer

PBS containing:

2% (v/v) FCS

0.05% (w/v) sodium azide

Borate Buffered Saline (BBS)

6.2g H_3BO_4

19g NaB_4O_7

9g NaCl

dissolved in 1 litre of dd H_2O

BBS/Tween

BBS containing 0.05% (v/v) Tween-20

CHAPTER 3

KINETIC STUDY OF SYSTEMIC ACUTE AND CHRONIC GvHD

Introduction

Previous studies have indicated that differential activation of $CD4^+$ T helper cell subsets may underlie the distinct diseases which occur in BDF_1 mice injected with either C57Bl/6 or DBA/2 parental spleen cells, by demonstrating that a Th1-type cytokine profile was apparent during acute GvHD while Th2-type cytokines were produced preferentially during chronic GvHD [42, 46, 47]. However, these studies examined cytokine production over a limited time course and did not attempt to determine when T cell polarisation occurred, or address the underlying mechanism.

The main aim of my project was to examine the factor(s) involved in the polarisation of Th cell responses in acute and chronic GvHD and, in particular, to assess the role played by the early non-specific immune response in determining the subsequent pattern of the two diseases. My initial series of experiments were therefore designed to determine at what point the cytokine response becomes skewed towards a preferential Th1 or Th2 phenotype by characterising the kinetics of acute and chronic GvHD in BDF_1 mice, using *in vivo* and *in vitro* indices of disease progression in parallel with measurements of cytokine production. For this study, I therefore mainly concentrated on events occurring during the first 3 weeks of disease.

Experimental Protocol

The GvHR was induced by i.v. injection of 10^8 viable C57Bl/6 or DBA/2 parental spleen cells into BDF_1 recipients. The intensity of the systemic GvHR *in vivo* was monitored by assessing body weight, splenomegaly and mortality. The effect of GvHD on the immune system was determined by counting the total

lymphocytes in the spleen and by measuring the proportions of splenic $CD4^+$ and $CD8^+$ T lymphocytes by FACS analysis.

Immune function was determined by measuring the proliferative capacity of spleen cells from GvHD mice either '*ex-vivo*', or in response to stimulation with T or B cell mitogens. IFN-γ, IL-2, IL-10, IL-5 and IL-4 production was measured by culturing spleen cells from GvHD mice either in medium alone or with Con A. I chose to measure these particular cytokines because they are characteristically associated with either Th1 (IFN-γ and IL-2) or Th2 (IL-4, IL-5 and IL-10) responses. Levels of total serum IgG and anti-ds DNA antibodies were measured on days 10, 20 and 30 of the GvHD.

Samples of kidney were taken from GvHD mice on day 48 (which was when chronic GvHD showed evidence of oedema and proteinuria) and examined for the presence of immune complex deposition by both light and electron microscopy.

Results

i) Weight Loss and Mortality

Consistent with previous studies in our own and other laboratories [7, 21, 23, 24, 27, 55, 98], BDF_1 mice injected with 10^8 B6 parental spleen cells developed an acute GvHD, with progressive weight loss from day 16 of the GvHD, which continued until the study was terminated on day 20 (Fig 3.1). Mortality was also apparent on day 16, with two deaths (Fig 3.1). A further three deaths occurred on day 18 and one on day 20, by which time an overall mortality rate of 80 % was observed (Fig. 3.1). In contrast, BDF_1 mice injected with 10^8 DBA/2 parental spleen cells showed no significant weight loss and none of these mice died during this phase of the disease (Fig. 3.1).

ii) Splenomegaly and Lymphoid Populations

a) Acute GvHD

B6 ⇒ BDF_1 mice exhibited significant splenomegaly by day 1 of the acute GvHD (Fig 3.2). This increased sharply, peaked on day 10, then returned towards control values, until no significant splenomegaly was observed in surviving mice on day 18 (Fig 3.2). A similar biphasic pattern of hyperplasia followed by lymphoid involution was evident when the total lymphocyte number in the spleens of these mice was assessed. By day 2, spleen cell numbers were already almost double that in control mice (Fig 3.3a and 3.3c). There was then a steady increase until day 10, when lymphocyte numbers began to return to control levels and by day 18 these were markedly reduced compared with control mice (Fig. 3.3a and 3.3c).

Phenotypic analysis of these spleen cell populations showed that on days 2 and 7, the proportions of $CD4^+$ and $CD8^+$ T cells in B6 ⇒ BDF_1 spleens were not different from those observed in control spleens (Table 3.1). Thus, there were dramatic increases in the absolute numbers of both populations in acute GvHD mice compared with controls (Fig. 3.3a and 3.3c). By day 10 and thereafter, the proportions of $CD4^+$ and $CD8^+$ splenic T cells in B6 ⇒ BDF_1 mice were strikingly different from those in control mice (Table 3.1). On days 10 and 14, there was a large increase in the percentage of T cells in the spleens of mice with acute GvHD compared with controls (60-70 % vs 25-30 %). This consisted mainly of $CD8^+$ T cells, with B6 ⇒ BDF_1 spleens containing up to 5 times as many $CD8^+$ T cells as controls. However, the percentage of $CD4^+$ T cells in the spleens of mice with acute GvHD was also higher than controls at all times up to the end of the study on day 18 (Table 3.1).

b) Chronic GvHD

Splenomegaly also developed in BDF_1 mice with chronic GvHD, initially following a similar initial pattern to that observed in mice with acute GvHD (Fig. 3.2). However, by day 7 and thereafter, the splenomegaly was less intense in chronic GvHD and it persisted at this level for the remainder of the study. In parallel, spleen lymphocyte numbers increased in DBA/2 $\Rightarrow$ BDF_1 mice during the first 10 days in a manner similar to that observed in B6 $\Rightarrow$ BDF_1 mice (Fig. 3.3b). However, after day 10, lymphocyte numbers continued to increase in mice with chronic GvHD, in contrast to the markedly reduced lymphocyte numbers evident in mice with acute GvHD after this time (Fig. 3.3a and 3.3b).

FACS analysis also revealed that the dramatic differences in the proportions of $CD4^+$ and $CD8^+$ T cells in the spleens of B6 $\Rightarrow$ BDF_1 mice were not apparent in DBA/2 $\Rightarrow$ BDF_1 mice (Table 3.1). Although the proportions of $CD4^+$ and $CD8^+$ T cells remained essentially the same as those in control mice throughout the GvHD (Table 3.1), the absolute numbers of splenic T cells did increase (Fig. 3.3b).

iii) Immune Function

I next examined how the effects of the different GvHD models *in vivo* correlated with parameters of immune function. Previous work had indicated that the B6 $\Rightarrow$ BDF_1 GvHD is associated with suppressed T and B cell function *in vitro* and *in vivo* [24, 27, 71, 73, 165], whereas DBA/2 $\Rightarrow$ BDF_1 GvHD is characterised by B cell hyperreactivity [23, 36-39]. I therefore assessed the ability of spleen cells from GvHD mice to proliferate, either spontaneously '*ex-vivo*' or in response to mitogenic stimulation *in vitro*, using Con A and LPS to stimulate proliferation of T cells and B cells respectively.

a) Spontaneous *Ex-Vivo* Proliferative Responses

Spleen cells from B6 ⇒ BDF_1 mice showed significantly increased spontaneous proliferation by day 2 after induction of GvHD (Fig 3.4). This peaked on day 10 and had returned to control levels by day 18. In contrast, the spontaneous proliferation of spleen cells from DBA/2 ⇒ BDF_1 mice was only modestly enhanced throughout the first 10 days after donor cell transfer, but continued to increase progressively thereafter (Fig 3.4). Thus, the changes in splenomegaly and spleen cell numbers in the two diseases are parallelled by alterations in the inherent proliferative capacity of lymphocytes.

b) Proliferative Responses to Con A Stimulation

Spleen cells from B6 ⇒ BDF_1 mice initially also showed significantly increased responsiveness to stimulation with Con A *in vitro* compared with control spleen cells (Fig 3.5). However, by day 7, these responses were significantly lower than those of controls and were almost negligible from day 10 onwards. In contrast, the Con A responses of DBA/2 ⇒ BDF_1 spleen cells were usually similar to those of control spleen cells (Fig 3.5), although a significant increase was observed on day 10.

c) Proliferative Responses to LPS Stimulation

Spleen cells from both B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice initially exhibited similarly enhanced responses to LPS compared with control cells. This was evident throughout the first 10 days after donor cell transfer in both models of GvHD (Fig. 3.6). However, the LPS response of B6 ⇒ BDF_1 spleen cells was subsequently suppressed, with complete ablation evident by day 14. This was in contrast to cells from DBA/2 ⇒ BDF_1 mice, which continued to show significant hyperresponsiveness to LPS throughout the chronic disease (Fig. 3.6).

Thus, both forms of GvHD are characterised by an initial period of B cell hyperresponsiveness, but this disappears in acute GvHD and persists in chronic

GvHD. To explore this further, I went on to examine a number of *in vivo* indices of B cell function.

iv) *In Vivo* Assessment of B cell Activity in Acute and Chronic GvHD

a) IgG Production

On day 10, significantly increased levels of total IgG were detected in the serum of mice with acute GvHD compared with controls (Fig. 3.7). However, by day 20 and thereafter, serum IgG levels were undetectable in these mice, consistent with their abrogated LPS responses observed at earlier time points *in vitro* (Fig3.6). In contrast, mice with chronic GvHD showed strikingly high IgG levels on both days 20 and 30, further supporting the persistent B cell stimulation throughout this disease (Fig. 3.7).

b) Anti-ds DNA Antibodies

B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice both displayed high levels of anti-ds DNA serum antibodies on day 10, with particularly high levels apparent in mice with acute GvHD (Fig 3.8). However, by day 20 and thereafter, no anti-ds DNA antibodies were detected in the serum of mice with acute GvHD, despite their continued presence in mice with chronic GvHD. No anti-ds DNA antibodies were found in control mice at any time.

c) Renal Immunopathology

To assess possible pathological consequences of the B cell hyperreactivity, I examined kidney sections for the presence of ICGN in mice surviving 7 weeks into GvHD, since this was when mice with chronic GvHD started to show clinical signs of kidney damage, such as oedema and proteinuria. At no time did mice from any other group exhibit physical evidence of kidney dysfunction and in

particular, the small number of B6 ⇒ BDF_1 mice which survived past day 21 of the acute GvHD remained visibly healthy.

Kidney sections from DBA/2 ⇒ BDF_1 mice showed widespread renal damage both by LM and EM at this time. By LM, the glomerular basement membrane appeared thickened, there was glomerular hypercellularity and evidence of focal protein casts (Fig. 3.9a). By EM, endothelial swelling was evident, resulting in loss of filtration space and there was ablation of the normal podocyte architecture (Fig. 3.10a). Numerous sub-epithelial electron-dense deposits were observed in most glomeruli and together these features were consistent with the presence of severe ICGN. In parallel with the severe kidney damage evident by microscopy, mice at this stage of chronic GvHD began to show progressive oedema and started to die on day 50 (33% mortality), with 77% mortality occurring by day 100 (Fig 3.11). In contrast, sections of kidney from B6 ⇒ BDF1 mice on day 48 of the acute GvHD showed no significant changes either by light microscopy (LM) or electron microscopy (EM) (Figs. 3.9b & 3.10b) and resembled kidneys from control mice (Figs. 3.9c & 3.10c).

Thus, the brief B cell hyperplasia observed in B6 ⇒ BDF1 mice does not lead to autoimmune pathology, but the persistently high levels of serum IgG and anti-ds DNA antibodies observed in mice with chronic GvHD provoke ICGN, as described previously [36-39].

v) Cytokine Production During Acute and Chronic GvHD

Although both forms of GvHD are clearly associated with expansion of T cell numbers, the results above showed that persistent B cell responses were apparent in chronic, but not acute GvHD. In addition, previous work has suggested that activation of different $CD4^+$ T helper cell subsets may cause the distinct outcomes of the GvHD in BDF_1 recipient mice [42, 46]. I therefore compared the pattern of cytokines produced by lymphocytes from mice with acute

or chronic GvHD *in vitro*, both spontaneously and in response to Con A stimulation.

IFN-γ

High levels of IFN-γ were produced spontaneously by B6 ⇒ BDF_1 splenocytes throughout the first 10 days of the acute GvHD (Fig. 3.12a). This was observed as early as day 2, peaked on day 10 and was undetectable after day 14. This was in direct contrast to cells from DBA/2 ⇒ BDF_1 mice, which spontaneously secreted similar low levels of IFN-γ to control cells at all time points examined (Fig. 3.12a).

B6 ⇒ BDF_1 spleen cells also exhibited enhanced IFN-γ production in response to Con A stimulation *in vitro* (Fig. 3.12b). Once again, elevated levels were apparent on day 2 and peaked on day 10, before falling below control levels at the later time points. In contrast to the lack of spontaneous IFN-γ production by DBA/2 ⇒ BDF_1 spleen cells, Con A induced the production of similar or significantly higher levels of IFN-γ by these cells compared with control cells at all times after day 2 of this study (Fig. 3.12b). Although these levels were never as high as those observed during the course of the acute GvHD, they remained elevated throughout the course of this study.

IL-2

IL-2 was not produced spontaneously by cells from any group (data not shown).

B6 ⇒ BDF_1 spleen cells produced significantly higher levels of IL-2 in response to Con A than control cells on day 2 (Fig. 3.13), but at all times thereafter, IL-2 levels were significantly lower than those produced by control cells and were negligible after day 10. In contrast, spleen cells from DBA/2 ⇒ BDF_1 mice produced similar levels of IL-2 in response to Con A stimulation to

control cells on day 2 (Fig. 3.13) and significantly higher levels of IL-2 than control cells at all time points thereafter (Fig. 3.13).

IL-5

Spontaneously produced IL-5 was not detected in any group during this study (data not shown).

B6 ⇒ BDF_1 cells produced significantly enhanced levels of IL-5 in reponse to Con A on both days 2 and 4 after donor cell transfer, compared with control cells (Fig. 3.14). However, the levels in acute GvHD mice then fell below the level of detection after day 10. In contrast, DBA/2 ⇒ BDF_1 spleen cells produced strikingly high levels of IL-5 in response to Con A throughout the time course examined (Fig. 3.14). These levels were consistently several fold greater than those produced by either control cells or B6 ⇒ BDF_1 cells after day 4 of the GvHD.

IL-4

No IL-4 production was detected by cells from any group at any of the time points examined (data not shown). It was unclear whether this reflected the fact that the levels of IL-4 produced were below the level of detection of this assay, or if IL-4 was produced transiently and then rapidly utilised by cells in culture.

IL-10

As with IL-5, IL-10 was not detected in supernatants from unstimulated cells of any group (data not shown).

From days 2-10 of the acute GvHD, B6 ⇒ BDF_1 spleen cells produced similar levels of IL-10 to control cells (Fig 3.15), but at later time points, IL-10 production fell to below control levels. DBA/2 ⇒ BDF_1 cells secreted elevated

levels of IL-10 at all time points examined, with significant increases compared to both control cells and B6 ⇒ BDF_1 cells after day 4 (Fig. 3.15).

Thus, acute GvHD is associated with spontaneous production of IFN-γ early in the disease, accompanied by very high levels of inducible IFN-γ and transient priming of IL-2 and IL-5 production. However, the production of all cytokines subsequently ceased as lymphoid atrophy developed. In contrast, DBA/2 ⇒ BDF_1 cells produced no IFN-γ spontaneously and displayed Con A induced IFN-γ production which was similar to, or only slightly above control levels. Conversely, cells from these mice produced increased levels of IL-2, IL-5 and IL-10.

Summary and Conclusions

The results of this kinetic study show that the first 10 days of both acute and chronic GvHD are characterised by an initial period of immune activation. In both diseases, this was manifest by splenomegaly, high levels of spontaneous '*ex-vivo*' proliferation and increased spleen cell numbers and both groups of mice showed heightened responsiveness to stimulation with B and T cell mitogens. However, the transfer of B6 donor cells provoked a more aggressive systemic GvHD than transfer of DBA/2 donor cells, since a much greater level of immunostimulation occurred early in acute GvHD. Furthermore, while the early period of immune activation in acute GvHD was rapidly replaced by involution, characterised by splenic atrophy, loss of T and B cell function, weight loss and death, mice with chronic GvHD continued to display evidence of immune activation throughout the study.

The pattern of early hyperplasia followed by immunosuppression in acute GvHD was also evidenced when I examined B cell responses *in vivo*. Accordingly, although enhanced levels of total IgG and anti-ds DNA antibodies were found early during acute GvHD, these were both absent at the later time

points and did not provoke renal immunopathology. This was again in contrast to my findings in chronic GvHD, where elevated IgG and anti-ds DNA antibodies were always observed and these mice developed lethal ICGN.

Phenotypic analysis of the T cell populations in the spleens of GvHD mice also revealed dramatic differences between the acute and chronic forms of disease. Thus, while DBA/2 ⇒ BDF_1 mice maintained normal proportions of splenic $CD4^+$ and $CD8^+$ T lymphocytes, expansion of $CD8^+$ T cells and, to a lesser extent, $CD4^+$ T cells was observed in B6 ⇒ BDF_1 mice from day 10 until the end of the study, thereby correlating with the onset of the destructive phase of acute GvHD.

Finally, my results confirmed that there is a dichotomy in the cytokines produced by cells from mice with acute and chronic GvHD. Thus, while acute GvHD was associated with early spontaneous production of IFN-γ, cells from chronic GvHD mice did not secrete IFN-γ spontaneously, but produced persistently high levels of IL-10 and IL-5 and IL-2, in response to stimulation with Con A.

Together, these results support the view that acute GvHD in B6 ⇒ BDF_1 mice is a biphasic disease, in which Th1 cytokines and generalised, intense lymphoid hyperreactivity evolve into a destructive disease characterised by death and immunosuppression. Chronic GvHD, on the other hand, is an initially milder, but ultimately more persistent disorder, which appears to involve a mainly Th2-mediated immune response. These findings highlight both the quantitative and qualitative differences between the two forms of GvHD and therefore form the basis for the remainder of my thesis. In the following chapters, I went on to use the parameters characterised here to examine the different cell types involved in the polarisation of the alloimmune response and how their cytokine products affect its outcome.

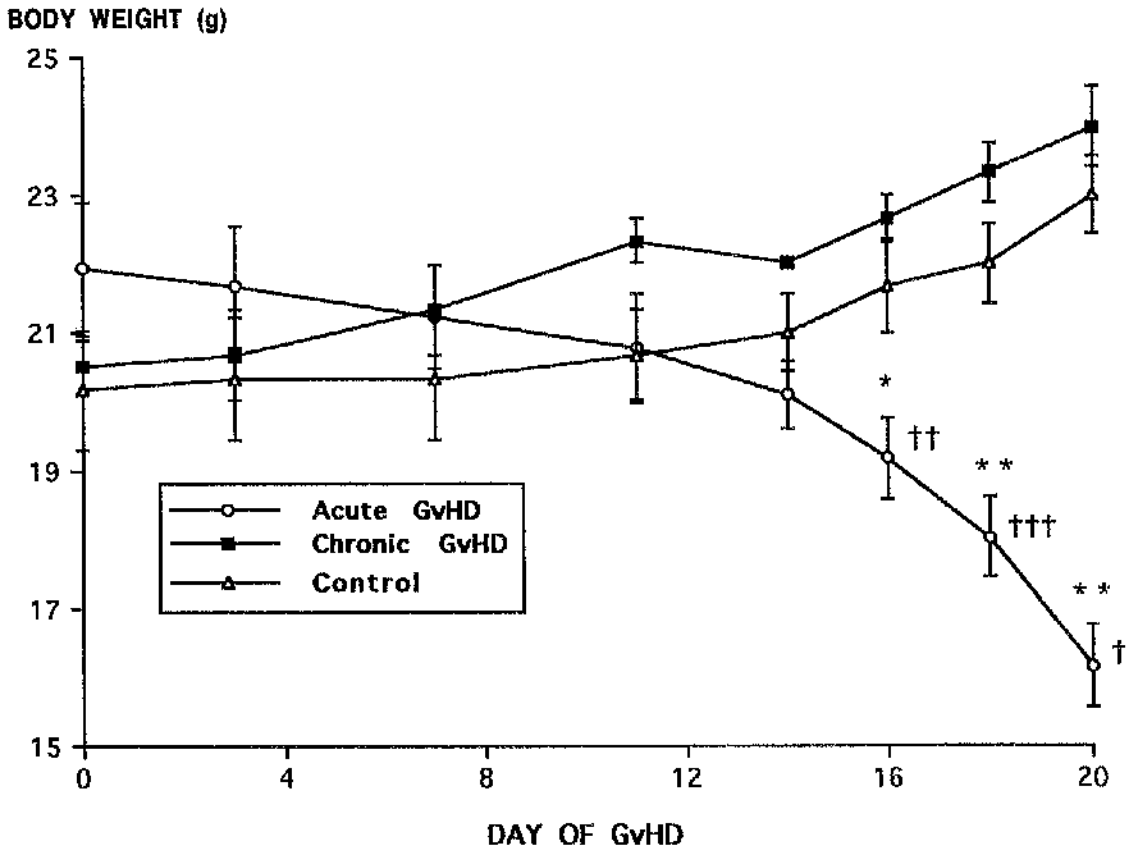


Fig. 3.1. Weight loss and mortality during acute and chronic GvHD. BDF_1 mice given 10^8 B6 spleen cells i.v. show progressive and significant weight loss and death from day 16 of the acute GvHD, while BDF_1 mice given DBA/2 cells i.v. show a similar increase in weight to control mice and do not die during this early period. Results show the mean body weight ± 1 SD for 9 mice per group.

(* $p < 0.05$; ** $p < 0.005$ vs controls and chronic GvHD mice; † = Death).

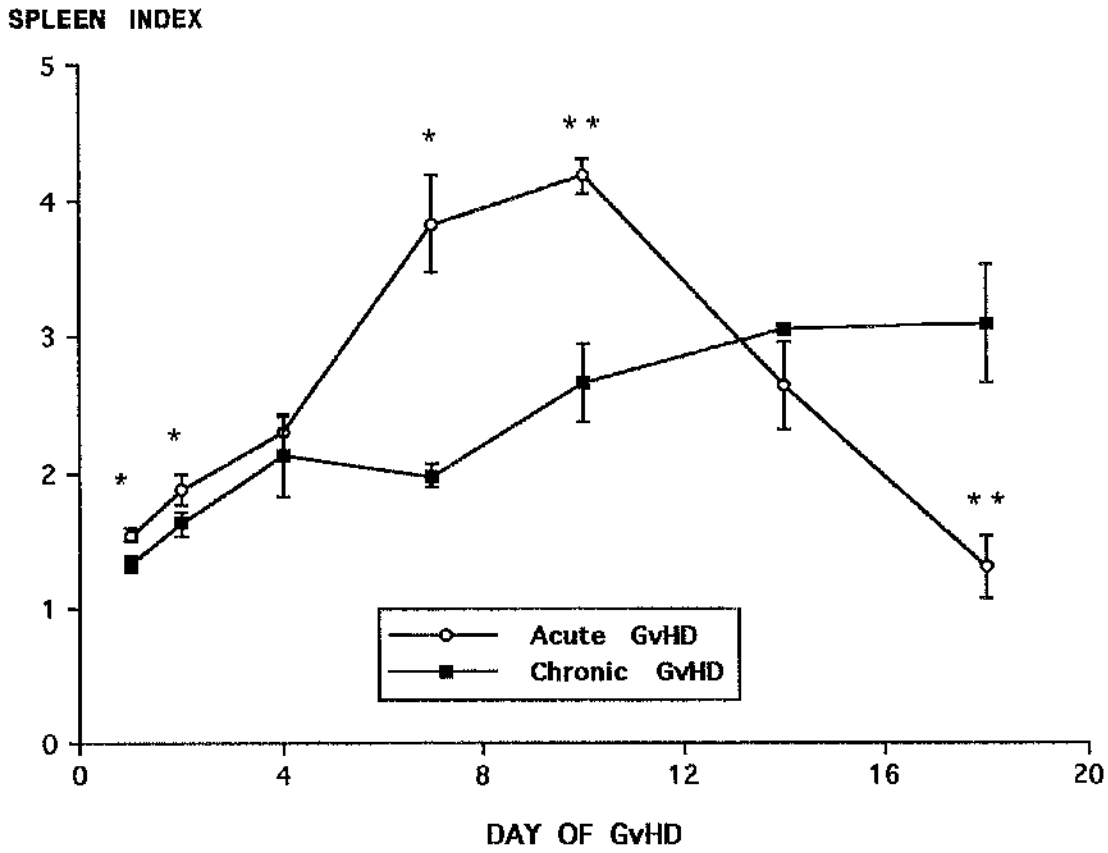


Fig. 3.2. Splenomegaly during acute and chronic GvHD.

BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. develop significant splenomegaly from day 2 of the GvHD. Results show the mean spleen index of 3 GvHD mice per group ± 1SD, calculated with reference to the mean spleen weights of 3 control mice.

(* $p < 0.05$ vs chronic GvHD; ** $p < 0.001$ vs chronic GvHD).

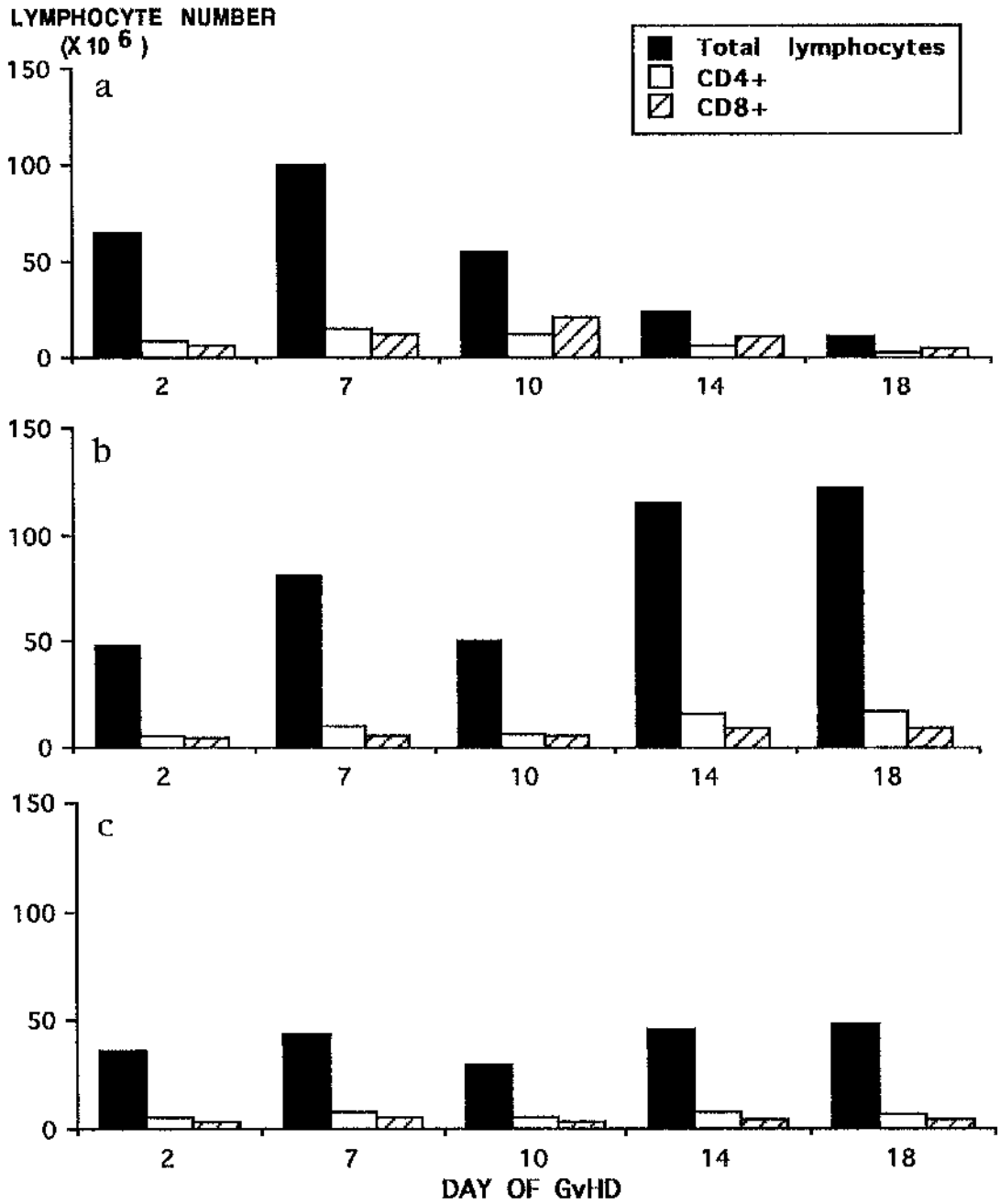


Fig. 3.3. Splenic lymphocyte numbers in mice with GvHD.

The data show the mean total lymphocyte number and the mean absolute number of $CD4^+$ and $CD8^+$ lymphocytes in the spleens of BDF_1 mice given either 10^8 B6 (Fig. 3.3a) or DBA/2 (Fig. 3.3b) spleen cells i.v. The number of splenic lymphocytes in control mice is also shown for comparison (Fig. 3.3c). The results are from pooled samples and are representative of the findings of 3 similar experiments.

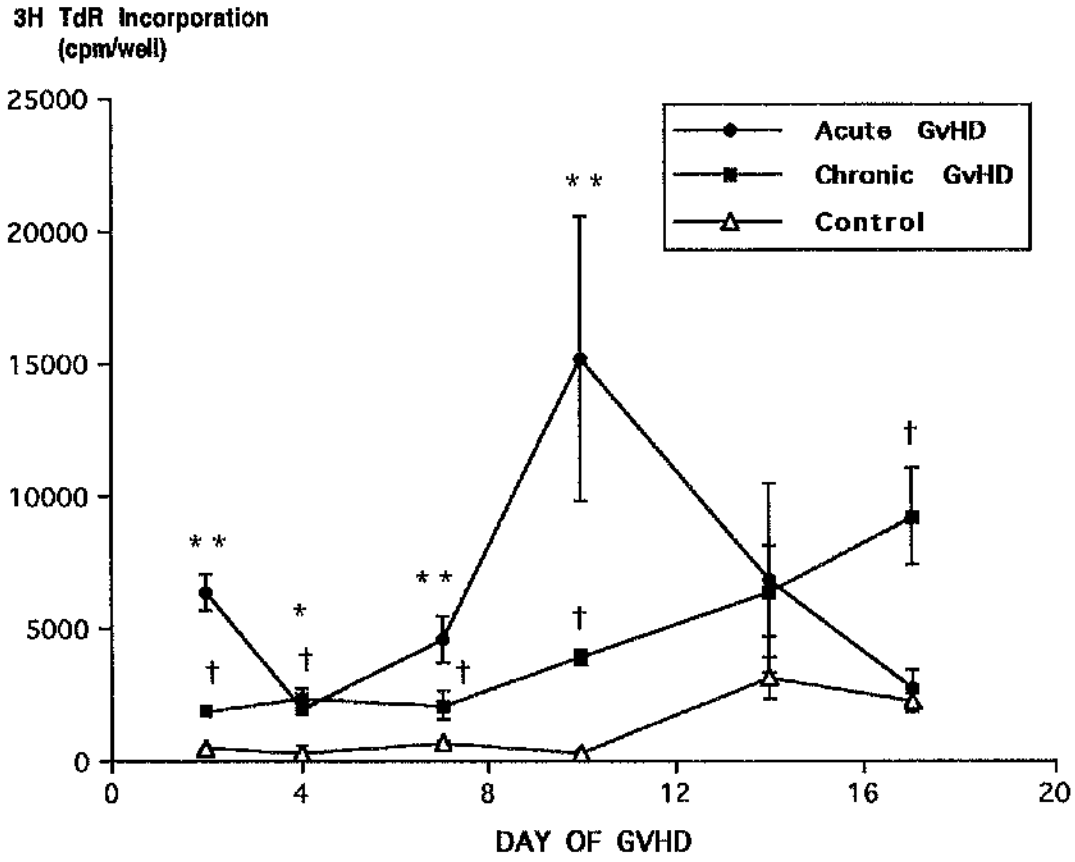


Fig. 3.4. Spontaneous '*ex-vivo*' proliferation by lymphocytes from mice with acute and chronic GvHD.

The results show the spontaneous proliferative capacity of splenocytes from BDF_1 mice given either 10^8 B6 or DBA/2 spleen cells i.v. The results are expressed as the mean uptake of 3H-TdR ± 1 SD after 4 hours in quadruplicate cultures, using spleen cells pooled from 3 mice per group. (* $p < 0.05$; ** $p < 0.01$ (acute GvHD vs controls); † $p < 0.05$ (chronic GvHD vs controls)).

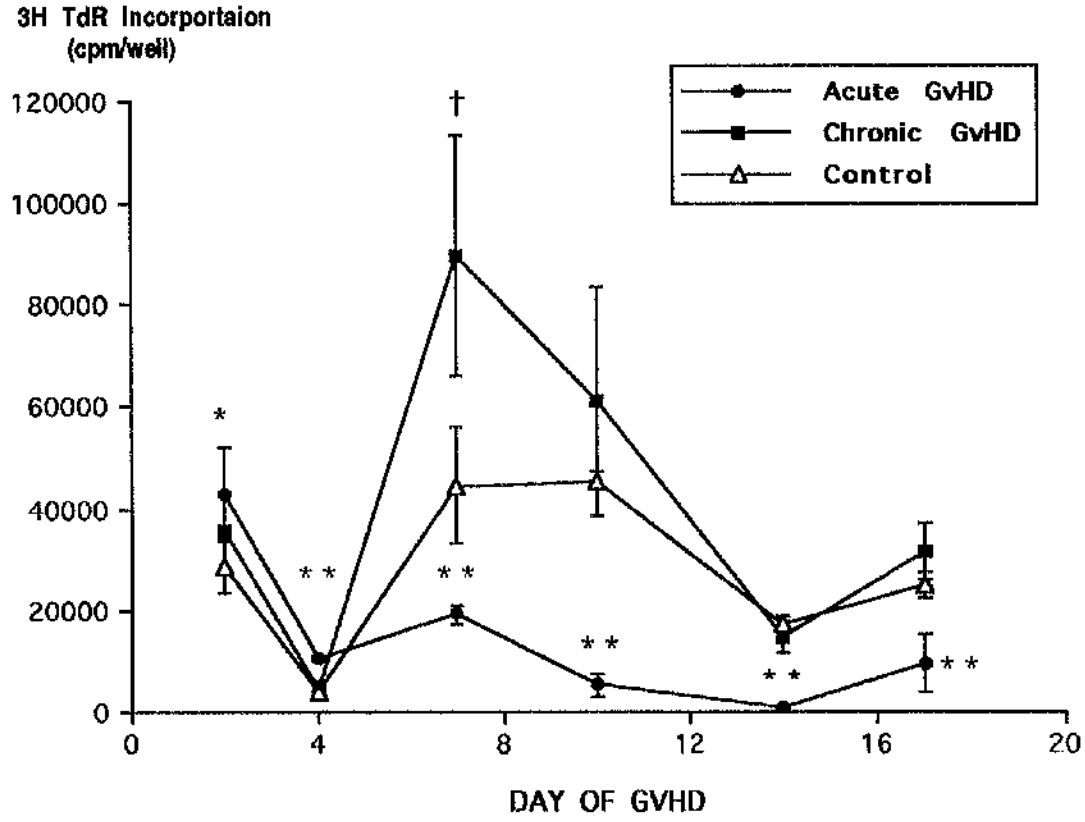


Fig. 3.5. Con A induced proliferation by lymphocytes from mice with acute and chronic GvHD.

The results show the proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. in response to mitogenic stimulation with Con A. Data are expressed as the mean uptake of 3H-TdR ± 1 SD after 48 hours in quadruplicate cultures, in the presence of 10μg/ml Con A using spleen cells pooled from 3-4 mice per group. (* $p < 0.05$ ** $p < 0.01$ (acute GvHD vs controls); † $p < 0.05$ (chronic GvHD vs controls).

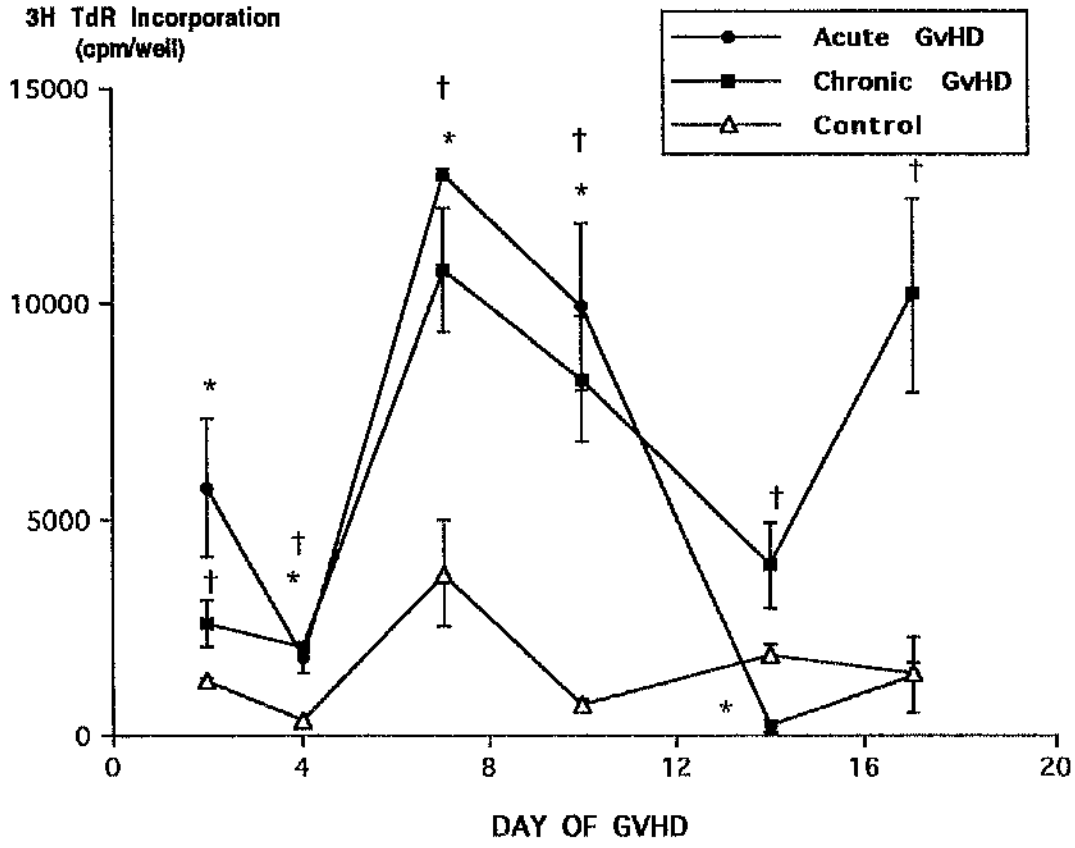


Fig. 3.6. LPS induced proliferation by lymphocytes from mice with acute and chronic GvHD.

The results show the proliferative capacity of splenocytes from BDF1 mice given 108 B6 or DBA/2 spleen cells i.v. in response to mitogenic stimulation with LPS. Data are expressed as the mean uptake of 3H-TdR ± 1 SD after 24 hours in quadruplicate cultures in the presence of 10μg/ml LPS, using spleen cells pooled from 3-4 mice per group. (* $p < 0.001$ (acute GvHD vs controls); † $p < 0.001$ (chronic GvHD vs controls).

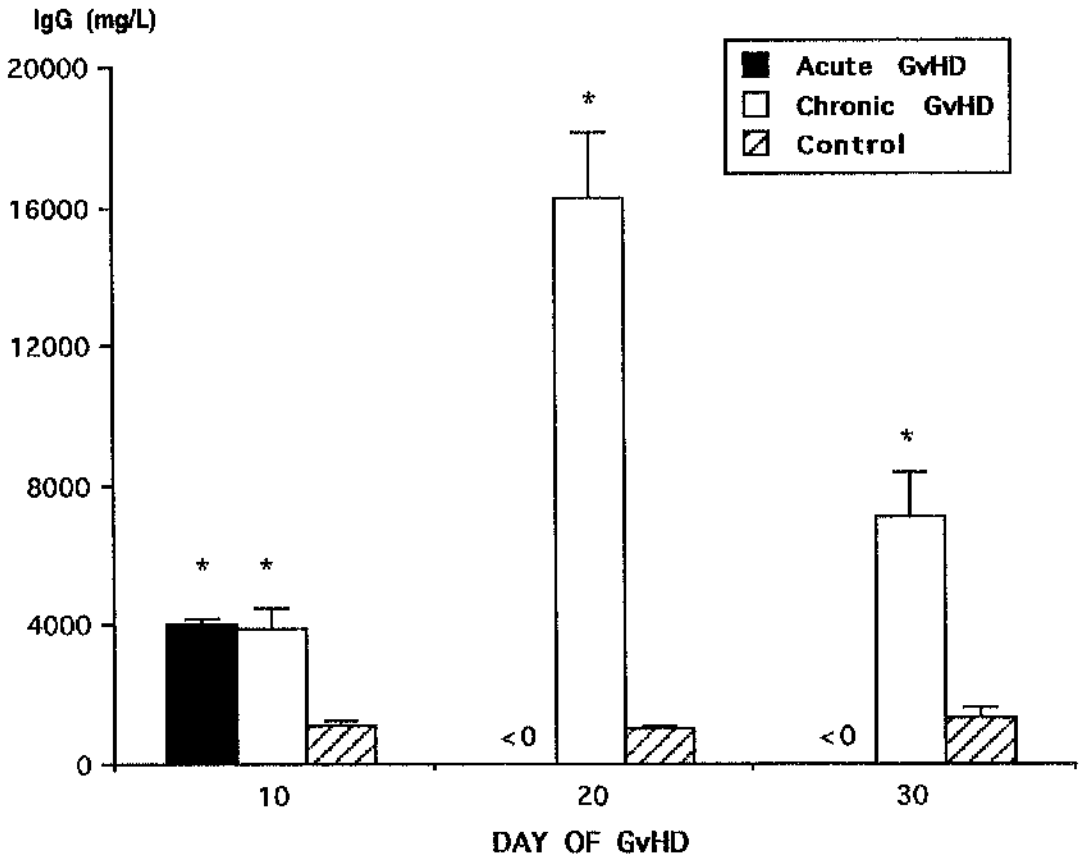


Fig. 3.7. Total serum IgG levels in GvHD mice.

Results show the mean total IgG levels ± 1SD in the serum of BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v., as determined by RID. The IgG levels of 5-6 mice per group were assessed at each time point. (* $p < 0.001$ vs controls).

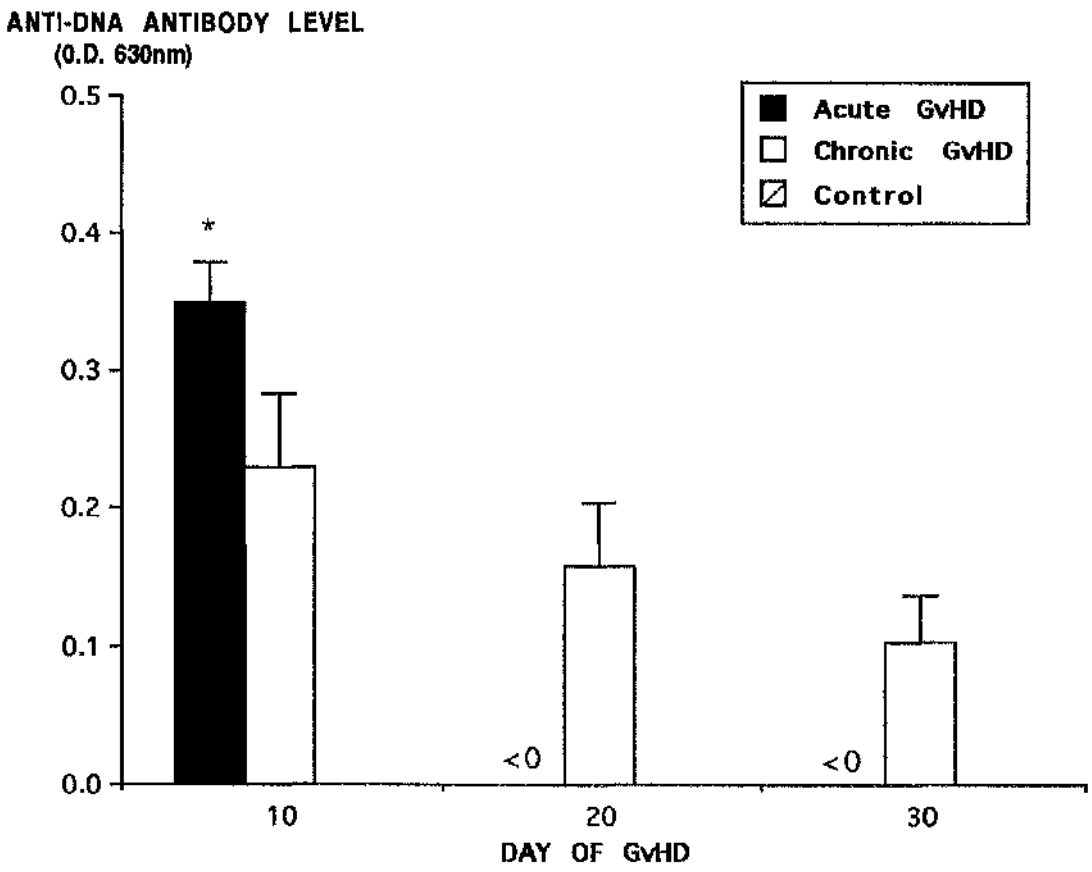


Fig. 3.8. Anti-ds DNA antibodies in the serum of GvHD mice.

The results show the mean level of anti-ds DNA specific IgG antibodies present in the serum of BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v., as determined by ELISA. The anti-ds DNA antibody levels of 6 mice per group were assessed at each time point and expressed as the mean OD value for positive sera at 1:50 ± 1SD for each group at 630nm. (* $p < 0.05$ vs chronic GvHD).

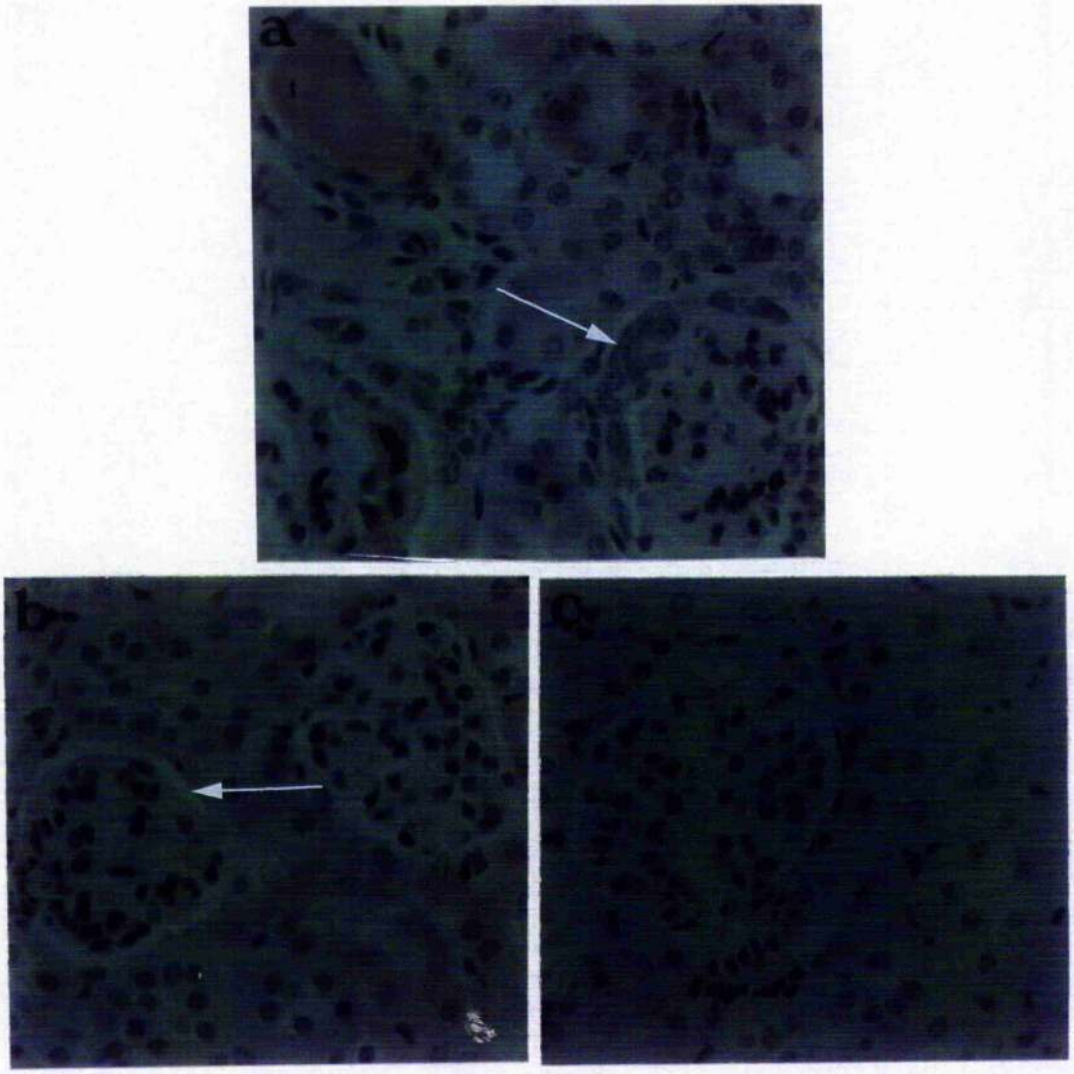


Fig. 3.9. Assessment of renal immunopathology in GvHD mice by light microscopy.

Histopathological analysis of kidneys from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells on day 48 of GvHD.

9a. Representative chronic GvHD kidney section. The glomerular basement membrane was significantly thickened and glomeruli appeared hypercellular (arrowed). Numerous protein casts were observed in the kidney tubules of these mice (t) (H&E x 40).

9b. Representative acute GvHD kidney section showing normal glomerular morphology (arrowed), with no thickening of the glomerular basement membrane. The tubules did not contain protein casts (H&E x40).

9c. Representative control kidney section (H&E x 40).

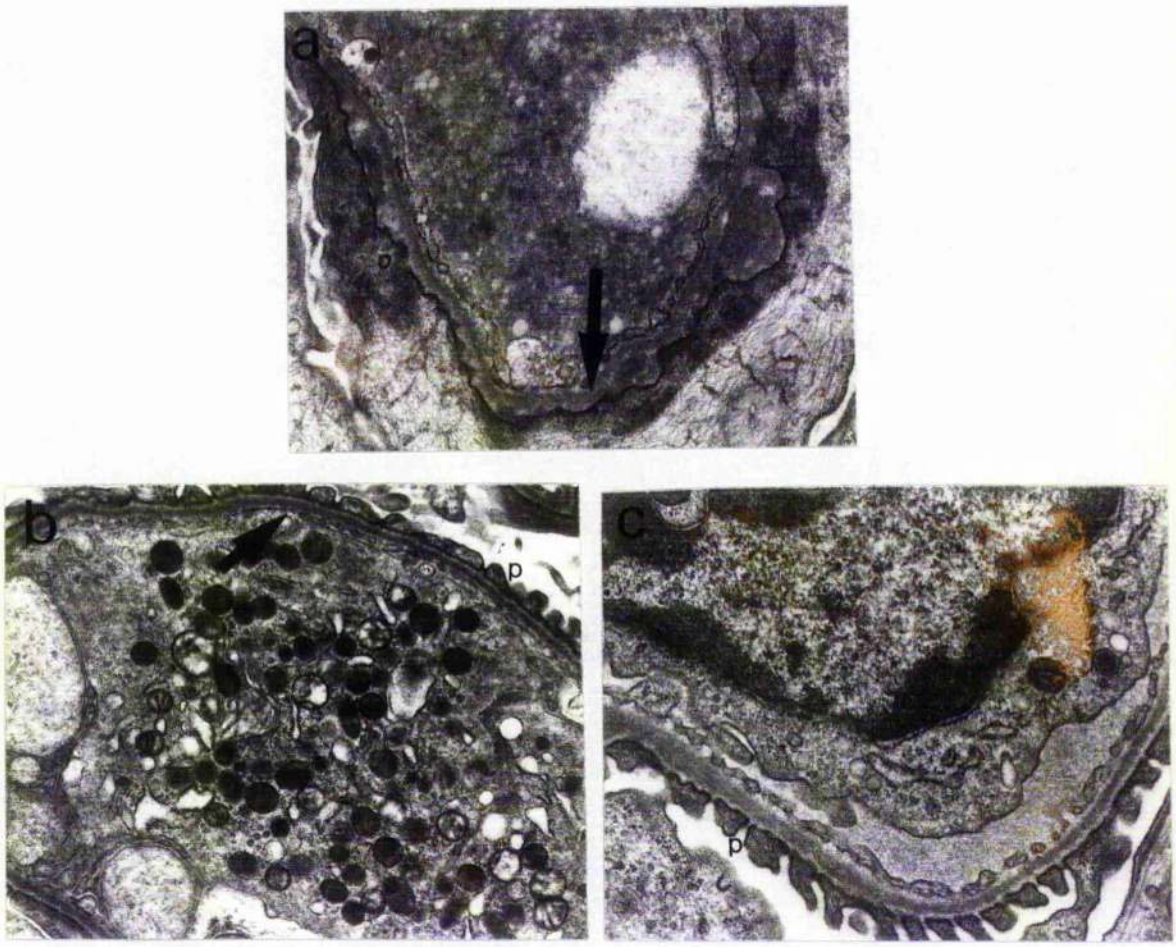


Fig. 3.10. Assessment of renal immunopathology in GvHD mice by electron microscopy.

Histopathological analysis of kidneys from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells on day 48 of GvHD.

10a. Representative chronic GvHD kidney section. Numerous sub-epithelial electron-dense deposits were observed in most glomeruli (arrowed), indicating the presence of immune complexes. Endothelial swelling was evident and the normal podocyte architecture was destroyed (x 14850)

10b. Representative acute GvHD kidney section showing normal glomerular morphology. No electron-dense immune complex deposits were observed and the endothelium (arrowed) and podocyte architecture (p) appeared normal (x 11700)

10c. Representative control kidney section (x 14875)

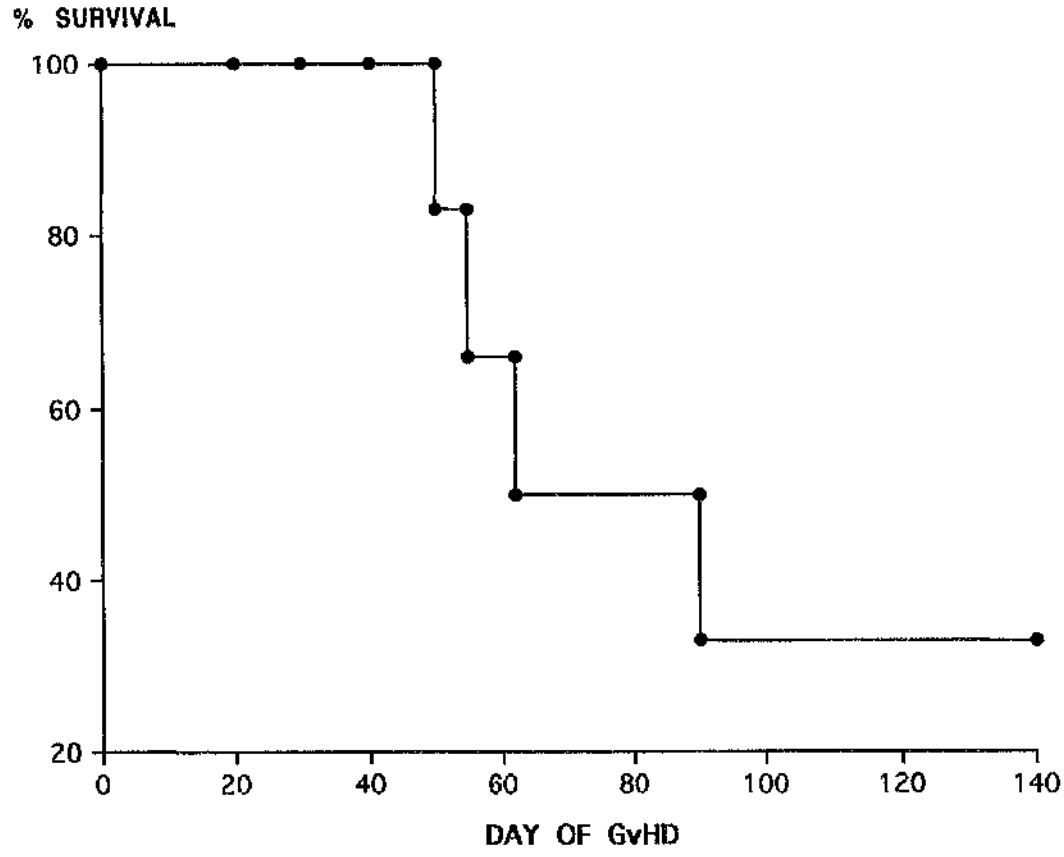


Fig. 3.11. Mice with chronic GvHD die late in disease.

BDF_1 mice given 10^8 DBA/2 spleen cells develop progressive oedema and start to die on day 50 from ICGN. The results show the percentage survival in a group of 9 chronic GvHD mice and are representative of the findings of 3 similar experiments.

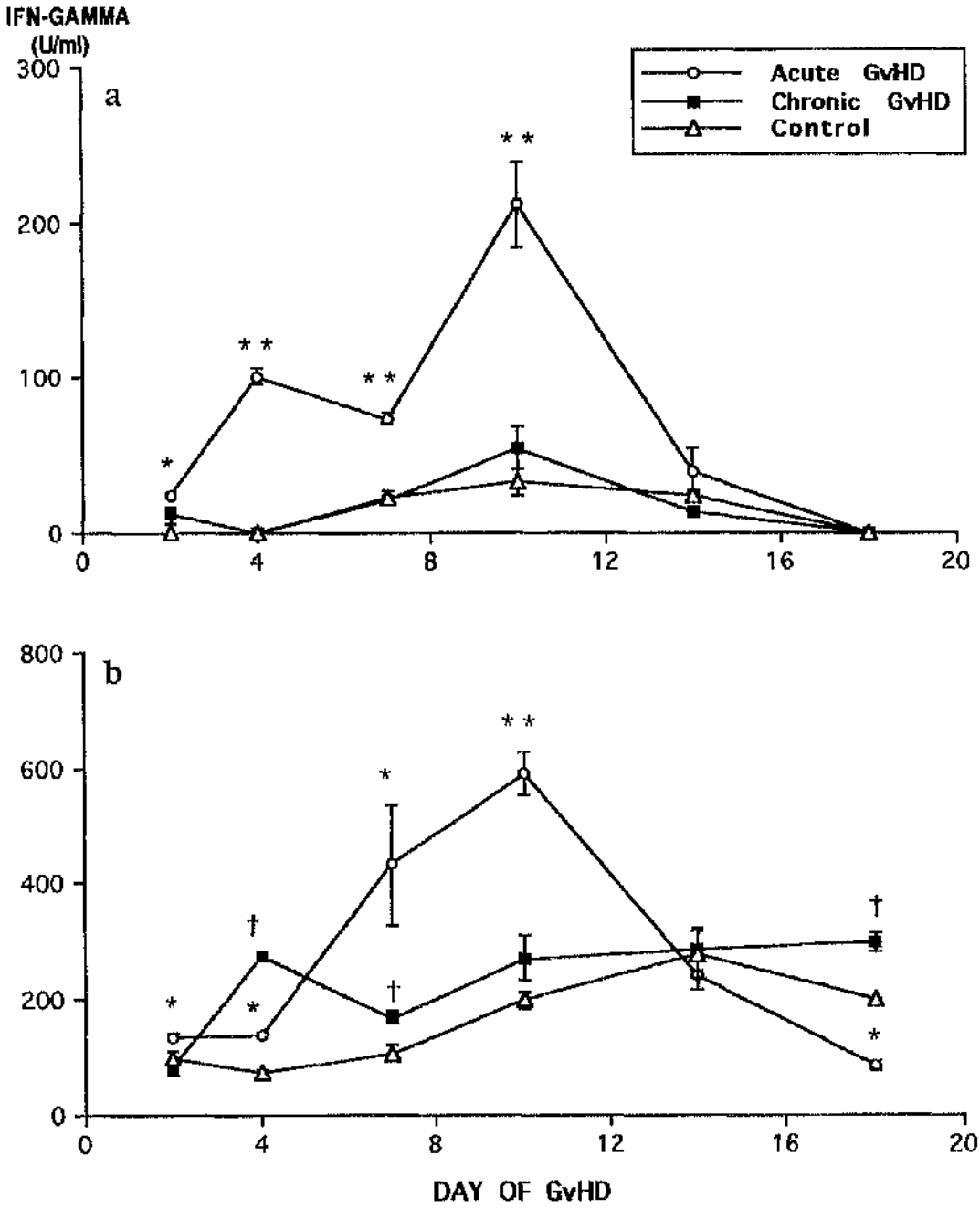


Fig 3.12. IFN-γ production during acute and chronic GvHD.

Splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells were cultured in medium (Fig. 3.12a) or in the presence of 10μg/ml Con A (Fig. 3.12b) for 48 hours, before the supernatants were removed and assayed for the presence of IFN-γ by ELISA. Results shown are means ± 1 SD of triplicate samples. (* $p < 0.05$ ** $p < 0.001$ (acute GvHD vs controls); † $p < 0.01$ (chronic GvHD vs controls)).

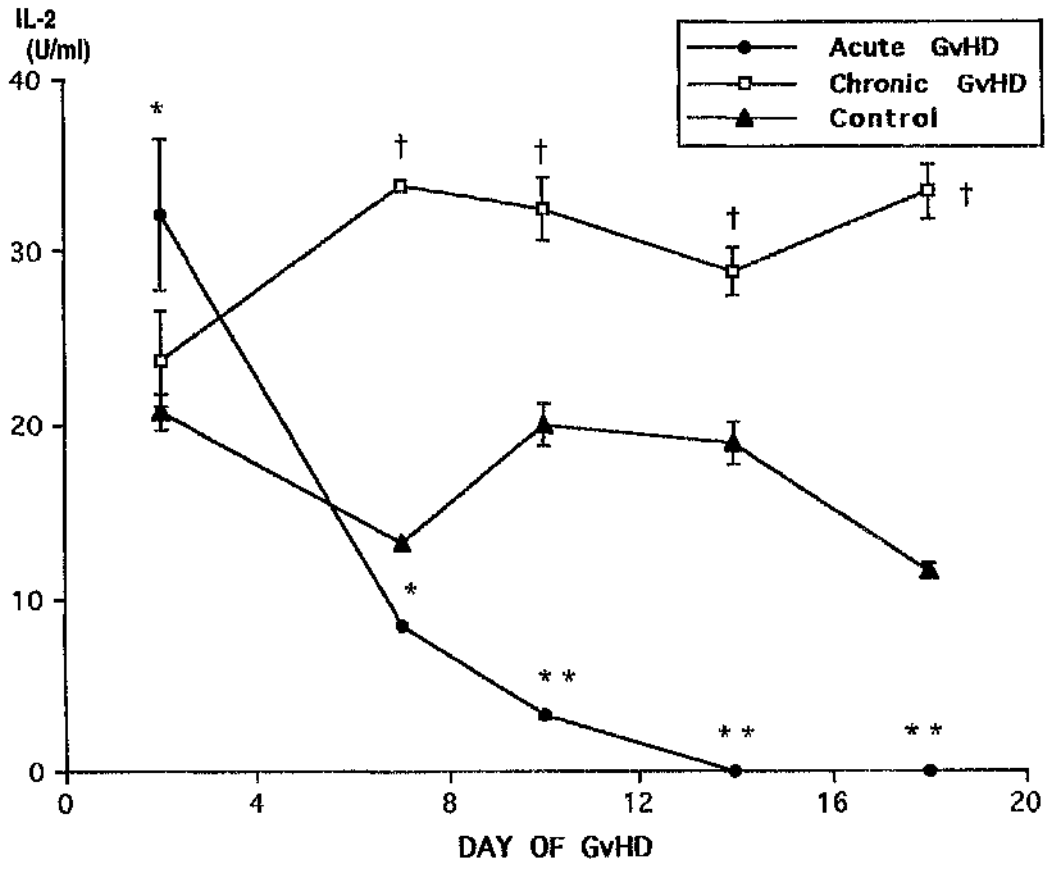


Fig. 3.13. IL-2 production during acute and chronic GvHD.

Splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells were cultured in the presence of 10μg/ml Con A for 24 hours, before the supernatants were removed and assayed for the presence of IL-2 by ELISA. Results shown are means ± 1 SD of triplicate samples. (* $p < 0.05$; ** $p < 0.001$ (acute GvHD vs controls); † $p < 0.001$ (chronic GvHD vs controls)).

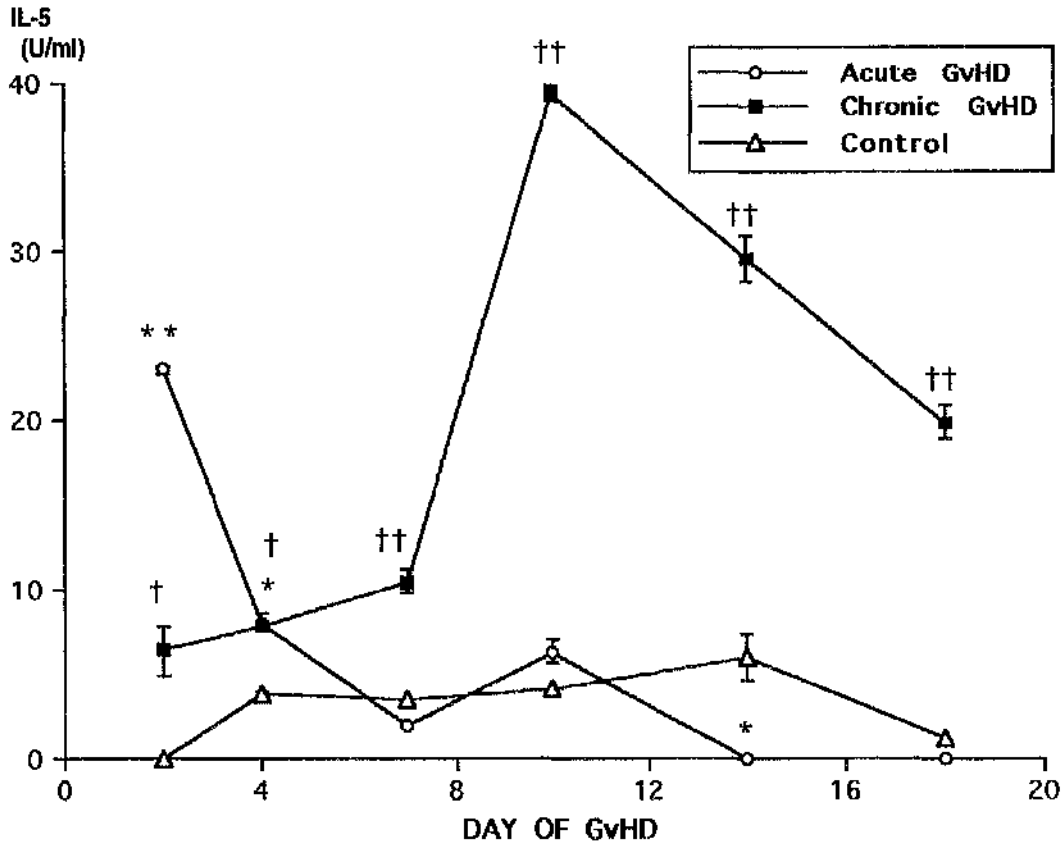


Fig. 3.14. IL-5 production during acute and chronic GvHD.

Splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells were cultured in the presence of 10μg/ml Con A for 96 hours, before the supernatants were removed and assayed for the presence of IL-5 by ELISA. Results shown are means ± 1 SD of triplicate samples. (* $p < 0.005$; ** $p < 0.001$ (acute GvHD vs chronic GvHD and controls); † $p < 0.05$; †† $p < 0.001$ (chronic GvHD vs acute GvHD and controls)).

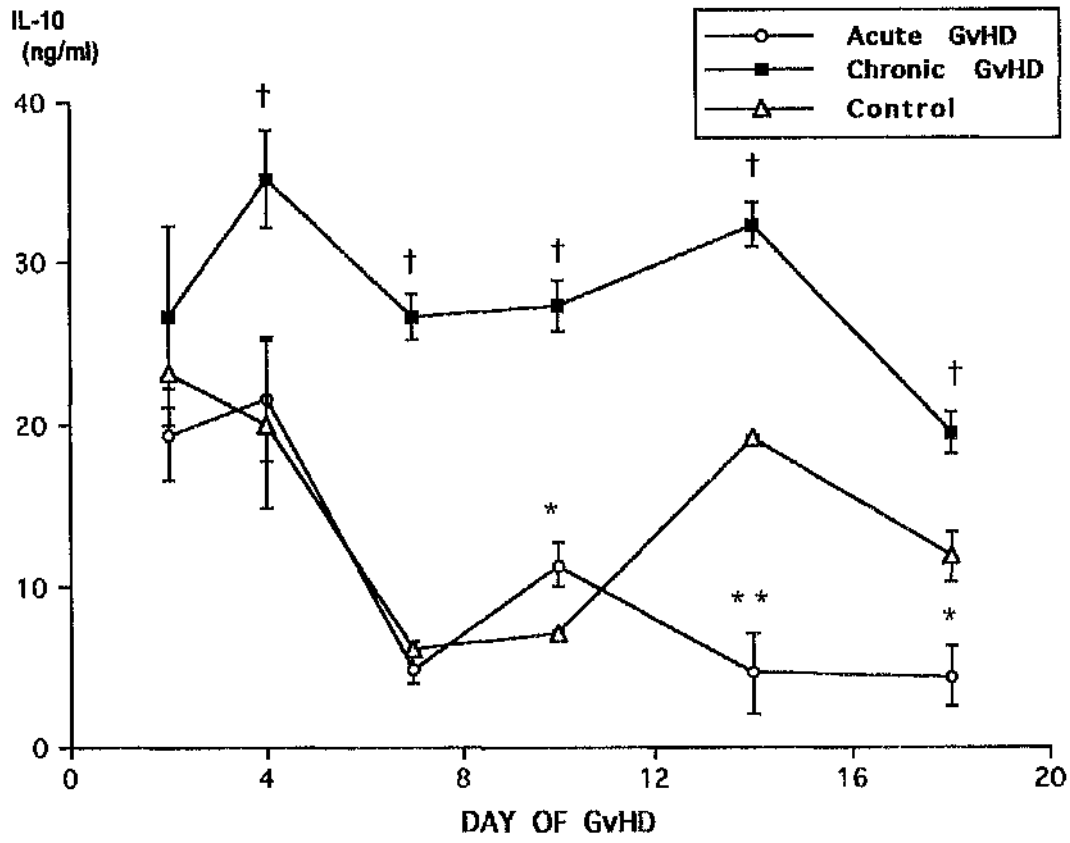


Fig. 3.15. IL-10 production during acute and chronic GvHD.

Splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells were cultured in the presence of 10μg/ml Con A for 48 hours, before the supernatants were removed and assayed for the presence of IL-5 by ELISA. Results shown are means ± 1 SD of triplicate samples. (* $p < 0.05$ ** $p < 0.01$ (acute GvHD vs chronic GvHD and controls); † $p < 0.001$ (chronic GvHD vs acute GvHD and controls)).

Table 3.1

Splenic T lymphocyte Populations in Mice with Acute and Chronic GvHD

Day of GvHD	B6 ⇒ BDF_1 (Acute GvHD)	DBA/2 ⇒ BDF_1 (Chronic GvHD)	Control
2	12.4	12.7	12.4
	9.7	9.6	10.5
7	14.1	12.3	17.6
	11.8	7.3	11.4
10	21.8	14.7	17.9
	39.1	11.9	10.5
14	26.6	13.6	15.7
	45.8	7.9	8.8
18	23.2	13.9	14.2
	50.0	7.5	7.2

Table 3.1 Percentages of splenic CD4+ and CD8+ T lymphocytes in GvHD mice.

The results show the proportions of splenic $CD4^+$ and $CD8^+$ T cells in BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v., assessed by flow cytometrical analysis and expressed as a percentage of the total gated splenic lymphocyte population. The upper number indicates the percentage of $CD4^+$ lymphocytes and the lower number indicates the percentage of $CD8^+$ cells.

CHAPTERS 4-7

ROLE OF ENDOGENOUS IL-12 IN GvHD

General Introduction

The study of cytokine production during acute and chronic GvHD in BDF_1 mice detailed in Chapter 3 showed a clear dichotomy in the cytokine responses of mice with the different forms of disease. Thus, the Th1 cytokine IFN-γ was produced spontaneously by cells from mice with acute GvHD, while high levels of IL-5 and IL-10 could only be induced by mitogenic stimulation of cells from mice with chronic GvHD. In agreement with previous studies [42, 46], these findings suggest that differential activation of $CD4^+$ T helper subsets may underlie the distinct diseases and also indicated that this polarisation of the specific response occurred early after transfer of the different donor cells.

In other models of T cell mediated immunity, the differentiation of Th1 and Th2 cells from naive $CD4^+$ T cells appears to be influenced by the early production of cytokines by cells of the innate immune system [115, 116]. Thus the presence of IL-4 during the initial period of immune stimulation promotes the development of a Th2 type response [133-135, 141], while IL-12 and IFN-γ, produced by macrophages and NK cells respectively, initiate Th1 cell differentiation [76-78, 159, 161]. Studies reported as I was performing my work showed that the autoimmune consequences of chronic GvHD in BDF_1 mice can be prevented by administration of rm IL-12 [166], suggesting that this non-specific cytokine may have an important influence on the nature of GvHD which develops in these animals. I therefore decided to examine the role of IL-12 in determining the outcome of the GvHD in BDF_1 mice, by investigating the effect of administering or neutralising IL-12 on the *in vivo* and *in vitro* parameters of acute and chronic GvHD characterised in Chapter 3. In addition, since IL-12 is an important growth factor for NK cells and specific CTL [118, 120, 121, 150] and

both cell types are thought to be involved in the pathogenesis of acute, but not chronic GvHD [30, 31, 49, 96], I also assessed the role of IL-12 in regulating specific and non-specific cytotoxic responses during GvHD.

The role of IL-12 in acute and chronic GvHD is described in the next four chapters. In the current chapter, I will show the effect of a single dose of neutralising anti-IL-12 antibody on the early phase of both forms of GvHD, while in Chapter 5, I conducted a more detailed study on the effect of repeated anti-IL-12 administration on the acute form of disease only. Chapter 6 then examines the long-term effects of neutralising IL-12 during acute or chronic GvHD and finally, Chapter 7 focuses on the effect of exogenous rm IL-12 on acute and chronic GvHD.

1) Effect of a Single Dose of Anti-IL-12 Antibody on Acute and Chronic GvHD

My initial experiments examined the effect of a single injection of neutralising anti-IL-12 antibody given 1 day prior to induction of acute or chronic GvHD. I chose to administer the antibody at this time because it is well established that the cytokine environment in which T cell priming occurs, strongly influences the nature of the subsequent specific immune response [76, 78, 132-135, 159]. Furthermore, the results of Chapter 3 indicated that polarisation of the GvHD occurred very rapidly after the transfer of parental spleen cells, suggesting that a single neutralisation of IL-12 early in the disease might be sufficient to alter the subsequent course of disease. For this series of experiments, I concentrated on days 2 and 10 of GvHD, since these time points give a good indication of the disease during the early proliferative phase and at the onset of the destructive late phase.

Experimental Protocol

The GvHR was induced by i.v. injection of 10^8 viable B6 or DBA/2 parental spleen cells into BDF_1 recipients as before. Endogenous IL-12 was neutralised *in vivo* by i.p. injection of 0.5mg goat IgG anti-mouse IL-12 antibody given 1 day before induction of GvHR. Control animals received 0.5 mg of normal goat IgG. The intensity of the systemic GvHD was assessed by measuring splenomegaly. Lymphocyte proliferation and cytokine production were determined as descibed in Chapter 3. Levels of splenic NK cell-mediated cytotoxicity were measured in GvHD mice on day 2 using YAC-1 cells as targets.

Results

i) Splenomegaly

As before, B6 ⇒ BDF_1 mice exhibited significant splenomegaly on day 2 of the acute GvHD (Fig. 4.1a) which continued to increase up to day 10 (Fig. 4.1b). Anti-IL-12 treatment reduced the early splenomegaly on day 2 of the acute disease (Fig. 4.1a), but by day 10, the splenomegaly in anti-IL-12 treated B6 ⇒ BDF_1 mice was similar to that in unmanipulated B6 ⇒ BDF_1 animals (Fig. 4.1b).

DBA/2 ⇒ BDF_1 mice also displayed significant splenomegaly on both day 2 and day 10 of the chronic disease (Figs. 4.1a & b), but as I found earlier, the splenomegaly in these mice was less marked than in B6 ⇒ BDF_1 mice at both time points. Anti-IL-12 treatment did not affect the splenomegaly evident in chronic GvHD mice on either day 2 or day 10 (Figs. 4.1a & b).

ii) NK cell activity

A characteristic early feature of several models of GvHD is enhanced NK cell-mediated cytotoxicity [7, 49, 95-98]. Since IL-12 was originally described as a growth factor for NK cells [118, 120, 121, 150], I decided to examine whether neutralising IL-12 *in vivo* affected NK cell activity early during the GvHD in

BDF_1 mice. B6 ⇒ BDF_1 mice showed enhanced splenic NK cell activity on day 2 of the acute GvHD compared with control mice (Fig. 4.2). This was reduced to below control levels by a single dose of anti-IL-12 antibody (Fig. 4.2).

In contrast, mice with chronic GvHD showed similar levels of NK cell mediated cytotoxicity to control mice and anti-IL-12 treatment did not affect these levels in either DBA/2 ⇒ BDF1 or control mice (Fig. 4.2).

It should be noted that in this chapter, I only examined the effect of anti-IL-12 on NK cell levels on day 2, since previous work had suggested that NK cell-mediated cytotoxicity was primarily a very early feature of the disease [49]. When I subsequently examined a more detailed time course study of NK cell activity during both diseases, I found that after day 4, mice with chronic GvHD also showed enhanced NK cell activity (detailed in Chapter 9).

iii) Immune function

I next went on to examine the effect of anti-IL-12 on immune function in both acute and chronic GvHD, by assessing its effect on the ability of cells from B6 ⇒ BDF1 and DBA/2 ⇒ BDF_1 mice to proliferate spontaneously, or in reponse to stimulation with either Con A or LPS *in vitro*.

a) Spontaneous '*ex-vivo*' proliferation

As before, on both days 2 and 10, splenocytes from B6 ⇒ BDF_1 mice showed enhanced spontaneous '*ex-vivo*' proliferation compared with control splenocytes (Figs. 4.3a & b). Anti-IL-12 treatment caused a significant reduction in these levels on both days 2 and 10 (Figs. 4.3a & b), although on day 10, the spontaneous proliferative capacity of splenocytes from anti-IL-12 treated B6 ⇒ BDF_1 mice remained higher than that of control cells.

In contrast, splenocytes from DBA/2 ⇒ BDF1 mice showed a similar ability to proliferate '*ex-vivo*' to cells from control mice on day 2 of chronic GvHD (Fig. 4.3a), but by day 10 their proliferation was significantly higher than

control levels (Fig. 4.3b). Anti-IL-12 treatment did not affect the levels of spontaneous *'ex-vivo'* proliferation of chronic GvHD spleen cells on day 2 (Fig. 4.3a), but on day 10, spleen cells from anti-IL-12 treated DBA/2 ⇒ BDF_1 mice had significantly increased spontaneous*'ex-vivo'* proliferation (Fig. 4.3b).

Anti-IL-12 antibody did not affect the ability of control cells to proliferate *'ex-vivo'* on either day 2 or day 10 (Figs. 4.3a & b).

b) Proliferative Responses to Con A Stimulation

As demonstrated in Chapter 3, on day 2 of GvHD, spleen cells from B6 ⇒ BDF_1 mice showed significantly increased responsiveness to stimulation with Con A *in vitro* compared with control spleen cells (Fig. 4.4a). By day 10, the Con A induced reponses of B6 ⇒ BDF1 mice were essentially the same as those of control mice (Fig. 4.4b). Anti-IL-12 treament of B6 ⇒ BDF_1 mice significantly reduced their heightened responsiveness to Con A on day 2 of acute GvHD (Fig. 4.4a), but this effect had disappeared by day 10, when the responses of unmanipulated and anti-IL-12 treated B6 ⇒ BDF_1 mice were similar (Fig. 4.4b).

DBA/2 ⇒ BDF_1 spleen cells showed very similar Con A induced responses to control spleen cells on day 2 (Fig. 4.4a), but by day 10, these were significantly increased above control levels (Fig. 4.4b). Anti-IL-12 treatment did not significantly affect the Con A responses of chronic GvHD mice on either day 2 (Fig. 4.4a) or day 10 (Fig. 4.4b) and similarily did not affect the Con A responses of control mice at either time point (Figs. 4.4a & 4.4b).

c) Proliferative Responses to LPS Stimulation

On day 2, spleen cells from B6 ⇒ BDF_1 mice showed significantly increased responsiveness to stimulation with LPS *in vitro* compared with control spleen cells (Fig. 4.5a). By day 10, the responses of these mice to LPS stimulation were significantly lower than those of control mice (Fig. 4.5b). Anti-IL-12 treatment caused a significant reduction in the enhanced LPS responses of

acute GvHD mice on day 2 (Fig. 4.5a), but by day 10, the responses of anti-IL-12 treated B6 ⇒ BDF_1 mice were identical to those of unmodified B6 ⇒ BDF_1 mice (Fig. 4.5b).

In contrast, DBA/2 ⇒ BDF_1 spleen cells showed similar LPS responses to control cells on day 2 (Fig. 4.5a), but significantly increased responses by day 10 (Fig. 4.5b) and these were unaffected by anti-IL-12 treatment. Similarly, anti-IL-12 did not affect the LPS responses of control mice at either time point (Figs. 4.5a & b).

iv) Cytokine Production

Given the critical role of IL-12 in T helper cell phenotype development [76-78, 161], I then examined the effect of anti-IL-12 treatment on the cytokines produced by cells from mice with either acute or chronic GvHD.

IFN-γ

In this study, IFN-γ was not produced spontaneously by cells from any group (data not shown).

On both days 2 and 10, spleen cells from B6 ⇒ BDF_1 mice produced significantly more IFN-γ in response to Con A stimulation than control cells (Figs. 4.6a & b). This increase was abolished on day 2 by treatment with anti-IL-12 antibody (Fig. 4.6a), but by day 10, similarly high levels of IFN-γ were produced by cells from both anti-IL-12 treated and unmanipulated B6 ⇒ BDF_1 mice (Fig. 4.6b).

Spleen cells from DBA/2 ⇒ BDF_1 mice produced significantly lower levels of IFN-γ in response to Con A stimulation than control spleen cells on both days 2 and 10 (Figs. 4.6a & b). Anti-IL-12 treatment did not affect these levels at either time point (Figs. 4.6a & b). Anti-IL-12 treatment of control mice did not affect the ability of spleen cells from these mice to produce IFN-γ on day 2 or 10 (Fig. 4.6a & b).

IL-2

IL-2 was not produced spontaneously by cells from any group at any time during this study (data not shown).

On day 2, spleen cells from B6 ⇒ BDF_1 mice produced similar levels of IL-2 in response to Con A to control cells (Fig. 4.7a). By day 10, however, IL-2 production by cells from B6 ⇒ BDF_1 mice was dramatically reduced and levels were severalfold lower than those produced by control cells (Fig. 4.7b). Anti-IL-12 treatment caused a significant reduction in the levels of IL-2 produced by spleen cells from B6 ⇒ BDF_1 mice on day 2 (Fig. 4.7a) and completely ablated IL-2 production in acute GvHD mice by day 10 (Fig. 4.7b).

IL-2 production by DBA/2 ⇒ BDF_1 spleen cells was significantly lower than that of control spleen cells on both days 2 and 10 (Figs. 4.7a & b). This was in contrast to the enhanced levels of IL-2 which these cells produced in the study described in Chapter 3 and may reflect altered consumption of IL-2 *in vitro.* Anti-IL-12 did not affect these levels (Figs. 4.7a & b). Similarly, anti-IL-12 treatment of control mice did not affect the ability of spleen cells from these mice to produce IL-2 on either day 2 or day 10 of this study (Figs. 4.7a & b).

IL-10

IL-10 was not produced spontaneously by cells from any group at any time during this study (data not shown).

On both days 2 and 10, spleen cells from B6 ⇒ BDF_1 mice produced significantly lower levels of IL-10 in response to Con A stimulation than control cells (Figs. 4.8a & b). Anti-IL-12 treatment restored IL-10 production to control levels on day 2 (Fig. 4.8a), but by day 10, spleen cells from anti-IL-12 treated and untreated B6 ⇒ BDF_1 mice produced similarly low levels of IL-10 (Fig. 4.8b).

Spleen cells from DBA/2 ⇒ BDF_1 mice produced significantly higher levels of IL-10 in response to Con A stimulation compared with controls on days 2 and 10 and this was unaffected by anti-IL-12 treatment (Figs. 4.8a & b). Spleen

cells from control mice given anti-IL-12 produced similar levels of IL-10 to unmodified controls on days 2 and 10 (Fig. 4.8a & b).

IL-5

IL-5 was not produced spontaneously by cells from any group at any time during this study (data not shown). Con A induced IL-5 production was not detected in cultures of cells from any group on day 2 (data not shown) and spleen cells from anti-IL-12 treated and unmanipulated B6 ⇒ BDF_1 mice did not produce detectable Con A induced IL-5 on day 10 (Fig. 4.9).

In contrast, on day 10, spleen cells from DBA/2 ⇒ BDF_1 mice produced significantly higher levels of Con A stimulated IL-5 than control cells (Fig. 4.9). Again, the levels of IL-5 produced by both control spleen cells or cells from DBA/2 ⇒ BDF_1 mice were not significantly affected by anti-IL-12 treatment.

IL-4

IL-4 production by cells from all groups was below the level of detection at all time points during this study (data not shown).

Summary

This study showed that a single dose of anti-IL-12 antibody abrogated many of the early proliferative features of acute GvHD, including splenomegaly, enhanced spontaneous and mitogen induced proliferation, elevated NK cell activity and increased IFN-γ and IL-2 production. In parallel, anti-IL-12 increased the levels of IL-10. Despite these effects on the early phase of the acute disease, a single injection of anti-IL-12 was unable to prevent acute GvHD completely and by day 10, anti-IL-12 treated B6 ⇒ BDF_1 mice showed the same degree of splenomegaly and reduced responses to Con A and LPS stimulation as unmanipulated B6 ⇒ BDF_1 mice. They also exhibited the characteristic Th1-type

cytokine pattern associated with the acute disease. This dose of anti-IL-12 was therefore sufficient to delay, but not to abrogate onset of acute GvHD.

In contrast anti-IL-12 treatment had no effect on the chronic form of GvHD. Anti-IL-12 treated DBA/2 ⇒ BDF_1 mice had identical splenomegaly, NK cell activity, and levels of mitogen induced proliferation to unmanipulated DBA/2 ⇒ BDF_1 mice on both days 2 and 10, although on day 10, the anti-IL-12 treated group had markedly higher levels of spontaneous '*ex-vivo*' proliferation than unmanipulated DBA/2 ⇒ BDF_1 mice, perhaps indicating a subtle regulatory role for IL-12 at this stage in the chronic GvHD. The cytokine profile of mice with chronic GvHD was also unaffected by anti-IL-12 treatment and cells from both unmanipulated and anti-IL-12 treated DBA/2 ⇒ BDF_1 mice produced significantly lower levels of IFN-γ and increased levels of IL-10 and IL-5 as cells from control mice.

Conclusions

These results suggest that endogenous IL-12 may be involved in the development of acute, but not chronic GvHD. However, the effects of anti-IL-12 were transient. I therefore decided to extend these initial studies by neutralising endogenous IL-12 in B6 ⇒ BDF_1 mice for a longer period to determine whether this would have a more dramatic effect on the development of acute GvHD.

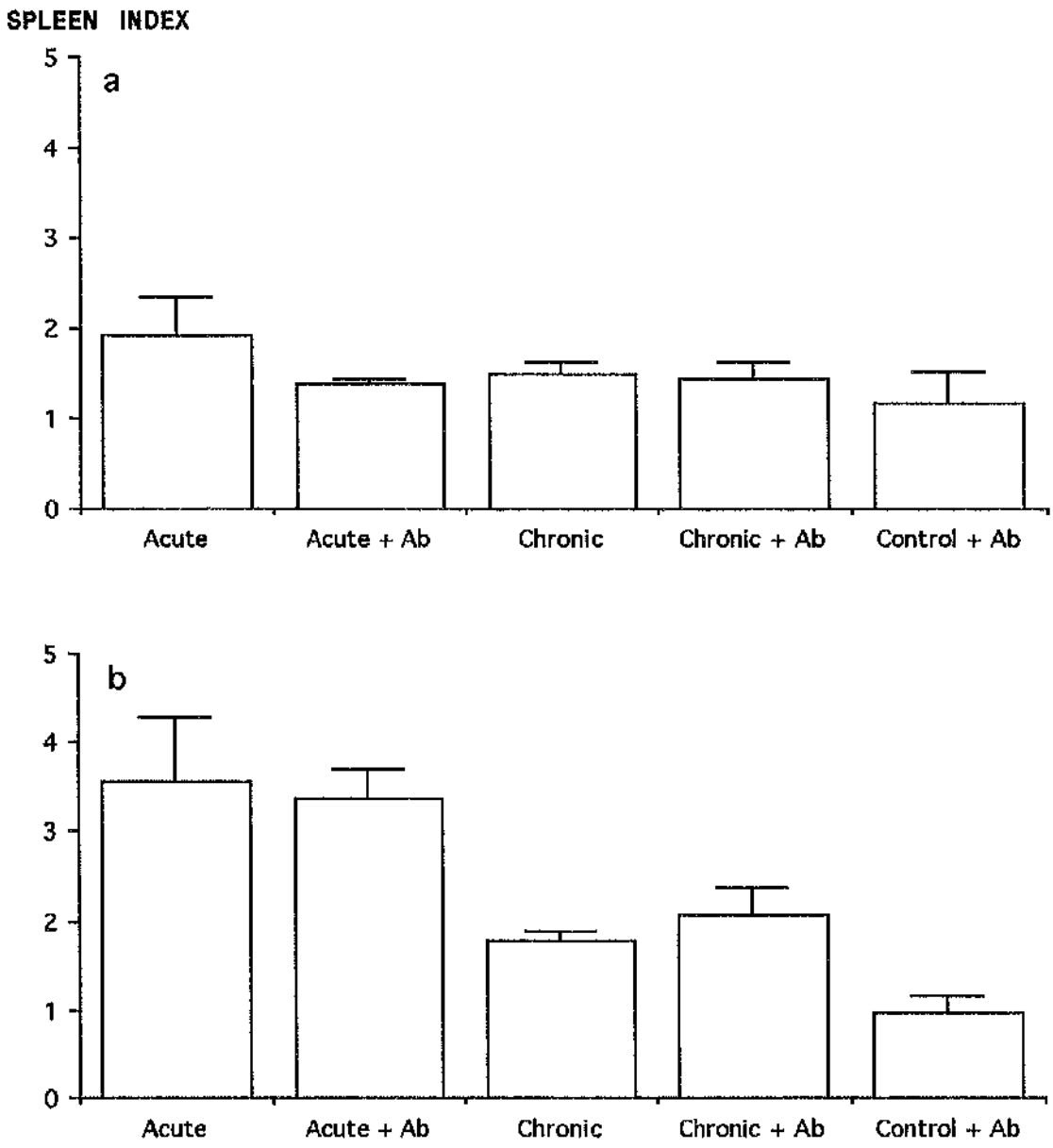


Fig. 4.1. Effects of anti-IL-12 treatment on splenomegaly during acute and chronic GvHD.

The results show the effect of a single dose of anti-IL-12 antibody on splenomegaly in BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. Results shown are the mean spleen indices of 3 GvHD mice per group ± 1SD relative to the mean spleen weights of 3 control mice on days 2 (Fig. 4.1a) and 10 (Fig. 4.1b) of GvHD. (* $p < 0.001$ vs controls). Ab = Anti-IL-12 treated.

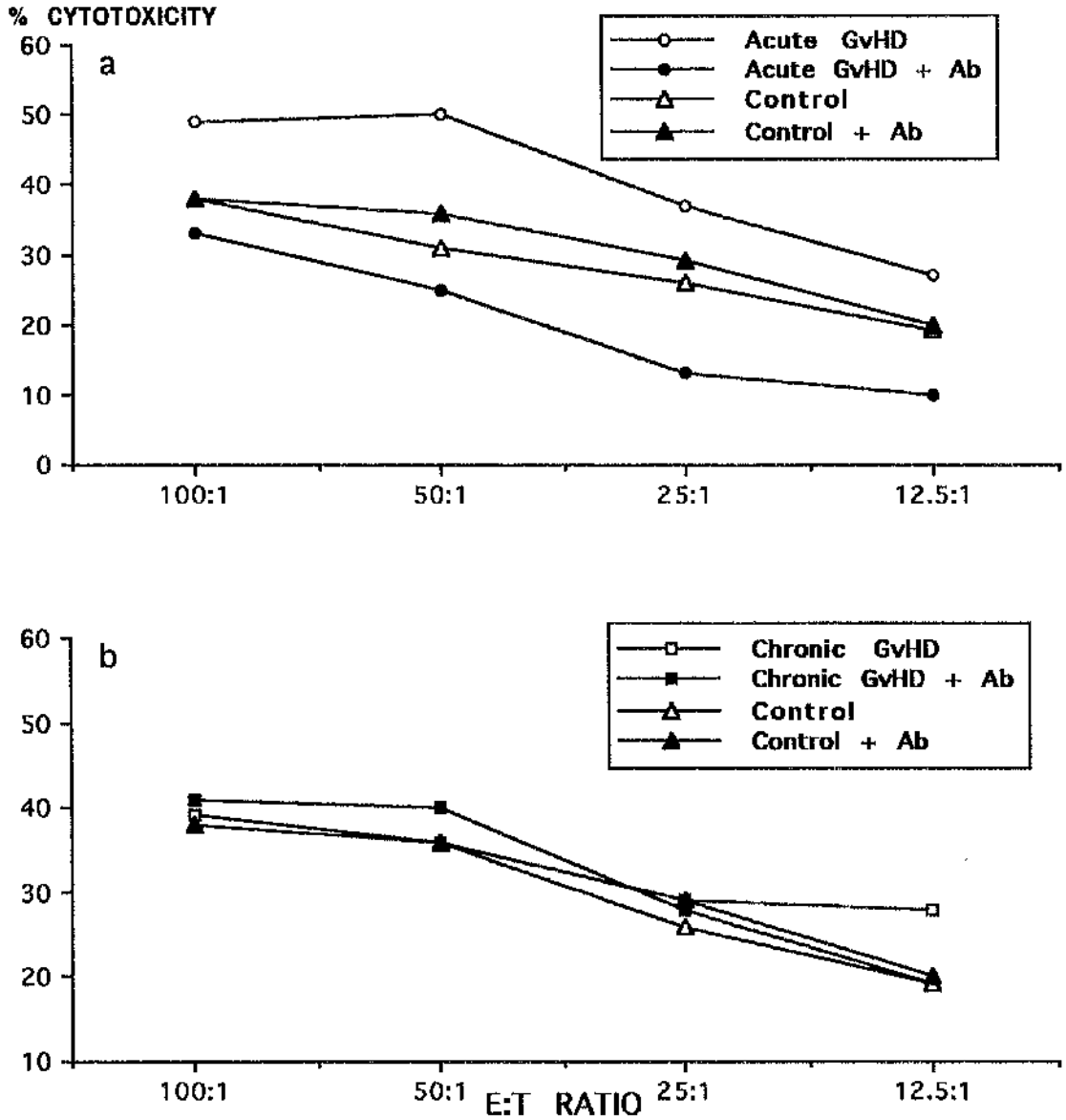


Fig. 4.2. Effects of anti-IL-12 treatment on NK cell activity during acute and chronic GvHD.

The results show the effect of a single dose of anti-IL-12 antibody on splenic NK cell activity in BDF_1 mice given 10^8 B6 (Fig. 4.2a) or DBA/2 (Fig. 4.2b) spleen cells i.v. The results shown are the % cytotoxicity against YAC-1 target cells from quadruplicate assays measured at effector : target (E:T) ratios from 100:1 to 12.5:1, using spleen cells pooled from 3 mice per group on day 2 of GvHD. Ab = Anti-IL-12 treated.

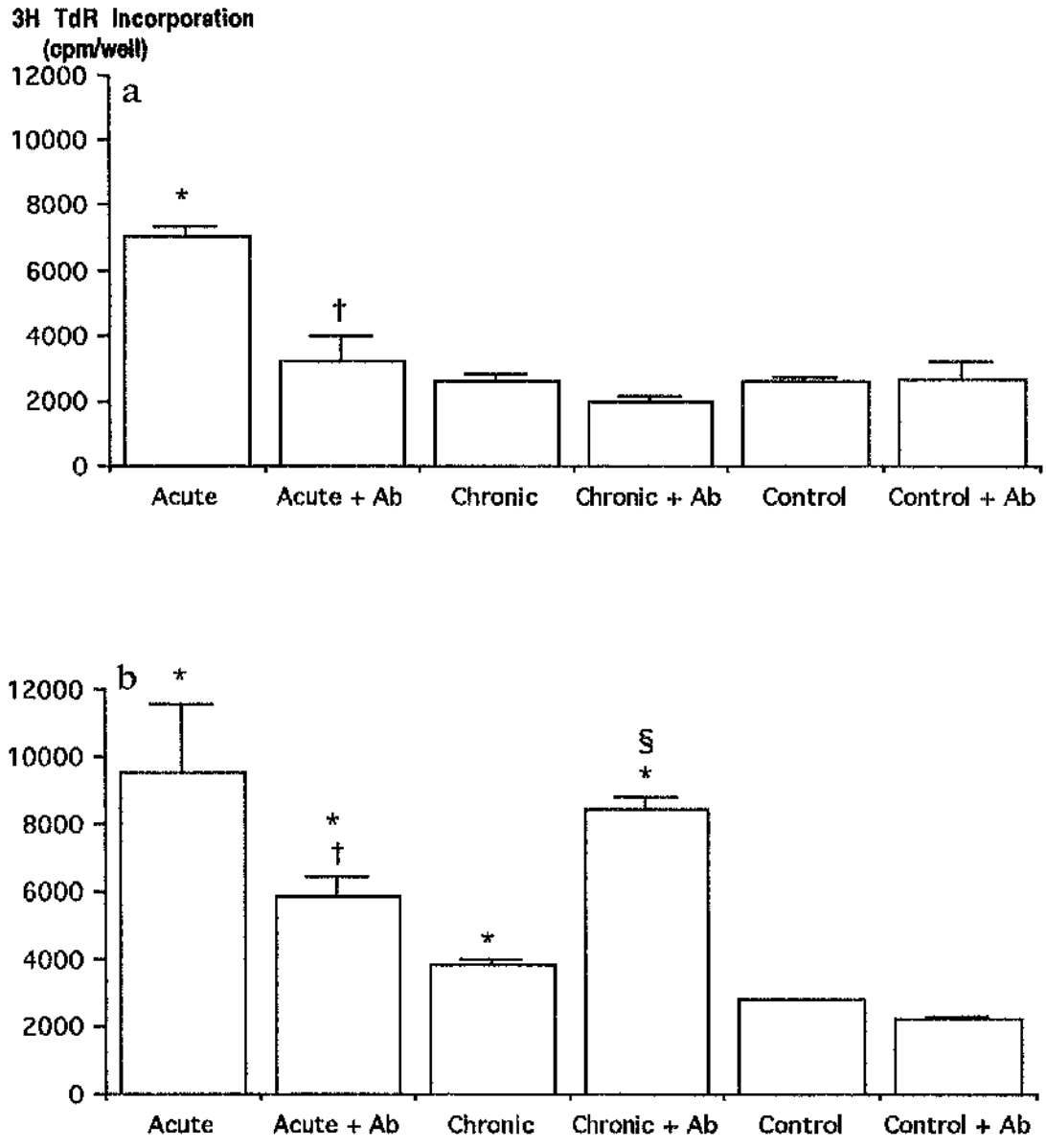


Fig. 4.3. Effects of anti-IL-12 treatment on spontaneous '*ex-vivo*' proliferation during acute and chronic GvHD.

The results show the effect of a single dose of anti-IL-12 antibody on the spontaneous proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. The results are expressed as the mean uptake of 3H-TdR ± 1 SD after 4 hours in quadruplicate cultures, using spleen cells pooled from 3 mice per group on days 2 (Fig. 4.3a) and 10 (Fig. 4.3b) of the GvHD. (* $p < 0.001$ vs controls; † $p < 0.01$ vs unmodified acute GvHD; § $p < 0.001$ vs unmodified chronic GvHD). Ab = Anti-IL-12 treated.

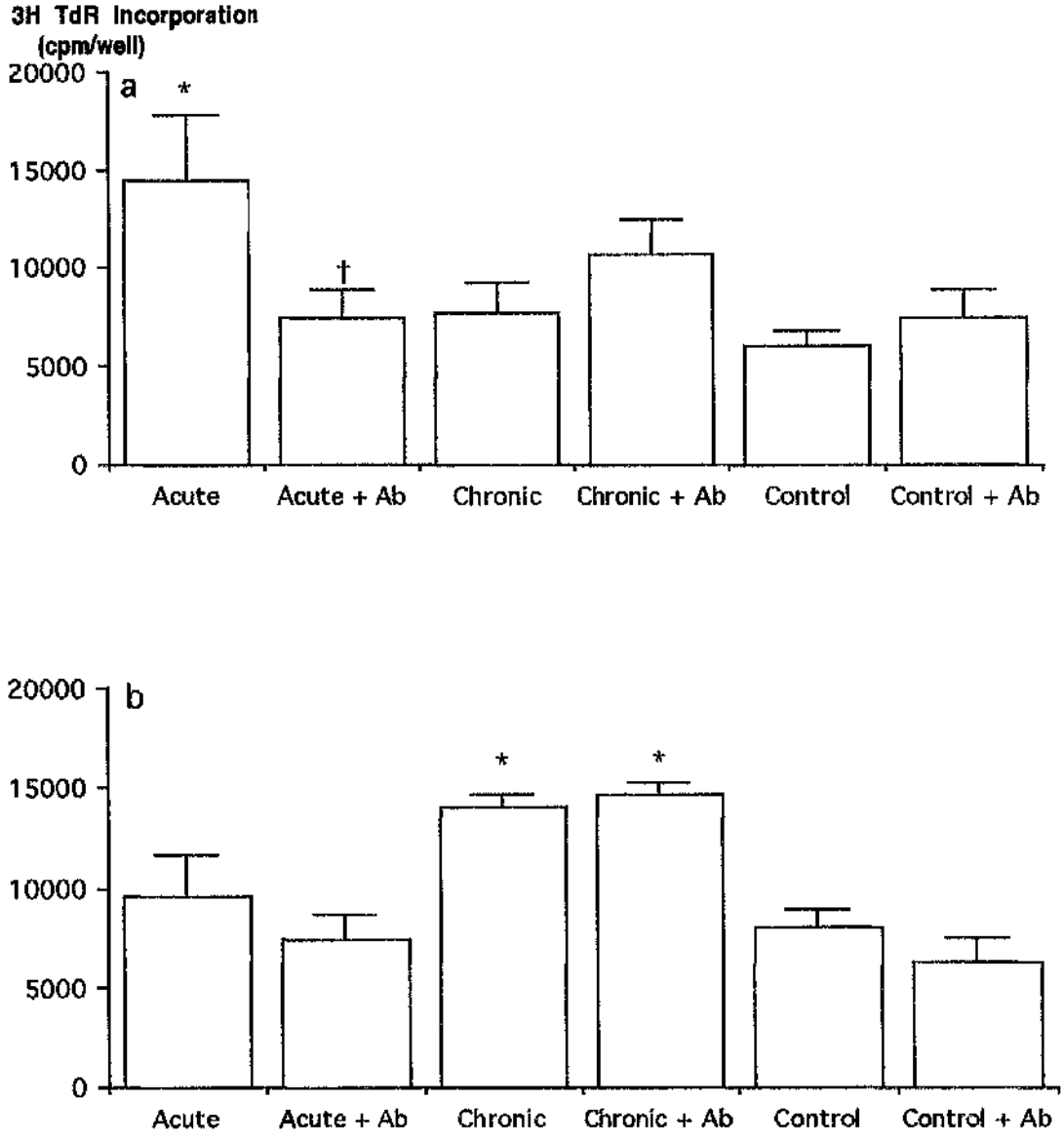


Fig. 4.4. Effects of anti-IL-12 treatment on Con A induced proliferation during acute and chronic GvHD.

The results show the effect of a single dose of anti-IL-12 antibody on the proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. in response to mitogenic stimulation with 10μg/ml Con A. The data are expressed as the mean uptake of 3H-TdR ± 1 SD after 48 hours in quadruplicate cultures, using spleen cells pooled from 3-4 mice per group on days 2 (Fig. 4.4a) and 10 (Fig. 4.4b) of the GvHD. (* $p < 0.01$ vs controls; † $p < 0.01$ vs unmodified acute GvHD). Ab = Anti-IL-12 treated

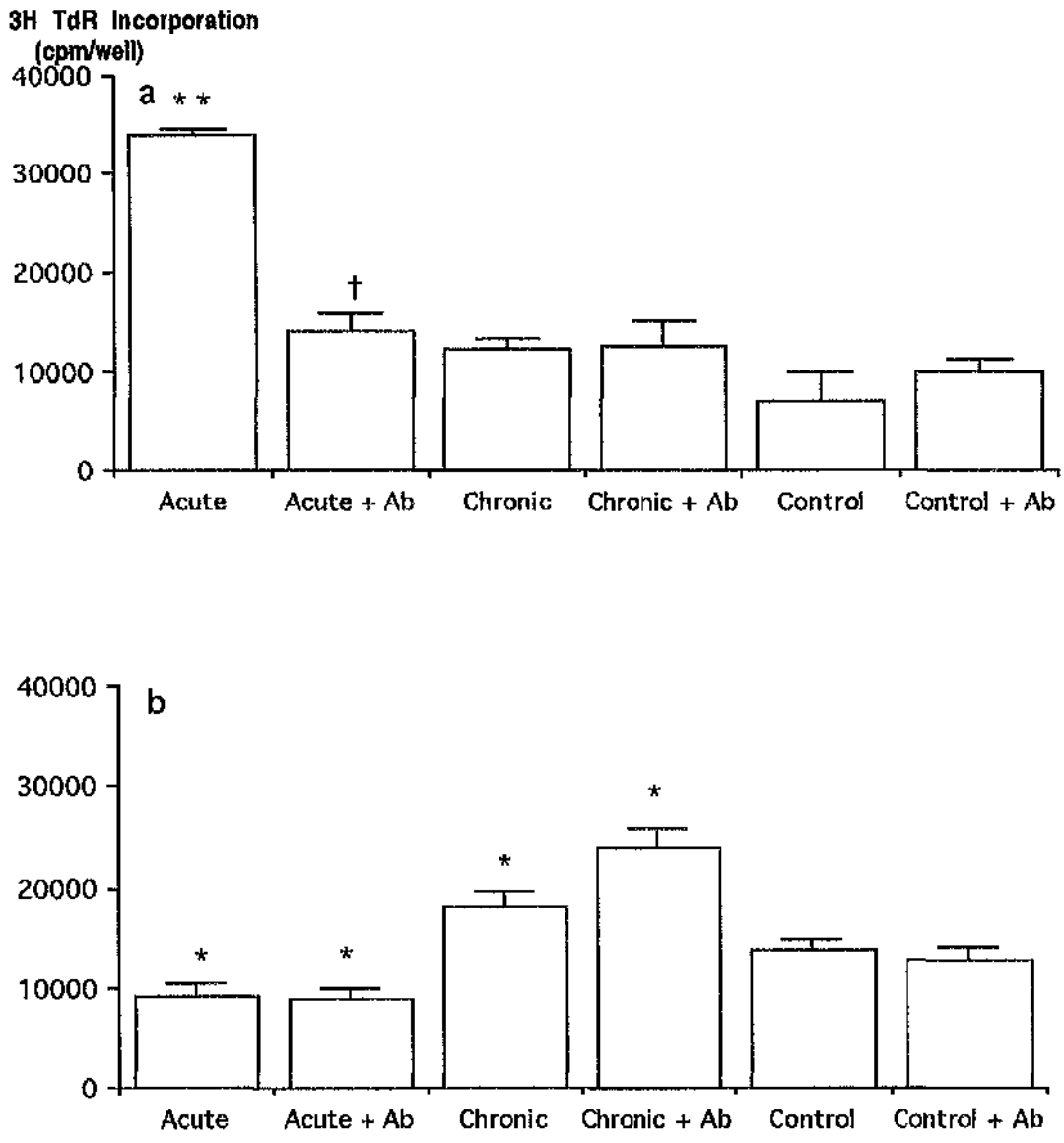


Fig. 4.5. Effects of anti-IL-12 treatment on LPS induced proliferation during acute and chronic GvHD.

The results show the effect of a single dose of anti-IL-12 antibody on the proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. in response to mitogenic stimulation with 10μg/ml LPS. Data are expressed as the mean uptake of 3H-TdR ± 1 SD after 24 hours in quadruplicate cultures, using spleen cells pooled from 3-4 mice per group on days 2 (Fig. 4.5a) and 10 (Fig. 4.5b) of the GvHD. (* $p < 0.05$ ** $p < 0.001$ vs controls; † $p < 0.01$ vs unmodified acute GvHD). Ab = Anti-IL-12 treated.

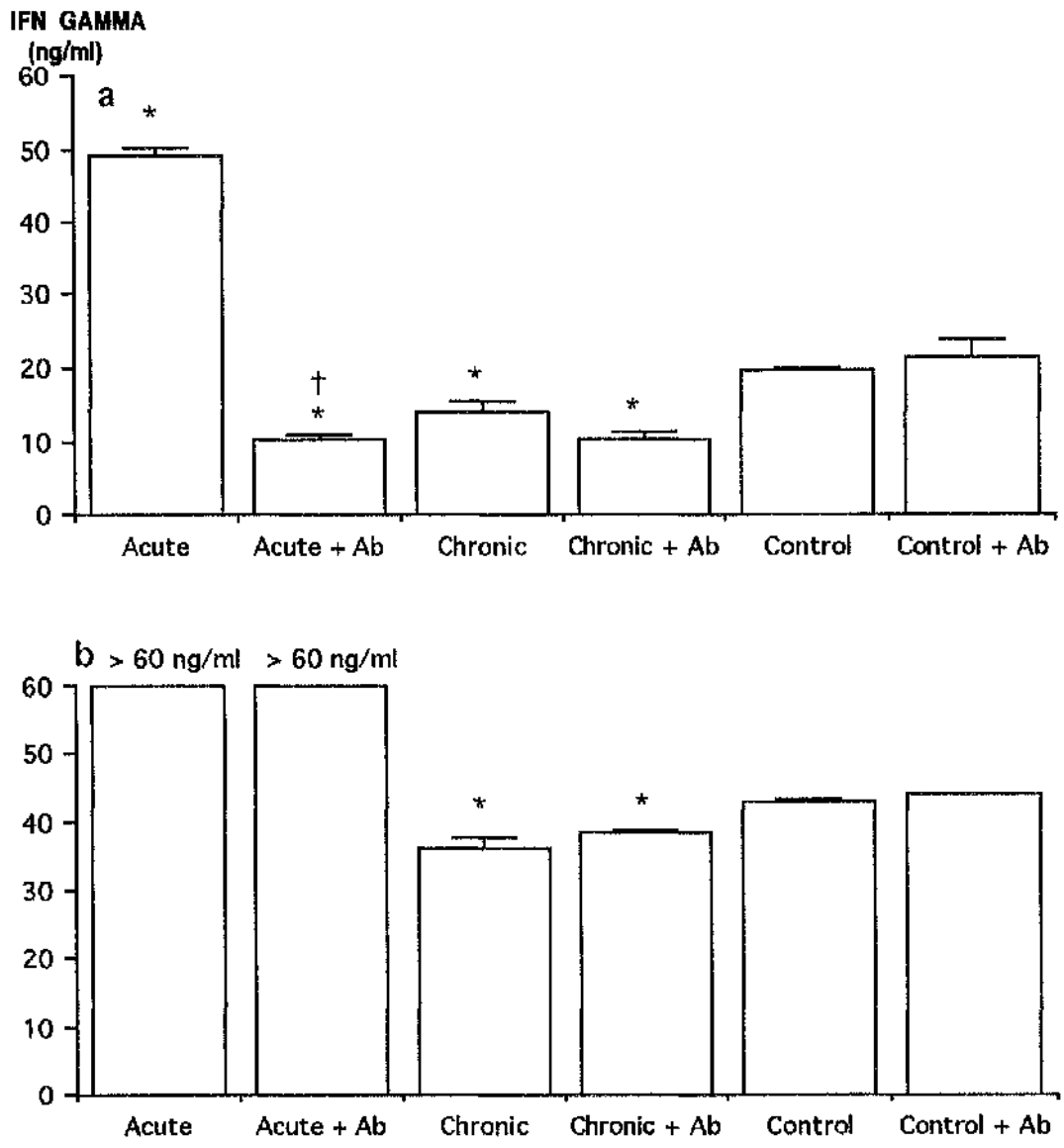


Fig. 4.6. Effects of anti-IL-12 treatment on IFN-γ production during acute and chronic GvHD.

The results show the effect of a single dose of anti-IL-12 on IFN-γ production by splenocytes from BDF_1 mice given B6 or DBA/2 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice were cultured with 10μg/ml Con A for 48 hours and the supernatants assayed for the presence of IFN-γ by ELISA. The results shown are the means ± SD of triplicate samples on days 2 (Fig. 4.6a) and 10 (Fig. 4.6b) of the GvHD. (* $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD).

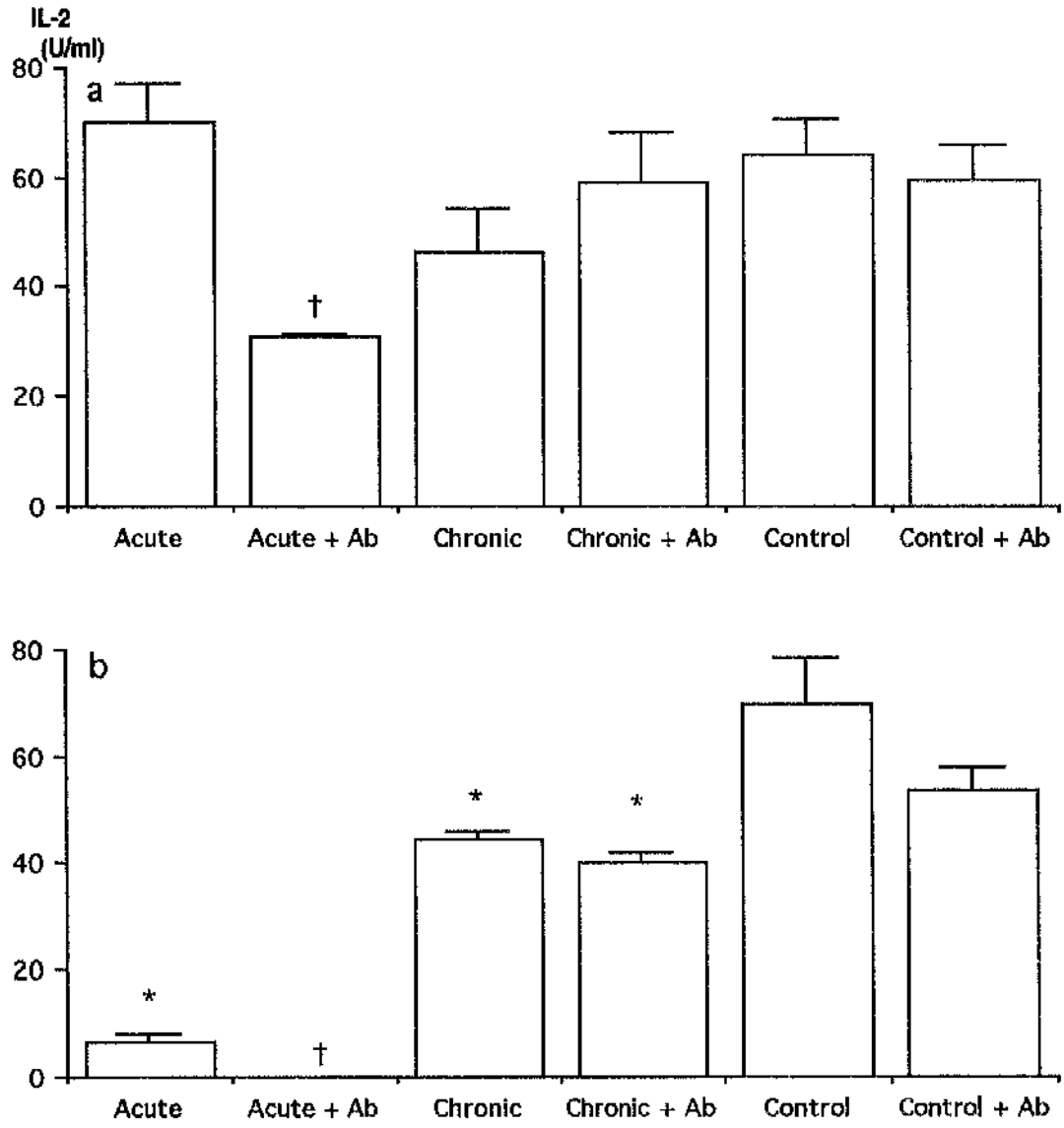


Fig. 4.7. Effects of anti-IL-12 treatment on IL-2 production during acute and chronic GvHD.

The results show the effect of a single dose of anti-IL-12 on IL-2 production by splenocytes from BDF_1 mice given B6 or DBA/2 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice were cultured with 10μg/ml Con A for 24 hours and the supernatants assayed for the presence of IL-2 by ELISA. The results shown are the means ± SD of triplicate samples on days 2 (Fig. 4.7a) and 10 (Fig. 4.7b) of the GvHD. (* $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD). Ab = Anti-IL-12 treated.

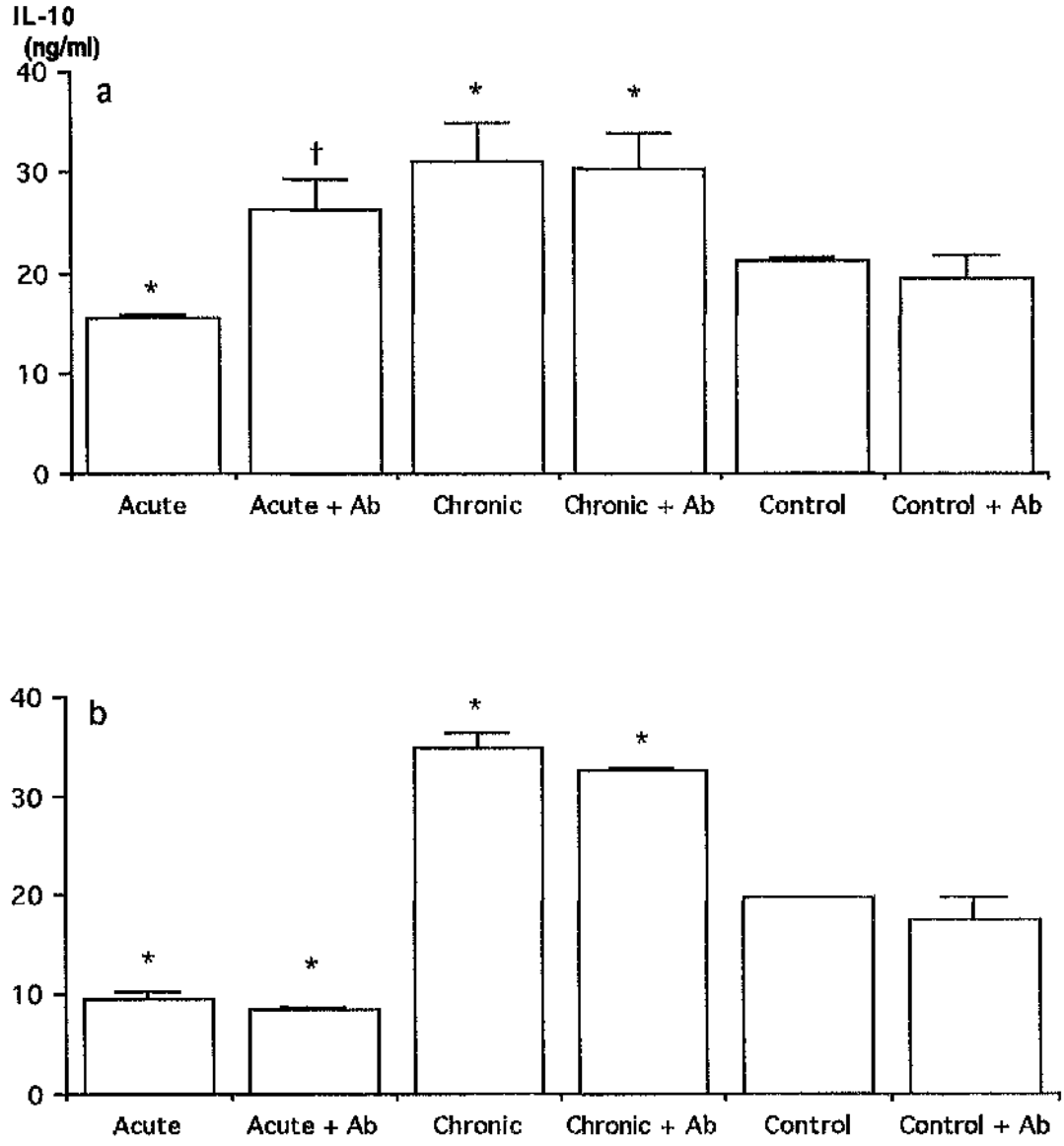


Fig. 4.8. Effects of anti-IL-12 treatment on IL-10 production during acute and chronic GvHD.

The results show the effect of a single dose of anti-IL-12 on IL-10 production by splenocytes from BDF_1 mice given B6 or DBA/2 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice were cultured with 10μg/ml Con A for 48 hours and the supernatants assayed for the presence of IL-10 by ELISA. The results shown are the means ± SD of triplicate samples on days 2 (Fig. 4.8a) and 10 (Fig. 4.8b) of the GvHD. (* $p < 0.01$ vs controls; † $p < 0.001$ vs unmodified acute GvHD). Ab = Anti-IL-12 treated.

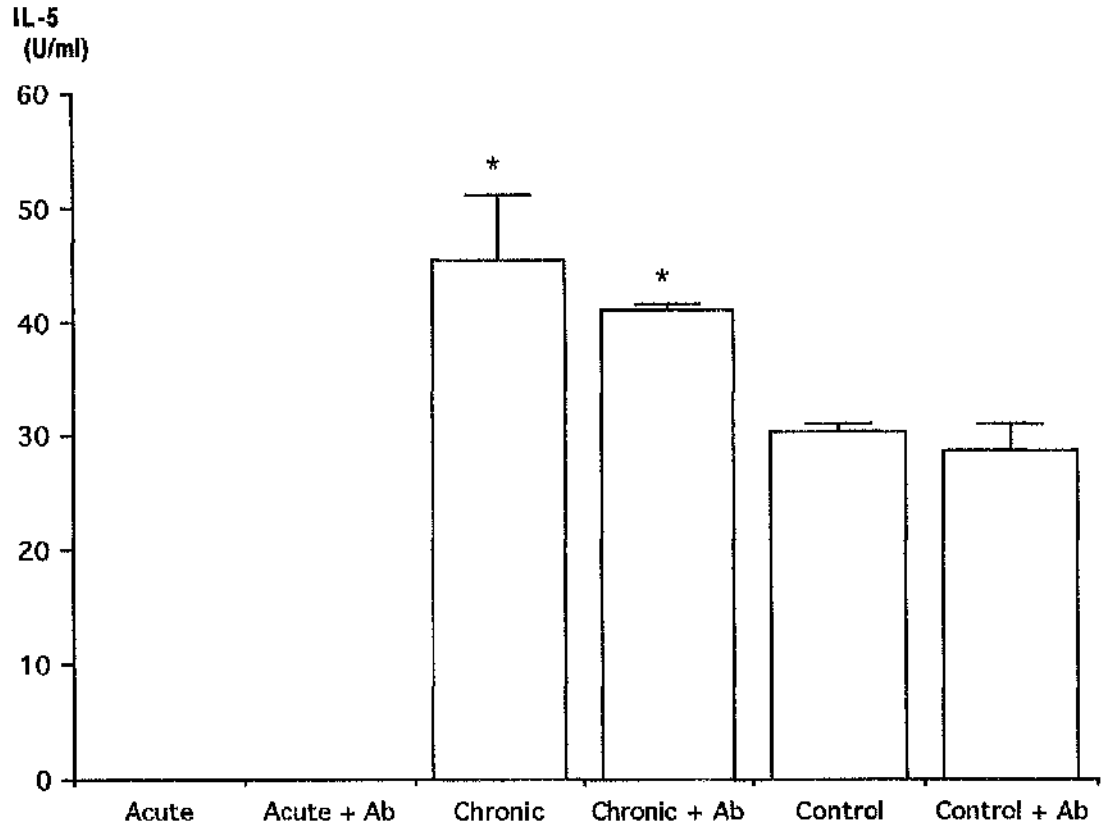


Fig. 4.9. Effects of anti-IL-12 treatment on IL-5 production during acute and chronic GvHD.

The results show the effect of a single dose of anti-IL-12 on IL-5 production by splenocytes from BDF_1 mice given B6 or DBA/2 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice were cultured with 10μg/ml Con A for 96 hours, and the supernatants assayed for the presence of IL-5 by ELISA. The results shown are the means ± SD of triplicate samples on day day 10 of the GvHD. (* $p < 0.05$ vs unmodified controls).

Ab = Anti-IL-12 treated.

CHAPTER 5

ROLE OF ENDOGENOUS IL-12 IN GvHD

Effects of Repeated Anti-IL-12 Administration on Acute GvHD

Introduction

In Chapter 4, I demonstrated that early production of IL-12 is important for the development of acute, but not chronic GvHD. My results also showed that while neutralising IL-12 during the afferent phase of acute GvHD abrogated many of the early features of the disease, it was unable to prevent its onset. In this chapter, I wanted to examine whether neutralising IL-12 in B6 $\Rightarrow$ BDF_1 mice for a longer period could inhibit development of acute GvHD. In addition to assessing the effects of repeated antibody treatment on the disease on days 2 and 10 as before, I also examined a later time point in the disease to determine whether repeated doses of anti-IL-12 antibody affected parameters associated with the more established disease, such as immunosuppression, weight loss and mortality.

Because the supplies of neutralising anti-IL-12 antibody were limiting and because anti-IL-12 treatment had little effect on the chronic form of GvHD, I restricted this more detailed study to the acute form of the GvHD. The long-term effects of neutralising endogenous IL-12 on the autoimmune consequences of chronic GvHD are detailed in Chapter 6.

Experimental protocol

The protocol used in this chapter was similar to that in Chapter 4, except that neutralising anti-IL-12 antibody was given on days -1, 2, 5 and 8. In addition, the effect of the antibody on acute GvHD was examined up to day 20 and CTL activity was measured in the spleens of GvHD mice on day 10 using P815 ($H\text{-}2^d$) target cells.

Results

i) Weight loss and mortality

As expected, BDF_1 mice injected with B6 parental cells developed an acute GvHD with progressive weight loss beginning on day 12 and continuing until the end of this study on day 20 (Fig. 5.1). Additionally, two of these mice died on day 17 and a further two on day 19. In contrast, B6 ⇒ BDF_1 mice given repeated doses of anti-IL-12 antibody during the first 8 days of acute GvHD showed no significant weight loss or mortality (Fig. 5.1).

ii) Splenomegaly

In parallel, unmanipulated B6 ⇒ BDF_1 mice with acute GvHD developed significant splenomegaly by day 2, which peaked on day 10 before returning towards control values (Fig. 5.2). Treatment with anti-IL-12 antibody significantly reduced the degree of splenomegaly on day 2, but, as in Chapter 4, on day 10 these mice had similar splenomegaly to that observed in unmodified mice with acute GvHD (Fig. 5.2). However, while the spleen weights of unmodified B6 ⇒ BDF_1 mice returned towards control levels by day 20, the splenomegaly in anti-IL-12 treated B6 ⇒ BDF_1 animals was prolonged and on day 20, the spleen weights of these mice were several fold greater than those of unmodified acute GvHD mice (Fig. 5.2).

iii) Immune function

a) Spontaneous 'ex-vivo' proliferation

Spleen cells from unmanipulated B6 ⇒ BDF_1 mice showed enhanced levels of spontaneous '*ex-vivo*' proliferation compared with control cells. This was apparent by day 2, peaked at day 10, but was markedly reduced by day 20 (Fig. 5.3). Treatment with anti-IL-12 antibody did not alter the enhanced

spontaneous proliferation seen on days 2 and 10, but on day 20, the proliferative capacity of spleen cells from anti-IL-12 treated B6 ⇒ BDF_1 mice remained significantly higher than both untreated acute GvHD and control levels (Fig. 5.3). In Chapter 4, I observed that cells from B6 ⇒ BDF_1 mice given a single injection of anti-IL-12 showed significantly reduced spontaneous proliferation on both days 2 and 10 compared with cells from unmodified B6 ⇒ BDF_1 mice. The reason why antibody treatment did not similarly affect proliferation in this study was unclear, although different batches of anti-IL-12 antibody were used in the two studies.

Anti-IL-12 did not significantly affect the spontaneous proliferative capacity of control cells at any time (Fig. 5.3).

b) Proliferative responses to Con A stimulation

In this experiment, I used Con A responsiveness as a measure of the immunosuppression which occurs in the established phase of the acute disease. As anticipated, on day 20, splenocytes from B6 ⇒ BDF_1 mice showed significantly reduced responses to Con A stimulation compared with cells from control mice (Fig. 5.4). This defect was prevented by treatment with anti-IL-12, as spleen cells from anti-IL-12 treated B6 ⇒ BDF_1 mice had Con A responses identical to those in anti-IL-12 treated controls (Fig. 5.4).

Anti-IL-12 caused a significant reduction in the responses of control cells to Con A stimulation (Fig. 5.4).

c) Proliferative responses to LPS stimulation

LPS induced proliferation was also assessed on day 20, in order to determine whether anti-IL-12 altered the B cell suppression observed late during acute GvHD. As expected, splenocytes from unmodified B6 ⇒ BDF_1 mice showed significantly reduced proliferative responses to LPS stimulation compared with cells from control mice (Fig. 5.5). The LPS responses of spleen cells from

anti-IL-12 treated B6 ⇒ BDF_1 mice were significantly higher than those of unmodified B6 ⇒ BDF_1 mice (Fig. 5.5). However, they were still significantly reduced compared with the responses of control cells (Fig. 5.5). Splenocytes from control mice given anti-IL-12 antibody exhibited proliferative responses to LPS similar to those of spleen cells from unmodified control mice (Fig. 5.5).

iv) Cytokine Production

I then went on to compare the cytokine profiles of cells from unmanipulated and anti-IL-12 treated B6 ⇒ BDF_1 mice.

IFN-γ

Spontaneous

On days 2 and 20, IFN-γ was not produced spontaneously by cells from any group (data not shown). On day 10, however, spleen cells from unmanipulated B6 ⇒ BDF_1 mice produced high levels of IFN-γ spontaneously (Fig. 5.6). Treatment with anti-IL-12 caused a significant reduction in this spontaneous IFN-γ production (Fig. 5.6).

Con A

As before, on both days 2 and 10, cells from unmodified B6 ⇒ BDF_1 mice produced significantly enhanced amounts of IFN-γ in response to Con A compared with control cells (Fig. 5.7a and b). By day 20, however, cells from these mice produced similar levels of IFN-γ to control cells (Fig. 5.7c). Repeated treatment with anti-IL-12 significantly reduced the levels of IFN-γ produced by cells from B6 ⇒ BDF_1 mice on both days 2 and 10 (Fig. 5.7a and b). This was in contrast to the effect of a single dose of anti-IL-12, which reduced IFN-γ levels on day 2, but not day 10 (see Chapter 4). Furthermore, on day 20, cells from anti-IL-

12 treated B6 ⇒ BDF_1 mice showed similar Con A stimulated IFN-γ production to cells from unmodified B6 ⇒ BDF_1 and control mice (Fig. 5.7c).

Anti-IL-12 had no effect on Con A stimulated IFN-γ production by control spleen cells at any time during this study (Figs. 5.7a-c).

IL-2

IL-2 was not produced spontaneously by cells from any group at any time during this study (data not shown).

On day 2, spleen cells from B6 ⇒ BDF_1 mice produced levels of IL-2 in response to Con A which were similar to those produced by control cells (Fig. 5.8a). However by day 10, IL-2 production by cells from GvHD mice was dramatically reduced, with levels severalfold lower than those produced by control cells (Fig. 5.8b) and on day 20, there was no IL-2 production at all (Fig. 5.8c). This paralleled the loss of Con A proliferative responses by these cells (Fig. 5.4). In contrast, cells from anti-IL-12 treated B6 ⇒ BDF_1 mice produced significantly lower levels of IL-2 than cells from unmodified B6 ⇒ BDF_1 mice on day 2 (Fig. 5.8a) and by day 10, similarly reduced IL-2 production by cells from unmodified and anti-IL-12 treated B6 ⇒ BDF_1 mice was apparent when compared with control cells (Fig. 5.8b). Thus, the effects of repeated anti-IL-12 administration were similar to those of a single injection of the antibody (see Chapter 4). However, on day 20, cells from anti-IL-12 treated B6 ⇒ BDF_1 mice retained the capacity to produce IL-2 in response to Con A stimulation, albeit at significantly lower levels than control cells (Fig. 5.8c).

Repeated administration of anti-IL-12 antibody to control mice resulted in significantly reduced IL-2 production at all time points examined (Figs. 5.8a-c), consistent with the lower Con A induced proliferative responses of cells from anti-IL-12 treated control mice compared with unmanipulated controls (Fig. 5.4). Reduced Con A induced IL-2 levels were a common feature of anti-IL-12 treated

GvHD and control mice, suggesting that IL-12 may play a regulatory role in IL-2 production.

IL-10

IL-10 was not produced spontaneously by cells from any group at any time during this study (data not shown). Similarly, on day 20, Con A stimulated IL-10 levels were also below the level of detection for all groups. Since IL-10 production in response to Con A was readily detectable on days 2 and 10 (see below), the reason for its absence on day 20 was not clear.

On both days 2 and 10, spleen cells from B6 ⇒ BDF_1 mice produced significantly lower levels of IL-10 in response to Con A stimulation compared with control cells (Figs. 5.9a & b). Repeated anti-IL-12 treatment resulted in a significant increase in the levels of Con A induced IL-10 production by B6 ⇒ BDF_1 spleen cells on both days 2 and 10 (Figs. 5.9a & b). This was in contrast to the effect of a single administration of anti-IL-12, which had no effect on IL-10 production by B6 ⇒ BDF_1 cells after day 2 (see Chapter 4).

Spleen cells from control mice given repeated injections of anti-IL-12 produced similar levels of IL-10 to cells from unmodified control mice at both time points examined (Figs. 5.9a & b).

IL-5

IL-5 production by cells from all groups was below the level of detection at all time points during this study (data not shown). Since IL-5 was detected in the previous studies detailed in Chapters 3 and 4, its absence in this study highlights the potential variability of cytokine production between experiments.

IL-4

IL-4 production by cells from all groups was below the level of detection at all time points during this study (data not shown).

v) Non-Specific and Specific Cell-Mediated Cytotoxicity

a) NK cell activity

As before, B6 ⇒ BDF_1 mice showed enhanced splenic NK cell activity on day 2 of the acute GvHD compared with control mice (Fig. 5.10). As I found in Chapter 4, this increased NK cell activity was reduced to below control levels by anti-IL-12 antibody treatment (Fig. 5.10).

b) CTL activity

Since IL-12 is also involved in facilitating specific CTL-mediated responses [118, 120, 121, 150], I next examined whether anti-IL-12 treatment also reduced the donor anti-host cytotoxicity which occurs later during acute GvHD.

On day 10 of acute GvHD, spleen cells from unmodified B6 ⇒ BDF_1 mice showed high levels of anti-host CTL activity (Fig. 5.11). Despite the dramatic effect of anti-IL-12 on early NK cell activation and its ability to ameliorate the associated weight loss and mortality, anti-IL-12 did not prevent the generation of anti-host CTL (Fig. 5.11).

Summary

These results indicate that neutralising endogenous IL-12 during the first 8 days of acute GvHD ameliorated the disease and prevented the immunosuppression, weight loss and mortality which occur in established acute GvHD. Thus repeated neutralisation of IL-12 extended the effects of a single injection of anti-IL-12 antibody and also converted the characteristic Th1-type cytokine profile associated with acute GvHD into a more Th2 dominated response, as evidenced by reduced IFN-γ production and increased levels of the Th2 cytokine, IL-10. In contrast, the single dose of anti-IL-12 only altered cytokine production on day 2. Despite its dramatic effect on weight loss and mortality,

however, anti-IL-12 treatment did not reduce the high levels of anti-host cytotoxicity in the spleens of B6 ⇒ BDF_1 mice.

Conclusions

Thus, many features of early acute GvHD such as IFN-γ production and NK cell activation are dependent on IL-12, as are the immunosuppression, weight loss and mortality which occur in the established disease. Initiation of acute GvHD is critically dependent on IL-12, supporting the view that this is a Th1-mediated disease. In the next chapter, I went on to examine the long-term consequences of neutralising IL-12.

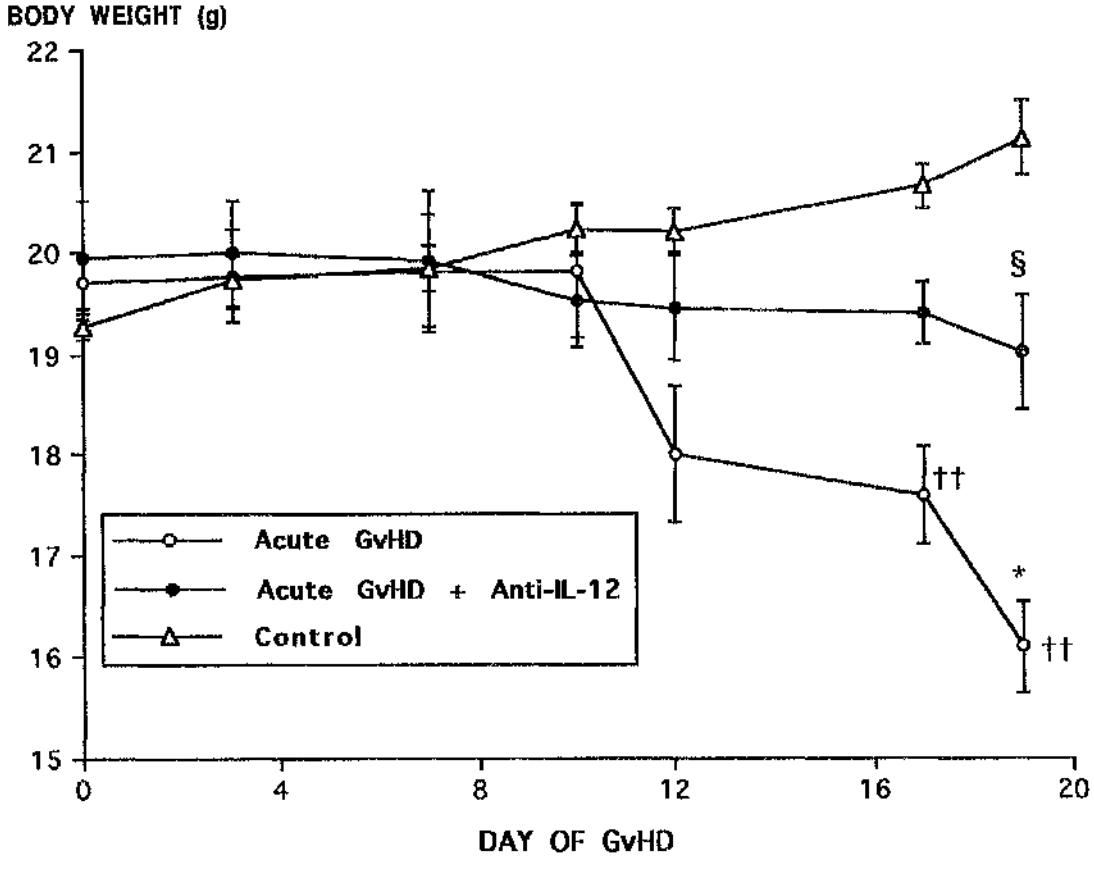


Fig. 5.1. Effects of anti-IL-12 treatment on weight loss and mortality during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on weight loss and mortality in BDF_1 mice given 10^8 B6 spleen cells i.v. The results shown are the mean body weights ± 1 SD for 6 mice per group. (* $p < 0.05$ vs controls; § $p < 0.05$ vs unmodified acute GvHD; † Death).

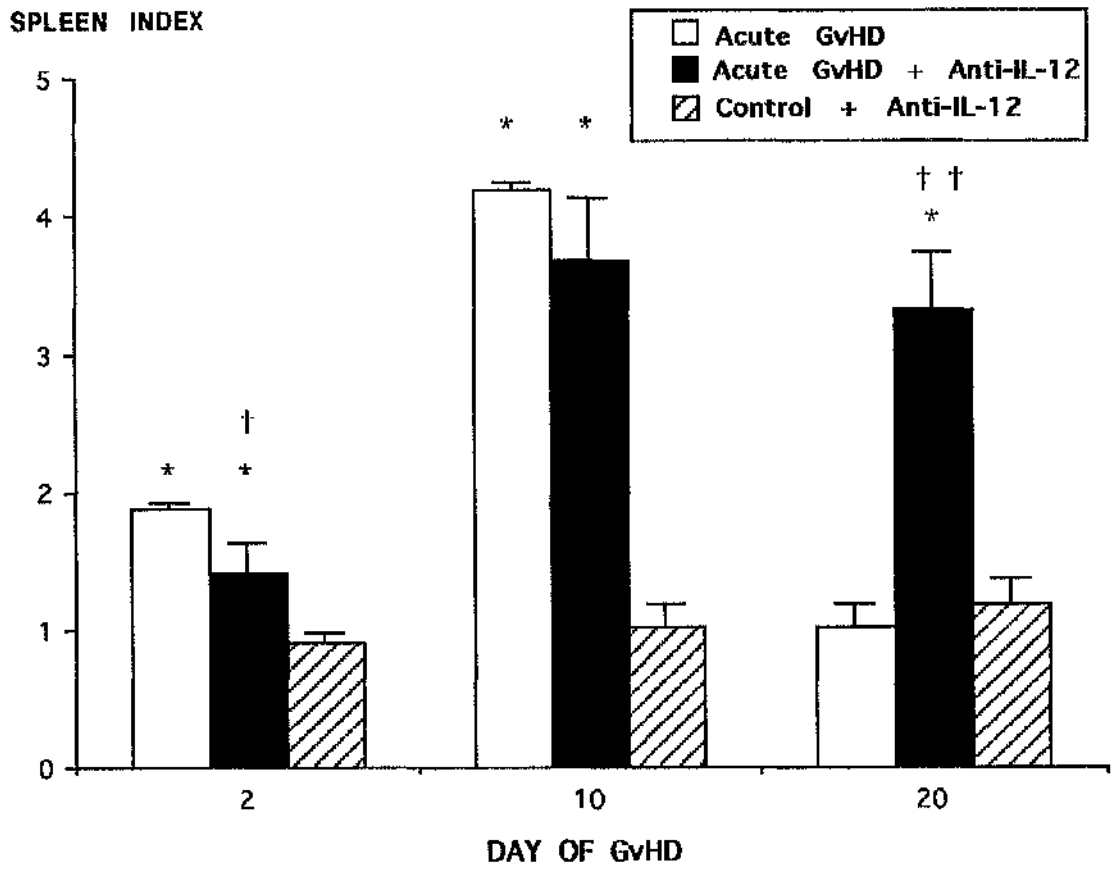


Fig. 5.2. Effects of anti-IL-12 treatment on splenomegaly during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on splenomegaly in BDF_1 mice given 10^8 B6 spleen cells i.v. The results shown are the mean spleen indices of 3 GvHD mice per group ± 1 SD relative to the mean spleen weights of 3 control mice. (* $p < 0.001$ vs controls; † $p < 0.05$ †† $p < 0.001$ vs unmodified acute GvHD).

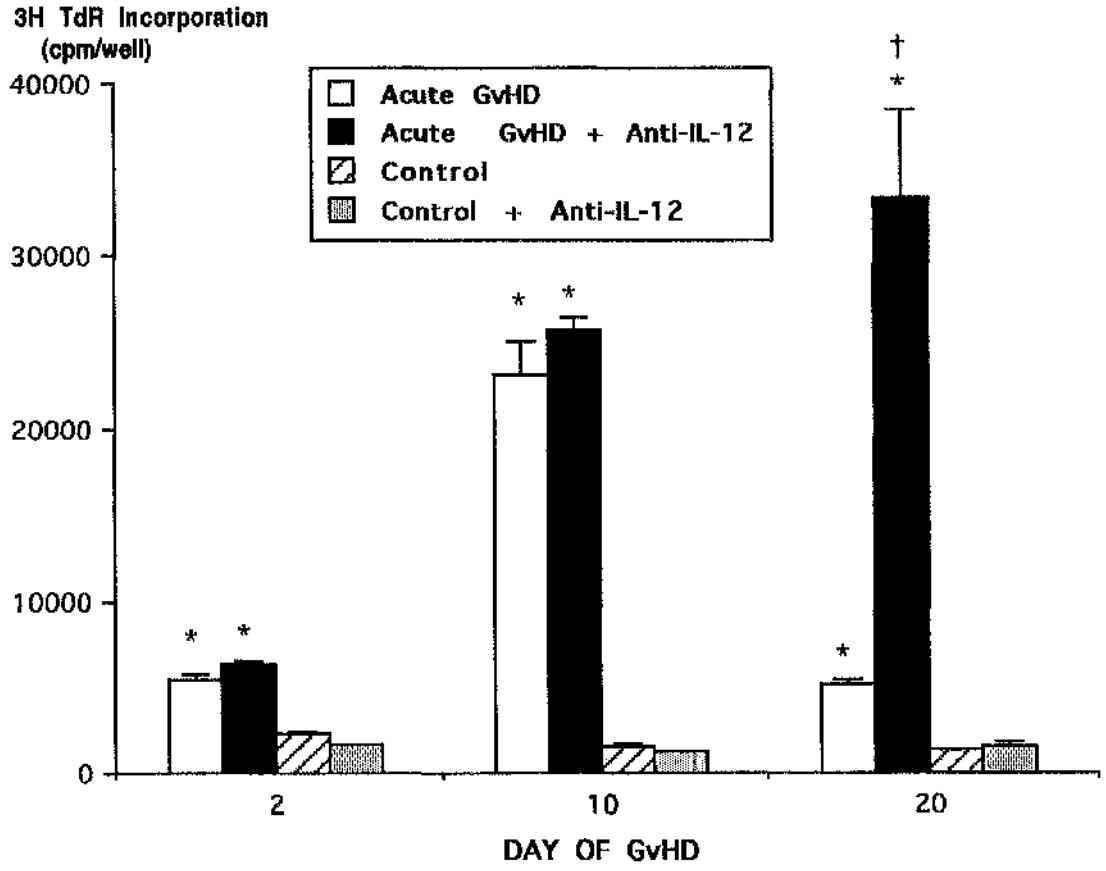


Fig. 5.3. Effects of anti-IL-12 treatment on spontaneous '*ex-vivo*' proliferation during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on the spontaneous proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. The results are expressed as the mean uptake of 3H-TdR ± 1 SD after 4 hours in quadruplicate cultures using spleen cells pooled from 3 mice per group. (* $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD).

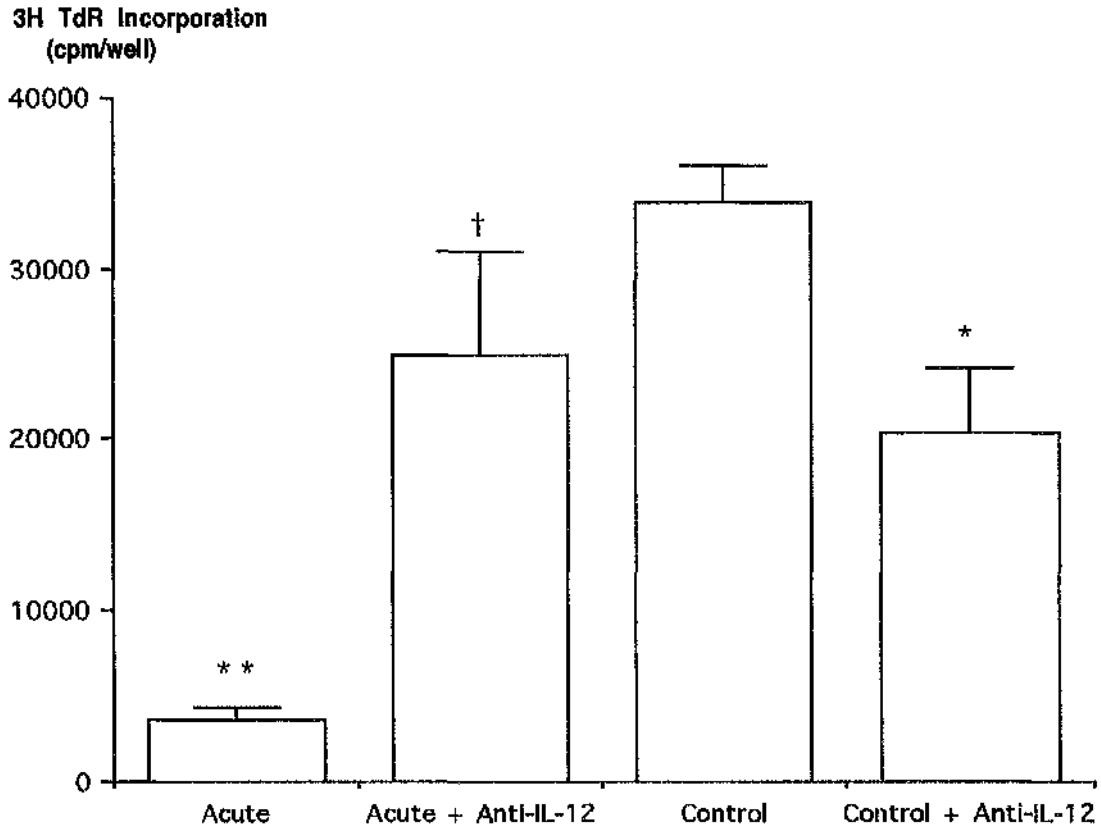


Fig. 5.4. Effects of anti-IL-12 treatment on Con A induced proliferation during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on the proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. in response to mitogenic stimulation with 10μg/ml Con A. The data are expressed as the mean uptake of 3H-TdR ± 1 SD after 48 hours in quadruplicate cultures, using spleen cells pooled from 3-4 mice per group on day 20 of the disease. (* $p < 0.005$ ** $p < 0.001$ vs controls; † $p < 0.005$ vs unmodified acute GvHD).

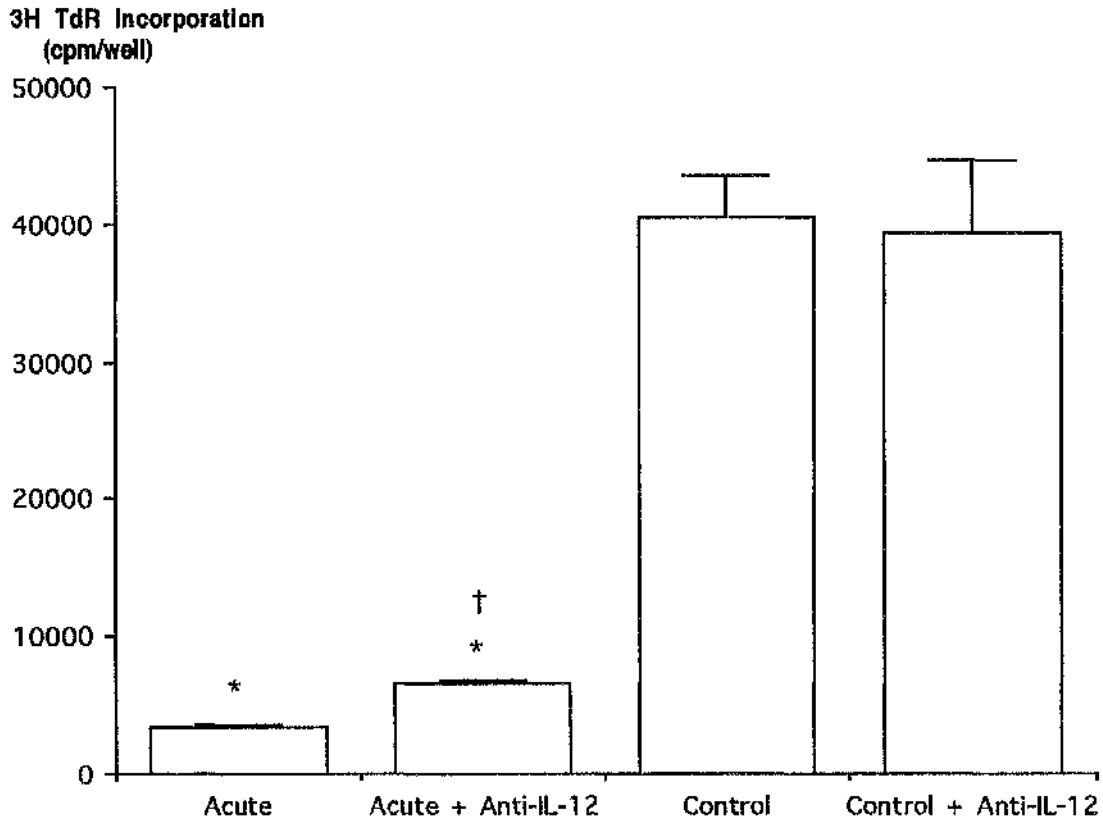


Fig. 5.5. Effects of anti-IL-12 treatment on LPS induced proliferation during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on the proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. in response to mitogenic stimulation with 10μg/ml LPS. The data are expressed as the mean uptake of 3H-TdR ± 1 SD after 24 hours in quadruplicate cultures, using spleen cells pooled from 3-4 mice per group on day 20 of the disease. (* $p < 0.001$ vs controls; † $p < 0.05$ vs unmodified acute GvHD).

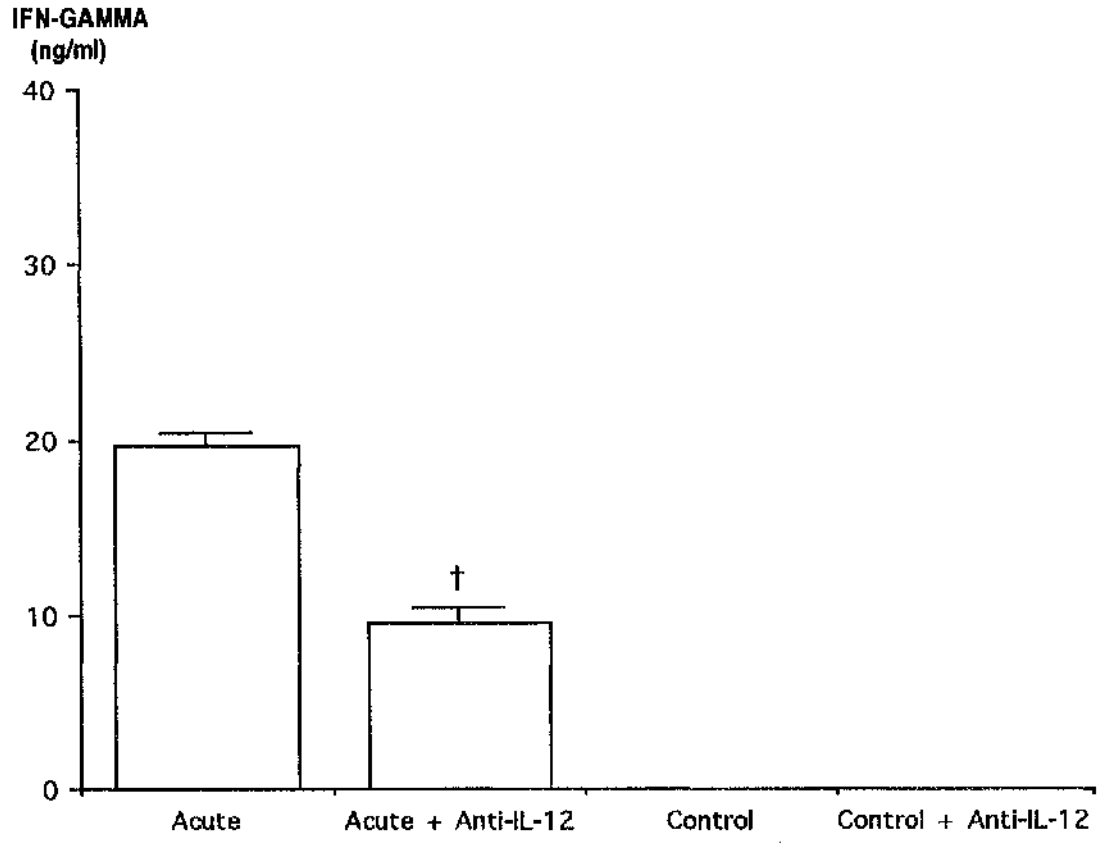


Fig. 5.6. Effects of anti-IL-12 treatment on spontaneous IFN-γ production during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on spontaneous IFN-γ production by splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 mice were cultured in medium for 48 hours and the supernatants assayed for the presence of IFN-γ by ELISA. The results shown are the means ± SD of triplicate samples on day 10 of the GvHD. († $p < 0.001$ vs unmodified acute GvHD).

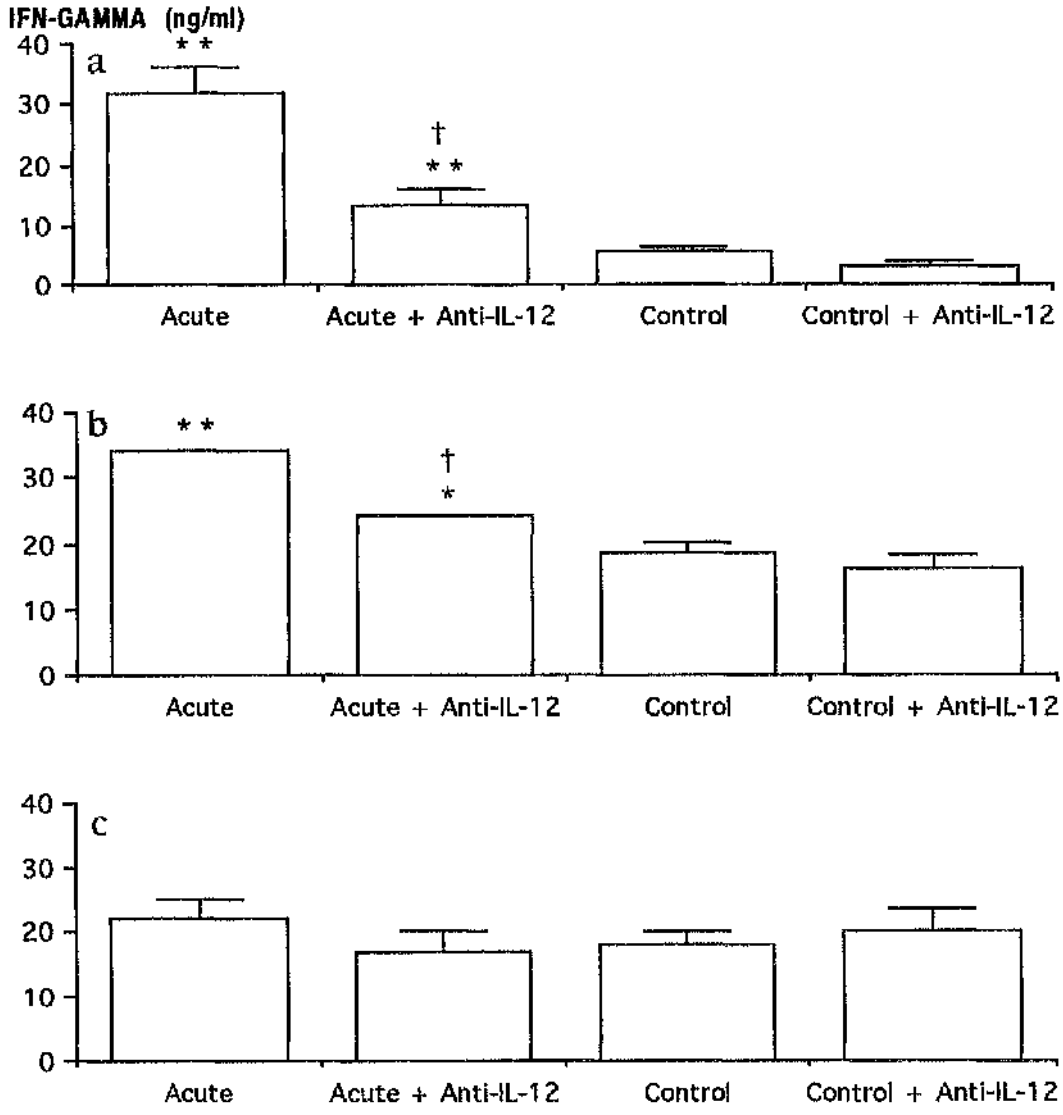


Fig. 5.7. Effects of anti-IL-12 treatment on Con A induced IFN-γ production during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on Con A induced IFN-γ production by cells from BDF_1 mice given 10^8 B6 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 mice were cultured with 10μg/ml Con A for 48 hours and the supernatants assayed for the presence of IFN-γ by ELISA. The results shown are the means ± SD of triplicate samples on days 2 (Fig. 5.7a), 10 (Fig. 5.7b) or 20 (Fig. 5.7c) of the GvHD. (* $p < 0.05$ ** $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD).

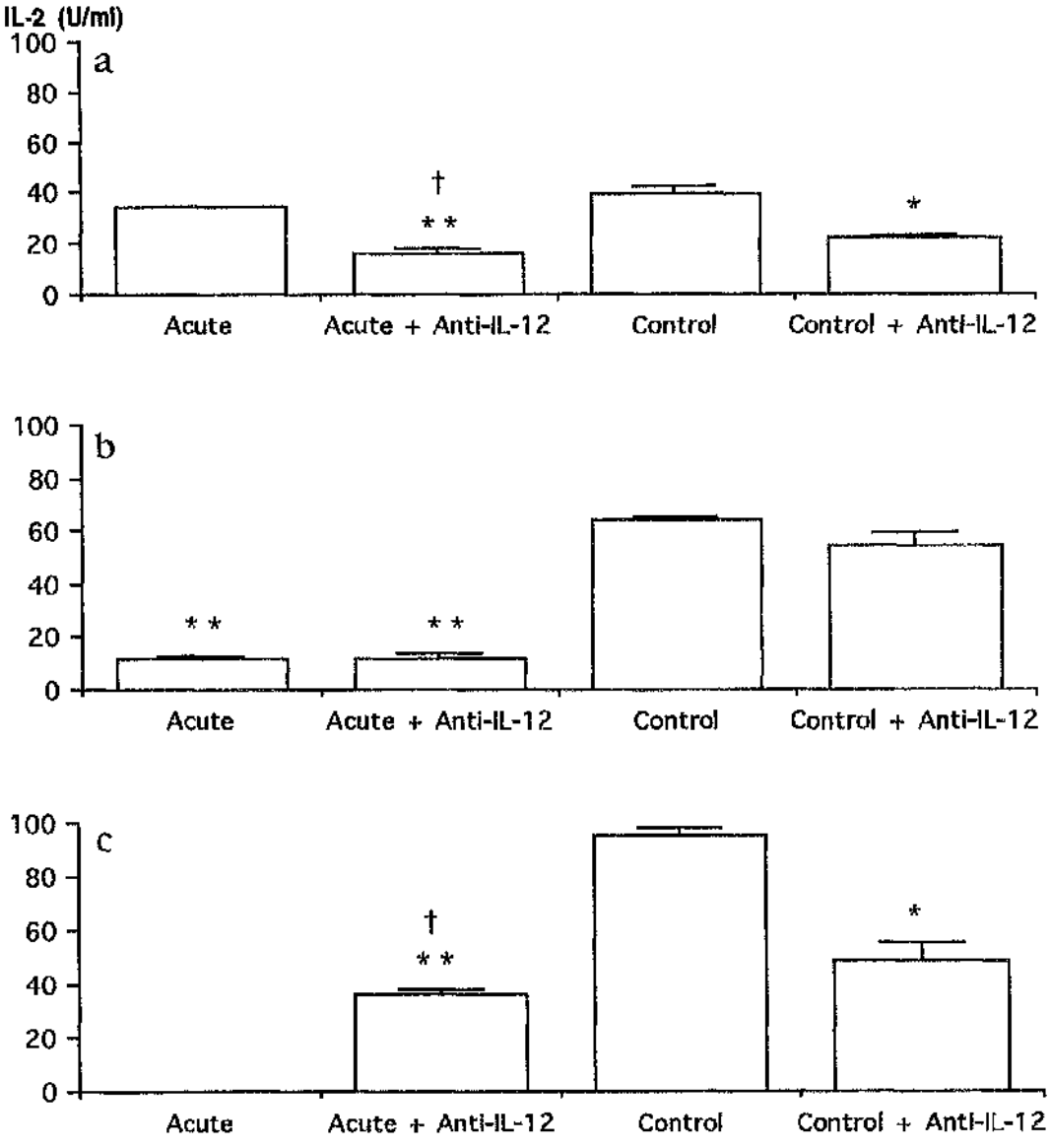


Fig. 5.8. Effects of anti-IL-12 treatment on IL-2 production during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on Con A induced IL-2 production by cells from BDF_1 mice given 10^8 B6 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 mice were cultured with 10μg/ml Con A for 24 hours and the supernatants assayed for the presence of IL-2 by ELISA. The results shown are the means ± SD of triplicate samples on days 2 (Fig. 5.7a), 10 (Fig. 5.7b) and 20 (Fig. 5.7c) of the GvHD. (* $p < 0.005$ ** $p < 0.001$ vs unmodified controls; † $p < 0.001$ vs unmodified acute GvHD).

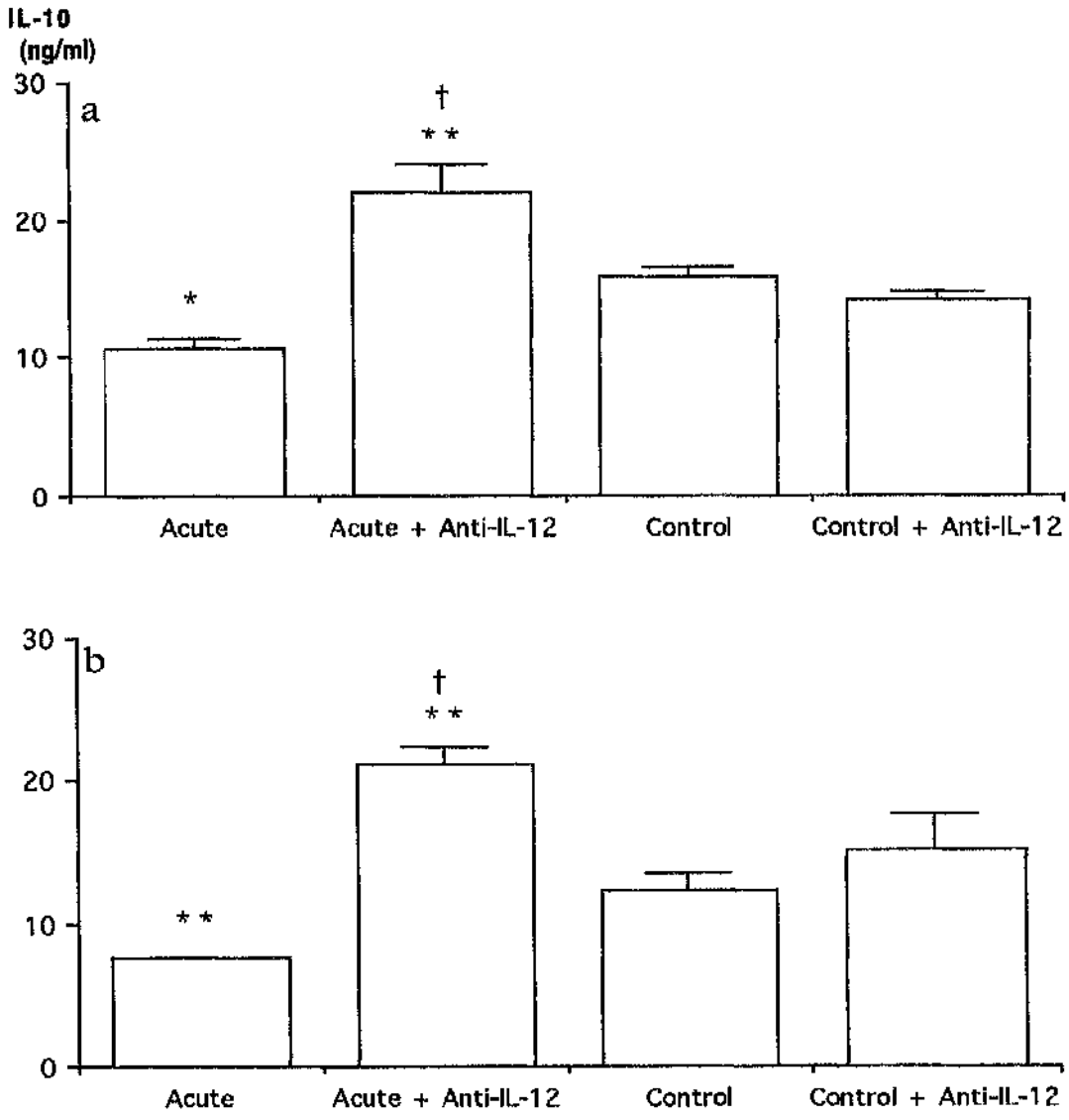


Fig. 5.9. Effects of anti-IL-12 treatment on IL-10 production during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on Con A induced IL-10 production by splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 were cultured with 10μg/ml Con A for 48 hours and the supernatants assayed for the presence of IL-10 by ELISA. The results shown are the means ± SD of triplicate samples on days 2 (Fig. 5.9a) and 10 (Fig. 5.9b) of the GvHD. (* $p < 0.01$ ** 0.001 vs controls; † $p < 0.001$ vs unmodified acute GvHD).

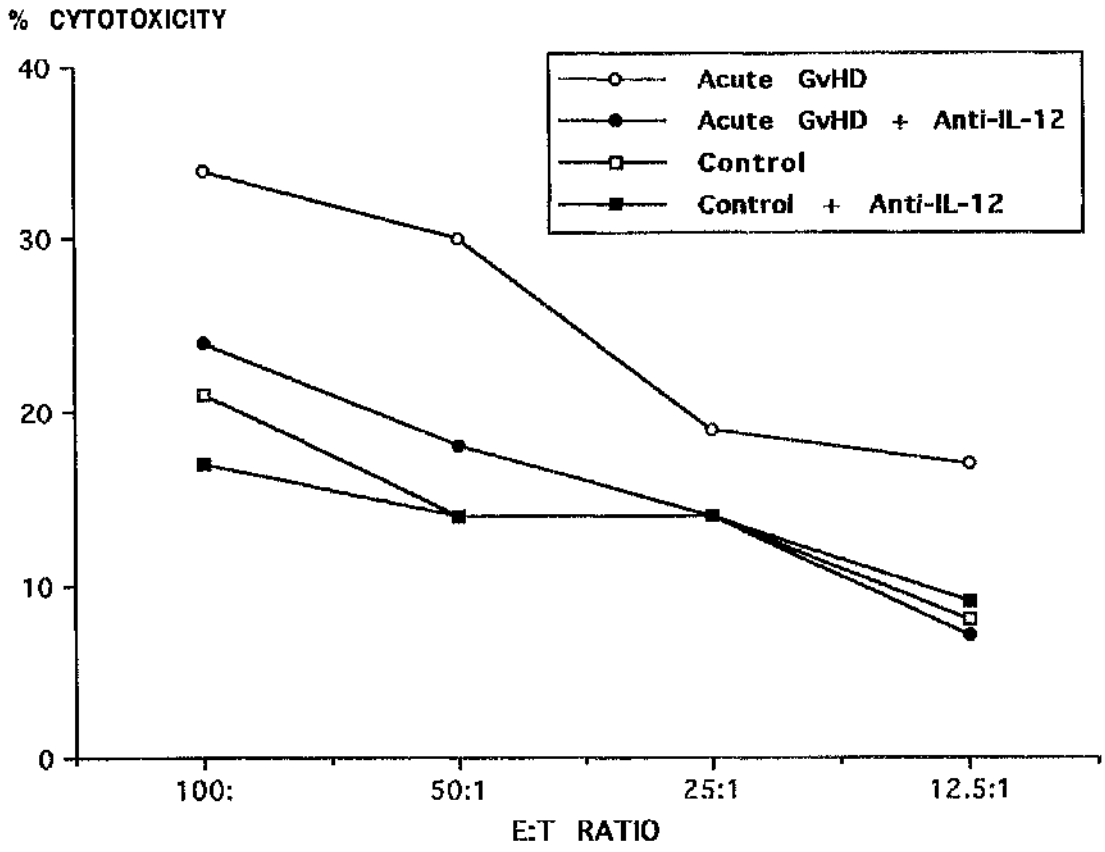


Fig. 5.10. Effects of anti-IL-12 treatment on NK cell activity during acute GvHD.

The results show the effect of a neutralising endogenous IL-12 during the first 8 days of acute GvHD on splenic NK cell activity in BDF_1 mice given 10^8 B6 i.v. The results shown are the % cytotoxicity against YAC-1 target cells from quadruplicate assays measured at effector : target (E:T) ratios from 100:1 to 12.5:1, using spleen cells pooled from 3 mice per group on day 2 of the GvHD.

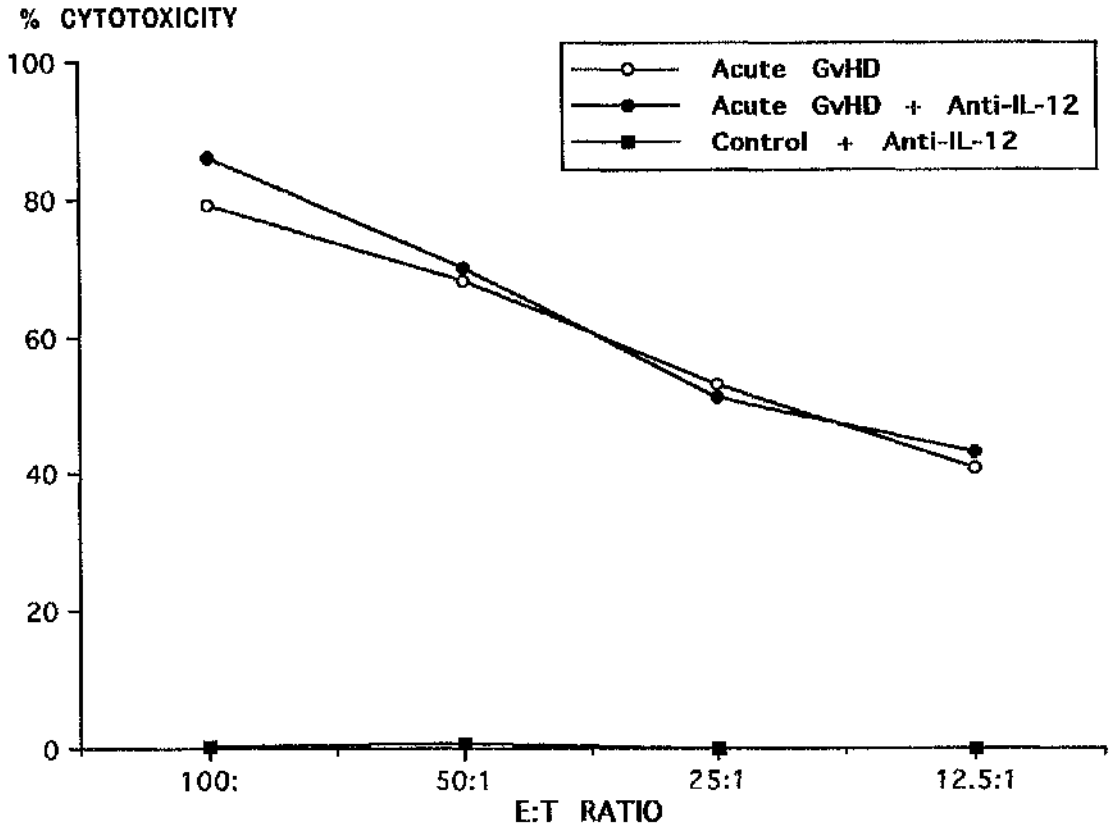


Fig. 5.11. Effects of IL-12 depletion on specific CTL activity during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on splenic CTL activity in BDF_1 mice given 10^8 B6 i.v. The results shown are the % cytotoxicity against P815 target cells from quadruplicate assays measured at effector : target (E:T) ratios from 100:1 to 12.5:1, using spleen cells pooled from 3 mice per group on day 10 of the GvHD.

CHAPTER 6

ROLE OF ENDOGENOUS IL-12 IN GvHD

Long-Term Effects of IL-12 Neutralisation on Acute and Chronic GvHD

Introduction:

In Chapters 4 & 5, I showed the importance of the cytokine IL-12 in mediating acute GvHD in BDF_1 mice, by demonstrating that *in vivo* administration of neutralising anti-IL-12 antibody inhibited the Th1 cytokine production, immunosuppression, weight loss and mortality which occur during the first 3 weeks of the disease. A number of important issues were raised by these earlier studies. Firstly, they did not address the critical question of whether neutralising IL-12 early in GvHD conferred long-term protection from acute disease, or simply delayed its onset. Secondly, I did not establish whether IL-12 depletion impaired the development of donor haemopoietic chimerism. If so, host cells might remain to allow continued stimulation of donor T cells. Finally, if neutralising IL-12 promotes the development of a Th2 cytokine response to alloantigen, this may eventually result in the development of a chronic GvHD, comparable to that observed in DBA/2 $\Rightarrow$ BDF_1 animals. In this chapter, I have addressed these issues in an extended time course of acute GvHD. To determine whether anti-IL-12 treated B6 $\Rightarrow$ BDF_1 mice go on to develop chronic GvHD, I examined whether long-term survivors exhibit any of the features of the chronic disease, such as high levels of Th2 cytokine production, B cell hyperplasia, enhanced serum immunoglobulin and anti-ds DNA antibody levels, and antibody-mediated kidney pathology. In addition, I investigated whether DBA/2 $\Rightarrow$ BDF_1 mice given anti-IL-12 antibody developed raised serum immunoglobulin and anti-

ds DNA antibody levels earlier than unmodified DBA/2 ⇒ BDF_1 mice and if the antibody treatment increased long-term mortality in chronic GvHD.

Experimental Protocol

The GvHR was induced by i.v. injection of 10^8 viable B6 or DBA/2 parental spleen cells into BDF_1 recipients, as usual. IL-12 was neutralised *in vivo* as described in Chapter 5.

The numbers of donor and host derived $CD4^+$ and $CD8^+$ T cells in the spleen were assessed by flow cytometry using mAbs directed against polymorphic determinants expressed by either H-$2D^d$ or H-$2D^b$. Lymphocytes expressing high levels of both H-$2D^d$ and H-2 D^b were identified as host lymphocytes, while cells which lacked expression of H-$2D^d$ Class I MHC were designated B6 donor-derived cells.

Immune function was determined on day 70 by assessing the ability of GvHD splenocytes to proliferate in response to Con A or LPS stimulation *in vitro* . Cytokine production was also measured at this time. Levels of total serum IgG were measured throughout the GvHD using radial immunodiffusion, while serum IgE and anti-ds-DNA antibody levels were both determined by ELISA.

Kidneys were harvested from GvHD mice on days 50, 70 and 130 and examined blind for evidence of immune complex deposition and glomerular damage by both light and electron microscopy.

1. Long-Term Effects of Anti-IL-12 Treatment on Acute GvHD

Results

i) Survival

Unmodified B6 ⇒ BDF_1 mice with acute GvHD showed the expected early mortality, with the first death occurring on day 16 and 78% mortality in this

group by day 23 (Fig. 6.1). Only two out of the 8 mice in the anti-IL-12 treated B6 ⇒ BDF group died (one on day 19 and one on day 28 - 25% mortality). No further deaths were observed in either group after day 28 until the study was terminated on day 130. Thus, early treatment with anti-IL-12 confers long-term protection from mortality.

ii) Effect of anti-IL-12 treatment on donor cell engraftment

As expected, unmodified B6 ⇒ BDF_1 mice showed progressive repopulation by donor-derived $CD4^+$ T cells (Fig. 6.2 and Table 6.1). This was already marked by day 9 (Fig. 6.2), had increased significantly by day 17 and was virtually complete in the small number of mice which survived until day 70 (Table 6.1). Engraftment of donor $CD8^+$ T cells occurred more slowly than that of donor $CD4^+$ cells and on day 9, only 4.7% of the $CD8^+$ T cells in the spleen of B6 ⇒ BDF_1 mice were of donor origin (Fig. 6.2). The percentage of donor-derived $CD8^+$ T cells in these mice increased dramatically between days 9-17 (Table 6.1), although no further increase was observed thereafter and in mice surviving on day 70, repopulation of the $CD8^+$ T cell compartment was still incomplete (Table 6.1). Insufficient mice were available thereafter to establish if complete repopulation by donor $CD8^+$ T cells occurred.

Anti-IL-12 treated B6 ⇒ BDF_1 mice showed equivalent engraftment of donor $CD4^+$ T cells to that seen in unmodified B6 ⇒ BDF_1 mice on days 9 (Fig. 6.2) and 17 (Table 6.1). However, on day 70, the percentage of $CD4^+$ lymphocytes in anti-IL-12 treated B6 ⇒ BDF_1 mice was markedly lower than that observed in unmodified B6 ⇒ BDF_1 mice (Table 6.1), suggesting that repopulation of the $CD4^+$ compartment occurred more slowly in anti-IL-12 treated mice. However, complete $CD4^+$ T cell chimerism was observed in anti-IL-12 treated mice by day 130 (Table 6.1). Engraftment of donor $CD8^+$ T cells occurred at a similar rate in untreated and anti-IL-12 treated B6 ⇒ BDF_1 mice (Fig. 6.2 and Table 6.1), but the larger numbers of surviving mice allowed me to establish that

by day 130, complete repopulation by donor derived $CD8^+$ cells had occurred in anti-IL-12 treated mice (Table 6.1).

Thus, the long-term survival observed in anti-IL-12 treated B6 ⇒ BDF_1 mice is accompanied by complete repopulation of their $CD4^+$ and $CD8^+$ T cell compartments by engrafted donor B6 cells.

iii) Immune function

In Chapter 5, I demonstrated that anti-IL-12 prevented the T cell immunosuppression observed on day 20 of acute GvHD, although it had a less dramatic effect on the B cell suppression observed at this time. To establish whether anti-IL-12 treated mice retain immunocompetence in the long-term, I examined the ability of their spleen cells to proliferate in response to Con A or LPS stimulation on day 70. I also assessed whether these mice developed the B cell hyperreactivity associated with chronic GvHD by comparing the responses of anti-IL-12 treated B6 ⇒ BDF_1 mice to those of DBA/2 ⇒ BDF_1 mice with chronic GvHD.

The high mortality rate of unmanipulated B6 ⇒ BDF_1 mice precluded analysis of their functional responses at this point.

a) Proliferative responses to Con A stimulation

On day 70, spleen cells from anti-IL-12 treated B6 ⇒ BDF_1 mice showed Con A responses identical to those of spleen cells from both control and DBA/2 ⇒ BDF_1 mice (Fig. 6.3). Anti-IL-12 treatment did not affect the Con A responses of control mice in the long-term (Fig. 6.3).

b) Proliferative responses to LPS stimulation

On day 70, the LPS induced responses of splenocytes from both anti-IL-12 treated B6 ⇒ BDF_1 mice and unmodified DBA/2 ⇒ BDF_1 mice were significantly enhanced compared to those of control cells (Fig. 6.4). Similarily

enhanced responses to LPS were found in control mice treated with anti-IL-12 (Fig. 6.4).

Administration of anti-IL-12 therefore appears to provide long-term preservation of T cell responses in B6 ⇒ BDF_1 mice, but induces a state of hyper-responsiveness to the B cell mitogen, LPS, in both acute GvHD and control animals. These features are similar to those observed in DBA/2 ⇒ BDF_1 mice with chronic GvHD.

iv) Cytokine production

In Chapter 5, I showed that IL-12 depletion reduced the high levels of IFN-γ produced during the early period of acute GvHD and this was associated with increased IL-10 production. I now examined whether antibody treatment polarised the cytokine response of long-term survivors of the acute GvHD towards a permanent Th2 phenotype, similar to that observed in DBA/2 ⇒ BDF_1 mice with the chronic disease. Again, the high mortality rate of unmanipulated B6 ⇒ BDF_1 mice precluded analysis of their cytokine phenotype at this point.

IFN-γ

IFN-γ was not produced spontaneously by cells from any group on day 70 (data not shown).

On day 70, spleen cells from anti-IL-12 treated B6 ⇒ BDF_1 survivors produced significantly lower levels of IFN-γ in response to Con A than cells from control animals (Fig. 6.5). The levels of IFN-γ produced by Con A stimulated cells from DBA/2 ⇒ BDF_1 mice were below the level of detection for this assay (Fig. 6.5). Anti-IL-12 did not affect the ability of control spleen cells to produced IFN-γ in response to Con A.

IL-2

IL-2 was not produced spontaneously by cells from any group on day 70 (data not shown).

Similar levels of IL-2 were produced by Con A stimulated cells from anti-IL-12 treated B6 ⇒ BDF_1, DBA/2 ⇒ BDF_1 and both unmanipulated and anti-IL-12 treated control mice (Fig. 6.6).

IL-10

IL-10 was not produced spontaneously by cells from any group on day 70 (data not shown).

Cells from anti-IL-12 treated B6 ⇒ BDF_1 mice produced significantly enhanced levels of IL-10 in response to Con A, in comparison with both controls and DBA/2 ⇒ BDF_1 mice (Fig. 6.7). Control mice given anti-IL-12 also showed significantly enhanced IL-10 production compared to unmanipulated controls (Fig. 6.7).

IL-5

IL-5 was not produced spontaneously by cells from any group on day 70 (data not shown).

Cells from anti-IL-12 treated B6 ⇒ BDF_1 mice produced significantly enhanced amounts of IL-5 in response to Con A compared with both controls (which did not produce any detectable IL-5) and with cells from DBA/2 ⇒ BDF_1 mice (Fig. 6.8). Control mice given anti-IL-12 also showed significantly enhanced Con A stimulated IL-5 production compared with unmanipulated controls (Fig. 6.8).

IL-4

IL-4 production by cells from all groups was below the level of detection at all time points during this study (data not shown).

v) Serum Immunoglobulin Levels

As IL-12 depleted B6 ⇒ BDF_1 mice exhibited a polarised Th2 cytokine profile and heightened LPS proliferative responses, I next assessed whether these mice also had the B cell hyperreactivity characteristic of chronic GvHD. The high mortality rate in B6 ⇒ BDF_1 mice prevented analysis of their immunoglobulin levels after day 30. Mice with chronic GvHD also started to die from ICGN from day 50 onwards and insufficient numbers of these mice remained to analyse on day 130.

a) IgG

DBA/2 ⇒ BDF_1 mice with chronic GvHD developed hypergammaglobulinaemia, with strikingly higher than normal levels of total serum IgG on days 20 and 30 of the disease (Fig. 6.9). Serum IgG levels in these mice declined thereafter, probably due to hypoproteinanaemia resulting from severe ICGN [36, 37, 39, 167]. In contrast, unmodified B6 ⇒ BDF_1 mice with acute GvHD had no detectable serum IgG on days 20 and 30, reflecting their profound immunosuppression (Fig. 6.9). Anti-IL-12 treated B6 ⇒ BDF_1 mice maintained normal levels of serum IgG until day 130, when levels became significantly elevated compared with those of controls (Fig. 6.9). However, these did not reach the strikingly high levels seen at the peak of chronic GvHD. Treatment of control mice with anti-IL-12 also resulted in a transient increase in the levels of IgG on days 20 to 70 (Fig. 6.9), consistent with their enhanced LPS response *in vitro* and the increased production of Th2 type cytokines. However, by day 130, IgG levels in these mice were identical to those in untreated control mice.

b) IgE

As anticipated, DBA/2 ⇒ BDF_1 mice with chronic GvHD also displayed very high levels of total serum IgE compared with control mice. This was evident on days 20 to 70 of the disease (Fig. 6.10). In contrast, untreated mice with acute

GvHD had no detectable serum IgE when examined on days 20 or 30 of the disease (Fig. 6.10). Anti-IL-12 treated B6 ⇒ BDF_1 mice had negligible levels of IgE on day 20, but levels gradually recovered and on day 70 and thereafter, their serum IgE levels were significantly elevated compared with control mice (Fig. 6.10). Again, these levels did not reach the same magnitude as those observed during the course of chronic GvHD.

c) Anti-ds DNA Antibodies

The increased levels of serum IgG and IgE present in mice with chronic GvHD was accompanied by high levels of anti-ds DNA antibodies (Table 6.2). These antibodies were not observed in the serum of anti-IL-12 treated B6 ⇒ BDF_1 mice on days 20 and 30, but on day 70 and thereafter, anti-ds DNA antibody levels were similar to those seen in mice with chronic GvHD (Table 6.2). Some of the unmodified and anti-IL-12 treated BDF_1 mice also had anti-ds DNA antibodies in their serum at later time points, which has previously been described in ageing mice [30].

Thus, in anti-IL-12 treated B6 ⇒ BDF_1 long-term survivors, there was some evidence of B cell hyperreactivity *in vivo*, but this was minor in comparison with that observed in DBA/2 ⇒ BDF_1 mice with chronic GvHD.

vi) Antibody Mediated Immunopathology

Finally, it was important to determine whether the B cell hyperplasia evident in anti-IL-12 treated B6 ⇒ BDF_1 long-term survivors was accompanied by the antibody-dependent immunopathology characteristically seen in DBA/2 ⇒ BDF_1 mice. I therefore examined for evidence of ICGN at different time points using both light and electron microscopy.

As I found previously, in the kidneys of all DBA/2 ⇒ BDF_1 mice examined, a severe and progressive ICGN was readily detectable by 50 days and was still present in surviving mice on day 70 (Figs. 6.11A & B). Light

microscopy revealed extensive glomerular damage and many of the renal tubules contained protein casts. There was thickening of basement membranes, mesangial prominence and hyperplasia of the epithelium of Bowman's capsule (Fig. 6.11A). Electron microscopy confirmed the presence of subepithelial immune complex deposits and focal endothelial swelling (Fig. 6.11B).

In contrast, kidneys from anti-IL-12 treated B6 ⇒ BDF_1 mice displayed no evidence of ICGN at any time point examined (Fig. 6.11C & D). The glomeruli appeared healthy and although occasional mesangial hyperplasia was evident, there was no thickening of the glomerular basement membrane or detectable immune complex deposits. However, there was evidence of a mild to moderate infiltration by chronic inflammatory cells which was perivascular in distribution, but was not associated with tissue injury (Fig.6.11C). The significance of this is unclear, but has been previously described in long-term survivors of unmodified acute GvHD [33].

2) Long-term effects of Anti-IL-12 on Chronic GvHD

In the final part of this chapter, I went on to assess the role of endogenous IL-12 in chronic GvHD by examining whether neutralising IL-12 during the initiation of the chronic GvHD affected the mortality and enhanced serum immunoglobulin production. The unmodified DBA/2 ⇒ BDF_1 GvHD data are the same as those shown in the first part of this chapter.

Results

i) Survival

As described in Chapter 3, DBA/2 ⇒ BDF_1 mice developed severe oedema which became evident in the majority of mice from day 40 onwards. Mice started to die on day 50 and by day 72, 67% mortality was observed (Fig. 6.12). Anti-IL-12 treated DBA/2 ⇒ BDF_1 mice also began to develop oedema die by day

50 (Fig. 6.12) and the overall mortality in this group was eventually even higher than in the unmodified DBA/2 ⇒ BDF_1 group, with 100% mortality observed by day 80 (Fig. 6.12).

ii) Serum Immunoglobulin levels

a) IgG

As before, unmodified DBA/2 ⇒ BDF_1 mice with chronic GvHD showed strikingly higher than normal levels of total serum IgG on days 20 and 30 of the disease, and again, these declined by day 70 (Fig. 6.13). Anti-IL-12 treated DBA/2 ⇒ BDF_1 mice exhibited similarly high serum IgG levels to unmodified DBA/2 ⇒ BDF_1 animals on day 20 (Fig.6.13). However, these IgG levels appeared to decline more rapidly than in unmodified mice, consistent with more pronounced proteinuria and they were significantly lower than in unmodified mice by day 30. In addition, by day 70, IgG levels in anti-IL-12 treated DBA/2 ⇒ BDF_1 mice were similar to, or lower than, those of controls (Fig. 6.13).

Treatment of control mice with anti-IL-12 resulted in mildly increased levels of IgG on days 20 to 70, but by day 130, IgG levels in these mice were identical to those in untreated control mice (Fig. 6.13).

b) IgE

DBA/2 ⇒ BDF_1 mice with chronic GvHD again displayed significantly higher serum IgE levels compared with control mice on days 20 to 70 of the disease (Fig. 6.14). Anti-IL-12 treatment of DBA/2 ⇒ BDF_1 mice resulted in a further significant increase in IgE levels on days 20 and 30, but on day 70, the levels of IgE in the serum of antibody treated DBA/2 ⇒ BDF_1 mice were similar to those in unmodified DBA/2 ⇒ BDF_1 mice (Fig. 6.14).

Anti-IL-12 did not affect the levels of IgE in the serum of control mice (Fig. 6.14).

c) Anti-ds DNA Antibodies

The high levels of anti-ds DNA antibodies present in the serum of DBA/2 $\Rightarrow$ BDF_1 mice on days 20 to 70 of the chronic GvHD were unaffected by anti-IL-12 treatment (Fig. 6.15). Both unmodified and anti-IL-12 treated control mice did not display detectable levels of anti-ds DNA antibodies on days 20 or 30, but by day 70, one mouse from each of these groups showed a high titer of these antibodies (Fig. 6.15).

iii) Antibody-Mediated Pathology

Kidneys were harvested from both unmanipulated and anti-IL-12 treated DBA/2 $\Rightarrow$ BDF_1 mice on day 70 and examined for ICGN by electron microscopy. The kidneys of both groups of mice showed characteristic glomerular damage at both time points, which, as before was evidenced by thickening of the glomerular basement membrane and immune complex deposition (Figs. 6.16 A & B). The severity of the kidney damage was not obviously different between the untreated and anti-IL-12 treated groups.

Summary and Conclusions

In this chapter I have shown that neutralising endogenous IL-12 for a brief period during the initiation of acute GvHD in B6 $\Rightarrow$ BDF_1 mice not only confers long-term protection from the disease but also permits full repopulation with donor B6 lymphocytes. The cytokine response of surviving mice was permanently polarised towards a Th2 phenotype similar to that observed in DBA/2 $\Rightarrow$ BDF_1 mice and long-term survivors showed B cell hyperplasia, as evidenced by heightened responses to the B cell mitogen LPS *in vitro*. However, compared with DBA/2 $\Rightarrow$ BDF_1 mice, anti-IL-12 treated B6 $\Rightarrow$ BDF_1 animals displayed only mildly increased serum IgG and IgE levels and moderate levels of anti-ds DNA antibodies. Furthermore, detailed examination of the kidneys from anti-IL-12 treated B6 $\Rightarrow$ BDF_1 mice by both light and electron microscopy did not reveal any

evidence of the ICGN apparent in mice with chronic GvHD. In parallel, anti-IL-12 treated mice did not develop oedema or die. These results therefore confirm the critical role of early IL-12 production in acute GvHD.

Consistent with its ability to polarise acute GvHD towards a Th2 phenotype, neutralising IL-12 appeared to slightly exacerbate chronic GvHD in DBA/2 ⇒ BDF_1 mice. Although antibody treated DBA/2 ⇒ BDF_1 mice developed oedema and started to die at exactly the same time as unmodified DBA/2 ⇒ BDF1 mice, the overall mortality rate was slightly higher in the antibody treated group than in the unmodified group. In parallel, IgE levels were significantly higher in anti-IL-12 treated DBA/2 ⇒ BDF_1 mice and serum IgG levels declined more rapidly in antibody treated animals, perhaps suggesting a more rapid onset of hypoproteinaemia. However these differences were relatively subtle and only small numbers of mice were analysed. In addition, anti-ds DNA antibody levels were similar in both groups of mice. Thus, although IL-12 may play a subtle regulatory role in the development of the chronic GvHD, these results need repeated.

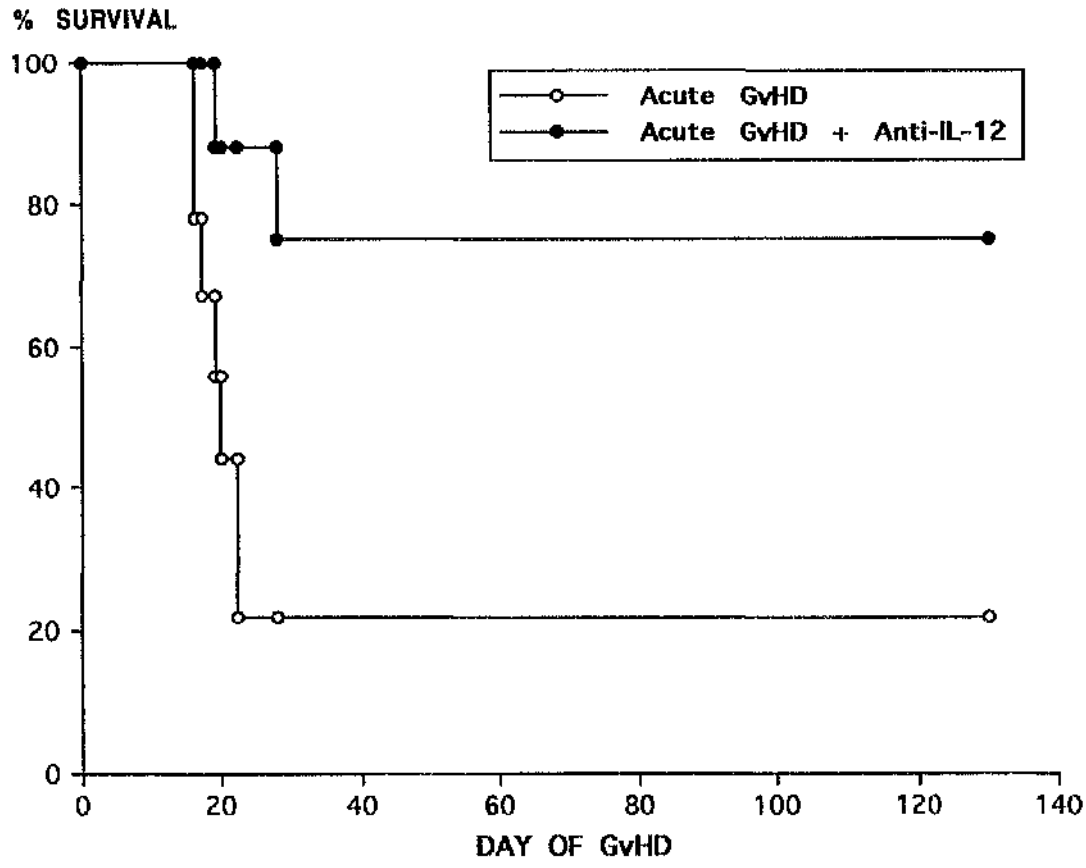
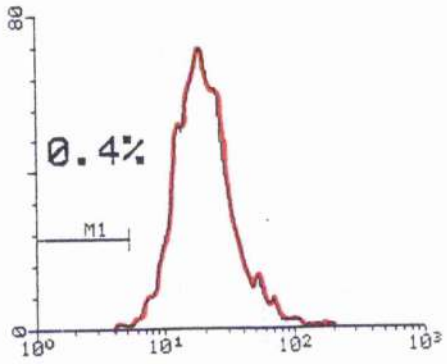


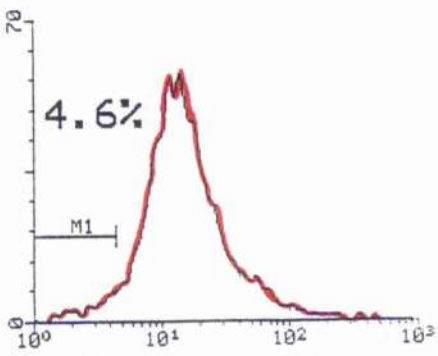
Fig. 6.1. Effect of neutralising IL-12 on long-term survival during acute GvHD.

The results show the effect of neutralising endogenous IL-12 during the first 8 days of acute GvHD on long-term survival in BDF_1 mice given 10^8 B6 spleen cells i.v. The data show the percentage of surviving mice in each group throughout the acute disease.

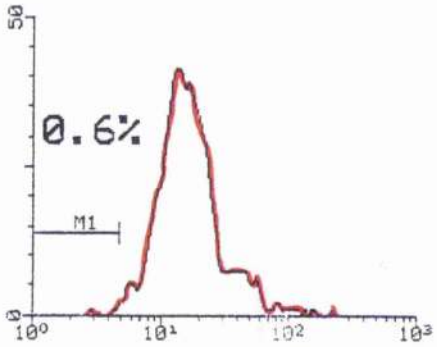
H-2^{d} Expression on CD4^{+} T cells



H-2^{b} Expression on CD4^{+} T cells



H-2^{d} Expression on CD8^{+} T cells



H-2^{b} Expression on CD8^{+} T cells

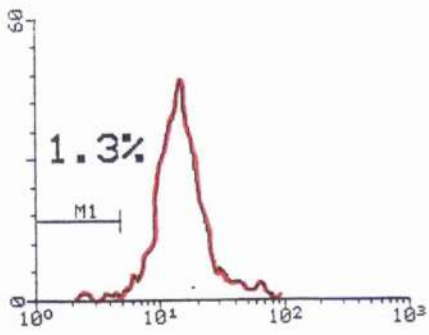


Fig. 6.2. Effect of neutralising IL-12 on donor cell chimerism in acute GvHD.

Donor cell chimerism was assessed throughout the GvHD by flow cytometry. The level of H-2D^{d} and H-2D^{b} expression on gated control CD4^{+} and CD8^{+} splenic lymphocytes was determined and the percentage of H-2D^{d} and H-2D^{b} negative cells defined by M1. These control histograms (shown in red) were then used to calculate the percentage of H-2D^{d} negative (donor derived) cells in GvHD mice, as illustrated on page 112. All histograms shown were obtained on day 9 of the GvHD, while the percentage of donor-derived cells at later time points are given in Table 6.1.

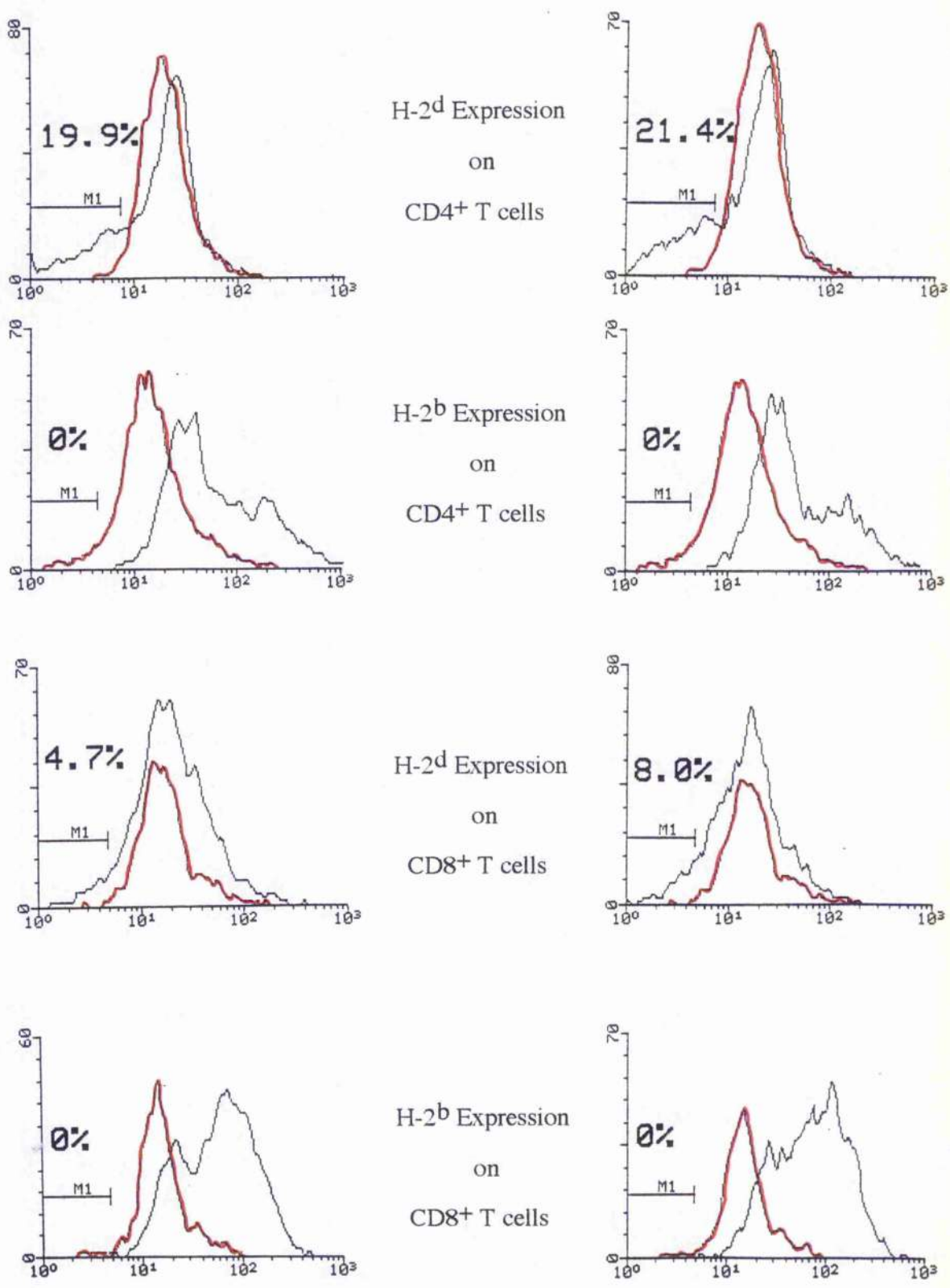


Fig. 6.2. Effect of neutralising IL-12 on donor cell chimerism in acute GvHD. The percentage of donor-derived cells is defined by region M1.

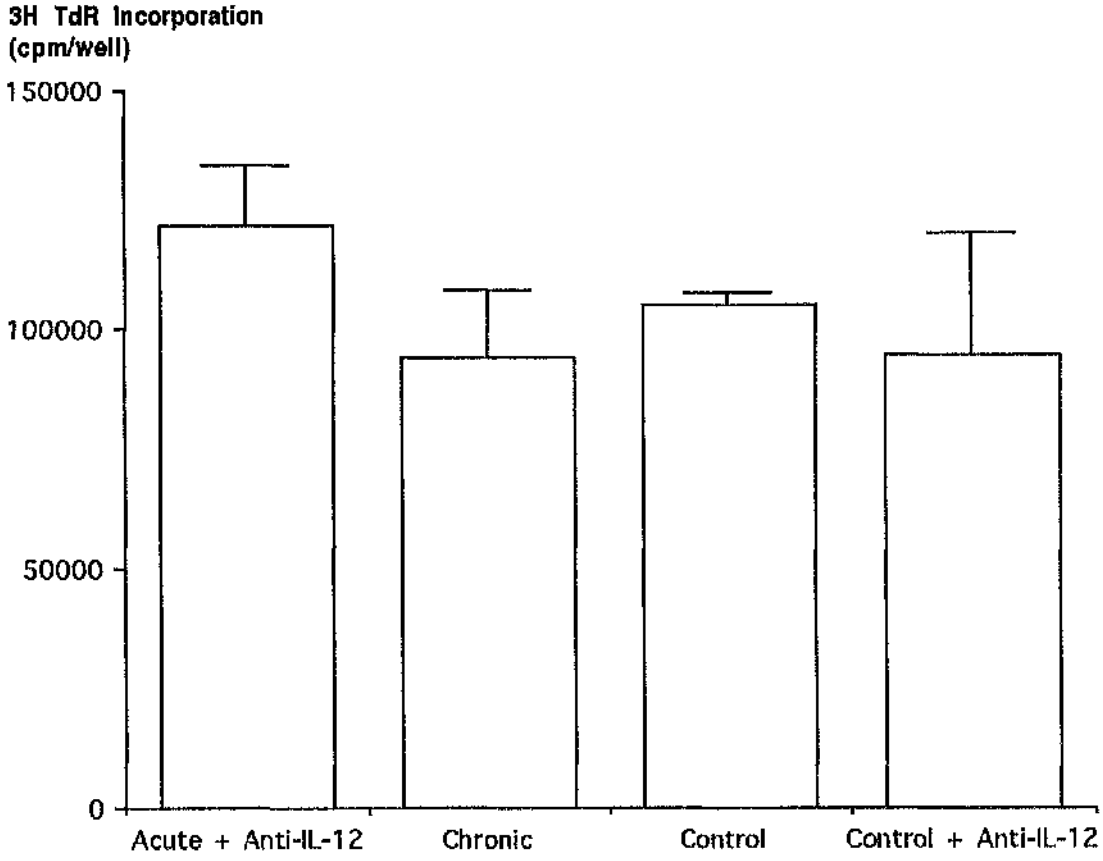


Fig. 6.3. Effect of neutralising IL-12 on T cell function in long-term survivors of acute GvHD.

The data show the prolifcrative responses of spleen cells after stimulation with 10μg/ml Con A on day 70 of acute or chronic GvHD and are expressed as the mean uptake of 3H-TdR ± 1 SD after 48 hours in quadruplicate cultures, using spleen cells pooled from 3-4 mice per group.

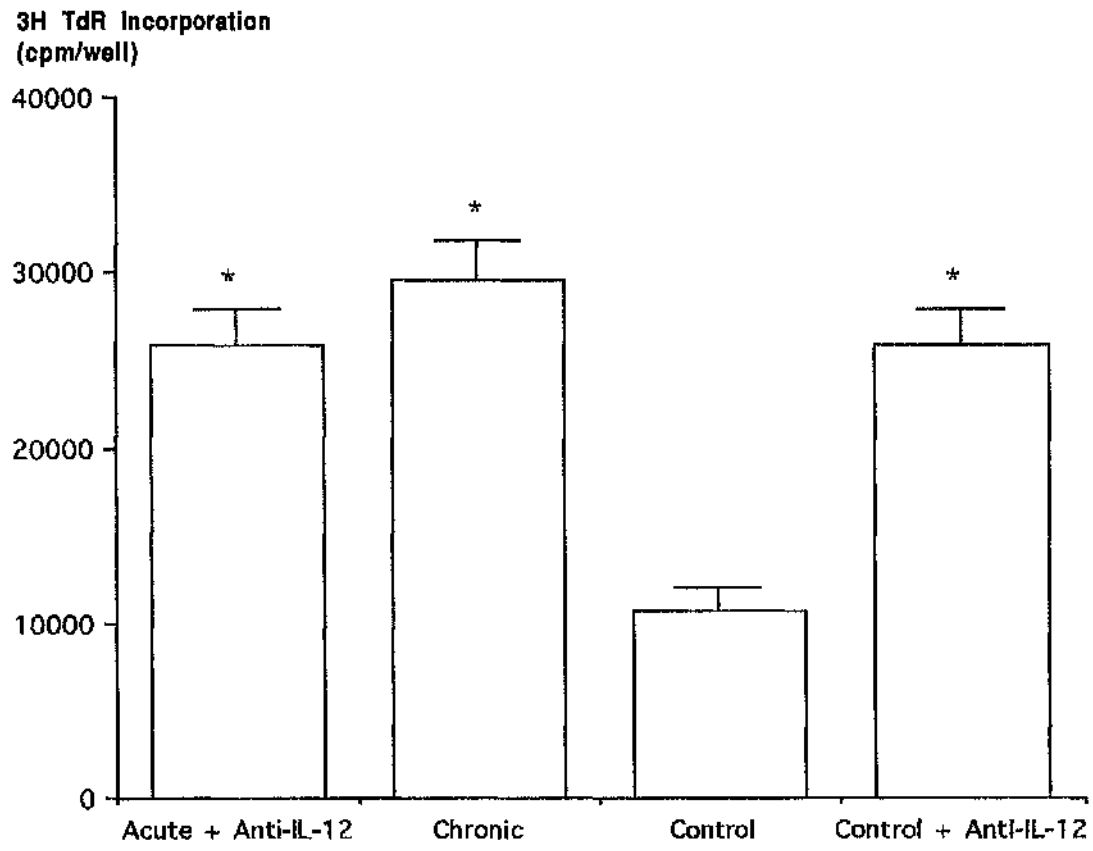


Fig. 6.4. Effect of neutralising IL-12 on B cell function in long-term survivors of acute GvHD.

The data show the proliferative responses of spleen cells after stimulation with 10μg/ml LPS on day 70 of acute or chronic GvHD and are expressed as the mean uptake of 3H-TdR ± 1 SD after 24 hours in quadruplicate cultures, using spleen cells pooled from 3-4 mice per group. (* $p < 0.001$ vs controls).

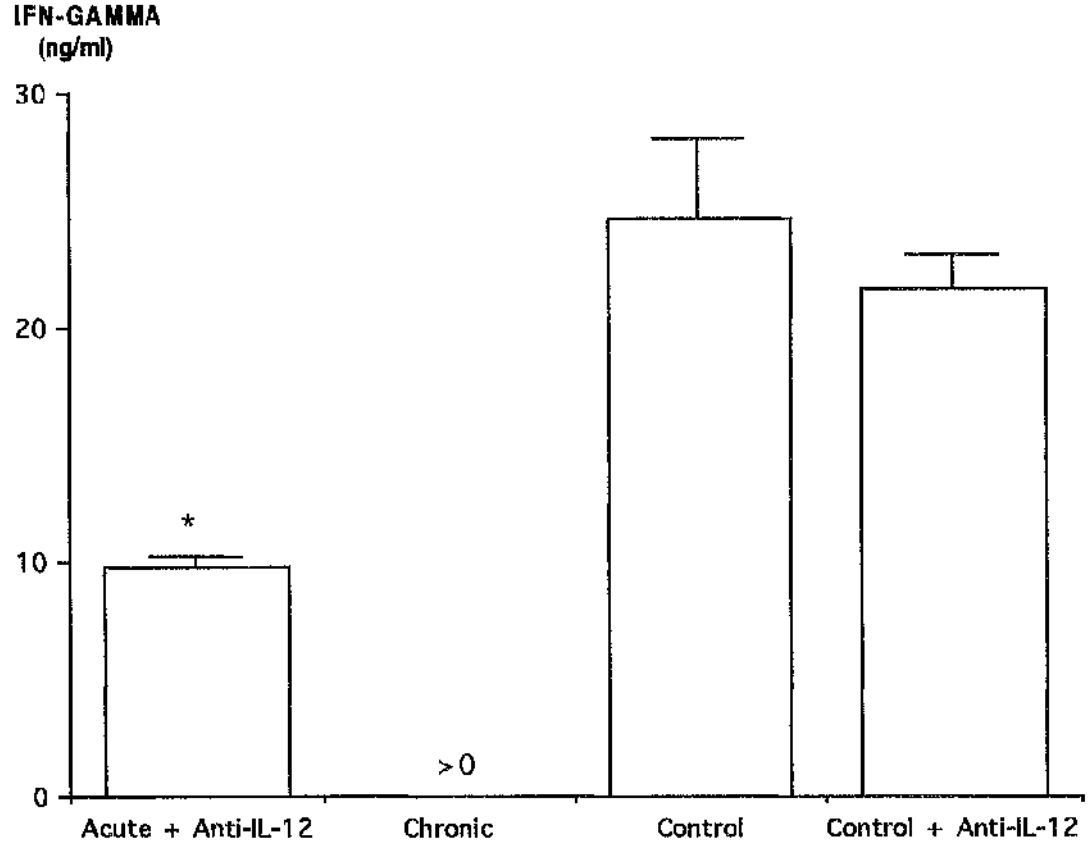


Fig. 6.5. Effect of neutralising IL-12 on IFN-γ production in long-term survivors of acute GvHD.

The results show IFN-γ production by spleen cells from mice on day 70 of acute or chronic GvHD after stimulation with 10μg/ml Con A for 48 hours and are the means ± SD of triplicate samples. (* $p < 0.001$ vs controls).

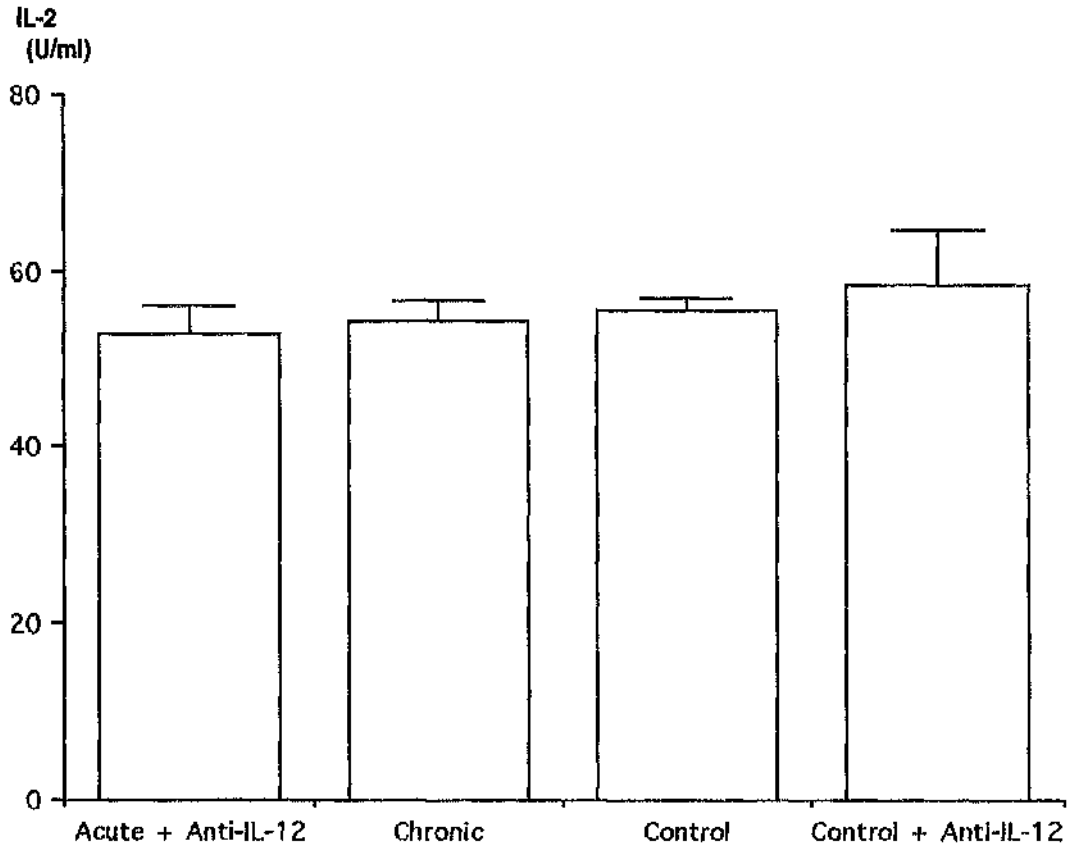


Fig. 6.6. Effect of neutralising IL-12 on IL-2 production in long-term survivors of acute GvHD.

The results show IL-2 production by spleen cells from mice on day 70 of acute or chronic GvHD after stimulation with 10μg/ml Con A for 24 hours and are the means ± SD of triplicate samples.

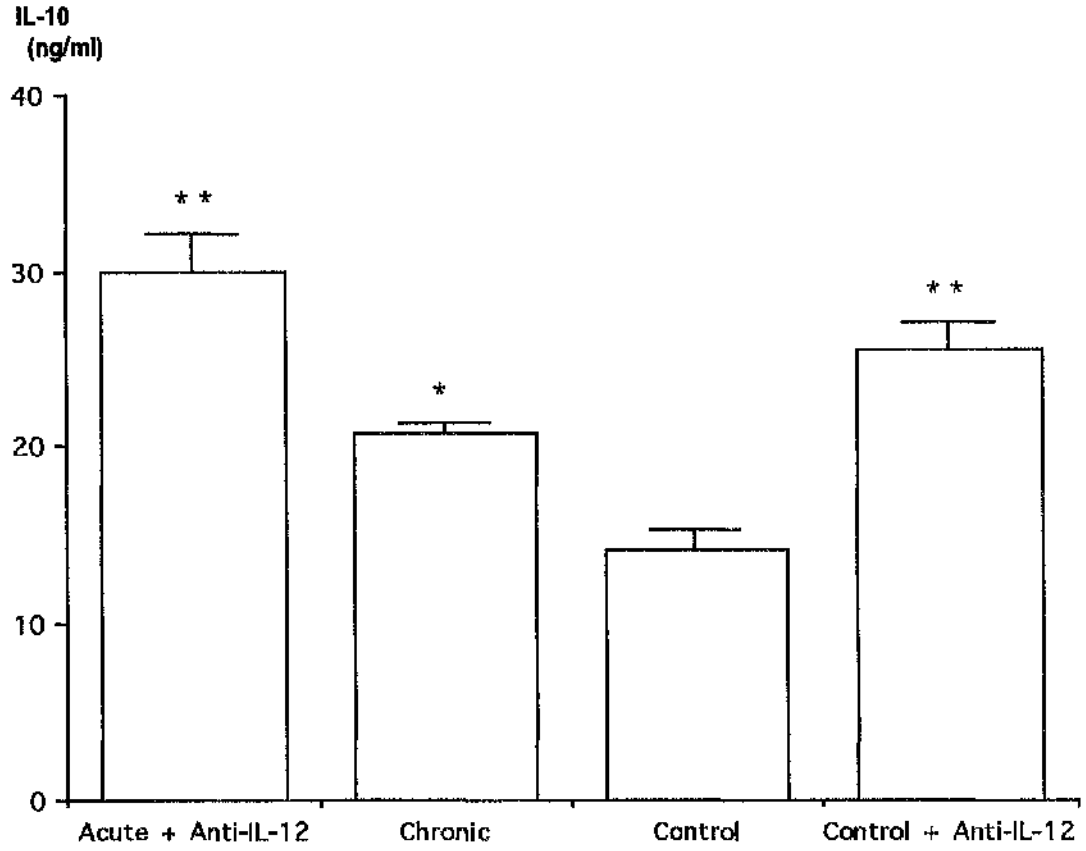


Fig. 6.7. Effect of neutralising IL-12 on IL-10 production in long-term survivors of acute GvHD.

The results show IL-10 production by spleen cells from mice on day 70 of acute or chronic GvHD after stimulation with 10μg/ml Con A for 48 hours and are the means ± SD of triplicate samples. (* $p < 0.01$ ** $p < 0.001$ vs controls).

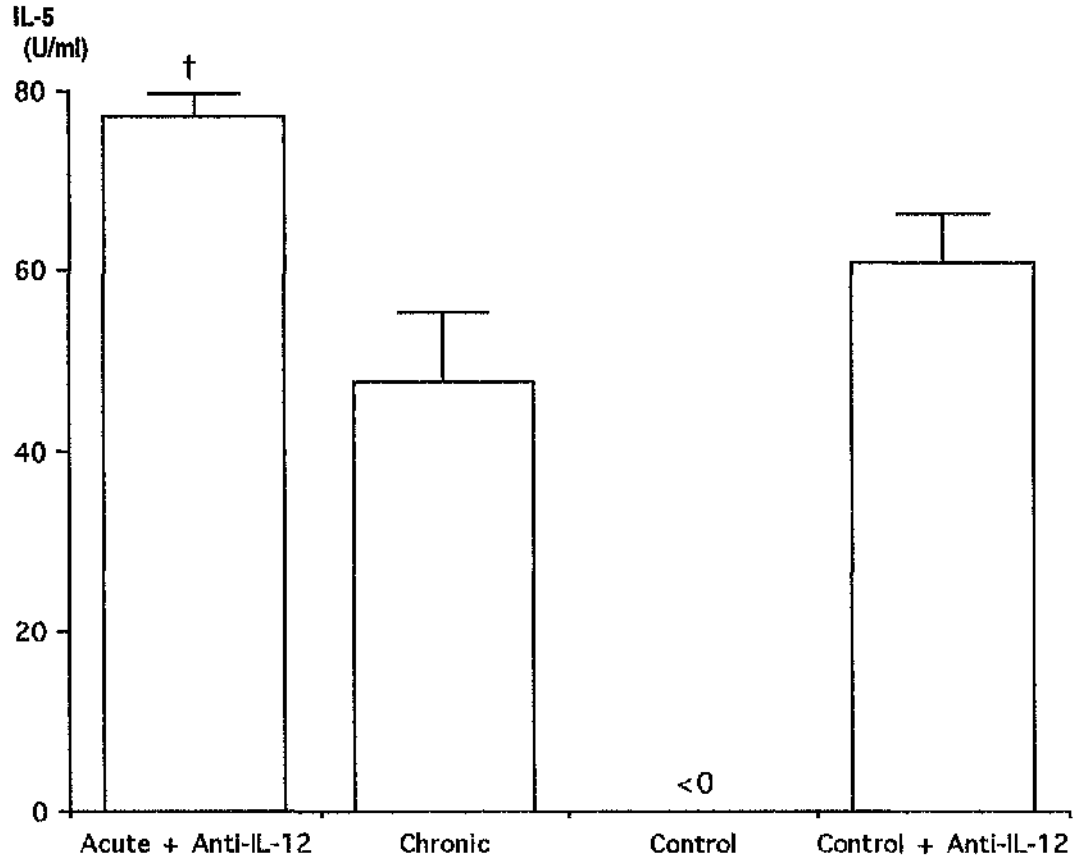


Fig. 6.8. Effect of neutralising IL-12 on IL-5 production in long-term survivors of acute GvHD.

The results show IL-5 production by spleen cells from mice on day 70 of acute or chronic GvHD after stimulation with 10μg/ml Con A for 96 hours and are the means ± SD of triplicate samples. († $p < 0.001$ vs chronic GvHD).

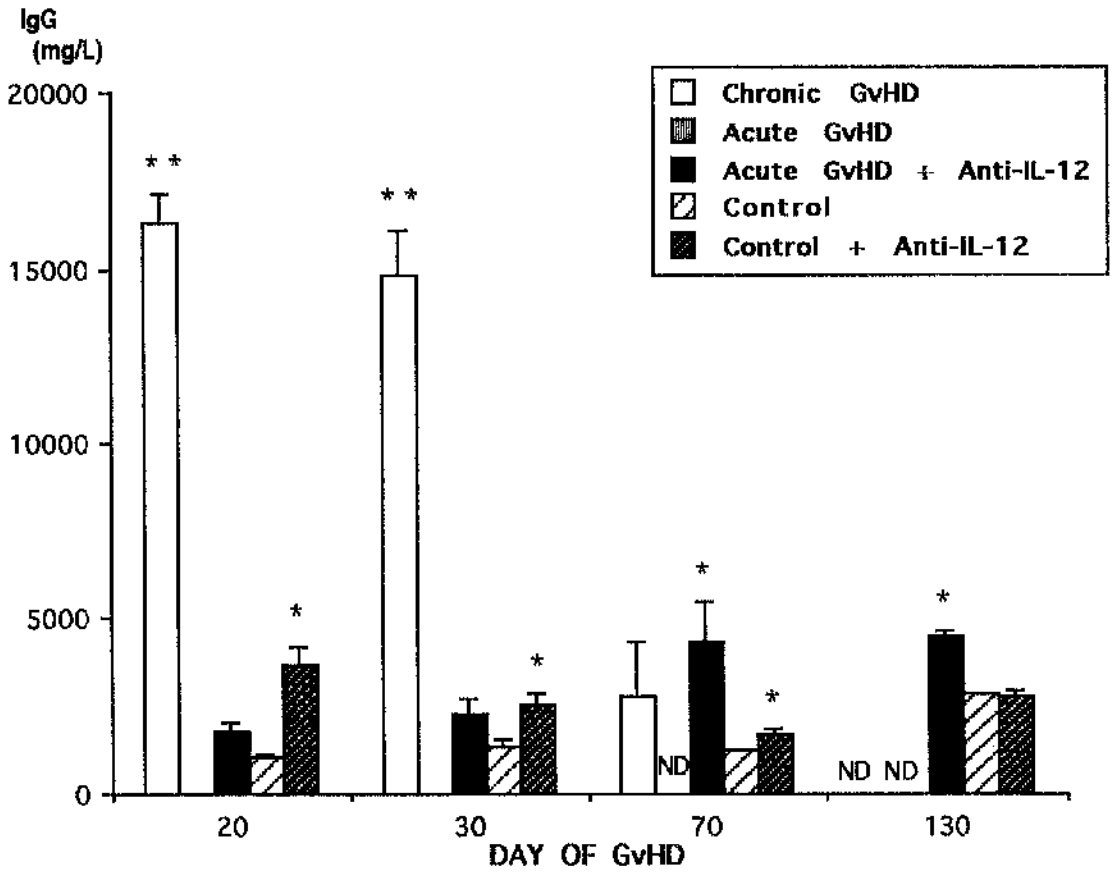


Fig. 6.9. Effect of neutralising IL-12 on total serum IgG levels in long-term survivors of acute GvHD.

The results shown are the levels of total serum IgG in 4-6 mice with acute or chronic GvHD and are expressed as the mean ± SD.

(* $p < 0.05$ ** $p < 0.001$ vs controls). ND = Not done.

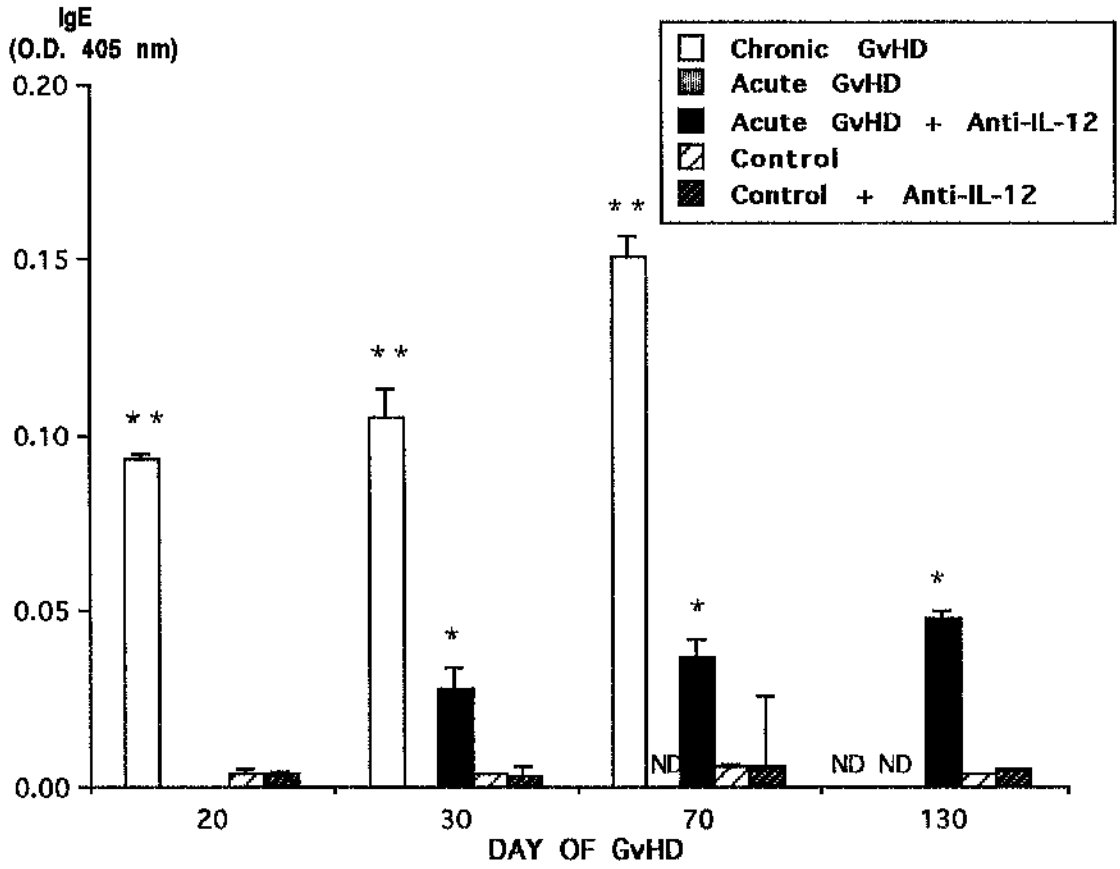


Fig. 6.10. Effect of neutralising IL-12 on total serum IgE levels in long-term survivors of acute GvHD.

The results shown are the levels of total serum IgE in 4-6 mice with acute or chronic GvHD and are expressed as the mean O.D. at 405 nm ± SD.

(* $p < 0.01$ ** $p < 0.001$ vs controls). ND = Not done.

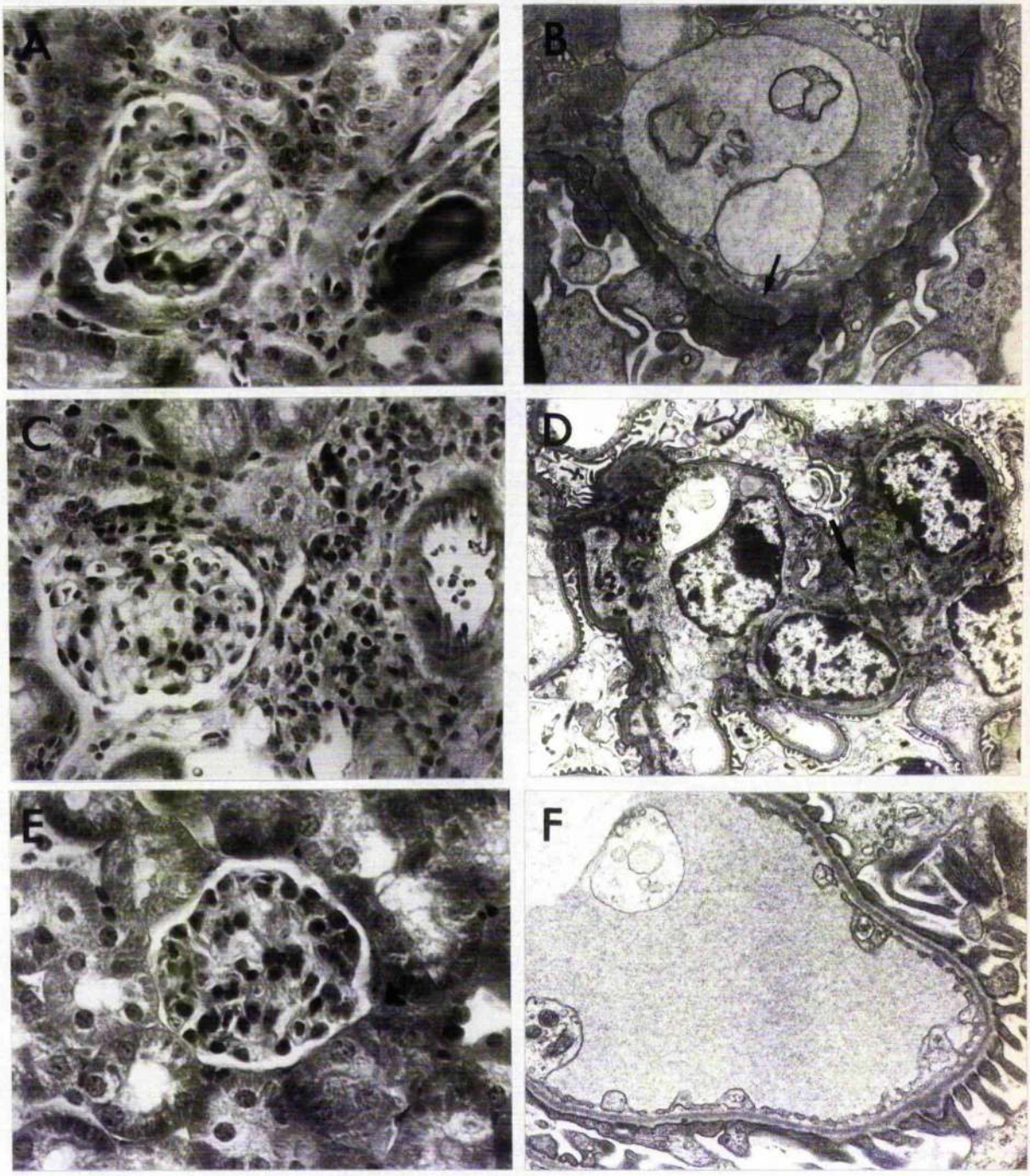


Fig. 6.11. Renal Immunopathology in GvHD.

Light (A, C, E) and electron (B, D, F) microscopic appearances of kidneys on day 70 of GvHD. (A, C, E : H &E x 400 ; B, F : x 11500 ; D : x 4000).

A, B. Mice with chronic GvHD have severe ICGN, characterised by thickening of the glomerular basement membrane and tubular casts (A) and subepithelial immune complex deposition (arrowed in B). **C, D.** Anti-IL-12 treated B6 ⇒ BDF_1 mice had normal kidney architecture apart from perivascular infiltration of mononuclear cells (C) and mild mesangial cell prominence (D). **E, F.** Controls.

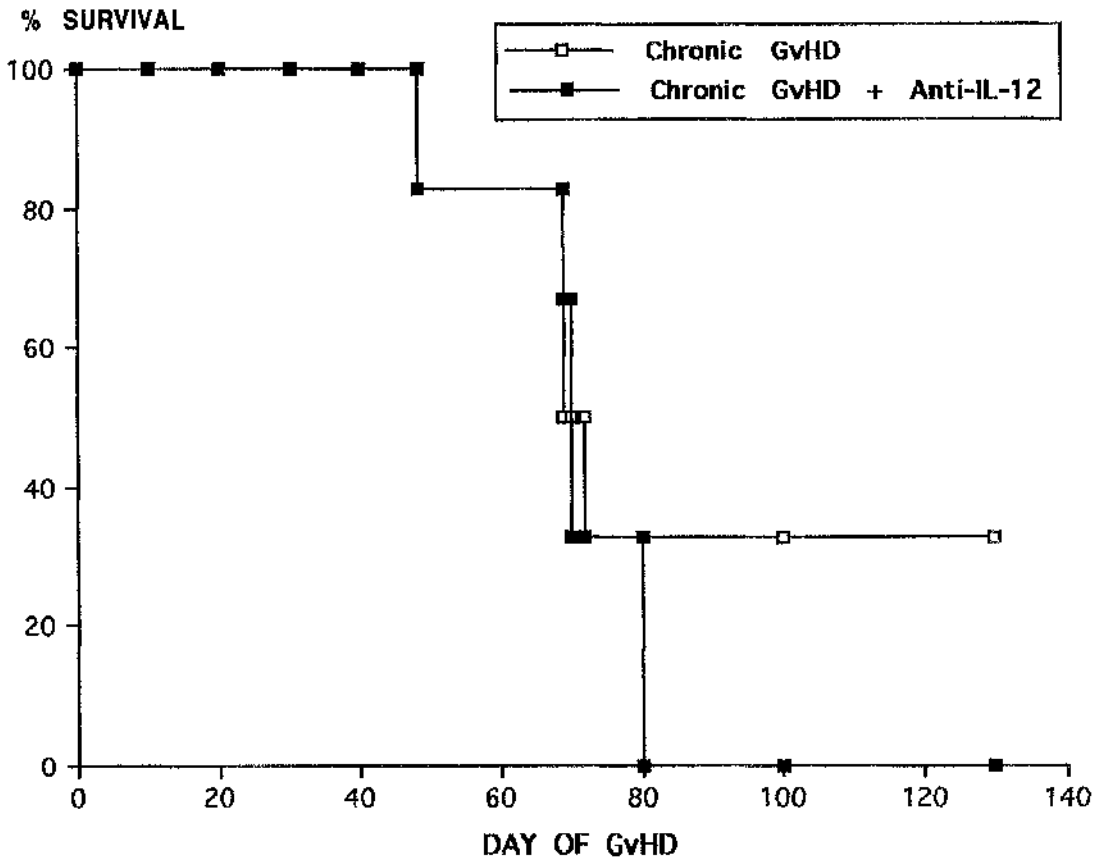


Fig. 6.12. Effect of neutralising IL-12 on long-term mortality in chronic GvHD.

Cumulative mortality in mice with chronic GvHD and treated with anti-IL-12 for the first 8 days of disease.

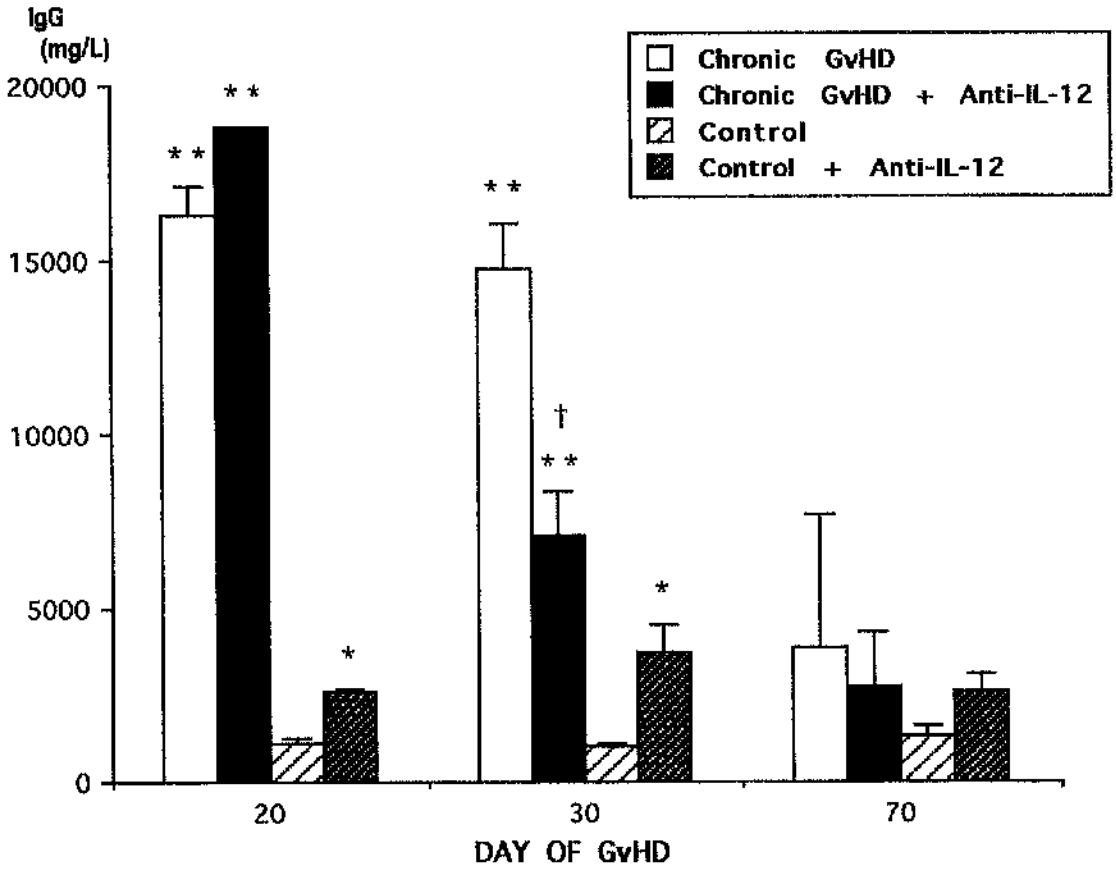


Fig. 6.13. Effect of neutralising IL-12 on total serum IgG levels in chronic GvHD.

The results shown are the levels of total serum IgG in 4-6 mice with chronic GvHD and are expressed as the mean ± SD.

(* $p < 0.05$ ** $p < 0.001$ vs controls † $p < 0.01$ vs unmodified chronic GvHD).

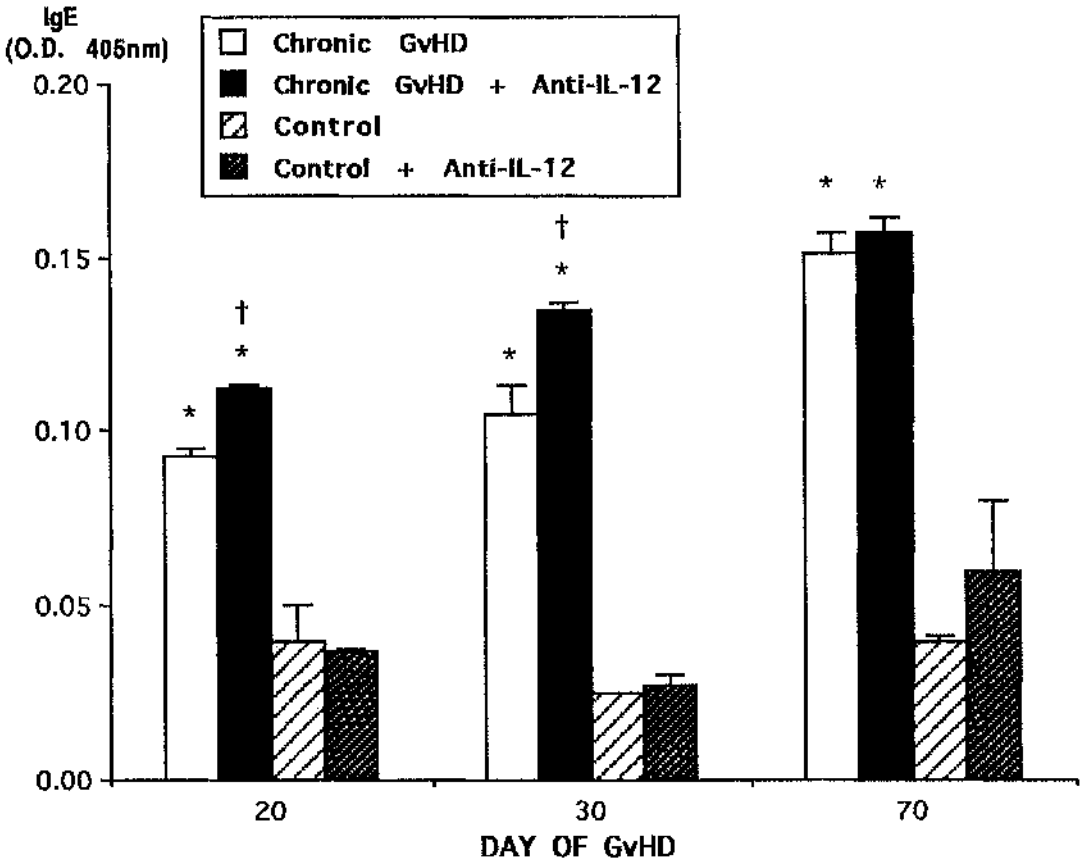


Fig. 6.14. Effect of neutralising IL-12 on total serum IgE levels in chronic GvHD.

The results shown are the levels of total serum IgE in 4-6 mice with chronic GvHD and are expressed as the mean O.D. at 405 nm ± SD.

(* $p < 0.001$ vs controls; † $p < 0.05$ vs unmodified chronic GvHD).

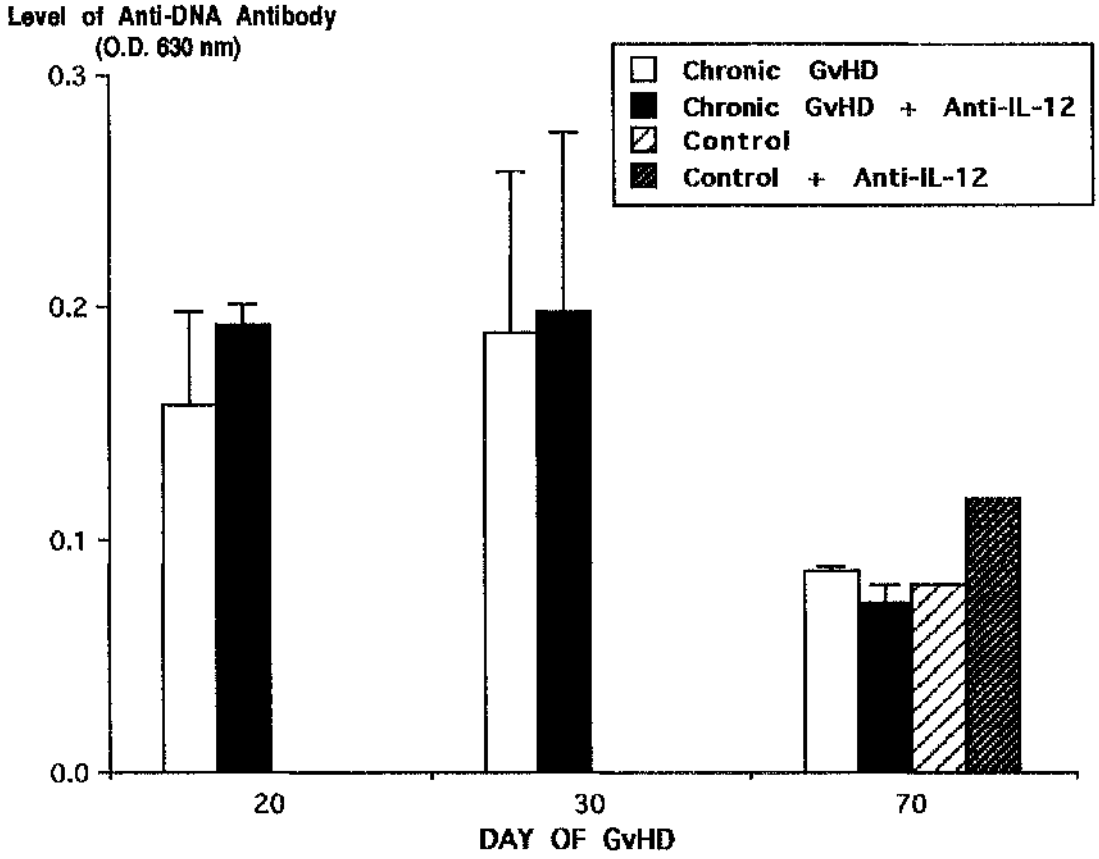


Fig. 6.15. Effect of neutralising IL-12 on anti-ds DNA antibody levels in chronic GvHD.

The results shown are the levels of anti-ds DNA antibodies in the serum of 6 mice with chronic GvHD and are expressed as the mean OD value ± 1SD at 630nm at a 1:50 dilution.

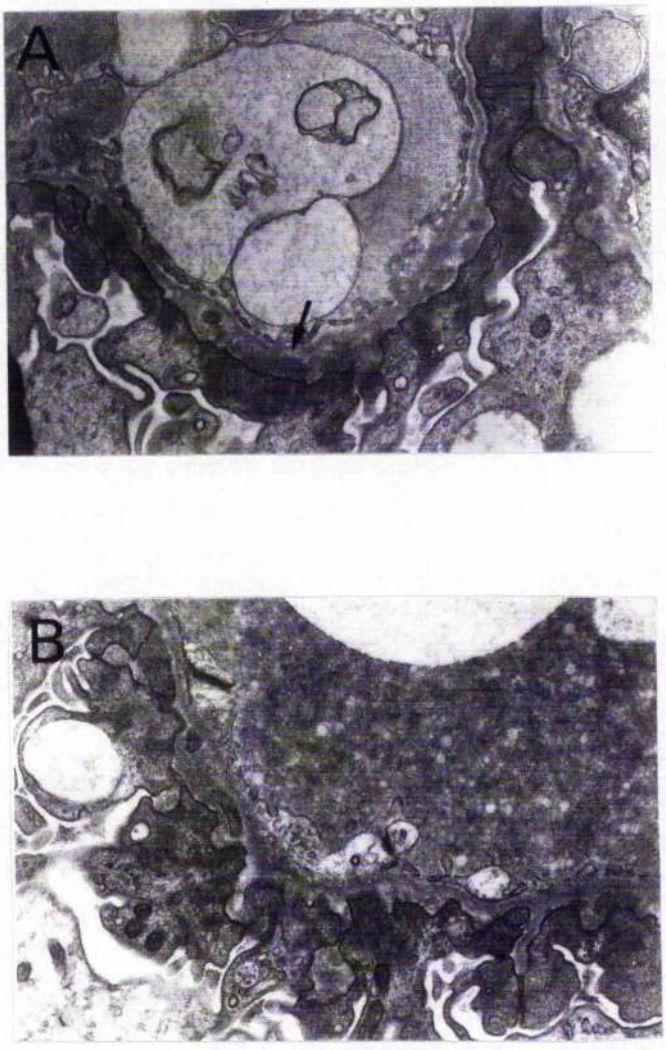


Fig. 6.16. Renal Immunopathology in GvHD.

Electron microscopic appearances of kidneys on day 50 of GvHD. Kidneys from unmodified (A) and anti-IL-12 treated (B) mice with chronic GvHD show a similar degree of kidney damage, characterised by thickening of the glomerular basement membrane and subepithelial immune complex deposition (x 11500).

Table 6.1.

Effect of Neutralising IL-12 on Donor Cell Chimerism in Acute GvHD.

	% Donor-Derived Lymphocytes			
Day of GvHD	**Unmodified B6 ⇒ BDF_1**		**Anti-IL-12 Treated B6 ⇒ BDF_1**	
	CD4	**CD8**	**CD4**	**CD8**
9	19.9	4.7	21.4	8.0
17	69.9	73.2	54.3	69.9
70	95.8	63.2	30.0	71.4
130	ND	ND	100	100

Table 6.1. Effect of Anti-IL-12 Treatment on Donor Cell Chimerism in Acute GvHD.

The levels of donor-derived $CD4^+$ and $CD8^+$ splenic lymphocytes were determined in GvHD mice throughout the disease, as described in Fig. 6.2. Cells expressing $H\text{-}2D^b$, but not $H\text{-}2D^d$ were designated as being of donor origin.

ND = not done.

Table 6.2.

Effect of Neutralising IL-12 on Anti-ds DNA Antibody Levels in GvHD

Experimental Group	Day 20	Day 30	Day 70	Day 130
Unmodified B6 ⇒ BDF_1	0/6	0/4	ND	ND
Anti-IL-12 treated B6 ⇒ BDF_1	0/6	0/6	6/6 (0.1 ± 0.02)	4/4 (0.145 ± 0.05)
DBA/2 ⇒ BDF_1	6/6 (0.16 ± 0.04)	6/6 (0.19 ± 0.07)	5/5 (0.09 ± 0.002)	ND
Control	0/6	0/6	1/6 (0.081)	1/4 (0.085)
Control + Anti-IL-12	0/6	0/6	1/6 (0.12)	2/4 (0.09)

The data show the number of mice/group with detectable anti-ds DNA antibody levels, while the results in parenthesis are the mean OD readings for the positive sera at a dilution of 1:50 ± 1SD for each group. ND = Not done.

CHAPTER 7

ROLE OF ENDOGENOUS IL-12 IN GvHD

Modulation of Acute and Chronic GvHD by Administration of Exogenous IL-12

Taken together, the results detailed Chapters 4-6 indicate a role for endogenous IL-12 in acute, but not chronic GvHD in BDF_1 mice. To explore further the influence of IL-12 in acute vs chronic GvHD, I next examined whether giving exogenous rm IL-12 to DBA/2 ⇒ BDF_1 mice could convert the chronic form of GvHD into an acute disease. In parallel, I also examined the effects of exogenous IL-12 on acute GvHD and tested the hypothesis that the different outcomes of the two diseases were due to differences in the production of endogenous IL-12 during GvHD.

Experimental Protocol

Exogenous IL-12 was administered by i.p. injection of 100ng rm IL-12 from day -1 to 3 and 6 to 10 of GvHD. Control mice received PBS containing 1% syngeneic mouse serum. The effects on GvHD were assessed as before, with EL-4 cells used as targets for measuring anti-host CTL activity in the spleens of DBA/2 ⇒ BDF_1 mice.

IL-12 production was measured by culturing spleen cells from GvHD mice in medium alone, or in the presence of 10μg/ml LPS. The supernatants were then harvested at 24, 48, 72 and 96 hours and levels of IL-12 assessed by ELISA. The results shown are for peak IL-12 production, which occurred at 48 hours.

Results

i) Weight loss and mortality

As described in Chapter 3, BDF_1 mice with chronic GvHD showed no evidence of weight loss or mortality for the duration of this study (Fig. 7.1). However, DBA/2 $\Rightarrow$ BDF_1 mice injected with 100ng of rm IL-12 for 10 consecutive days developed an acute GvHD-like syndrome, with weight loss evident by day 12, 50% mortality by day 14 (Fig. 7.1) and 100% mortality by day 15 (data not shown). Due to this high mortality rate, the study was terminated at this point. Rm IL-12 also exacerbated the systemic consequences of acute GvHD, with weight loss and mortality appearing in the IL-12 treated group, but not in the unmodified B6 $\Rightarrow$ BDF_1 mice by the time the experiment was terminated (Fig. 7.1). Rm IL-12 did not provoke weight loss or mortality in control animals (Fig. 7.1).

ii) Splenomegaly

On day 1, mice with chronic GvHD had not yet developed significant splenomegaly (Fig. 7.2a). By day 10, the spleens of mice from this group were significantly enlarged compared with controls, but the splenomegaly was less than that found in mice with the acute form of the disease (Fig. 7.2b). IL-12 treatment significantly increased the degree of spleen enlargement in mice with chronic GvHD at both days 1 and 10, reaching levels similar to those observed in mice with acute GvHD (Figs. 7.2a & b). IL-12 also significantly enhanced the splenomegaly seen in mice with acute GvHD on both days 1 and 10 (Figs. 7.2a & b) and had provoked significant splenomegaly in control animals by day 10, perhaps reflecting the increased extramedullary haematopoiesis observed in previous sudies of IL-12 treated mice [121].

iii) Lymphocyte Function

a) Spontaneous 'ex-vivo' proliferation

Splenocytes from unmodified DBA/2 ⇒ BDF_1 mice showed significantly enhanced spontaneous proliferation compared with control cells on both days 1 (Fig. 7.3a) and 10 (Fig. 7.3b) of GvHD, although this was less marked than that found in acute GvHD at either time point. Treatment with rm IL-12 had no effect on the proliferation of chronic GvHD spleen cells on day 1 (Fig. 7.3a), but by day 10, the rm IL-12 treated chronic GvHD spleen cells showed a marked increase in their spontaneous proliferative capacity which resembled that observed in mice with acute GvHD (Fig. 7.3b).

Treatment with rm IL-12 had no effect on the enhanced spontaneous proliferation of acute GvHD spleen cells on day 1 (Fig. 7.3a), but by day 10, the proliferative capacity of splenocytes from rm IL-12 treated acute GvHD mice was significantly reduced compared with untreated mice with acute GvHD (Fig. 7.3b). Rm IL-12 injected control animals exhibited significantly enhanced spontaneous proliferative responses on both day 1 (Fig. 7.3a) and day 10 (Fig. 7.3b).

b) Proliferative responses to Con A stimulation

To determine whether DBA/2 ⇒ BDF_1 mice given exogenous IL-12 developed immunosuppression similar to that usually found in B6 ⇒ BDF_1 mice with acute GvHD, I assessed Con A responses on day 15, as this was when significant weight loss and mortality were evident in DBA/2 ⇒ BDF_1 mice given exogenous IL-12.

At this time, spleen cells from unmodified DBA/2 ⇒ BDF_1 mice showed Con A responses similar to those of control cells (Fig. 7.4). Conversely, the responses of cells from DBA/2 ⇒ BDF_1 mice given IL-12 were markedly suppressed at this time, with responses similar to those found in unmodified B6 ⇒ BDF_1 mice with acute GvHD (Fig. 7.4). Exogenous IL-12 actually slightly

increased the low Con A responses of acute GvHD cells on day 15, although these remained significantly suppressed compared with controls (Fig. 7.4).

The Con A responses of splenocytes from control mice were unaffected by rm IL-12 treatment (Fig. 7.4).

c) Proliferative repsonses to LPS stimulation

The low numbers of lymphocytes recovered from the spleeens of IL-12 treated B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice precluded comparison of their LPS responsiveness on day 15.

iv) Cytokine production

I now went on to examine the effect of IL-12 on cytokine production. This was carried out only on days 1 and 10 of acute and chronic GvHD, as the low numbers of lymphocytes recovered from the spleeens of IL-12 treated B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice precluded analysis at later times.

Spontaneous IFN-γ

On days 1 and 10, spleen cells from mice with chronic GvHD produced similarly low levels of spontaneous IFN-γ to control cells (Fig. 7.5a & b). Administration of rm IL-12 to DBA/2 ⇒ BDF_1 mice resulted in a large increase in spontaneous IFN-γ production by splenocytes at both time points and these reached levels equal to, or above those produced by acute GvHD cells (Figs. 7.5a & b). IL-12 had little effect on the already high levels of IFN-γ produced spontaneously by cells from mice with the acute form of GvHD at both time points (Figs. 7.5a & b). Although exogenous IL-12 had no effect on IFN-γ production by control cells on day 1 (Fig. 7.5a), it induced the spontaneous production of IFN-γ from control spleen cells by day 10 (Fig. 7.5b).

Con A Induced IFN-γ

On day 1, spleen cells from unmodified DBA/2 ⇒ BDF_1 mice produced similar levels of IFN-γ in response to Con A to control cells (Fig. 7.6a), whereas by day 10, IFN-γ production by DBA/2 ⇒ BDF_1 cells was significantly lower than that of control cells (Fig. 7.6b). IL-12 did not affect Con A induced IFN-γ production by DBA/2 ⇒ BDF_1 cells on day 1 (Fig. 7.6a), but significantly increased that observed on day 10 to levels even greater than the high amounts produced by cells from B6 ⇒ BDF_1 mice (Fig. 7.6b). In contrast, IL-12 did not significantly increase the already high levels of IFN-γ produced by Con A stimulated B6 ⇒ BDF_1 spleen cells on either days 1 or 10 (Figs. 7.6a & b). Cells from control mice given IL-12 produced similar levels of IFN-γ in response to Con A to cells from unmodified controls on day 1 (Fig. 7.6a), but by day 10, these mice had significantly enhanced levels of IFN-γ production compared with unmodified controls (Fig. 7.6b).

IL-2

On both days 1 and 10, spleen cells from unmodified mice with chronic GvHD produced significantly higher levels of IL-2 in response to Con A than cells from control mice (Figs. 7.7a & b). This was in contrast to cells from mice with the acute form of the disease, which produced higher levels of IL-2 in response to Con A than control cells on day 1, but by day 10 had undetectable IL-2 production (Figs. 7.7a), consistent with the suppressed Con A responses of these mice. Administration of exogenous IL-12 significantly reduced the capacity of cells from mice with chronic GvHD to produce IL-2 in response to Con A on day 1 (Fig. 7.7a) and by day 10, IL-2 production by IL-12 treated DBA/2 ⇒ BDF_1 mice was abolished (Figs. 7.7b). IL-12 also reduced the early enhancement in IL-2 production by cells from B6 ⇒ BDF_1 mice on day 1 (Fig. 7.7a) and by day 10, IL-12 treated B6 ⇒ BDF_1 mice did not produce any detectable IL-2 (Figs. 7.7b).

Cells from IL-12 treated control mice also produced significantly lower levels of IL-2 on day 1 compared with cells from unmodified control mice (Fig. 7.7a). However, on day 10, both unmodified and IL-12 treated control mice produced similar levels of IL-2 (Figs. 7.7b).

IL-10

IL-10 was not produced spontaneously by cells from any group at any time during this study (data not shown). Similarly, on day 1, IL-10 production by Con A stimulated cells from all groups was below the level of detection (data not shown).

On day 10, cells from mice with chronic GvHD produced significantly higher levels of IL-10 in response to Con A than cells from control mice, although these levels were lower than those found using chronic GvHD splenocytes in earlier experiments (Fig. 7.8). Treatment with exogenous IL-12 resulted in a significant reduction in IL-10 production by DBA/2 $\Rightarrow$ BDF_1 splenocytes to below control levels (Fig. 7.8). However it had no effect on the levels of IL-10 produced by cells of any other group.

IL-5

IL-5 production by cells from all groups was below the level of detection at all time points during this study (data not shown).

IL-4

IL-4 production by cells from all groups was below the level of detection at all time points during this study (data not shown).

v) Non-specific and specific cell-mediated cytotoxicity

Finally, I examined whether administration of exogenous IL-12 to DBA/2 $\Rightarrow$ BDF_1 mice could elicit the high levels of NK cell-mediated and specific CTL-mediated cytotoxicity usually associated with acute GvHD.

a) NK cell activity

As before, the early phase of acute GvHD in B6 $\Rightarrow$ BDF_1 mice was associated with increased NK cell activity on day 1 (Fig. 7.9a). This increase was not evident in mice with chronic GvHD at this time (Fig. 7.9b). Administration of IL-12 did not induce enhanced NK cell activity in DBA/2 $\Rightarrow$ BDF_1 mice, (Fig. 7.9b), although it did slightly increase the levels of NK cell activity observed in B6 $\Rightarrow$ BDF_1 and control mice (Fig. 7.9a).

b) CTL activity

As before, untreated mice with acute GvHD exhibited high levels of anti-host CTL activity on day 10 (Fig. 7.10a), but little or no anti-host CTL activity was found in mice with chronic GvHD at this time (Fig. 7.10b). Administration of rm IL-12 induced high levels of CTL activity in mice with the chronic form of the disease (Fig. 7.10b), but suppressed those observed in acute GvHD (Fig. 7.10a). This may have been because the peak of CTL activity had already passed in the highly aggressive GvHD which occurred in IL-12 treated B6 $\Rightarrow$ BDF_1 mice (Fig. 7.10a). No lysis of P815 or EL4 cells was induced in control mice by IL-12 (Figs. 7.10a & b).

vi) IL-12 production during acute and chronic GvHD

In view of evidence that manipulating the levels of endogenous and exogenous IL-12 modified the course of GvHD, I examined whether the different outcomes of acute and chronic GvHD were reflected by differences in IL-12 production during the critical early period of disease.

No IL-12 was detected in unstimulated spleen cell cultures of any group at any time (data not shown), while control cells produced consistently low amounts in response to LPS throughout the study (Fig. 7.11). However, splenocytes from B6 ⇒ BDF_1 mice produced significantly more IL-12 in response to LPS than control splenocytes from day 1 of the GvHD, reaching a peak on day 7 of the disease (Fig. 7.11). Splenocytes from DBA/2 ⇒ BDF_1 mice also produced significantly higher levels of LPS stimulated IL-12 than controls at all time points examined, but these levels remained similar at all times after day 1 and did not rise to the same peak observed with B6 ⇒ BDF_1 splenocytes (Fig. 7.11).

Summary and Conclusions

Thus, administration of exogenous IL-12 converts chronic GvHD into a more acute type of disease, with a characteristic biphasic pattern of early hyperplasia followed by late immunosuppression and destruction. In addition to increasing the early splenomegaly and spontaneous lymphocyte proliferation normally observed in DBA/2 ⇒ BDF_1 mice up to levels similar to those found in the early period of acute GvHD, exogenous IL-12 also provoked intense immunosuppression, anti-host CTL activity, weight loss and mortality, features not normally associated with this form of disease. Furthermore, administering IL-12 to these mice converted the cytokine profile towards the Th1 pattern usually seen acute GvHD, with strikingly high levels of IFN-γ and reduced IL-10 production. Finally, my results show that although cells from both B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice produced similarly enhanced IL-12 up to day 2, B6 ⇒ BDF_1 splenocytes produced significantly higher IL-12 than DBA/2 ⇒ BDF_1 cells thereafter.

These results confirm the findings of Chapters 4, 5 & 6 by showing that IL-12 is critical for the development of acute, but not chronic GvHD. However, despite the apparent IL-12 independence of chronic GvHD, enhanced levels of IL-

12 were produced by spleen cells from DBA/2 ⇒ BDF_1 mice in response to LPS *in vitro*, albeit at lower levels than those produced by B6 ⇒ BDF_1 cells.

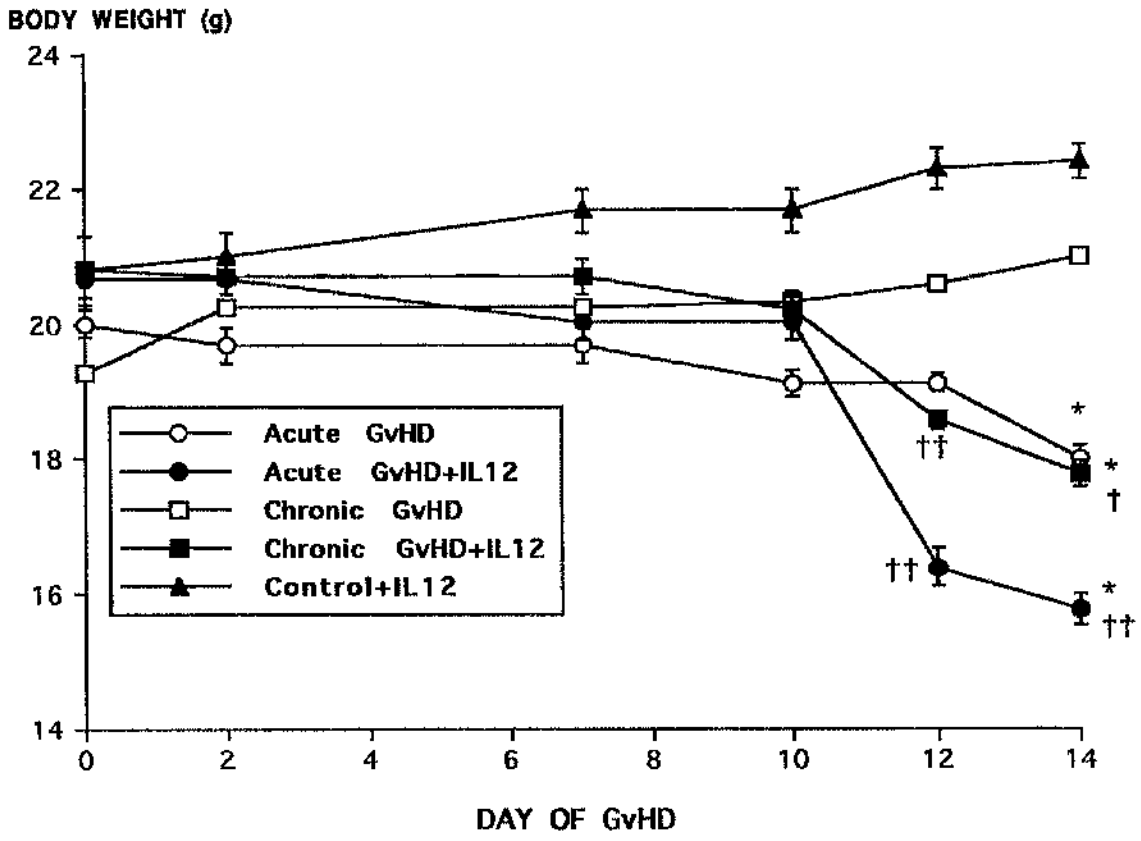


Fig. 7.1. Effects of exogenous IL-12 on weight loss and mortality in acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on weight loss and mortality in BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. and are the mean body weight ± 1 SEM for 6 mice per group. The weights of unmodified control mice were similar to IL-12 treated controls at all times and are not shown for clarity. (* $p < 0.05$ vs controls; † Death).

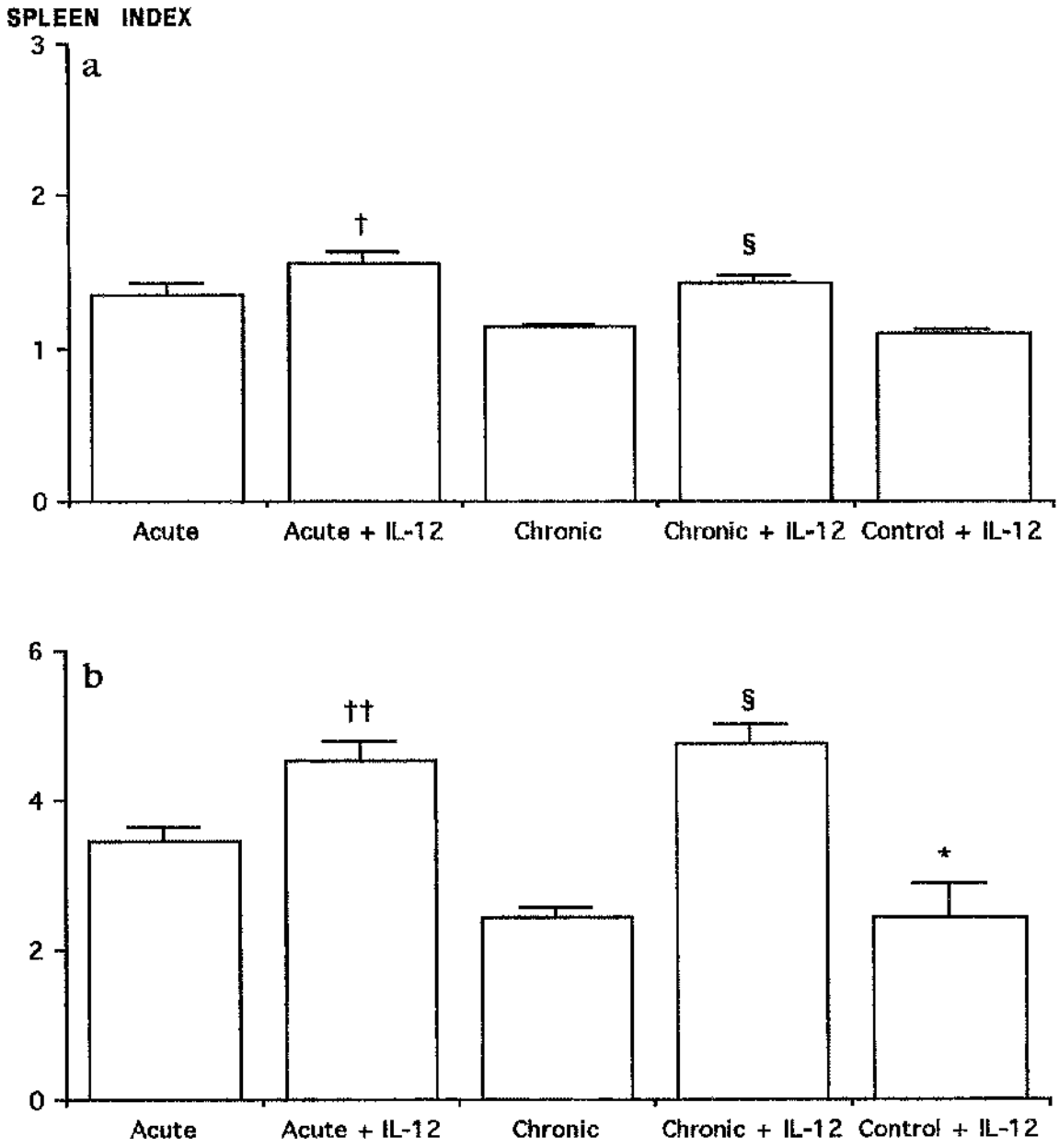


Fig. 7.2. Effects of exogenous IL-12 on splenomegaly during acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on splenomegaly in BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. and are the mean spleen indices ± 1 SD for 3 mice per group on days 1 (Fig. 7.2a) and 10 (Fig. 7.2b) of the GvHD. (* $p < 0.005$ vs controls; † $p < 0.05$ †† $p < 0.001$ vs unmodified acute GvHD; § $p < 0.001$ vs unmodified chronic GvHD).

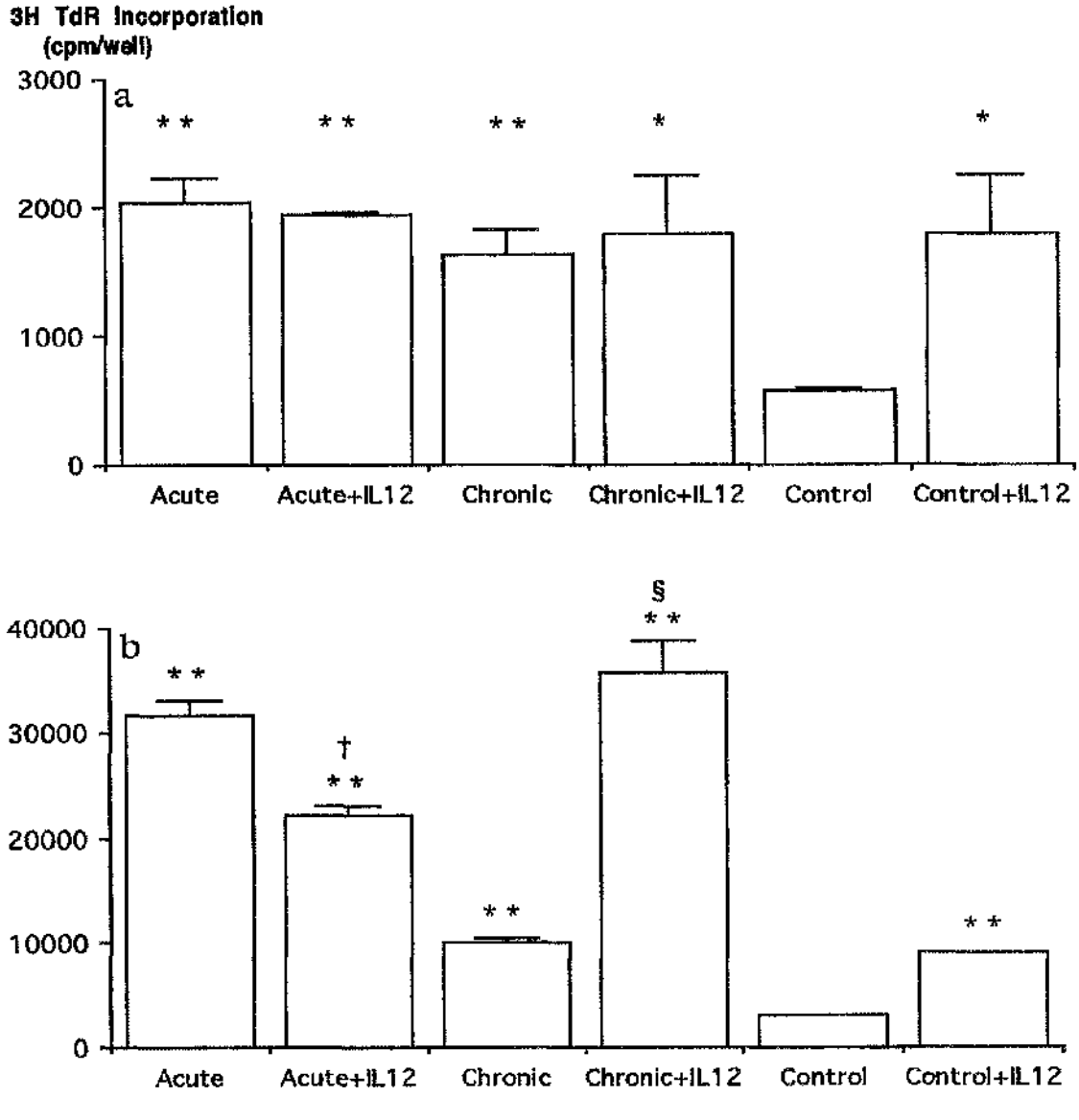


Fig. 7.3. Effects of exogenous IL-12 on spontaneous *'ex-vivo'* proliferation during acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on the spontaneous proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. The results are expressed as the mean uptake of 3H-TdR ± 1 SD after 4 hours in quadruplicate cultures using spleen cells pooled from 3 mice per group on days 1 (Fig. 7.3a) and 10 (Fig. 7.3b) of the GvHD. (* $p < 0.05$ ** $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD; § $P < 0.001$ vs unmodified chronic GvHD).

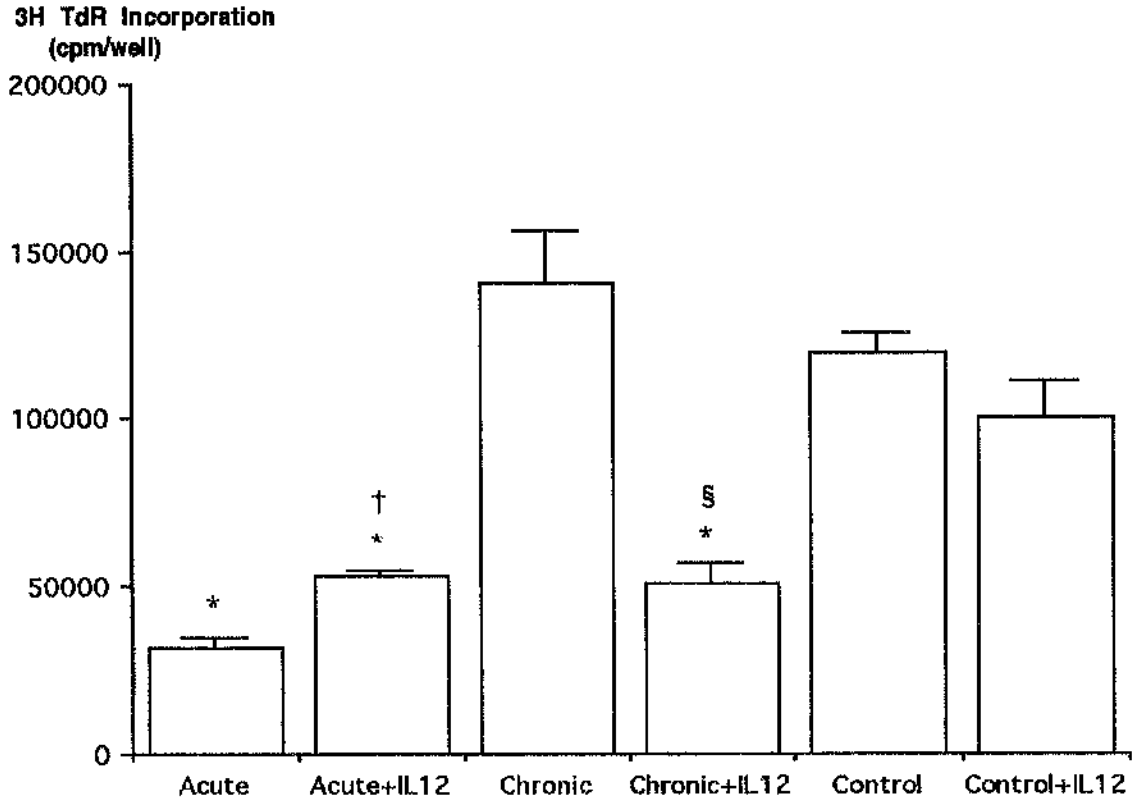


Fig. 7.4. Effects of exogenous IL-12 on Con A induced proliferation during acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on the proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. in response to mitogenic stimulation with 10μg/ml Con A. The data are expressed as the mean uptake of 3H-TdR ± 1 SD after 48 hours in quadruplicate cultures, using spleen cells pooled from 3-4 mice per group on day 15 of the GvHD. (* $p < 0.001$ vs controls; † $p < 0.005$ vs unmodified acute GvHD; § $p < 0.001$ vs unmodified chronic GvHD).

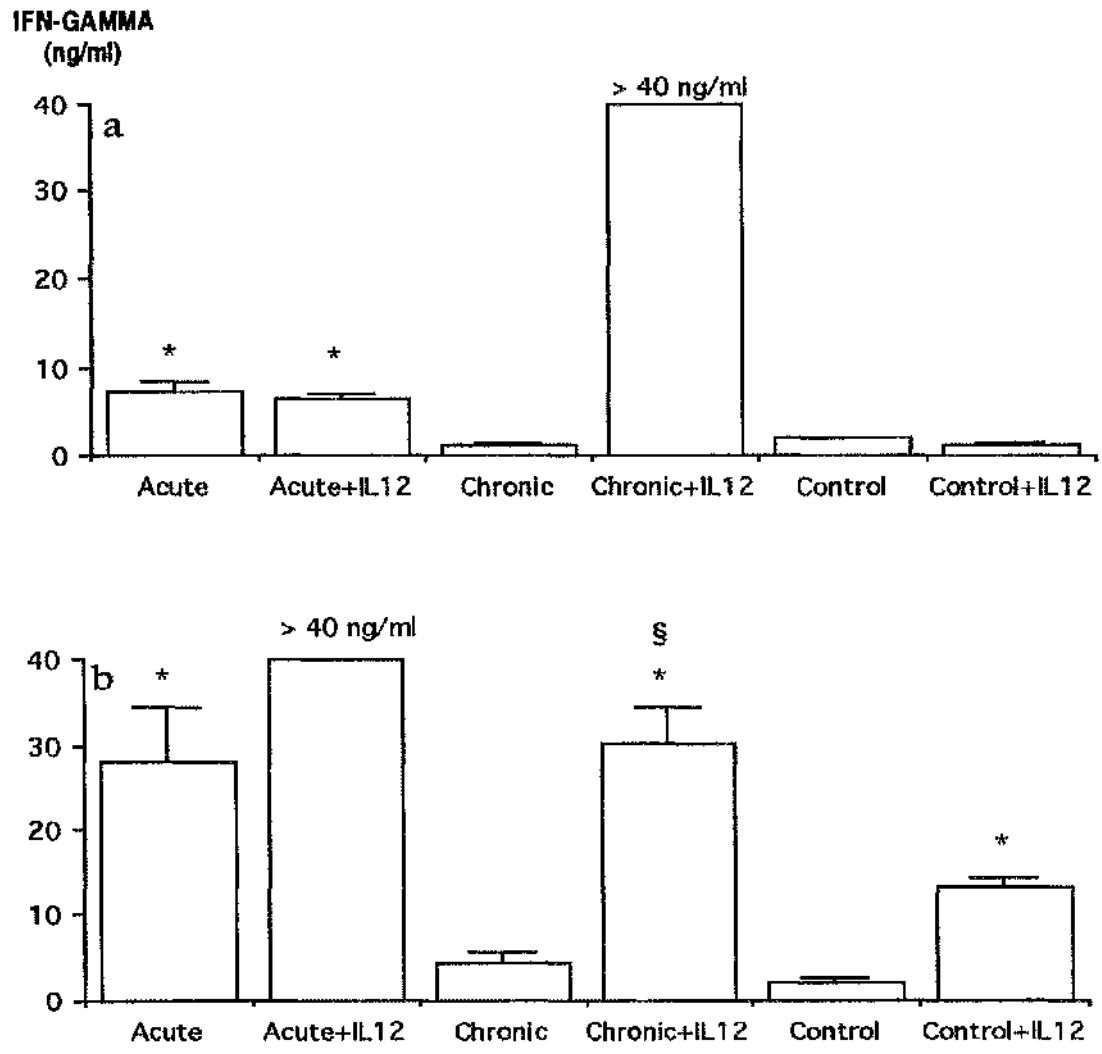


Fig. 7.5. Effects of exogenous IL-12 on spontaneous IFN-γ production during acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on spontaneous IFN-γ production by splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice were cultured in medium for 48 hours and the supernatants assayed for the presence of IFN-γ by ELISA. The results shown are the means ± SD of triplicate samples on days 1 (Fig. 7.5a) and 10 (Fig. 7.5b) of the GvHD. (* $p < 0.001$ vs controls; § $p < 0.001$ vs unmodified chronic GvHD).

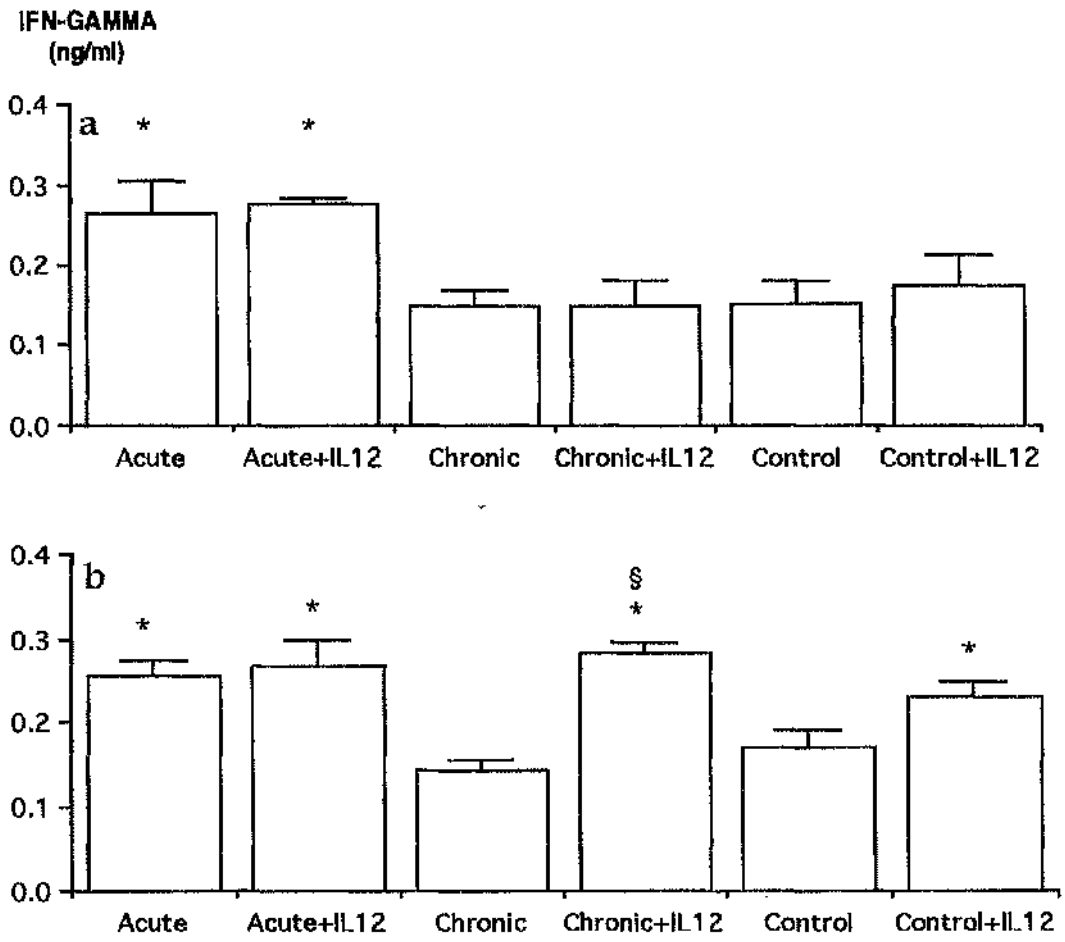


Fig. 7.6. Effects of exogenous IL-12 on Con A induced IFN-γ production during acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on IFN-γ production by Con A stimulated splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells *i.v.* Cells from B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice were cultured with 10μg/ml Con A for 48 hours and the supernatants assayed for the presence of IFN-γ by ELISA. The results shown are the means ± SD of triplicate samples on days 1 (Fig. 7.6a) and 10 (Fig. 7.6b) of the GvHD. (* $p < 0.005$ vs controls; § $p < 0.001$ vs unmodified chronic GvHD). **N.B.** Con A induced IFN-γ production is expressed as OD units at 405nm, since the levels for all groups were greater than the highest standard on the standard curve (40ng/ml).

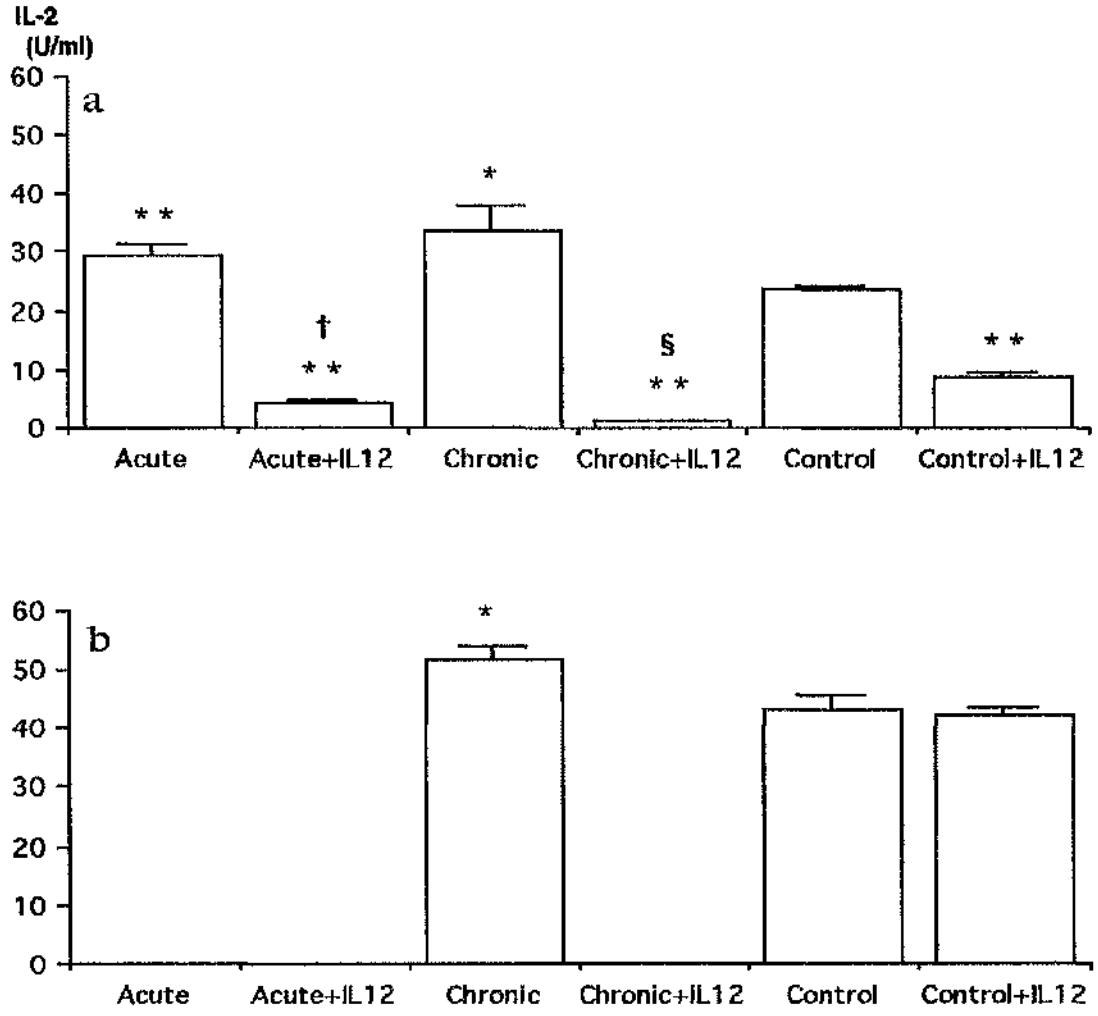


Fig. 7.7. Effects of exogenous IL-12 on IL-2 production during acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on Con A induced IL-2 production by cells from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice were cultured with 10μg/ml Con A for 24 hours and the supernatants were assayed for the presence of IL-2 by ELISA. The results shown are the means ± SD of triplicate samples on days 1 (Fig. 7.7a) and 10 (Fig. 7.7b) of the GvHD. (* $p < 0.05$ ** $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD; § $p < 0.001$ vs unmodified chronic GvHD).

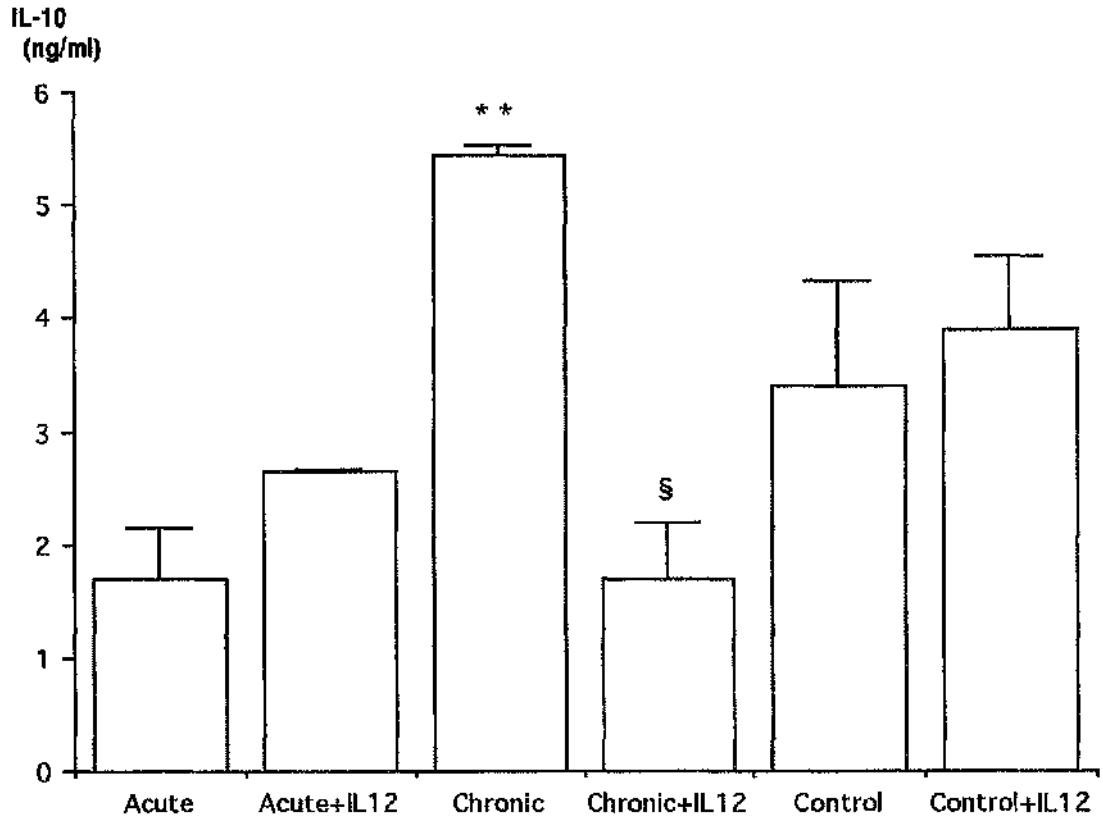


Fig. 7.8. Effects of exogenous IL-12 administration on IL-10 production during acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on Con A induced IL-10 production by splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. Splenocytes from B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice were cultured with 10μg/ml Con A for 48 hours and the supernatants assayed for the presence of IL-10 by ELISA. The results shown are the means ± SD of triplicate samples on day 10 of the GvHD. (* $p < 0.001$ vs controls; † $p < 0.01$ vs unmodified acute GvHD; § $p < 0.001$ vs unmodified chronic GvHD).

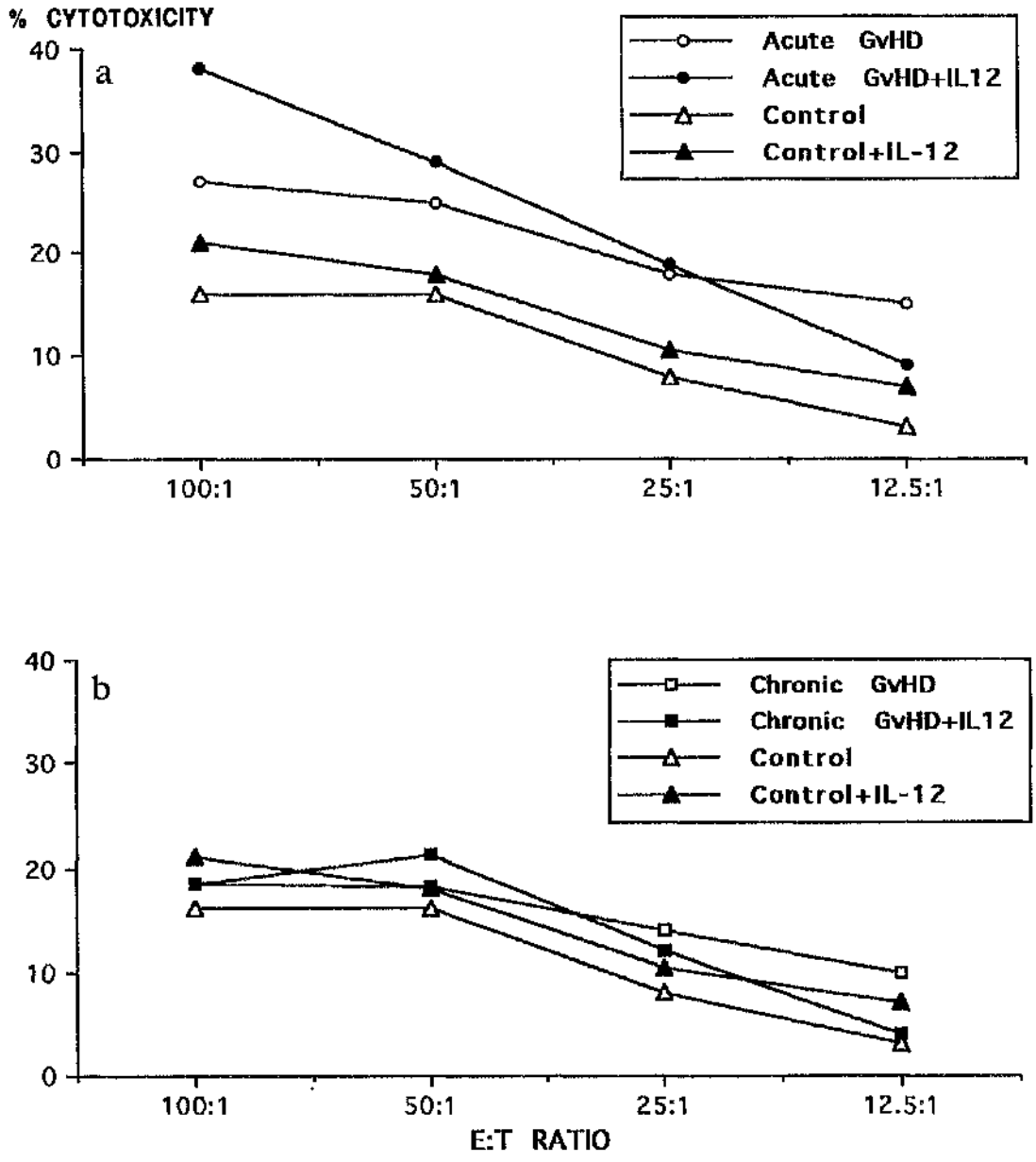


Fig. 7.9. Effect of exogenous IL-12 on NK cell activity during acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on splenic NK cell activity during acute GvHD in BDF_1 mice given 10^8 B6 spleen cells i.v. (Fig. 7.9a) and chronic GvHD in BDF_1 mice given 10^8 DBA/2 spleen cells i.v. (Fig. 7.9b). The results shown are the % cytotoxicity against YAC-1 target cells from quadruplicate assays measured at effector : target (E:T) ratios from 100:1 to 12.5:1, using spleen cells pooled from 3 mice per group on day 1 of the GvHD.

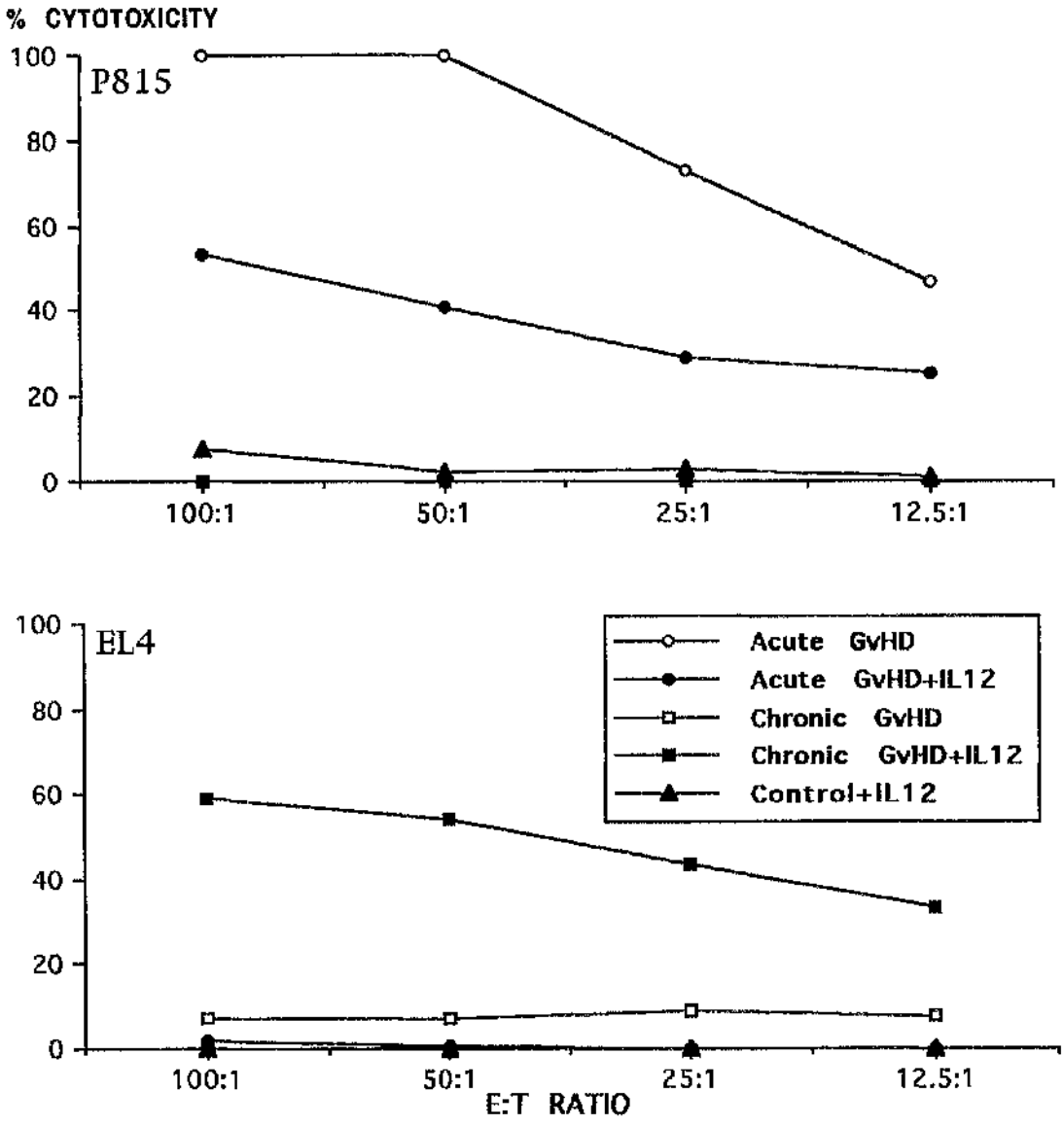


Fig. 7.10. Effect of exogenous IL-12 on specific CTL activity during acute and chronic GvHD.

The results show the effect of administering exogenous rm IL-12 on splenic CTL activity during acute and chronic GvHD in BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. The results shown are the % cytotoxicity against EL-4 ($H\text{-}2^b$) and P815 ($H\text{-}2^d$) target cells from quadruplicate assays measured at effector : target (E:T) ratios from 100:1 to 12.5:1, using spleen cells pooled from 3 mice per group on day 10 of the GvHD.

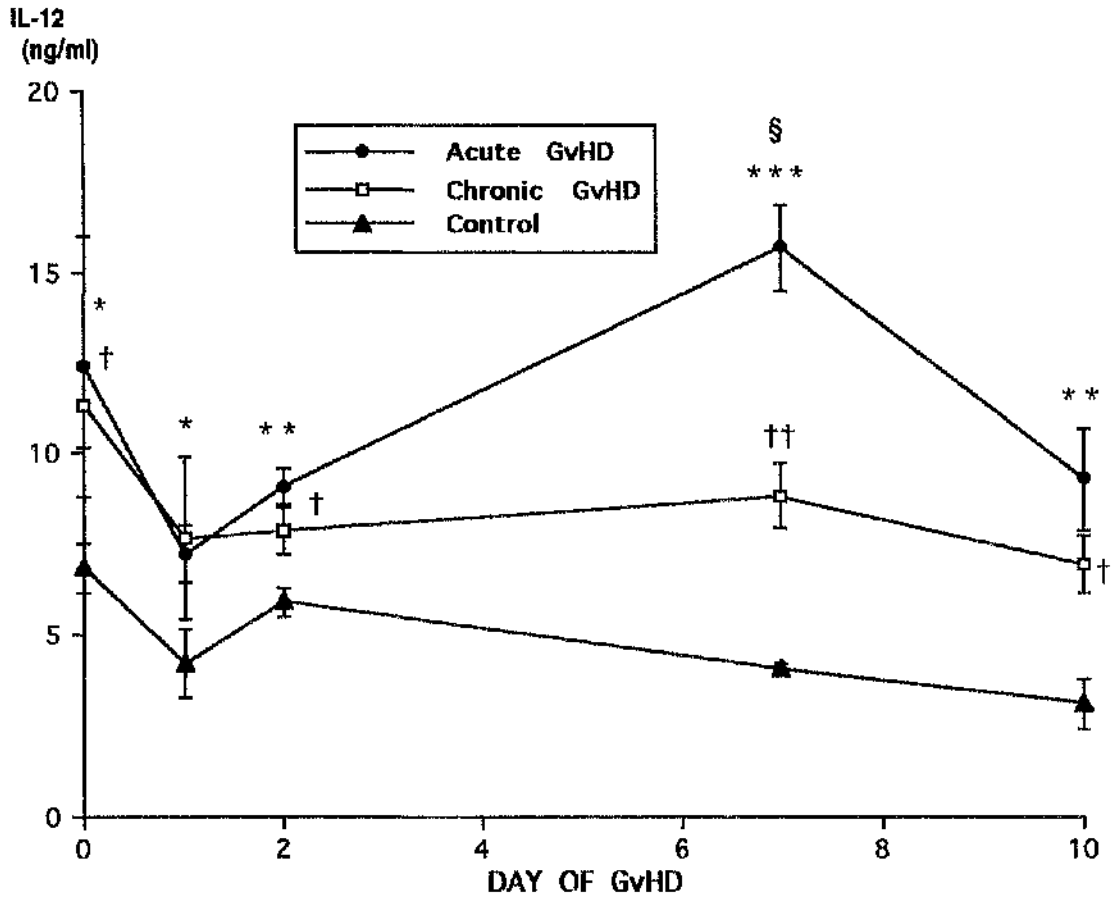


Fig. 7.11. IL-12 production during acute and chronic GvHD.

Splenocytes from BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v. were cultured in the presence of 10μg/ml LPS for 48 hours, before the supernatants were removed and assayed for the presence of IL-12 by ELISA. Results shown are means ± 1 SD of triplicate samples. Day 0 represents IL-12 production by GvHD splenocytes harvested 4 hours after disease induction. (* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ acute GvHD vs controls; † $p < 0.05$ †† $p < 0.001$ chronic GvHD vs controls; § $p < 0.001$ acute GvHD vs chronic GvHD).

CHAPTER 8

THE ROLE OF IFN-γ IN IL-12 DEPENDENT ACUTE GvHD.

Introduction

In Chapters 4-7, I demonstrated that IL-12 is critical for the development of acute GvHD. IL-12 has a variety of potent effects on cells of the lymphohaemopoietic system, but its principal immunomodulatory role is to induce IFN-γ production by $CD4^+$ and $CD8^+$ T cells and NK cells [118, 120, 121, 123, 147, 150, 151]. A number of aspects of my studies suggest that this may be the role of IL-12 in acute GvHD. IFN-γ production is enhanced very early during the acute GvHD and its kinetics follow a similar pattern to those of IL-12. Furthermore, the beneficial effects of IL-12 depletion in acute GvHD were associated with down-regulated production of IFN-γ, while the acute GvHD-like syndrome which appeared in DBA/2 ⇒ BDF_1 mice given exogenous IL-12 was characterised by high levels of IFN-γ. That modulation of IFN-γ production could account for the beneficial effects of anti-IL-12 is suggested by previous studies which demonstrated that neutralisation of IFN-γ *in vivo* prevented immunopathology in the small intestine of B6 ⇒ BDF_1 mice [55] and by the involvement of IFN-γ in GvHD-associated immunosuppression [71-73].

In this chapter, I therefore examined directly the role of IFN-γ in acute GvHD, both in unmanipulated B6 ⇒ BDF_1 mice and in DBA/2 ⇒ BDF_1 mice given IL-12, using the parameters which I had characterised in previous chapters.

Experimental Protocol

The GvHR was induced by i.v. injection of 10^8 viable B6 or DBA/2 parental spleen cells into BDF_1 recipients, as before. Endogenous IFN-γ was neutralised in B6 ⇒ BDF_1 mice *in vivo* by i.p. injection of 0.5mg H22 mAb

(hamster IgG anti-mouse IFN-γ), given 1 day before induction of GvHR and subsequently on days 3 and 7.

To induce acute GvHD in DBA/2 ⇒ BDF_1 mice, animals received 100ng of exogenous IL-12 on days -1 to 3 and 6 to 10, as described in Chapter 4. The effects of anti-IFN-γ on this disease were examined by coadministration of 0.5mg H22 anti-IFN-γ mAb on days -2, 1, 5 and 8. In all cases, controls received 0.5 mg of hamster IgG isotype control antibody.

The effect of the antibody on systemic GvHD was monitored by assessing body weight, splenomegaly and mortality as before, while lymphocyte proliferation, cytokine production, NK and CTL activity were determined as descibed in Chapters 3-7.

Results

1) Effects of Neutralising IFN-γ on Acute GvHD in Unmodified B6 ⇒ BDF_1 Mice.

i) Weight Loss and mortality

Unmodified B6 ⇒ BDF_1 mice displayed significant weight loss from day 18 of the GvHD, which continued until the study was terminated on day 22 (Fig.8.1). One of these mice died on day 18 and a further two deaths occurred on day 20. (The remaining 3 mice, which had all lost weight, were sacrificed on day 22 for use in functional studies). In contrast, B6 ⇒ BDF_1 mice which received anti-IFN-γ antibody showed no significant weight loss or mortality (Fig. 8.1).

ii) Splenomegaly

Unmodified B6 ⇒ BDF_1 mice showed the characteristic pattern of splenomegaly described in Chapter 3. It was evident on day 2, peaked on day 10 and returned to control levels by day 22 (Fig. 8.2). Anti-IFN-γ antibody had no

effect on the splenomegaly observed on day 2, but significantly reduced the splenomegaly observed on day 10 (Fig. 8.2). Interestingly, splenomegaly was maintained in anti-IFN-γ treated B6 ⇒ BDF_1 mice on day 22 and did not show the atrophy usually seen in untreated mice with acute GvHD.

iii) Immune Function

a) Spontaneous '*ex-vivo*' proliferation

Spleen cells from unmodified B6 ⇒ BDF_1 mice showed enhanced levels of spontaneous '*ex-vivo*' proliferation compared with control cells on days 2 and 10 of GvHD (Fig. 8.3). This paralleled the splenomegaly, as did the return to control levels by day 22.

On day 2, cells from anti-IFN-γ treated B6 ⇒ BDF_1 mice showed significantly increased levels of spontaneous proliferation compared with cells from unmodified B6 ⇒ BDF_1 mice (Fig. 8.3). Although similar levels of proliferation were observed in both groups on day 10, the proliferative capacity of cells from anti-IFN-γ treated mice continued to be greatly increased on day 22, when these cells exhibited levels of proliferation several-fold higher than those of any other group (Fig. 8.3).

Anti-IFN-γ treatment increased the spontaneous'*ex-vivo*' proliferative capacity of control spleen cells on days 2 and 10, but by day 22, the spontaneous proliferative capacity of cells from anti-IFN-γ treated and unmodified control mice were similar (Fig. 8.3).

b) Mitogen induced proliferative responses

To examine the effects of anti-IFN-γ treatment on immunosuppression in acute GvHD, I examined the responses of spleen cells to Con A and LPS on day 22 of disease.

As expected, splenocytes from unmodified B6 ⇒ BDF_1 mice showed significantly reduced proliferative responses to Con A (Fig. 8.4) and LPS (Fig.

8.5) compared with controls. Cells from anti-IFN-γ treated B6 ⇒ BDF_1 mice showed a significantly increased ability to respond to both Con A (Fig. 8.4) and LPS (Fig. 8.5) compared with cells from unmodified B6 ⇒ BDF_1 animals, although in both cases, these responses were still significantly suppressed compared with controls. Anti-IFN-γ had no effect on the Con A or LPS responses of control mice (Figs. 8.4 & 8.5).

iv) Cytokine Production

IFN-γ

Splenocytes from unmodified B6 ⇒ BDF_1 mice produced IFN-γ spontaneously on both days 2 (Fig. 8.6a) and 10 (Fig. 8.6b) of acute GvHD. Neutralisation of IFN-γ caused a significant increase in the levels of spontaneous IFN-γ production in B6 ⇒ BDF_1 mice on day 2, but had no effect on this parameter on day 10. No IFN-γ could be detected in unstimulated control cell cultures on either day 2 or day 10 and anti-IFN-γ treatment did not affect this.

IFN-γ production in response to Con A followed a slightly different pattern. On day 2, the levels produced by cells from unmodified B6 ⇒ BDF_1 mice were similar to those produced by control cells (Fig. 8.6a), but by day 10, IFN-γ production was significantly enhanced above control levels (Fig. 8.6b). Treatment with anti-IFN-γ *in vivo* significantly increased the levels of IFN-γ produced by cells from B6 ⇒ BDF_1 mice in response to Con A *in vitro* on day 2 (Fig. 8.6a), but did not affect those observed on day 10 (Fig. 8.6b). Neutralisation of IFN-γ significantly increased Con A induced IFN-γ production by control cells on both days 2 and 10 (Fig. 8.6a and b).

IL-2

Splenocytes from both unmodified and anti-IFN-γ treated B6 ⇒ BDF_1 mice produced IL-2 spontaneously on day 2 of GvHD (Fig. 8.7a). This was in contrast to my previous experiments and was not found at later times in GvHD.

IL-2 production in response to Con A was normal in unmanipulated acute GvHD spleen cells on day 2 (Fig. 8.7a), but was then considerably reduced compared with control cells (Figs. 8.7b & c). A similar pattern was seen with anti-IFN-γ treated cells, with normal IL-2 production on day 2 (Fig. 8.7a) and suppression on days 10 (Fig. 8.7b) and 22 (Fig. 8.7c). However, on day 22, the antibody treated group produced significantly higher amounts of IL-2 than cells from unmodified B6 ⇒ BDF_1 mice (Fig. 8.7c).

Anti-IFN-γ treatment significantly increased the levels of IL-2 produced by Con A stimulated control cells on days 2 and 10 (Figs. 8.7a and b), but on day 22, cells from both unmodified and antibody treated control mice produced similar levels of IL-2 (Fig. 8.7c).

IL-5

High levels of IL-5 were produced spontaneously by cells from anti-IFN-γ treated B6 ⇒ BDF_1 mice on day 2 (Fig. 8.8a), but this was transient and was not observed at any other time or with cells from any other group.

On day 2, cells from unmodified B6 ⇒ BDF_1 mice produced similar levels of IL-5 in response to Con A as control cells (Fig. 8.8a), while on day 10, IL-5 production by cells from both unmodified B6 ⇒ BDF_1 and control mice was below the level of detection (Fig. 8.8b). Anti-IFN-γ treatment significantly increased the levels of IL-5 produced by cells from both B6 ⇒ BDF_1 and control mice on days 2 (Fig. 8.8a) and 10 (Fig. 8.8b).

IL-4 and IL-10

IL-4 and IL-10 production by all groups was below the level of detection at all times in this experiment.

v) Non-specific and specifle cell-mediated cytotoxicity

a) NK cell activity

I first attempted to assess whether the enhanced NK cell activity I had observed early in acute GvHD in previous experiments was IFN-γ-dependent. However in this experiment, all groups showed little or no NK cell activity in two separate assays (data not shown). This appeared to be the result of technical problems and time did not permit me to repeat the experiment.

b) CTL activity

As before, spleen cells from unmodified B6 ⇒ BDF_1 mice showed high levels levels of specific anti-host cytotoxicity on day 10 of GvHD and this was only very slightly reduced by anti-IFN-γ treatment (Fig. 8.9).

These experiments indicate that IFN-γ is responsible for many of the characteristic features of acute GvHD in unmanipulated B6 ⇒ BDF_1 mice, including weight loss, mortality, and T cell immunosuppression. The effects of neutralising IFN-γ were therefore very similar to those of neutralising IL-12. I therefore next investigated the role of IFN-γ in the IL-12 induced acute GvHD in DBA/2 ⇒ BDF_1 mice.

2) Effects of Neutralising IFN-γ in IL-12 Induced Acute GvHD in DBA/2 ⇒ BDF_1 mice.

i) Weight loss and mortality

As expected, unmodified BDF_1 mice given DBA/2 spleen cells did not lose weight or die during the early period of the GvHD, whereas the mice given exogenous IL-12 showed significant weight loss from day 10 of the disease (Fig. 8.10). These mice also started to die from this time onward, with one mouse dying on day 10, a further three on day 11 and the last mouse on day 14 (Fig. 8.10). In contrast, DBA/2 ⇒ BDF_1 mice given exogenous IL-12 together with anti-IFN-γ did not lose weight and none of these mice had died up to the time the experiment had to be terminated on day 14 (Fig. 8.10).

Because of the rapid mortality observed in IL-12 treated mice, I was also unable to analyse splenomegaly and lymphocyte function after day 10.

ii) Splenomegaly

DBA/2 ⇒ BDF_1 mice showed significant splenomegaly on days 2 and 10 of the chronic GvHD and, as before, IL-12 significantly increased this splenomegaly (Figs. 8.11a & b). On day 2, this increase in splenomegaly was reduced by neutralising IFN-γ at the same time as administering exogenous IL-12 (Fig. 8.11a). However, on day 10, mice treated this way had a similar degree of splenomegaly as IL-12 treated DBA/2 ⇒ BDF_1 animals (Fig. 8.11a).

Control mice given IL-12 also developed splenomegaly at both time points, but this was not prevented by neutralising IFN-γ *in vivo* (Figs. 8.11a & b).

iii) Immune function

a) Spontaneous 'ex-vivo' proliferation

On day 10, spleen cells from unmodified DBA/2 ⇒ BDF_1 mice showed significantly enhanced spontaneous proliferation compared with controls and as I found previously, IL-12 significantly increased spontaneous proliferation (Fig. 8.12). Administration of anti-IFN-γ resulted in a further significant increase in the spontaneous proliferation by cells from IL-12 treated DBA/2 ⇒ BDF_1 mice (Fig. 8.12).

Exogenous IL-12 also increased the spontaneous proliferative capacity of control spleen cells, but this was not affected by anti-IFN-γ antibody (Fig. 8.12).

b) Proliferative responses to Con A stimulation

T cell function was assessed on day 10 by determining the ability of splenocytes to respond to Con A stimulation. I was unable to examine the LPS responsiveness due to low cell yields from IL-12 treated mice with GvHD.

Unmodified DBA/2 ⇒ BDF_1 mice showed similar levels of Con A induced proliferation to control cells, but cells from IL-12 treated DBA/2 ⇒ BDF_1 mice had dramatically suppressed Con A responses (Fig. 8.13). Neutralisation of IFN-γ had little effect on these responses. IL-12 also significantly reduced the responses of control cells to Con A stimulation and this was not influenced by anti-IFN-γ (Fig. 8.13).

iv) Cytokine production

IFN-γ

On day 2, IFN-γ was not produced spontaneously by cells from any group (data not shown). On day 10, cells from unmodified DBA/2 ⇒ BDF_1 mice also had no spontaneous production of IFN-γ, but this was provoked by administration

of IL-12 (Fig. 8.14a). Neutralisation of IFN-γ significantly reduced, but did not totally ablate this spontaneous IFN-γ production (Fig. 8.14a). Cells from control mice did not spontaneously produce IFN-γ on either day 2 or day 10 irrespective of whether treated with anti-IFN-γ or not (Fig. 8.14a).

On both days 2 and 10, cells from DBA/2 ⇒ BDF_1 mice produced slightly higher levels of IFN-γ in response to Con A than control cells (Fig. 8.14b & c). IL-12 treatment resulted in a dramatic increase in the levels of Con A induced IFN-γ production by cells from DBA/2 ⇒ BDF_1 mice on day 2 (Fig. 8.14b), but did not affect those observed on day 10 (Fig. 8.14c), perhaps because cells from IL-12 treated DBA/2 ⇒ BDF_1 mice were already producing maximal levels of IFN-γ spontaneously and could not be stimulated further with Con A. Neutralising IFN-γ in IL-12 treated DBA/2 ⇒ BDF_1 mice did not significantly affect their IFN-γ production in response to Con A on either day 2 or 10 (Fig. 8.14b &c).

IL-12 treatment significantly increased the levels of Con A stimulated IFN-γ production by control cells on both days 2 (Fig. 8.14b) and 10 (Fig. 8.14c). Neutralising IFN-γ significantly reduced, but did not totally inhibit the ability of exogenous IL-12 to increase IFN-γ production on day 2 (Fig. 8.14b). By day 10, in contrast, anti-IFN-γ had no effect on IL-12 induced IFN-γ production by control cells (Fig. 8.14c).

IL-2

IL-2 was not produced spontaneously by cells from any group on either day 2 or day 10 (data not shown).

On day 2, cells from unmodified DBA/2 ⇒ BDF_1 mice produced significantly higher levels of IL-2 in response to Con A than controls (Fig. 8.15a), but by day 10, GvHD cells had significantly reduced levels of IL-2 compared with controls (Fig. 8.15b). As I did not find this in previous experiments and because these mice had increased spontaneous '*ex-vivo*' proliferative responses (see

above), it is likely that this apparent reduction in IL-2 production may reflect increased IL-2 consumption by the DBA/2 ⇒ BDF_1 cells.

Administration of IL-12 significantly reduced the levels of IL-2 produced by DBA/2 ⇒ BDF_1 mice in response to Con A on day 2 (Fig. 8.15a), and completely ablated IL-2 production on day 10 (Fig. 8.15b). Neutralising IFN-γ prevented the IL-12-induced suppression of IL-2 production on day 2 (Fig. 8.15a), but not on day 10 (Fig. 8.15b).

Cells from IL-12 treated control mice produced significantly higher levels of Con A induced IL-2 on day 2 than cells from unmodified controls (Fig. 8.15a). In contrast, on day 10, the levels of IL-2 produced by cells from IL-12 treated control mice were significantly lower than those produced by cells from unmodified control mice (Fig. 8.15b). Cells from control mice which had received IL-12 together with anti-IFN-γ mAb produced strikingly high levels of IL-2 on day 2, which were significantly higher than those in any other group (Fig. 8.15a). However, this enhancing effect of anti-IFN-γ was not present on day 10 (Fig. 8.15b).

IL-10

IL-10 was not produced spontaneously by cells from any group (data not shown).

Cells from unmodified DBA/2 ⇒ BDF_1 mice produced significantly higher levels of Con A induced IL-10 compared with cells from control mice on both days 2 (Fig. 8.16a) and 10 (Fig. 8.16b). Administration of IL-12 to DBA/2 ⇒ BDF_1 mice significantly reduced IL-10 production on day 2 (Fig. 8.16a) and totally inhibited it on day 10 (Fig. 8.16b). Neutralisation of IFN-γ prevented the ability of IL-12 to reduce IL-10 production by DBA/2 ⇒ BDF_1 spleen cells on day 2 (Fig. 8.16a) and animals treated in this way actually had IL-10 levels significantly higher than unmodified DBA/2 ⇒ BDF_1 mice. However anti-IFN-γ

had no effect on the ability of IL-12 to inhibit IL-10 production on day 10 (Fig. 8.16b).

IL-12 did not affect Con A induced IL-10 production by control cells on either day 2 (Fig. 8.16aa) or day 10 (Fig. 8.16b). As in GvHD mice, neutralisation of IFN-γ significantly increased the levels of IL-10 produced by Con A stimulated, IL-12 treated control cells on day 2 (Fig. 8.16a), but had no significant effect on day 10 (Fig. 8.16b).

IL-5

In this study, high levels of IL-5 were produced spontaneously by cells from unmodified DBA/2 ⇒ BDF_1 mice on day 2 (13.84 ± 2.02 U/ml), but not by cells from any other group (data not shown). Cells from unmodified DBA/2 ⇒ BDF_1 mice also produced significantly higher levels of IL-5 in response to Con A than control cells on day 2 (Fig. 8.17a) and similar levels of IL-5 to control cells on day 10 (Fig. 8.17b). IL-12 treatment totally inhibited IL-5 production by cells from DBA/2 ⇒ BDF_1 mice on both days 2 (Fig. 8.17a) and 10 (Fig. 8.17b). Concomitant administration of anti-IFN-γ mAb restored the ability of cells from DBA/2 ⇒ BDF_1 mice to produce high levels of IL-5 on day 2 (Fig. 8.17a) and partially restored IL-5 levels on day 10 (Fig. 8.17b).

IL-12 significantly increased the levels of IL-5 produced by Con A stimulated control cells on day 2 (Fig. 8.17a), but not day 10 (Fig. 8.17b). Administration of anti-IFN-γ significantly increased IL-5 production by cells from IL-12 treated controls on both days 2 and 10.

IL-4

IL-4 production was not detectable in this experiment.

Thus, as I found previously, exogenous IL-12 converts the pattern of cytokines produced by spleen cells from DBA/2 ⇒ BDF_1 mice from a Th2

phenotype to a more Th1-dominated response and induces immunosppression. These effects are at least partially dependent on IFN-γ.

v) Non-specific and specific cell-mediated cytotoxicity

a) NK cell activity

As in the experiments described earlier in this chapter, I was unable to assess NK cell activity in this study, due to technical problems.

b) CTL activity

As before, there was virtually no anti-host CTL activity detectable in the spleens of DBA/2 ⇒ BDF_1 mice on day 10 (Fig. 8.18). In contrast, similar mice given exogenous IL-12 showed specific cytotoxicity against EL-4 target cells at all effector : target (E:T) ratios examined (Fig. 8.18). DBA/2 ⇒ BDF_1 mice which had received both IL-12 and anti-IFN-γ mAb showed even higher levels of specific cytotoxicity than mice which received IL-12 alone (Fig. 8.18). Thus, the CTL activity in this model of acute GvHD is not dependent on endogenous IFN-γ production.

vi) Effects of Neutralising IFN-γ on Long-Term Consequences of GvHD in IL-12 Treated DBA/2 ⇒ BDF_1 Mice.

As neutralising IFN-γ prevented the development of IL-12 induced lethal GvHD in DBA/2 ⇒ BDF_1 mice and restored the levels of Th2 cytokines produced by cells from these mice, I thought it was important to determine whether DBA/2 ⇒ BDF_1 mice given IL-12 and anti-IFN-γ developed classical chronic GvHD. I therefore monitored these mice throughout the following three months, to assess whether they developed lethal chronic GvHD, hypergammaglobulinaemia and anti-ds DNA antibodies. Of course, there were no surviving IL-12 treated DBA/2 ⇒ BDF_1 mice for direct comparison.

As expected, unmodified DBA/2 ⇒ BDF_1 mice developed oedema and started to die on day 80 and by day 90, 50% of this group had died (Fig. 8.19). In contrast, none of the DBA/2 ⇒ BDF_1 mice given IL-12 and anti-IFN-γ mAb displayed oedema or died. This confirms the ability of anti-IFN-γ to protect against the IL-12 induced acute disease and also shows that it does not provoke development of clinical chronic GvHD.

As described previously, unmodified DBA/2 ⇒ BDF_1 mice displayed high levels of total serum IgG on days 30 (Fig. 8.20a) and 60 (Fig. 8.20b) compared with controls. DBA/2 ⇒ BDF_1 mice given both IL-12 and anti-IFN-γ mAb showed mildly elevated serum IgG levels on day 30, but on day 60, the IgG levels in the serum of these mice were not significantly different than control levels (Fig. 8.20b). Furthermore, at both time points, the levels were not as high as those observed in the serum of unmodified DBA/2 ⇒ BDF_1 mice (Fig. 8.20a and b).

Finally, all of the unmodified DBA/2 ⇒ BDF_1 mice had detectable anti-ds DNA antibodies in their serum on both days 30 (Fig. 8.21a) and 60 (Fig. 8.21b). In contrast, anti-ds DNA antibodies were not detectable in the serum of any other group (Fig. 8.21a and b).

In conclusion, therefore, anti-IFN-γ mAb promoted long-term survival from the acute lethal GvHD in DBA/2 ⇒ BDF_1 mice given IL-12, but did not provoke the consequences of classical chronic GvHD, such as progressive oedema and death and high levels of serum IgG and anti-ds DNA antibodies. Thus, it would appear that IL-12 induces acute GvHD in DBA/2 ⇒ BDF_1 mice mainly by eliciting production of high levels of IFN-γ.

Summary

The results of this chapter indicate that IFN-γ plays an integral role in acute GvHD induced by B6 cells and that found in BDF_1 mice given IL-12 and DBA/2 cells. Neutralising IFN-γ during the early period of acute GvHD had very similar effects on both models of disease. Most importantly, anti-IFN-γ prevented GvHD-

associated weight loss and mortality and in the case of the extremely aggressive GvHD in IL-12 treated DBA/2 ⇒ BDF_1 mice, promoted long-term survival. Antibody treatment reduced the degree of splenomegaly observed in both unmodified B6 ⇒ BDF_1 and IL-12 treated DBA/2 ⇒ BDF_1 mice during the proliferative phase of the disease. However, anti-IFN-γ treated B6 ⇒ BDF_1 mice showed prolonged splenomegaly and did not develop the lymphoid atrophy exhibited by their untreated counterparts during the final stages of acute GvHD. In both disease models, anti-IFN-γ antibody also increased the levels of spontaneous '*ex-vivo*' proliferation exhibited by GvHD splenocytes, consistent with the known cytostatic properties of IFN-γ *in vitro*. This was particularly obvious during the late stage of disease, when although the spontaneous responses of untreated B6 ⇒ BDF_1 mice returned to control levels, those of anti-IFN-γ treated similar mice remained several fold higher than controls. Once again, this highlights the ability of anti-IFN-γ treatment to maintain the proliferative features of acute GvHD. Despite these beneficial effects, neutralising endogenous IFN-γ had no effect on the high levels of anti-host CTL activity observed in either B6 ⇒ BDF_1 mice, or DBA/2 ⇒ BDF_1 mice given IL-12.

There were some differences between the effects of anti-IFN-γ in the two models of acute GvHD. Whereas anti-IFN-γ treatment was able to significantly increase Con A induced proliferative responses in unmodified B6 ⇒ BDF_1 mice, it had little effect on the suppressed Con A responses in IL-12 treated DBA/2 ⇒ BDF_1 mice. This may reflect the fact that the disease induced in IL-12 treated DBA/2 ⇒ BDF_1 mice is more aggressive than that observed in unmodified B6 ⇒ BDF_1 animals.

The effect of anti-IFN-γ mAb on cytokine production by acute GvHD spleen cells was more complex. Anti-IFN-γ treatment *in vivo* had little effect on the ability of acute GvHD cells to produce IFN-γ *in vitro*, and, in the case of unmanipulated B6 ⇒ BDF_1 mice, actually increased early (day 2) levels. This may reflect a compensatory mechanism activated in the absence of biologically

active IFN-γ. The effects of anti-IFN-γ mAb on IL-2 production differed slightly between the two models. In B6 ⇒ BDF_1 mice, anti-IFN-γ had no effect on Con A stimulated IL-2 on either day 2 or 10, but restored the suppressed IL-2 levels seen at later times. In contrast, anti-IFN-γ prevented the IL-12 induced reduction in IL-2 levels observed in DBA/2 ⇒ BDF_1 mice on day 2, but had no effect on day 10. The reason for the different effects of anti-IFN-γ mAb in these two models was not clear and the effect on end-stage IL-2 levels was not examined in IL-12 treated DBA/2 ⇒ BDF_1 mice. Anti-IFN-γ had a more pronounced effect on the level of Th2 cytokines produced by acute GvHD splenocytes *in vitro*. In particular, neutralising IFN-γ dramatically increased Con A induced IL-5 production by cells from both acute GvHD groups at all times examined. This suggested that IFN-γ may directly regulate IL-5 production during acute GvHD. IFN-γ may also regulate IL-10 production, since anti-IFN-γ prevented the IL-12 induced reduction in IL-10 levels in DBA/2 ⇒ BDF_1 mice. However, this was only observed on day 2 and not day 10, possibly because antibody treatment was terminated on day 8 and biologically active IFN-γ will presumably be present by day 10.

Taken together, these results show that the effects of neutralising IFN-γ during acute GvHD are broadly similar to those observed in anti-IL-12 treated mice. Both anti-IFN-γ and anti-IL-12 antibodies reduced the early splenomegaly, but prolonged the proliferative period of the disease. Both antibody treatments reduced T cell suppression and had a less marked effect on B cell immune deficiency. In addition, anti-IFN-γ and anti-IL-12 shared the ability to prevent weight loss and mortality, but did not reduce the high levels of anti-host CTL activity associated with the disease. Finally, it is important to note that anti-IFN-γ shares with anti-IL-12 the ability to convert the pattern of cytokine production in acute GvHD mice from a Th1 to a Th2 phenotype. In both cases, however, acquisition of a Th2 dominated response was insufficient to promote classic chronic GvHD with hypergammaglobulinaemia, anti-ds DNA antibodies, oedema and death.

Conclusions

IFN-γ is an important effector cytokine in acute GvHD. In particular, IFN-γ appears to mediate many of the destructive features of the disease, such as weight loss, mortality and suppression of lymphoid responses. However, it also contributes to the inflammatory response which results in splenomegaly and regulates Th2 cytokine production. The high levels of IFN-γ required to elicit the acute GvHD are critically dependent on IL-12 and there are a variety of cell types which may be the source of IL-12 induced IFN-γ production. This issue is explored in more detail in Chapters 9 and 10.

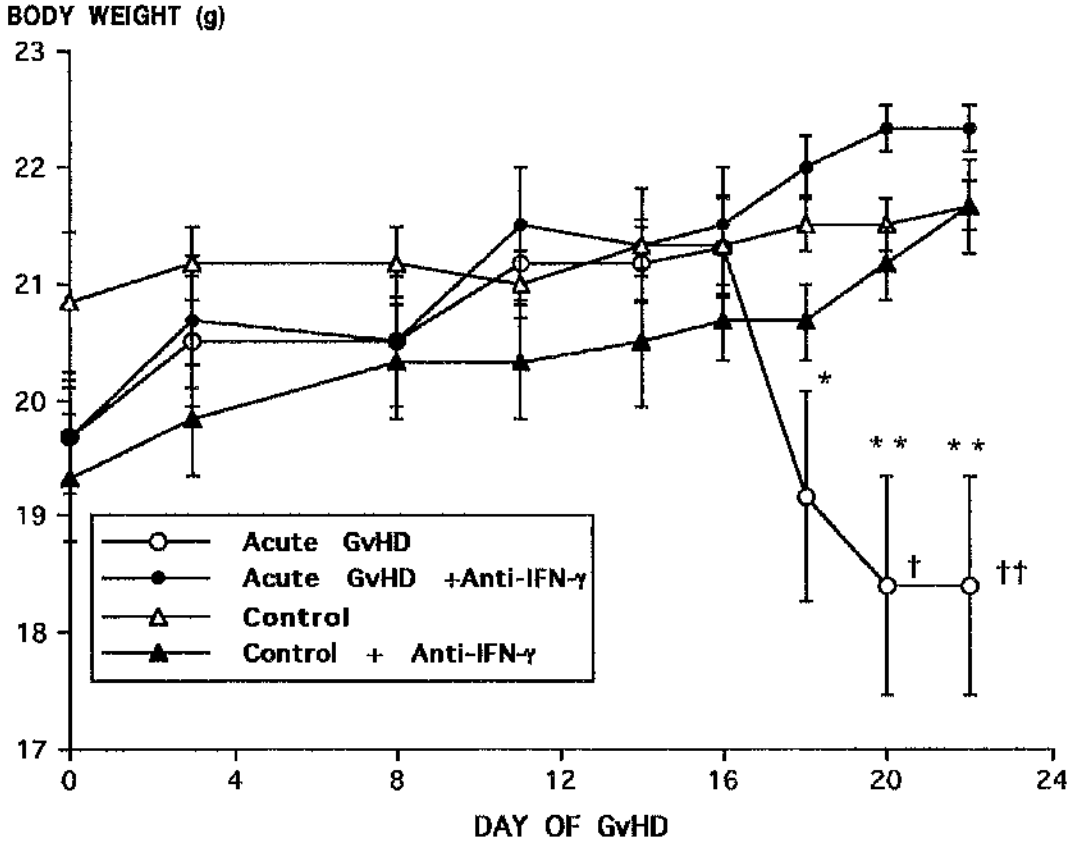


Fig. 8.1. Effects of neutralising IFN-γ on acute GvHD in B6 ⇒ BDF_1 mice.

Weight loss and mortality in BDF_1 mice given 10^8 B6 spleen cells i.v. and treated with anti-IFN-γ mAb. The results shown are mean body weight ± 1 SD for 6 mice per group. (* $p < 0.05$ ** $p < 0.001$ vs all other groups; † Death).

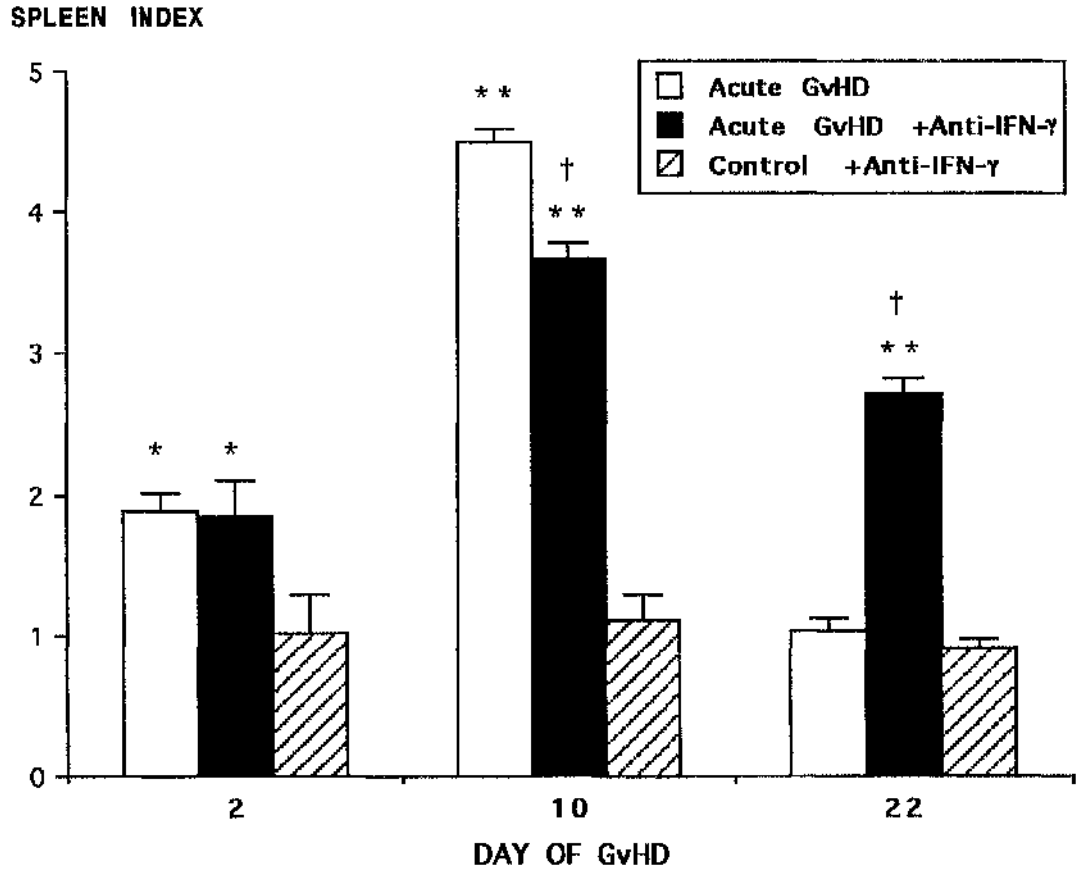


Fig. 8.2. Effects of neutralising IFN-γ on splenomegaly in acute GvHD in B6 ⇒ BDF_1 mice.

Splenomegaly in BDF_1 mice given 10^8 B6 spleen cells i.v. and treated with anti-IFN-γ mAb. The results shown are mean spleen indices ± 1 SD for 3 mice per group. (* $p < 0.05$ ** $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD).

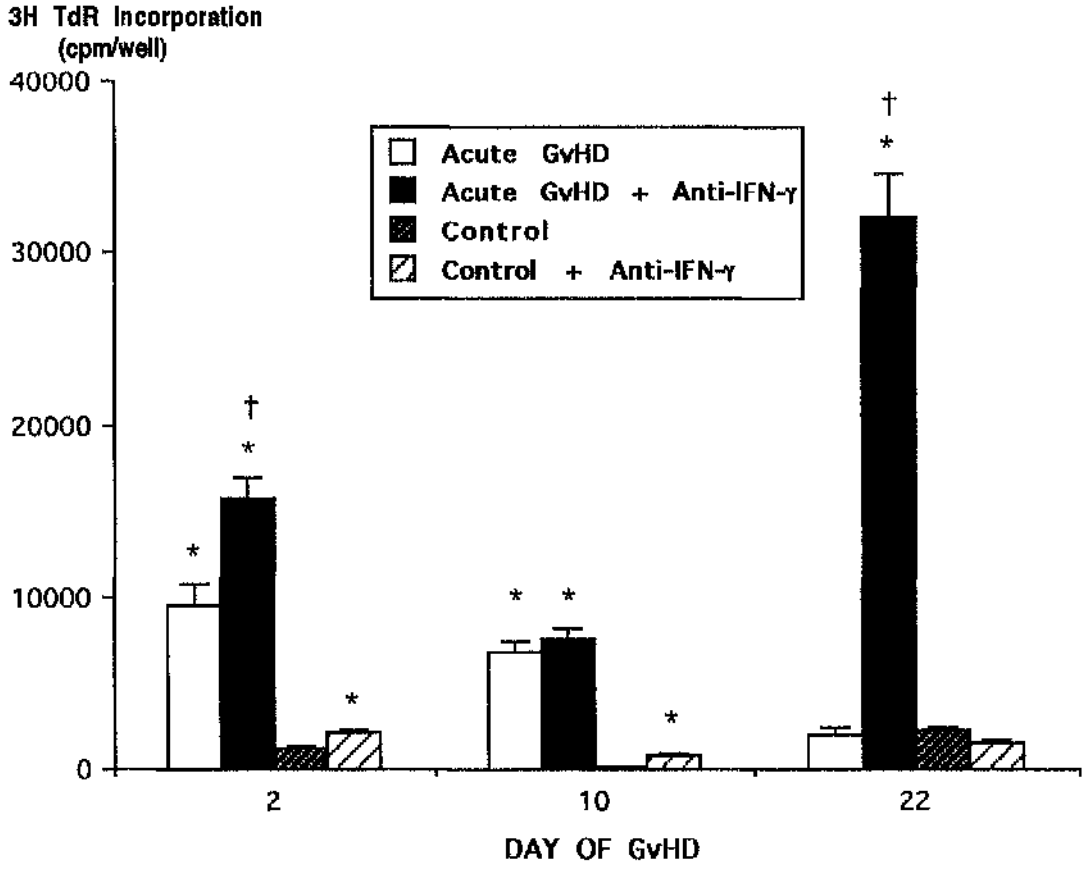


Fig. 8.3. Effects of neutralising IFN-γ on spontaneous *'ex-vivo'* proliferative responses in B6 ⇒ BDF_1 mice.

Spontaneous proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. and treated with anti-IFN-γ mAb. The results shown are the mean uptake of3H-TdR ± 1 SD after 4 hours in quadruplicate cultures, using spleen cells pooled from 3-4 mice per group. (* $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD)

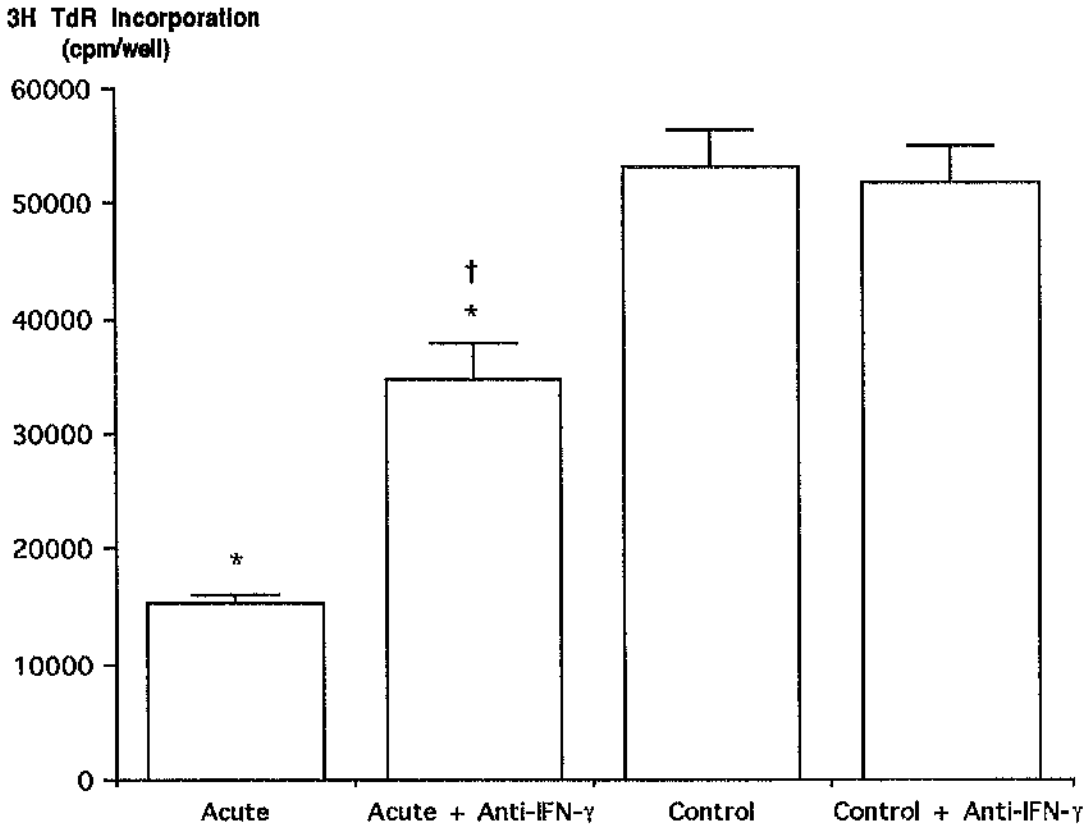


Fig. 8.4. Effects of neutralising IFN-γ on T cell function in B6 ⇒ BDF_1 mice.

Proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. and treated with anti-IFN-γ mAb. The results shown are the mean uptake of 3H-TdR ± 1 SD after 48 hours in quadruplicate cultures with 10μg/ml Con A, using spleen cells pooled from 3-4 mice per group on day 22 of GvHD. (* $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD)

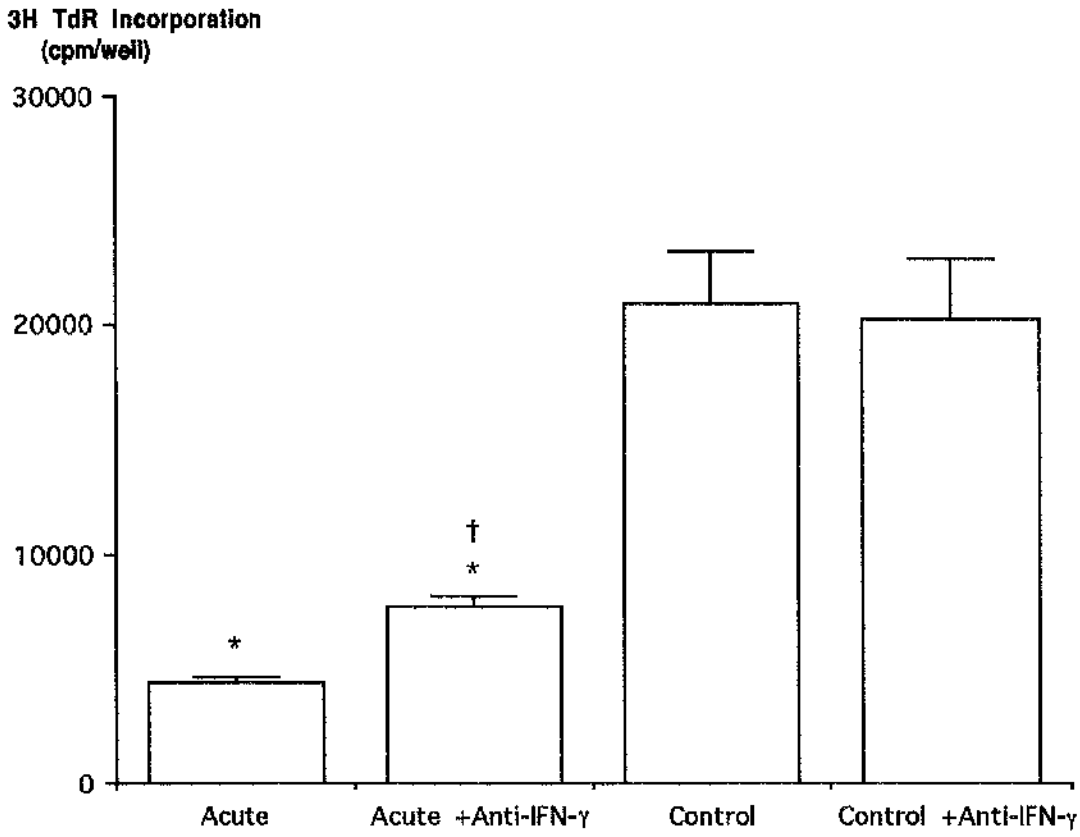


Fig. 8.5. Effects of neutralising IFN-γ on B cell function in B6 ⇒ BDF_1 mice.

Proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. and treated with anti-IFN-γ mAb. The results shown are the mean uptake of 3H-TdR ± 1 SD after 24 hours in quadruplicate cultures with 10μg/ml LPS, using spleen cells pooled from 3-4 mice per group on day 22 of GvHD. (* $p < 0.001$ vs controls; † $p < 0.001$ vs unmodified acute GvHD)

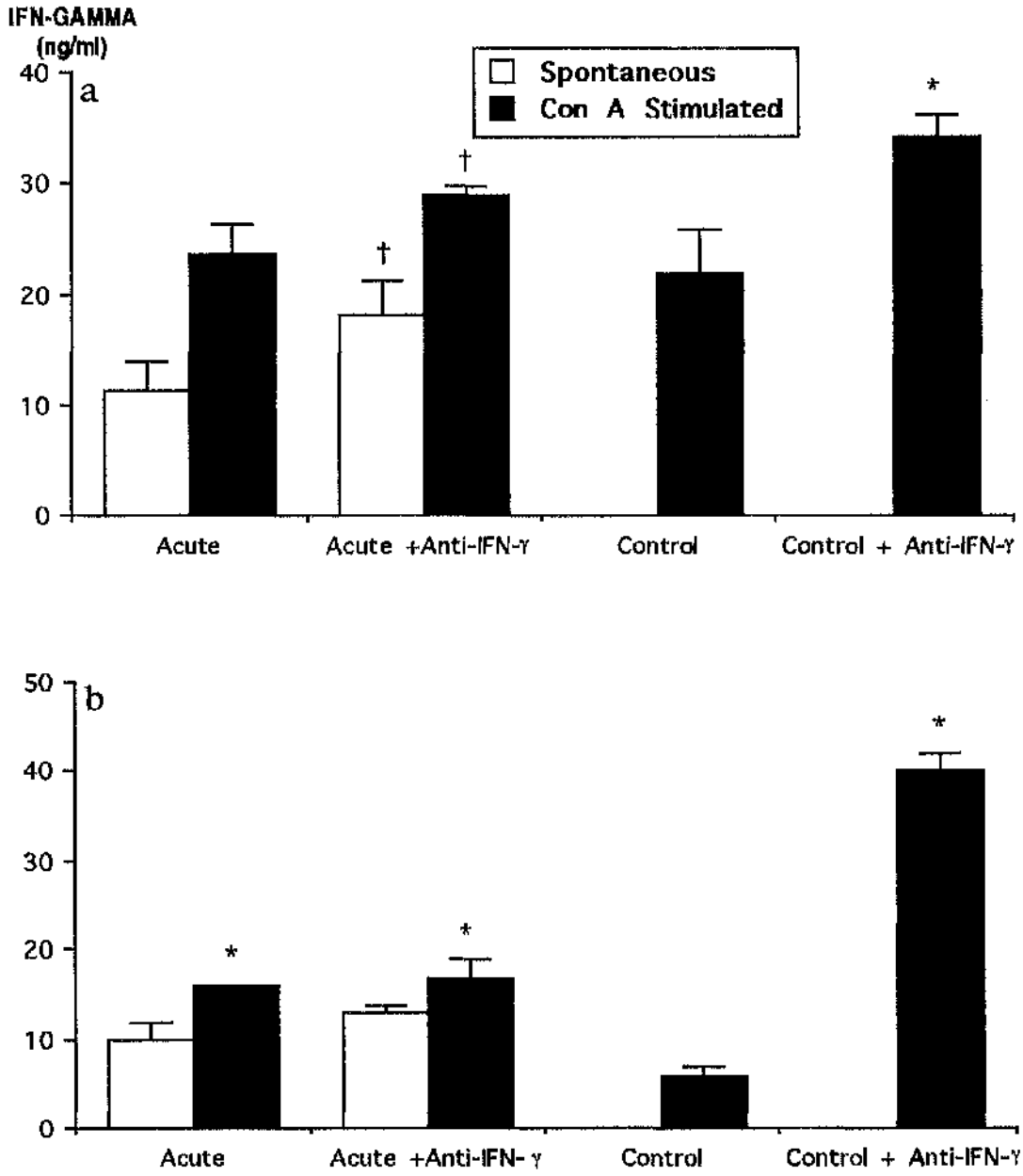


Fig. 8.6. Effects of neutralising IFN-γ on IFN-γ production in acute GvHD in B6 ⇒ BDF_1 mice.

IFN-γ production by splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. and treated with anti-IFN-γ mAb after culture in medium alone, or with 10μg/ml Con A for 48 hours. The results shown are the means ± 1SD of triplicate samples on day 2 (Fig. 8.6a) and day 10 (Fig. 8.6b) of GvHD. (* $p < 0.05$ ** $p < 0.001$ vs unmodified controls; † $p < 0.05$ vs unmodified acute GvHD).

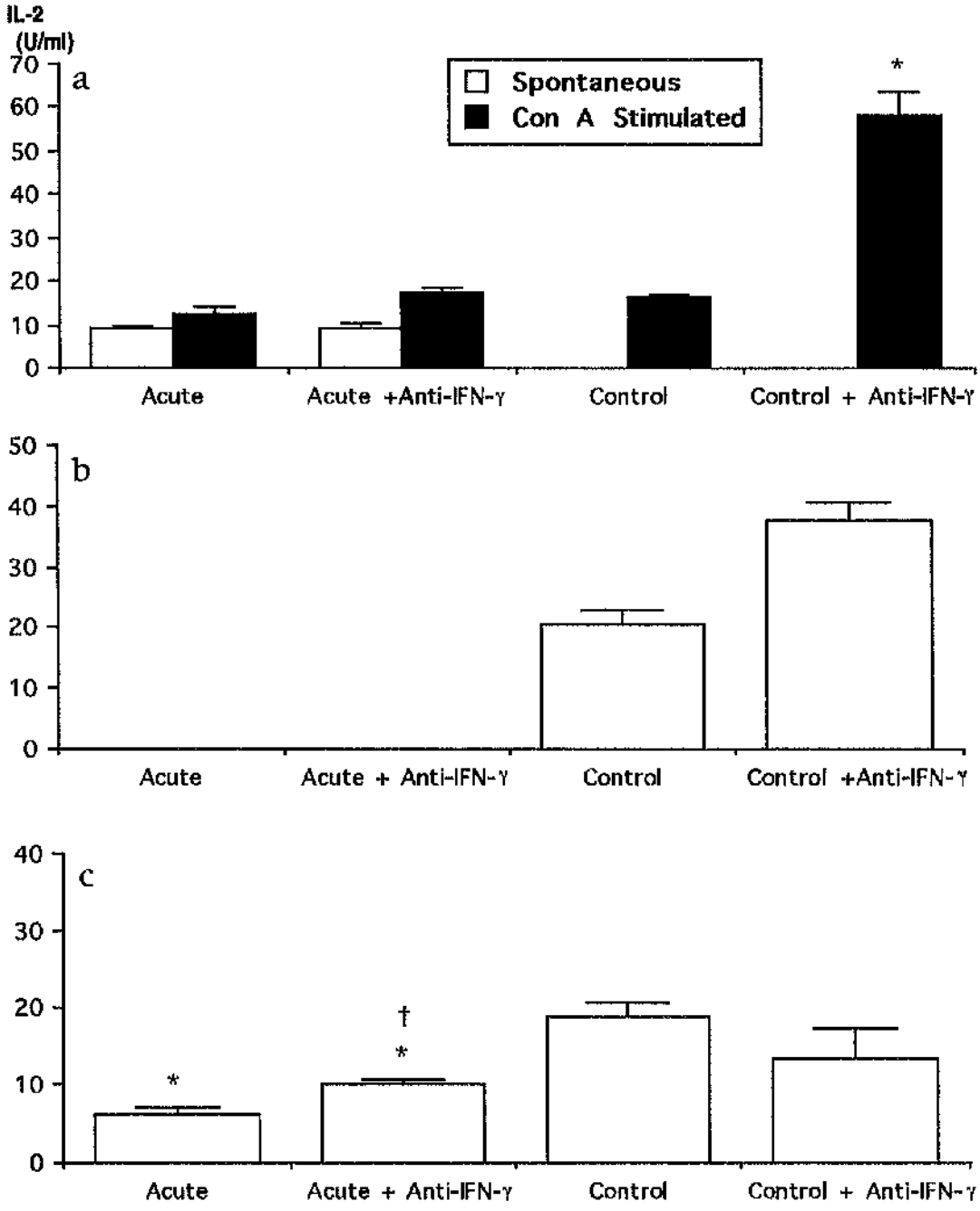


Fig. 8.7. Effects of neutralising IFN-γ on IL-2 production in acute GvHD in B6 ⇒ BDF_1 mice.

IL-2 production by splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. and treated with anti-IFN-γ mAb after culture in medium alone, or with 10μg/ml Con A for 24 hours. The results shown are the means ± 1SD of triplicate samples on day 2 (Fig. 8.7a), day 10 (Fig. 8.7b) and day 22 (Fig. 8.7c) of GvHD. (* $p < 0.001$ vs unmodified controls; † $p < 0.01$ vs unmodified acute GvHD).

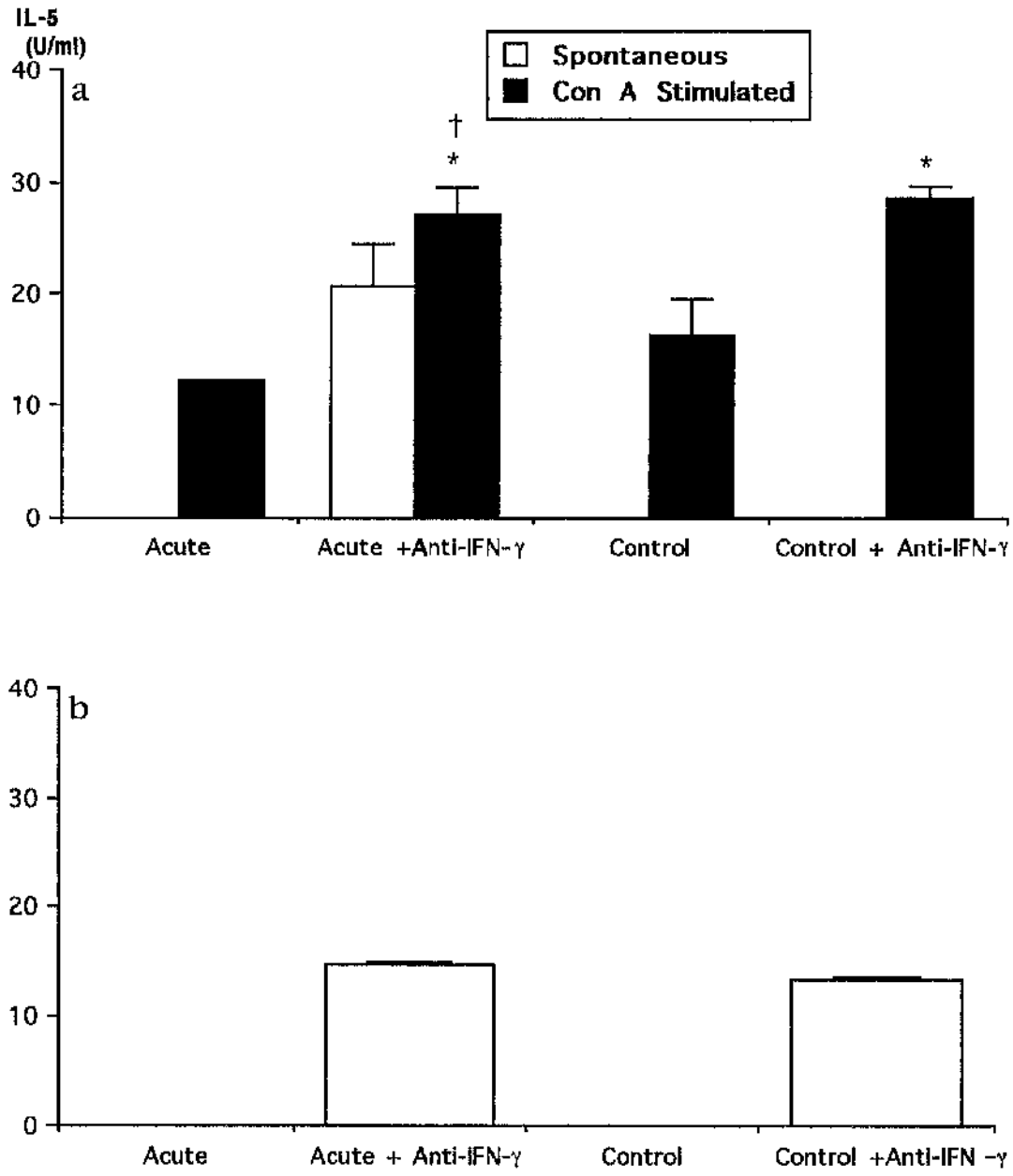


Fig. 8.8. Effects of neutralising IFN-γ on IL-5 production in acute GvHD in B6 ⇒ BDF_1 mice.

IL-5 production by splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. and treated with anti-IFN-γ mAb after culture in medium alone, or with 10μg/ml Con A for 120 hours. The results shown are the means ± 1SD of triplicate samples on day 2 (Fig. 8.8a) and day 10 (Fig. 8.8b) of GvHD. (* $p < 0.001$ vs unmodified controls; † $p < 0.001$ vs unmodified acute GvHD)

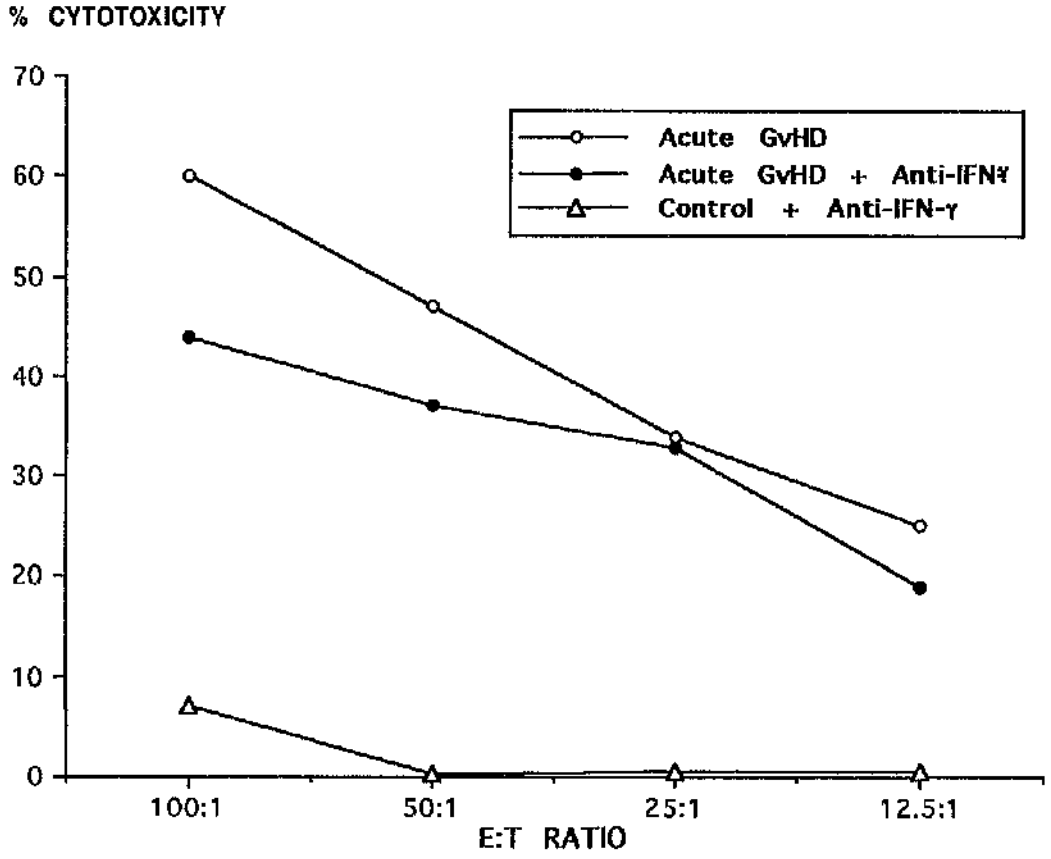


Fig. 8.9. Effects of neutralising IFN-γ on CTL activity in acute GvHD in B6 ⇒ BDF_1 mice.

Splenic CTL activity in BDF_1 mice given 10^8 B6 spleen cells i.v. and treated with anti-IFN-γ mAb. The results shown are the % cytotoxicity against P815 target cells from quadruplicate assays, using spleen cells pooled from 3 mice per group on day 10 of GvHD.

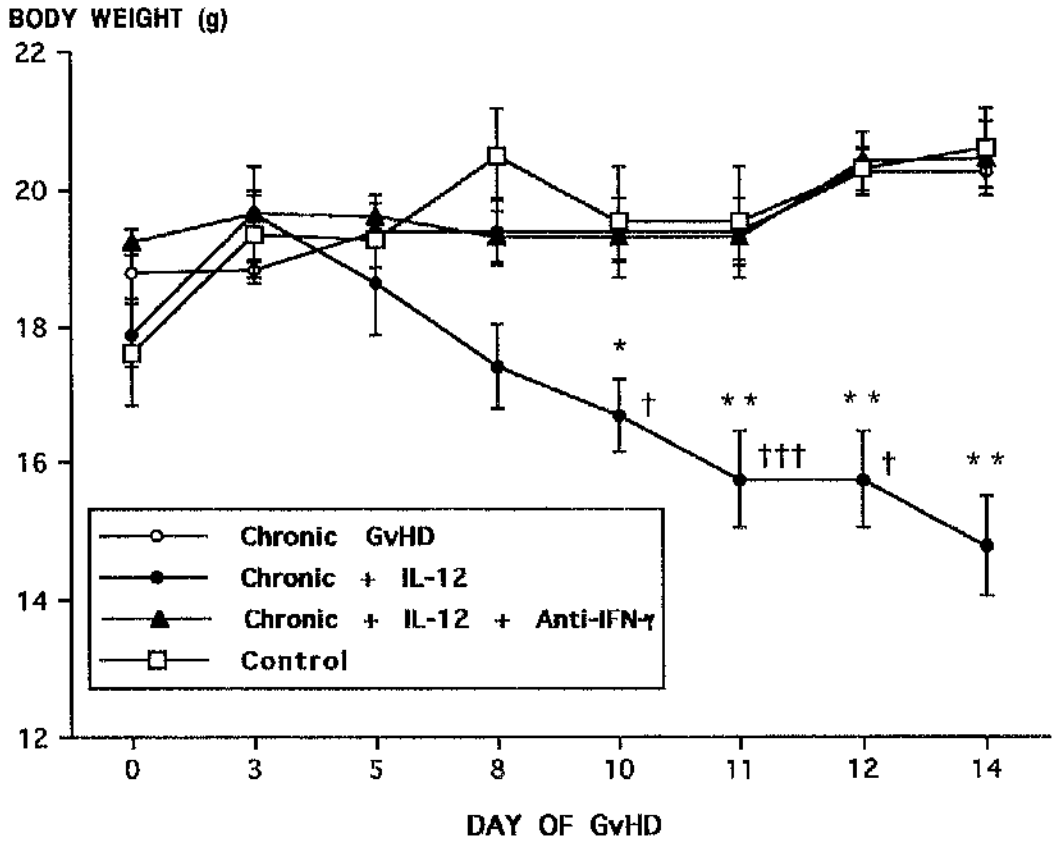


Fig. 8.10. Effects of neutralising IFN-γ on acute GvHD in DBA/2 ⇒ BDF_1 mice given IL-12.

Weight loss and mortality in BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb. The results shown are mean body weight ± 1 SD for 6 mice per group. Control mice given IL-12 ± anti-IFN-γ mAb did not lose weight or die and are not shown for clarity (* $p < 0.01$ ** $p < 0.001$ vs all other groups; † Death).

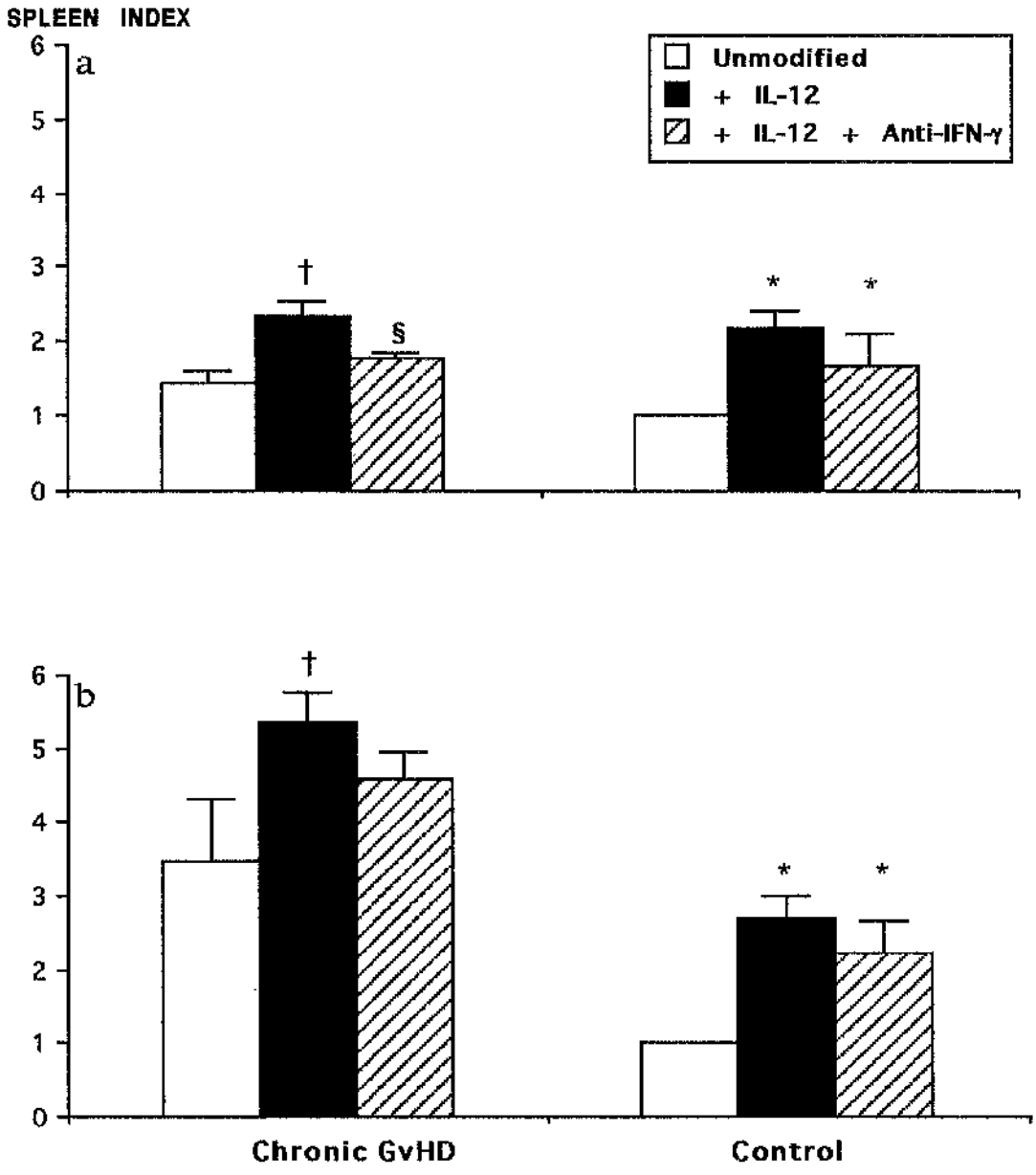


Fig. 8.11. Effects of neutralising IFN-γ in acute GvHD in DBA/2 ⇒ BDF_1 mice given IL-12.

Splenomegaly in BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb. The results shown are the mean spleen index ± 1 SD for 3 mice per group on day 2 (Fig. 8.11a) and day 10 (Fig. 8.11b) of GvHD. (* $p < 0.001$ vs unmodified controls; † $p < 0.01$ vs unmodified chronic GvHD; § $p < 0.05$ vs chronic + IL-12).

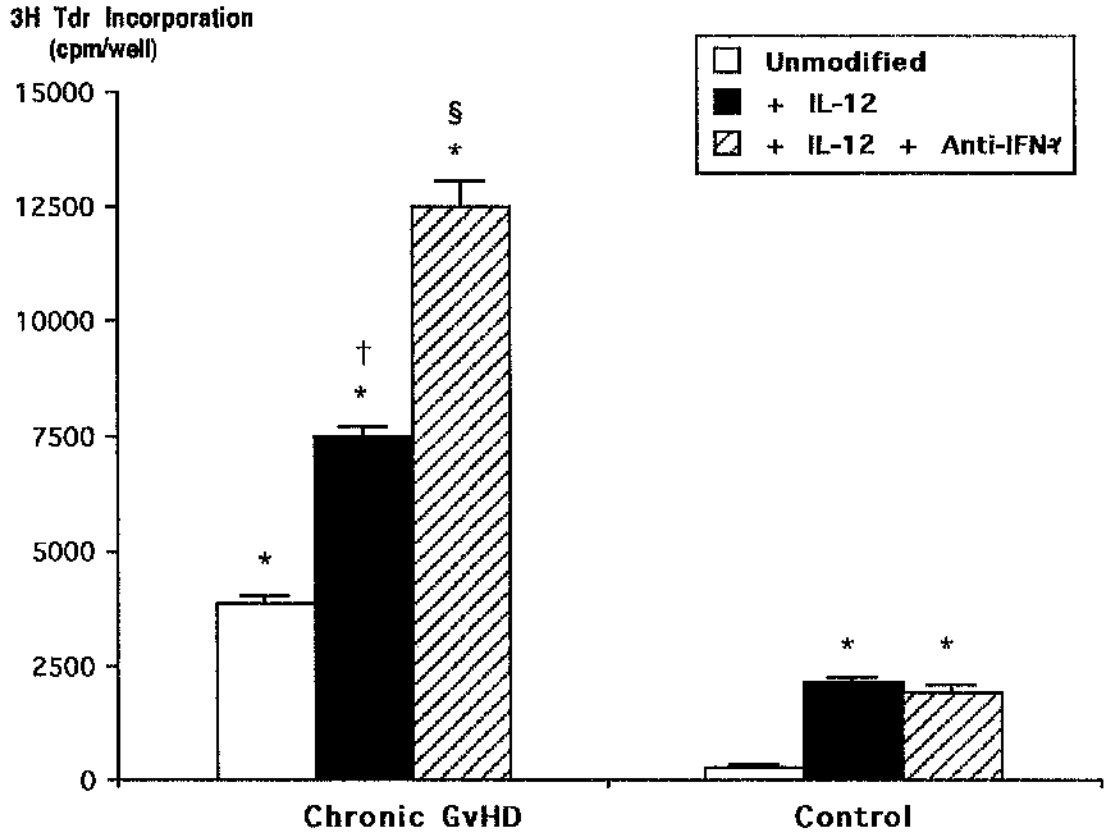


Fig. 8.12. Effects of neutralising IFN-γ on spontaneous '*ex-vivo*' proliferative responses in DBA/2 ⇒ BDF_1 mice given IL-12.

Spontaneous proliferative capacity of splenocytes from BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb. The results shown are the mean uptake of 3H-TdR ± 1 SD after 4 hours in quadruplicate cultures, using spleen cells pooled from 3-4 mice per group on day 10 of GvHD. (* $p < 0.001$ vs unmodified controls; † $p < 0.001$ vs unmodified chronic GvHD; § $p < 0.001$ vs chronic + IL-12).

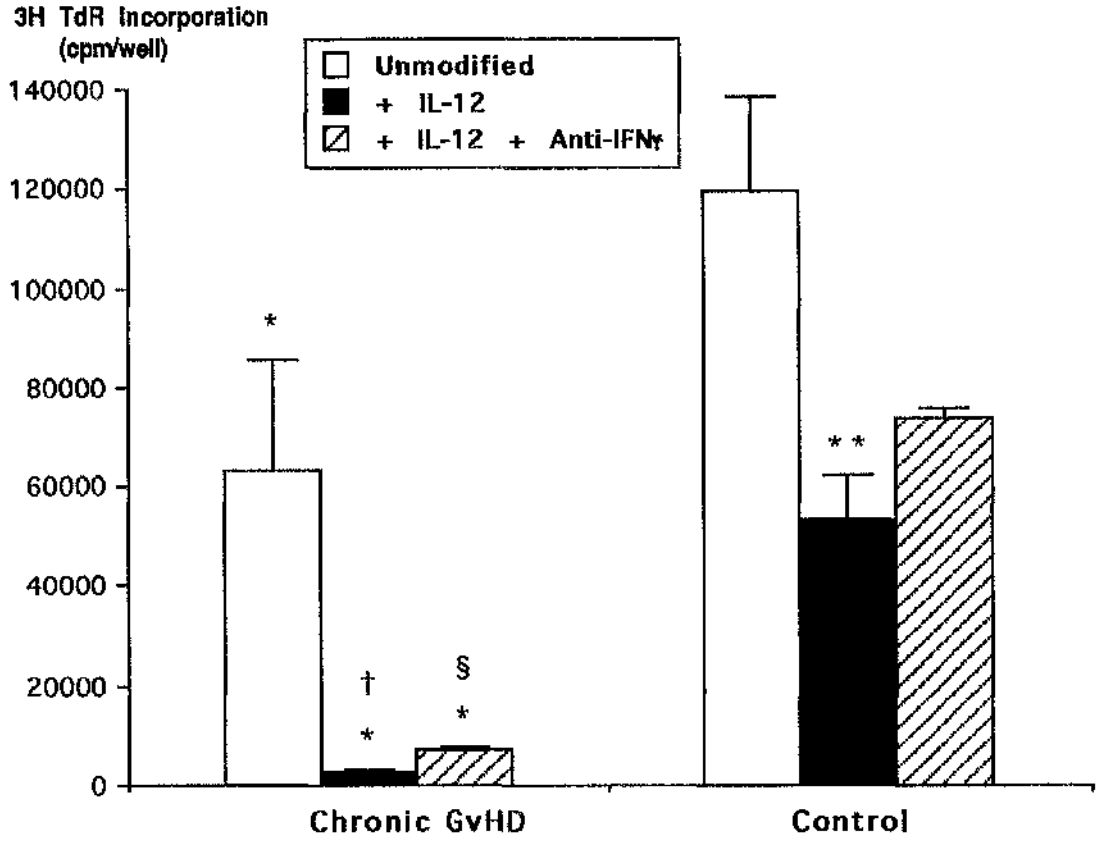


Fig. 8.13. Effects of neutralising IFN-γ on T cell function in DBA/2 ⇒ BDF_1 mice given IL-12.

Proliferative capacity of splenocytes from BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb. The results shown are the mean uptake of 3H-TdR ± 1 SD after 48 hours in quadruplicate cultures with 10μg/ml Con A, using spleen cells pooled from 3-4 mice per group on day 10 of GvHD. (* $p < 0.005$ ** $p < 0.001$ vs unmodified controls; † $p < 0.001$ vs unmodified chronic GvHD; § $p < 0.001$ vs chronic + IL-12).

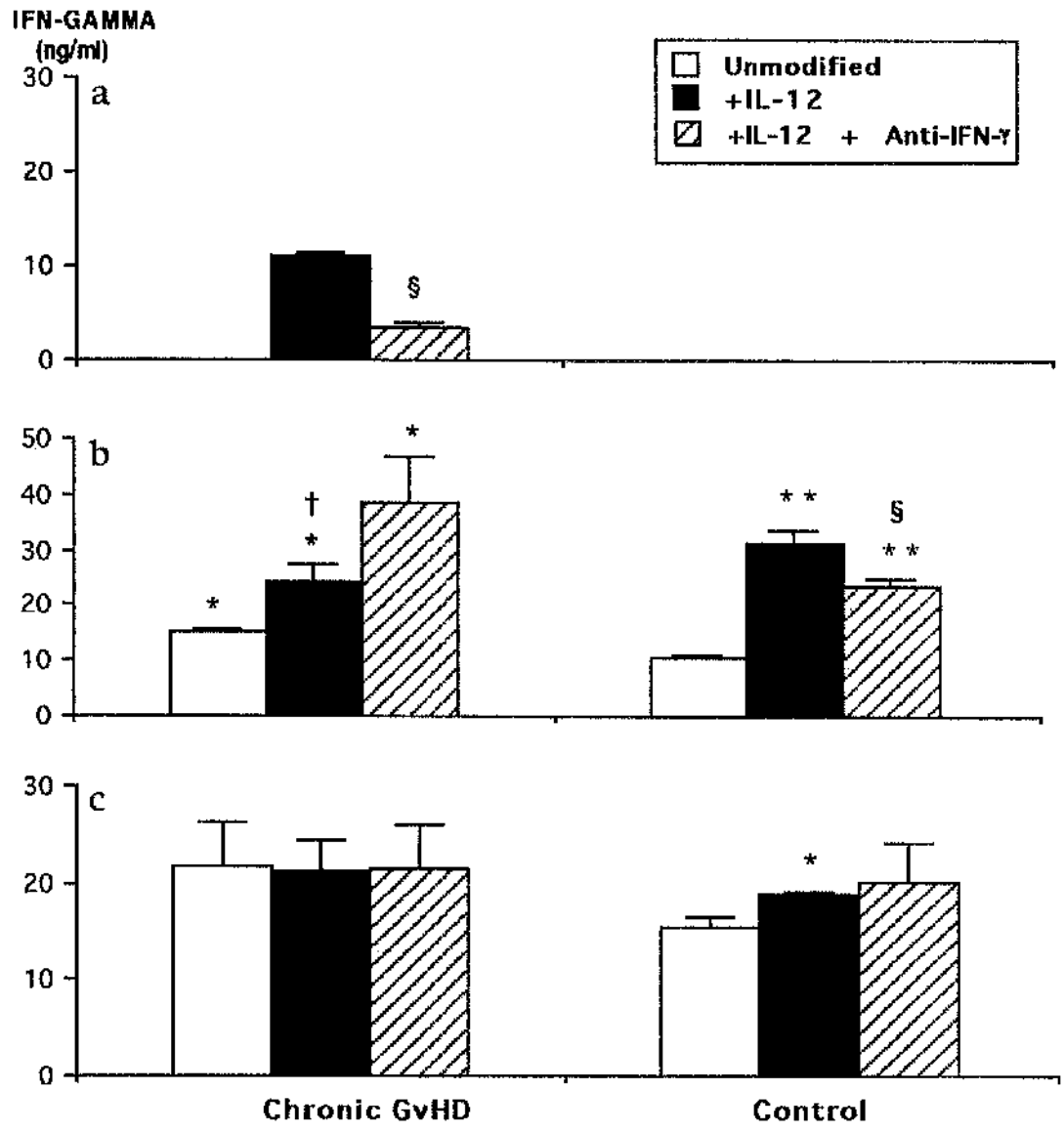


Fig. 8.14. Effects of neutralising IFN-γ on IFN-γ production in DBA/2 ⇒ BDF_1 mice given IL-12.

IFN-γ production by splenocytes from BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb. The results shown are for spontaneous IFN-γ production on day 10 of GvHD (Fig. 8.14a) and Con A induced IFN-γ on days 2 (Fig. 8.14b) and 10 (Fig. 8.14c) of GvHD. The results are the means ± 1SD of triplicate samples. (* $p < 0.05$ ** $p < 0.001$ vs unmodified controls; † $p < 0.05$ vs unmodified chronic GvHD § $p < 0.001$ vs chronic + IL-12).

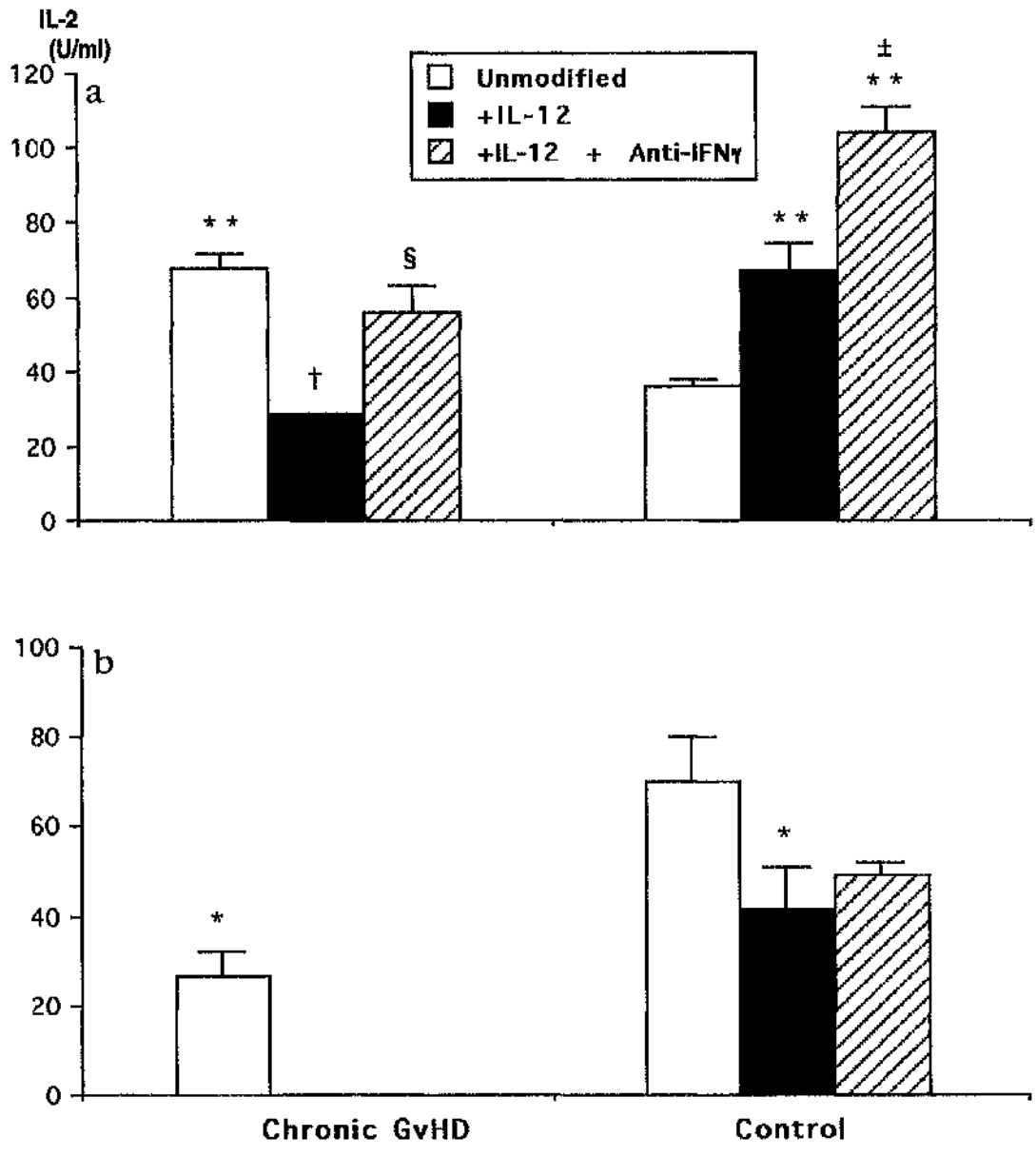


Fig. 8.15. Effects of neutralising IFN-γ on IL-2 production in DBA/2 ⇒ BDF_1 mice given IL-12.

IL-2 production by splenocytes from BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb, after culture with 10μg/ml Con A for 24 hours. The results shown are the means ± 1SD of triplicate samples on days 2 (Fig. 8.15a) and 10 (Fig. 8.15b) of GvHD. (* $p < 0.005$ ** $p < 0.001$ vs unmodified controls; † $p < 0.001$ vs unmodified chronic GvHD; § $p < 0.001$ vs chronic + IL-12; ± $p < 0.001$ vs control + IL-12).

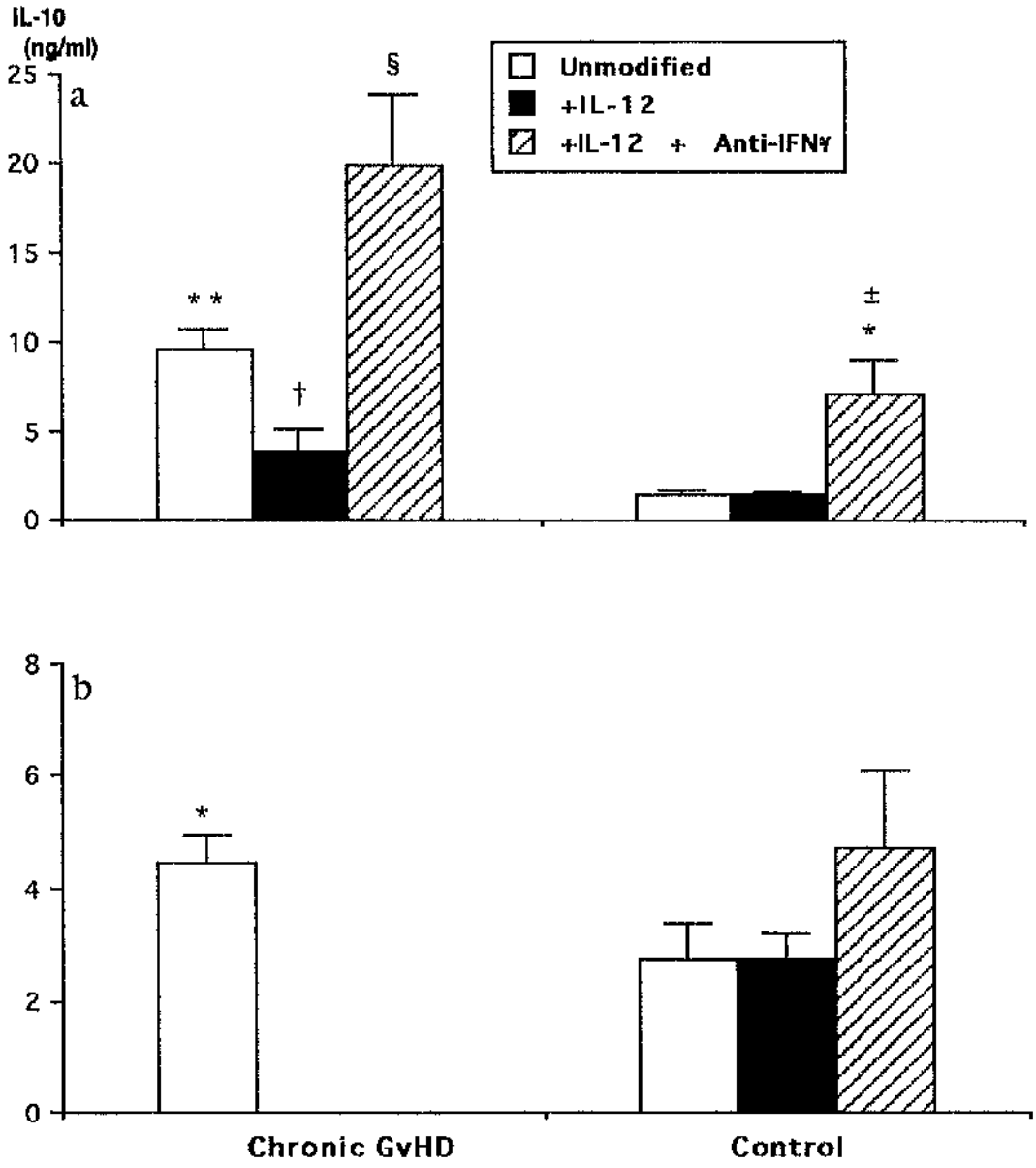


Fig. 8.16. Effects of of neutralising IFN-γ on IL-10 production in DBA/2 ⇒ BDF_1 mice given IL-12.

IL-10 production by splenocytes from BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb, after culture with 10μg/ml Con A for 48 hours. Results shown are means ± SD of triplicate samples on days 2 (Fig. 8.16a) and 10 (Fig. 8.16b) of GvHD. (* $p < 0.01$ ** $p < 0.001$ vs unmodified controls; † $p < 0.005$ vs unmodified chronic GvHD; § $p < 0.005$ vs chronic + IL-12).

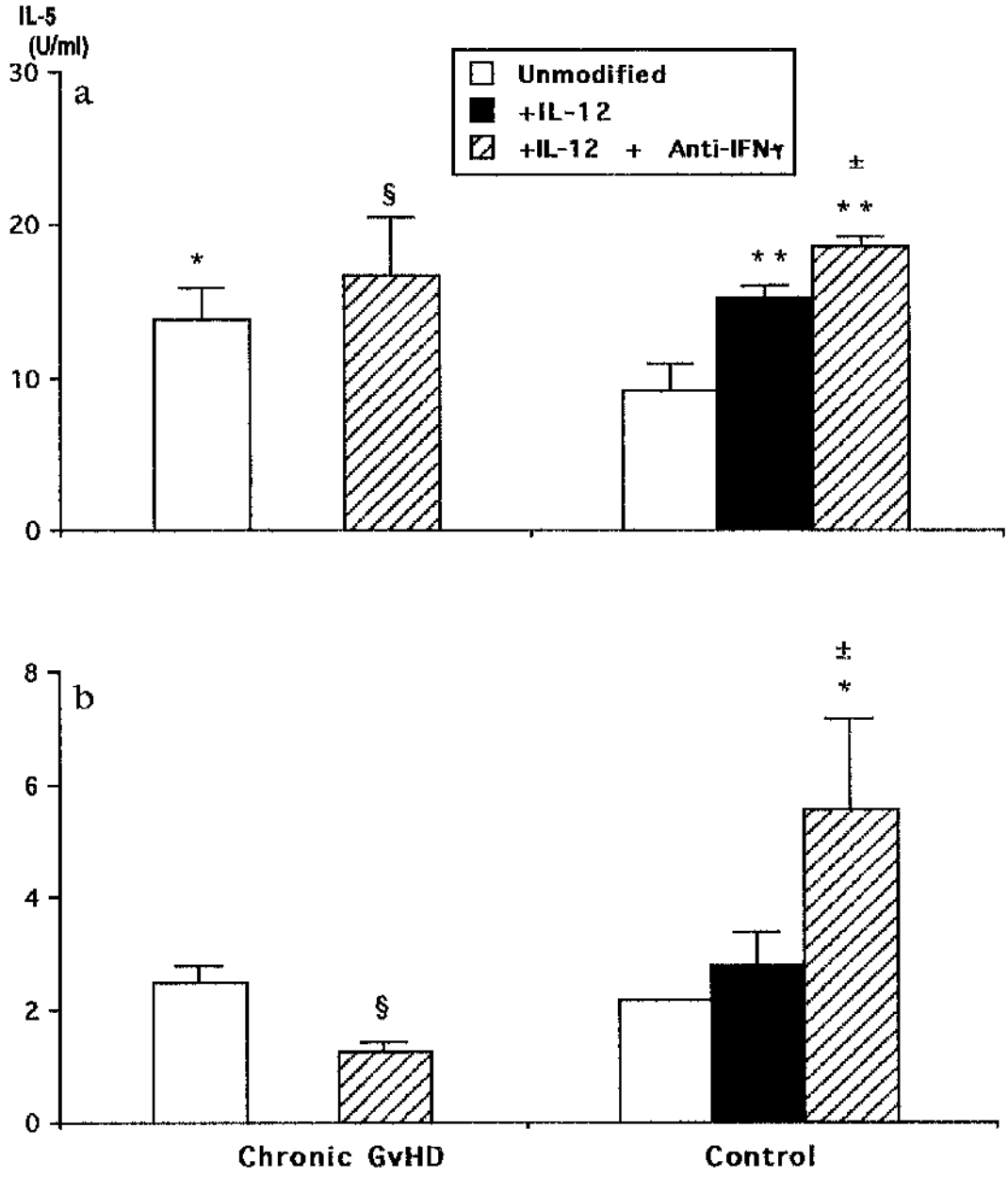


Fig. 8.17. Effects of neutralising IFN-γ on IL-5 production in DBA/2 ⇒ BDF_1 mice given IL-12.

IL-5 production by splenocytes from BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb, after culture with 10μg/ml Con A for 120 hours. The results shown are the means ± 1SD of triplicate samples on days 2 (Fig. 8.16a) and 10 (Fig. 8.16b) of GvHD. (* $p < 0.05$ ** $p < 0.001$ vs unmodified controls; § $p < 0.001$ vs chronic + IL-12; ± $p < 0.05$ vs control + IL-12).

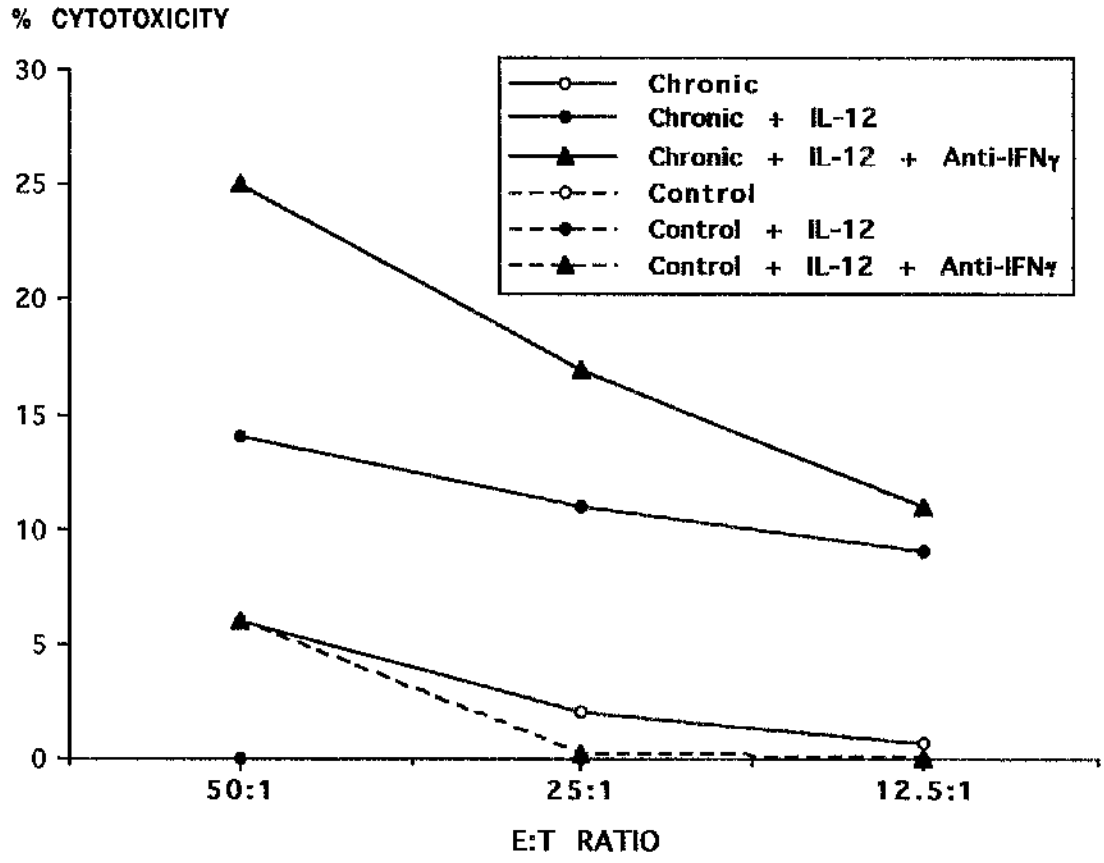


Fig. 8.18. Effects of neutralising IFN-γ on CTL activity in DBA/2 ⇒ BDF_1 mice given IL-12.

Splenic CTL activity in BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb. The results shown are the % cytotoxicity against P815 target cells from quadruplicate assays, using spleen cells pooled from 3 mice per group on day 10 of GvHD.

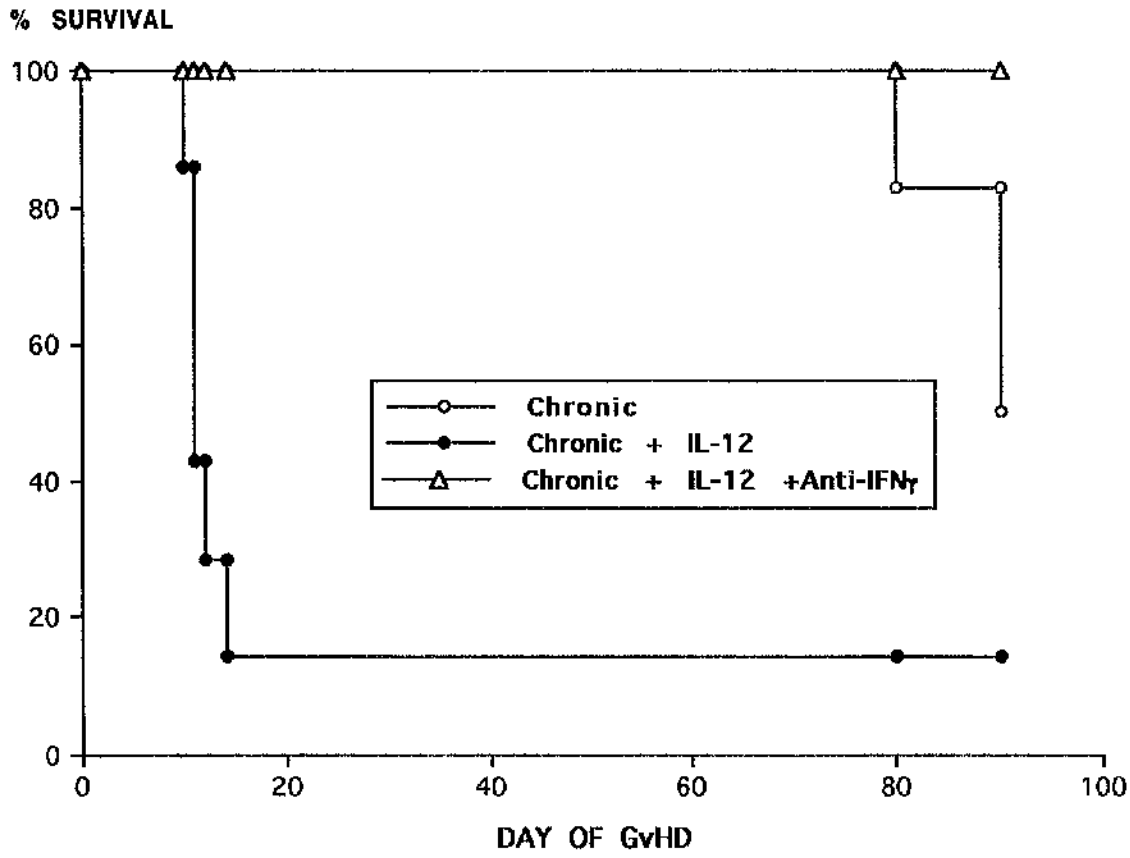


Fig. 8.19. Effects of neutralising IFN-γ on long-term consequences of IL-12 induced acute GvHD.

The results show the percentage of surviving mice in each group throughout the disease.

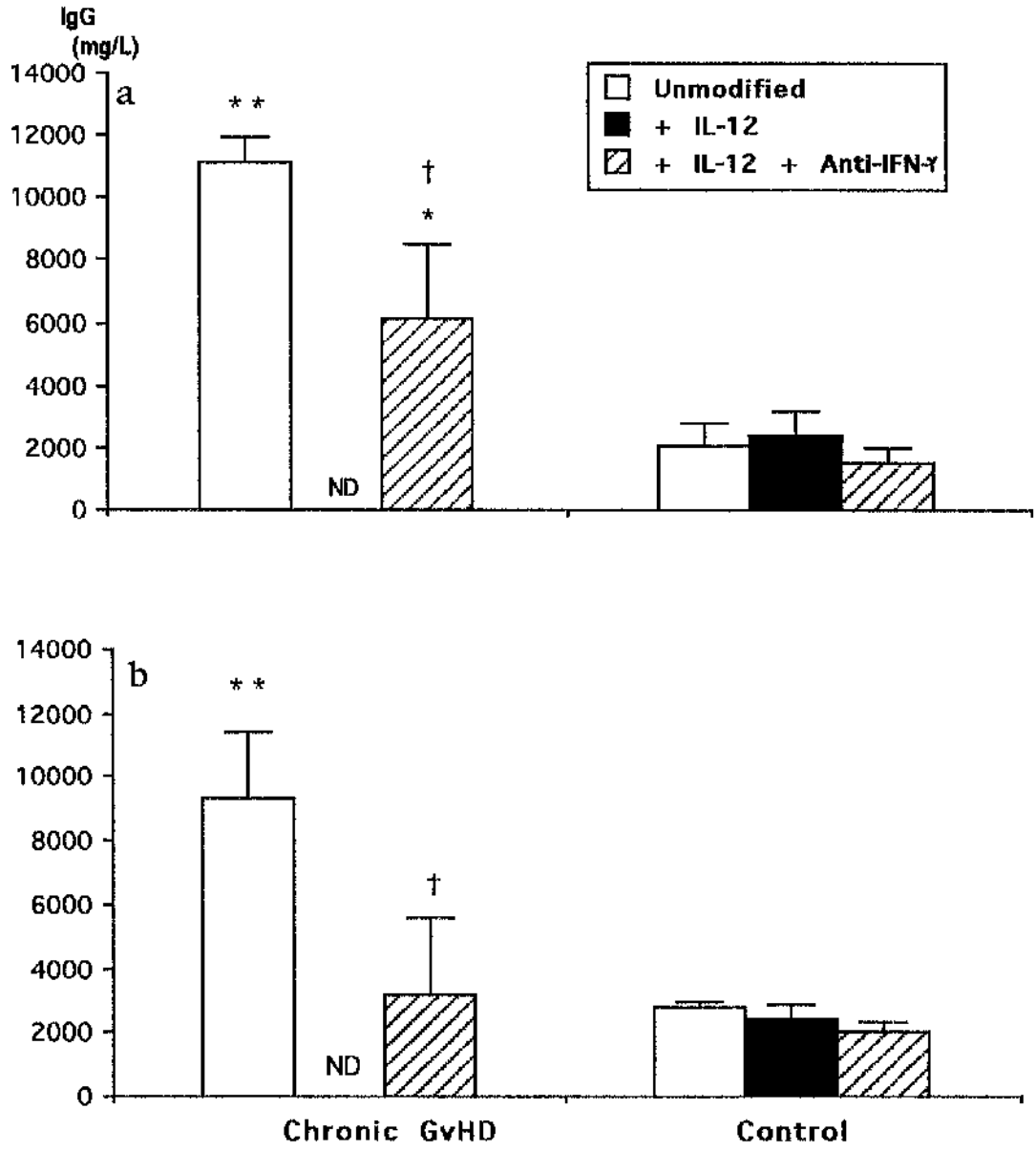


Fig. 8.20. Effects of neutralising IFN-γ on long-term consequences of IL-12 induced acute GvHD.

Levels of serum IgG in BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with anti-IFN-γ mAb. The results shown are the mean total IgG levels ± 1SD of 5-6 mice per group on days 30 (Fig. 8.20a) and 60 (Fig. 8.20b) of GvHD. (* $p < 0.05$ ** $p < 0.001$ vs unmodified controls; † $p < 0.05$ vs unmodified chronic GvHD). ND = Not done.

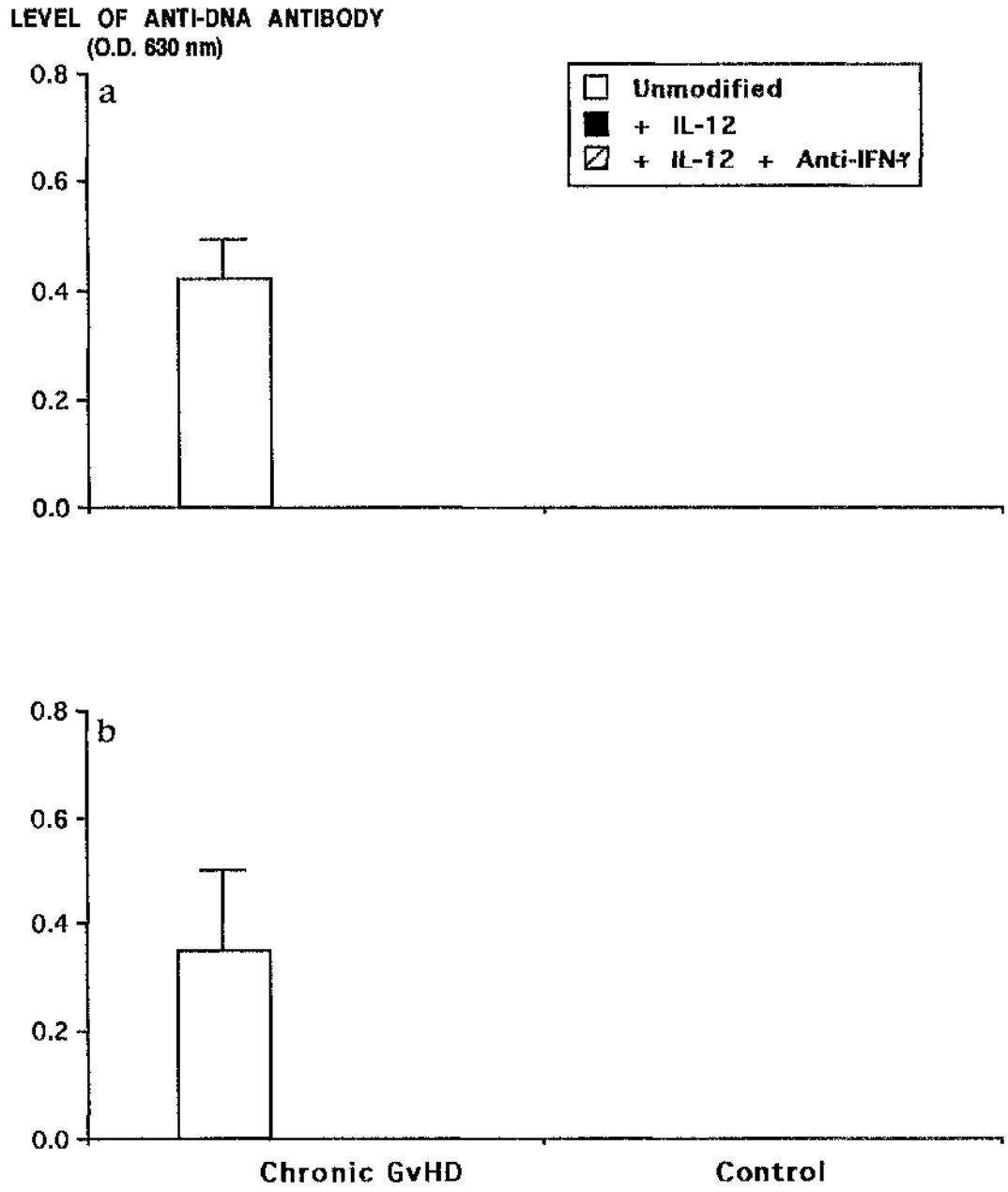


Fig. 8.21. Effects of neutralising IFN-γ on long-term consequences of IL-12 induced acute GvHD.

Levels of anti-ds DNA antibodies in BDF_1 mice given 10^8 DBA/2 spleen cells i.v. and treated with rm IL-12 and anti-IFN-γ mAb. The results shown are the mean OD value ± 1SD at 630nm of 5-6 mice per group on days 30 (Fig. 8.21a) and 60 (Fig. 8.21b) of GvHD.

CHAPTER 9

THE CELLULAR SOURCE OF CYTOKINES DURING GvHD. CD4+ AND CD8+ T CELLS

Introduction

In Chapters 3-8, I have shown that acute and chronic GvHD are associated with different patterns of cytokine production, with a Th1 or Th2 response predominating in acute or chronic GvHD respectively. These results suggest that the different diseases induced by B6 or DBA/2 donor cells may reflect expansion of distinct subsets of CD4+ Th cells *in vivo*. However, cells other than CD4+ Th lymphocytes are capable of producing several of the relevant cytokines and could therefore be important for the polarisation of GvHD. Two cell types which warrant particular attention are CD8+ T lymphocytes and NK cells.

In Chapter 3, I demonstrated that CD8+ T cells expanded preferentially during acute, but not chronic GvHD and become a major component of the lymphoid tissues of B6 $\Rightarrow$ BDF_1 mice. Previous studies have also highlighted the importance of CD8+ cells in the pathogenesis of acute GvHD, and their depletion from the B6 spleen cell inoculum prevents the lethality usually associated with the disease [30]. However, it is not known whether CD8+ T cells contribute to cytokine production during disease, despite their capacity to produce several cytokines in other systems, including IFN-γ [85, 86, 88], IL-4 [90, 94], IL-5 [91, 168] and IL-10 [94].

NK cells are a further potential source of cytokines during GvHD. In other experimental systems they have been shown to be important early producers of IFN-γ [112-114, 117, 119, 153, 169] and are believed to promote differentiation towards Th1-type responses [115-117]. Furthermore, NK cells are important for development of GvHD in other experimental models of the disease [97, 102, 104-106, 108]. In the experiments described in Chapters 4-6, it appeared that early NK

cells activation may be restricted to the acute form of disease, supporting the idea that these cells could be critical for the subsequent polarisation of acute GvHD, perhaps via production of IFN-γ.

In the next two chapters, I have therefore examined directly the cellular source of the cytokines produced during acute and chronic GvHD. This chapter will address the involvement of $CD4^+$ and $CD8^+$ cells in cytokine production *in vitro*, while Chapter 9 examines the role of NK cells in both cytokine production *in vitro* and disease progression *in vivo*.

Experimental Protocol

As usual, the GvHR was induced by i.v. injection of 10^8 viable B6 or DBA/2 parental spleen cells into BDF_1 recipients, and its intensity was monitored by assessing splenomegaly and mortality.

To examine the source of cytokines in vitro, $CD4^+$ and $CD8^+$ T cell subsets were depleted from pooled spleen cell suspensions isolated on days 2, 7, 10 and 14 of GvHD by complement-mediated lysis using YTS191.1 (anti-CD4) or YTS169.4 (anti-CD8) mAbs and rabbit complement. The percentage of residual $CD4^+$ or $CD8^+$ cells remaining after depletion was consistently $\leq 0.2\%$. A typical example of the efficiency of the depleting mAbs is shown in Fig. 9.1.

IFN-γ, IL-2, IL-10, IL-5 and IL-4 production was measured by culturing spleen cells from GvHD mice either in medium alone or with Con A.

Results

Systemic GvHD

Splenomegaly was assessed in GvHD mice to provide an indication of disease progression. As before, B6 ⇒ BDF_1 mice had significant splenomegaly by day 2 (Fig. 9.2). This increased throughout the early period of the acute disease, peaked on day 10 and had returned to control levels by day 16. The first deaths in this group also occurred on day 16, when two of the five mice died (data not shown). As insufficient lymphocytes were available from the surviving B6 ⇒ BDF_1 mice for analysis of proliferation or cytokine production on day 16, the last data point for all groups in this study was day 14.

The splenomegaly observed in DBA/2 ⇒ BDF_1 mice was characteristically less intense than that observed in B6 ⇒ BDF_1 mice, but as in my previous studies, it persisted for the remainder of the study (Fig. 9.2). Furthermore, none of these mice died during the period of study.

Role of CD4+ and CD8+ T cells in Production of IFN-γ in GvHD

a) Spontaneous

Unseparated spleen cells from B6 ⇒ BDF_1 mice produced IFN-γ spontaneously at all time points examined (Fig. 9.3a). On day 2, depletion of either $CD4^+$ or $CD8^+$ T cells did not affect these levels, suggesting that a non-T cell population was producing this early IFN-γ. In contrast, by day 7, $CD4^+$ cells appeared to be the main source of spontaneous IFN-γ, since depletion of $CD4^+$, but not $CD8^+$ cells resulted in significantly reduced IFN-γ production (Fig. 9.3a). On day 10, removal of $CD4^+$ cells also resulted in a significant reduction in spontaneous IFN-γ production. At this time, depletion of $CD8^+$ cells actually increased IFN-γ production, suggesting that an inhibitory $CD8^+$ population was present, or that removal of $CD8^+$ cells had enriched the population of IFN-γ

producing cells in culture. On day 14, both $CD4^+$ and $CD8^+$ populations appeared to be required for spontaneous IFN-γ production, since levels were totally ablated when either population was depleted (Fig. 9.3a).

Cells from DBA/2 ⇒ BDF_1 mice produced some spontaneous IFN-γ on days 2 and 10 of the GvHD (Fig. 9.3b). Although this was in contrast to earlier experiments, these levels were very low compared to those produced by B6 ⇒ BDF_1 cells and were dependent entirely on $CD4^+$ T cells at both time points.

Control cells did not produce IFN-γ spontaneously at any time during this study and depletion of either T cell subset did not affect this (data not shown).

b) Con A Induced

As anticipated, unseparated B6 ⇒ BDF_1 splenocytes produced significantly higher levels of Con A stimulated IFN-γ than control cells at all times examined (Figs. 9.4a & c respectively). In contrast to the T cell-independent spontaneous IFN-γ production during the initial phase of disease, on day 2, depletion of $CD4^+$ cells from these cultures significantly reduced Con A induced IFN-γ levels. $CD8^+$ cell depletion had no effect (Fig. 9.4a). Depletion of either $CD4^+$ or $CD8^+$ populations on either day 7 or 14 reduced the level of Con A induced IFN-γ production (Fig. 9.4a), suggesting that both populations were producing IFN-γ in response to Con A stimulation at these times. However, on day 10, $CD4^+$ cell depletion did not affect IFN-γ levels, while depletion of $CD8^+$ cells provoked a significant increase (Fig. 9.4a), consistent with the effects of $CD4^+$ and $CD8^+$ depletion on spontaneous IFN-γ production at this time. Thus, a regulatory $CD8^+$ T cells may be present at this stage of GvHD and a transient population of cells other than $CD4^+$ or $CD8^+$ T cells may also contribute to the levels of IFN-γ produced spontaneously or via Con A stimulation. If time had allowed, this could have been confirmed by total T cell depletion or combined depletion of $CD4^+$ and $CD8^+$ populations.

Unseparated splenocytes from DBA/2 ⇒ BDF_1 mice produced similar levels of Con A stimulated IFN-γ to control cells at all times examined (Figs. 9.4b & c). At all time points, both $CD4^+$ and $CD8^+$ populations appeared to be contributing to Con A induced IFN-γ production by control cells and cells from DBA/2 ⇒ BDF_1 mice, as depletion of either of these cells types resulted in significantly reduced levels (Figs. 9.4b & c).

Role of $CD4^+$ and $CD8^+$ T cells in IL-2 Production

IL-2 was not produced spontaneously by cells from any group at any time (data not shown).

On days 2 and 7, unseparated spleen cells from B6 ⇒ BDF_1 mice produced similar levels of IL-2 in response to Con A to those found with control mice (Fig. 9.5a & c). By day 10, IL-2 levels were significantly lower than those produced by controls and by day 14, no IL-2 production was detectable (Fig. 9.5a). Depletion of $CD4^+$ cells from B6 ⇒ BDF_1 cultures significantly reduced, or totally ablated IL-2 production at all time points examined (Fig. 9.5a). This procedure had no effect on the inability of total B6 ⇒ BDF_1 splenocytes to produce IL-2 on day 14. Depletion of $CD8^+$ cells also significantly reduced IL-2 levels on day 2, but did not affect those on days 7 and 10 (Fig. 9.5a). However, by day 14, depletion of $CD8^+$ T cells revealed low levels of IL-2 production, when none was observed using unseparated GvHD cells (Fig. 9.5a). This may be because the highly activated $CD8^+$ population were consuming any available IL-2, or because they act as regulatory cells.

Unseparated spleen cells from DBA/2 ⇒ BDF_1 mice produced control levels of IL-2 in response to Con A on days 2, 7 and 14 (Fig. 9.5b & c). However, on day 10, chronic GvHD cells showed reduced IL-2 production compared with controls, perhaps reflecting enhanced consumption of IL-2 by the activated GvHD cells. Depletion of $CD4^+$ cells from DBA/2 ⇒ BDF_1 cultures significantly reduced or ablated IL-2 production at all time points examined.

Depletion of $CD8^+$ cells also significantly reduced IL-2 levels on day 2, but did not affect those observed thereafter.

Depletion of $CD4^+$ cells from control splenocyte cultures significantly reduced the levels of IL-2 produced in response to Con A at all time points examined, while depletion of $CD8^+$ cells had no effect on days 2-10, but significantly increased the levels of IL-2 on day 14 (Fig. 9.5c).

Role of CD4+ and CD8+ T cells in IL-10 Production

IL-10 was not produced spontaneously by the cells of any group at any time (data not shown).

Unseparated splenocytes from B6 $\Rightarrow$ BDF_1 mice produced levels of IL-10 in response to Con A similar to those produced by control cells at all time points examined (Figs. 9.6a & c). On day 2, depletion of $CD4^+$ cells from B6 $\Rightarrow$ BDF_1 cultures significantly reduced the levels of IL-10, whereas depletion of the $CD8^+$ population had no effect (Fig. 9.6a). By days 7 and 10, depletion of $CD4^+$ cells from B6 $\Rightarrow$ BDF_1 cultures totally ablated IL-10 production. Thus $CD4^+$ cells are the main source of IL-10 early in acute GvHD. On day 14, however, depleting $CD4^+$ cells had no effect on IL-10 production, despite the clear efficacy of depletion and by its effects on other cytokines. Removal of the $CD8^+$ population on both days 10 and 14 significantly increased IL-10 levels in acute GvHD.

As I found previously, unseparated splenocytes from DBA/2 $\Rightarrow$ BDF_1 mice produced significantly higher levels of IL-10 in response to Con A than control cells at all time points examined (Figs. 9.6b & c). On days 2 and 7, depletion of either $CD4^+$ or $CD8^+$ cells from DBA/2 $\Rightarrow$ BDF_1 cultures significantly reduced IL-10 levels, suggesting that both populations may be capable of IL-10 production in these cultures (Fig. 9.6b). However on day 10, while depletion of $CD4^+$ cells totally ablated IL-10 levels, depletion of $CD8^+$ cells markedly increased IL-10 production, indicating that IL-10 was now being produced exclusively by $CD4^+$ cells and confirming the possible presence of

regulatory $CD8^+$ T cells. Similarly to my finding with B6 ⇒ BDF_1 cells, on day 14, depletion of either $CD4^+$ or $CD8^+$ cell populations had no effect on IL-10 production, although once again, the depletion appeared to be effective. The source of IL-10 at this time was therefore unclear and needs to be addressed in future work.

On day 2, depletion of either $CD4^+$ or $CD8^+$ populations did not significantly affect the low levels of IL-10 produced by control splenocytes in response to Con A (Fig. 9.6c). In contrast, on days 7 and 10, depletion of $CD4^+$ cells significantly reduced IL-10 levels, while depletion of $CD8^+$ cells had no effect. On day 14, depletion of $CD4^+$ cells again significantly reduced IL-10 levels, whereas $CD8^+$ depletion significantly increased them.

Role of CD4+ and CD8+ T cells in IL-5 Production

IL-5 was not produced spontaneously by the cells of any group at any time (data not shown).

Unseparated splenocytes from B6 ⇒ BDF_1 mice did not produce detectable IL-5 in response to Con A at any time point examined and this was not affected by depleting $CD4^+$ cells (Fig. 9.7a). On day 2, however, depleting $CD8^+$ cells from B6 ⇒ BDF_1 cultures revealed moderate levels of IL-5 (Fig. 9.7a), suggesting that $CD8^+$ cells may be directly inhibiting IL-5 production in the undepleted cultures at this time. On day 7 and thereafter, depletion of $CD8^+$ cells did not reveal any IL-5 production.

As before, unseparated splenocytes from DBA/2 ⇒ BDF_1 mice produced high levels of IL-5 in response to Con A throughout the period of study (Fig. 9.7b). At all time points, these levels were totally ablated by depletion of the $CD4^+$ population (Fig. 9.7b). On day 2, depletion of $CD8^+$ cells did not affect the levels of IL-5 produced by splenocytes from DBA/2 ⇒ BDF_1 mice, but on day 7, $CD8^+$ depletion resulted in a significant reduction in IL-5. On day 10, depletion of $CD8^+$ cells significantly increased IL-5 levels, while on day 14, $CD8^+$ depletion had no

effect on IL-5 production in chronic GvHD. Thus, $CD4^+$ cells appear to be the main IL-5 producing cell type in chronic GvHD, while $CD8^+$ cells play a minor role in the production of this cytokine and are frequently regulatory.

Unseparated control splenocytes did not produce detectable IL-5 in response to Con A at any point during this study and depletion of $CD4^+$ cells had no effect on this (Fig. 9.7c). In contrast, depletion of $CD8^+$ cells, revealed the presence of IL-5 in control splenocytes cultures, again suggesting that $CD8^+$ cells may play a regulatory role in IL-5 production.

IL-4

IL-4 production was below the level of detection for all groups at all time points examined (data not shown).

Summary and Conclusions

These results indicate that while $CD4^+$ Th cells are clearly a major source of a variety of different cytokines during acute and chronic GvHD, $CD8^+$ T cells and other cell types may also be important for the pattern of cytokines produced. During acute GvHD, $CD4^+$, $CD8^+$ and non-$CD4^+CD8^+$ populations all appear to contribute to the high levels of both spontaneous and Con A induced IFN-γ produced by spleen cells from B6 ⇒ BDF_1 mice. During chronic GvHD and also when control cells are stimulated with Con A *in vitro*, both $CD4^+$ and $CD8^+$ cells seem to produce IFN-γ and there was no evidence for the involvement of other non-T cells. In contrast, in all groups, $CD4^+$ cells were the main population which produced IL-2 in response to Con A stimulation, with $CD8^+$ cells playing little or no role in its production. Con A stimulated IL-10 again appeared to be derived from a number of cellular sources during the two diseases. While both $CD4^+$ and $CD8^+$ cells were capable of producing IL-10 during days 2-10 of acute and chronic GvHD, on day 14 of either disease, depletion of $CD4^+$ or $CD8^+$ cells did not affect the levels of Con A induced IL-10. Although this may indicate that the

cell populations had not been effectively removed on day 14, FACS analysis showed a similar degree of depletion at this time as had been observed at the earlier time points. Furthermore, the levels of the other cytokines were affected by depletion of $CD4^+$ or $CD8^+$ cells at this time. Thus, IL-10 may be produced by cells other than $CD4^+$ or $CD8^+$ lymphocytes, although the nature of this Con A responsive cell is unclear. This phenomenon was restricted to GvHD cells, since the levels of IL-10 produced by Con A stimulated control splenocytes could be reduced by depletion of $CD4^+$ cells at all times. During chronic GvHD, the high levels of IL-5 were almost entirely produced by $CD4^+$ cells and depletion of $CD8^+$ T cells was required to reveal IL-5 production by acute GvHD or control cells, indicating that $CD8^+$ T cells were inhibiting IL-5 production in these groups. The fact that this was evident at all times for control cells, but only on day 2 for B6 $\Rightarrow$ BDF_1 cells suggests that acute GvHD cells may have lost the capacity to produce IL-5 at the later time points. A regulatory effect of $CD8^+$ T cells was a frequent finding in these studies, in agreement with their generally suppressive role during immune responses [87, 91]. In particular, $CD8^+$ T lymphocytes appeared to inhibit Th2 cytokine production in B6 $\Rightarrow$ BDF_1 cultures, since $CD8^+$ depletion increased levels of both IL-5 and IL-10.

In summary, it is clear that the cellular interactions involved in cytokine production during acute and chronic GvHD are extremely complex. Several populations can produce or regulate the production of many of the critical cytokines involved in GvHD and the purification of these cell types may provide a more definitive answer as to their importance.

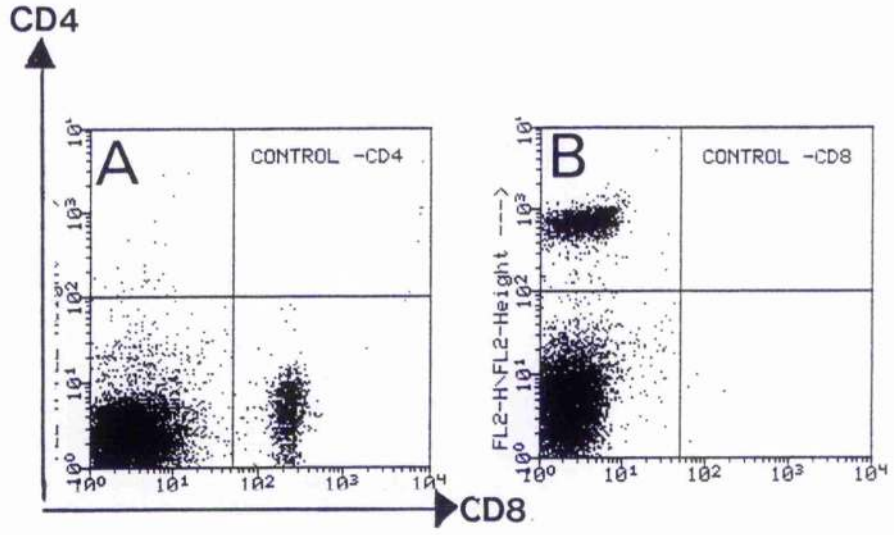


Fig. 9.1. Depletion of T cell subsets *in vitro*.

$CD4^+$ (Fig. 9.1A) and $CD8^+$ (Fig. 9.1B) splenic lymphocyte populations were depleted *in vitro* by complement mediated lysis using either YTS 191.1 anti-CD4 or YTS 169.4 anti-CD8α mAbs together with rabbit complement.

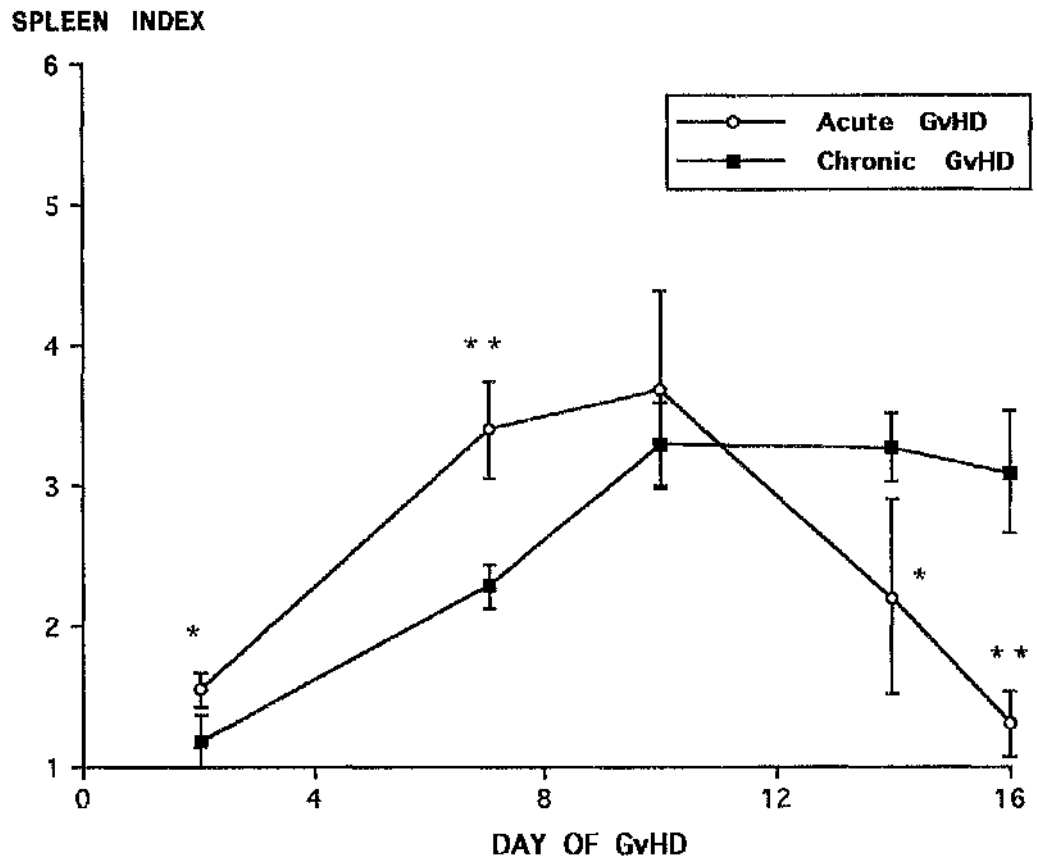


Fig. 9.2. Splenomegaly during acute GvHD and chronic GvHD.

The results show the splenomegaly in BDF_1 mice given 10^8 B6 or DBA/2 spleen cells i.v., expressed as the mean spleen index of 3 GvHD mice per group ± 1SD and calculated with reference to the mean spleen weights of 3 control mice.

(* $p < 0.05$ ** $p < 0.001$ vs chronic GvHD).

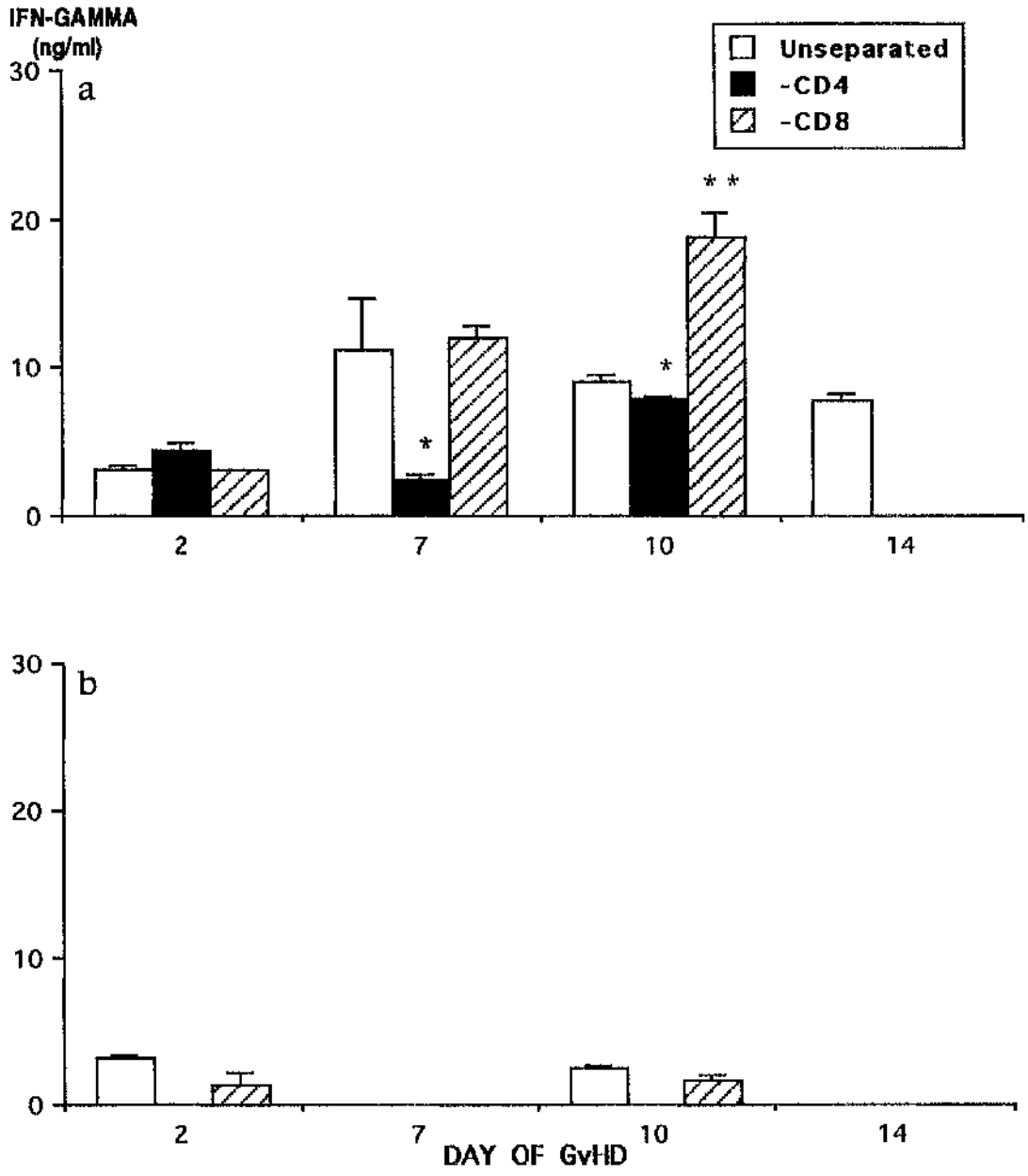


Fig. 9.3. Effects of depleting CD4+ and CD8+ cells *in vitro* on spontaneous IFN-γ production during acute and chronic GvHD.

The results show the effect of depleting CD4+ or CD8+ lymphocytes on IFN-γ levels in unstimulated cultures of splenocytes from mice with (a) acute GvHD and (b) chronic GvHD. CD4+ or CD8+ cells were depleted *in vitro* using complement mediated lysis and the cells cultured in medium for 48 hours before the supernatants were assayed for the presence of IFN-γ by ELISA. (* $p < 0.05$ ** $p < 0.001$ vs unseparated).

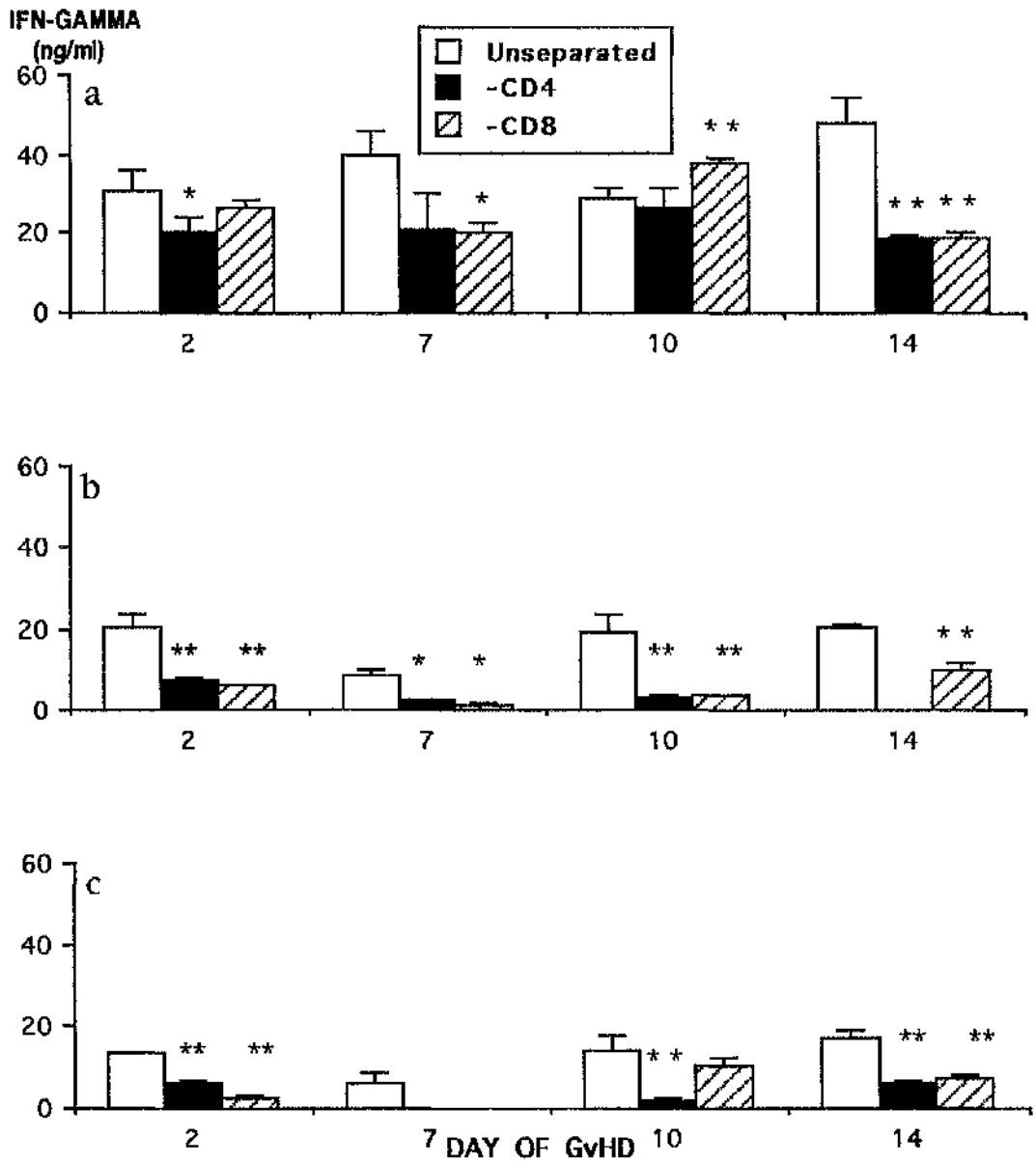


Fig. 9.4. Effects of depleting CD4⁺ and CD8⁺ cells *in vitro* on Con A induced IFN-γ production during acute and chronic GvHD.

The results show the effect of depleting CD4⁺ or CD8⁺ lymphocytes on IFN-γ levels in Con A stimulated spleen cell cultures from mice with acute GvHD (a), mice with chronic GvHD (b) and control mice (c). CD4⁺ or CD8⁺ cells were depleted *in vitro* using complement mediated lysis and the cells cultured with 10μg/ml Con A for 48 hours before the supernatants were assayed for the presence of IFN-γ by ELISA. (* $p < 0.05$ ** $p < 0.001$ vs unseparated).

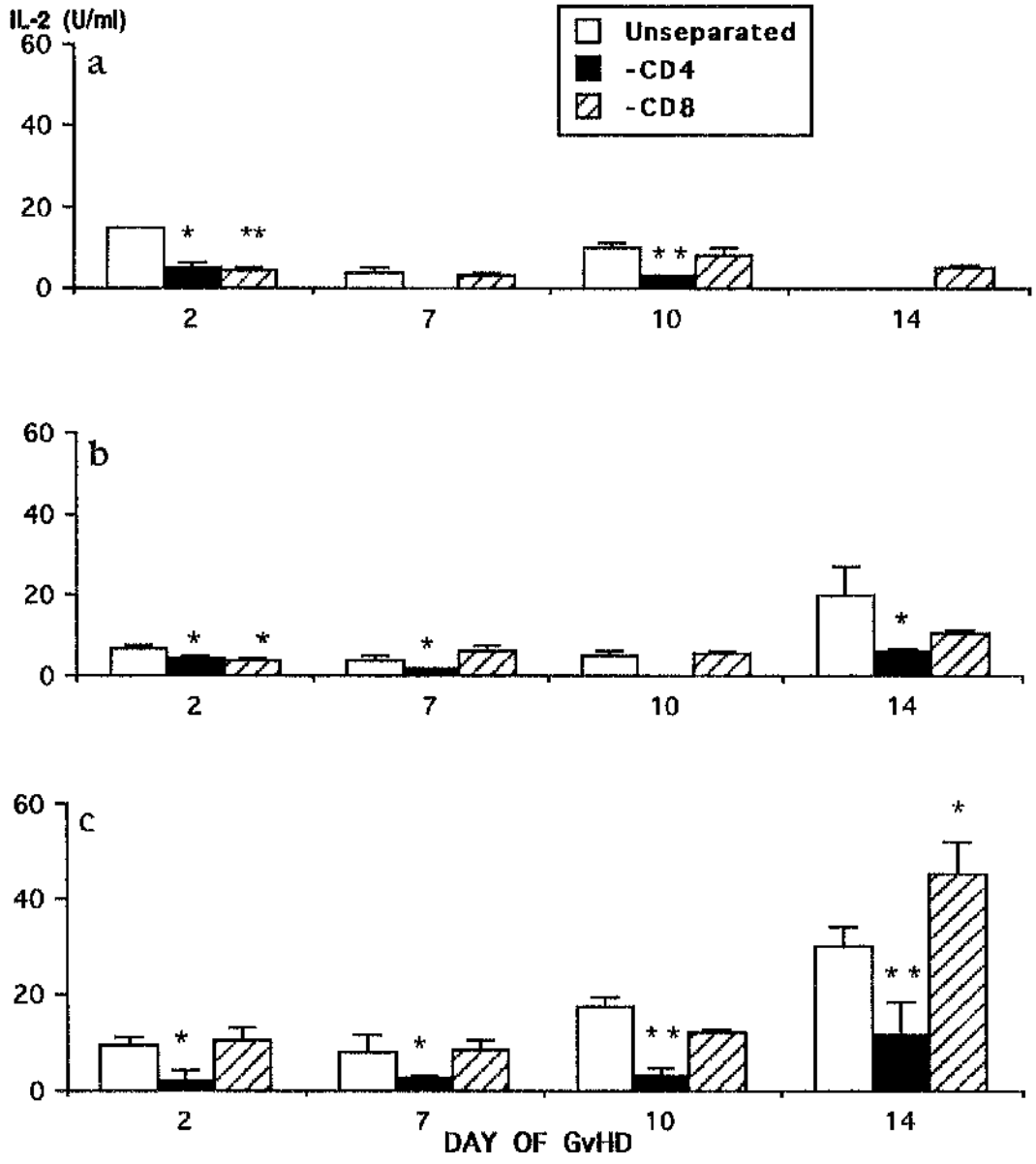


Fig. 9.5. Effects of depleting CD4+ and CD8+ cells *in vitro* on Con A induced IL-2 production during acute and chronic GvHD.

The results show the effect of depleting CD4+ or CD8+ lymphocytes on IL-2 levels in Con A stimulated spleen cell cultures from mice with acute GvHD (a), mice with chronic GvHD (b) and control mice (c). CD4+ or CD8+ cells were depleted *in vitro* using complement mediated lysis and the cells cultured with 10μg/ml Con A for 24 hours before the supernatants were assayed for the presence of IL-2 by ELISA. (* $p < 0.05$ ** $p < 0.005$ vs unseparated).

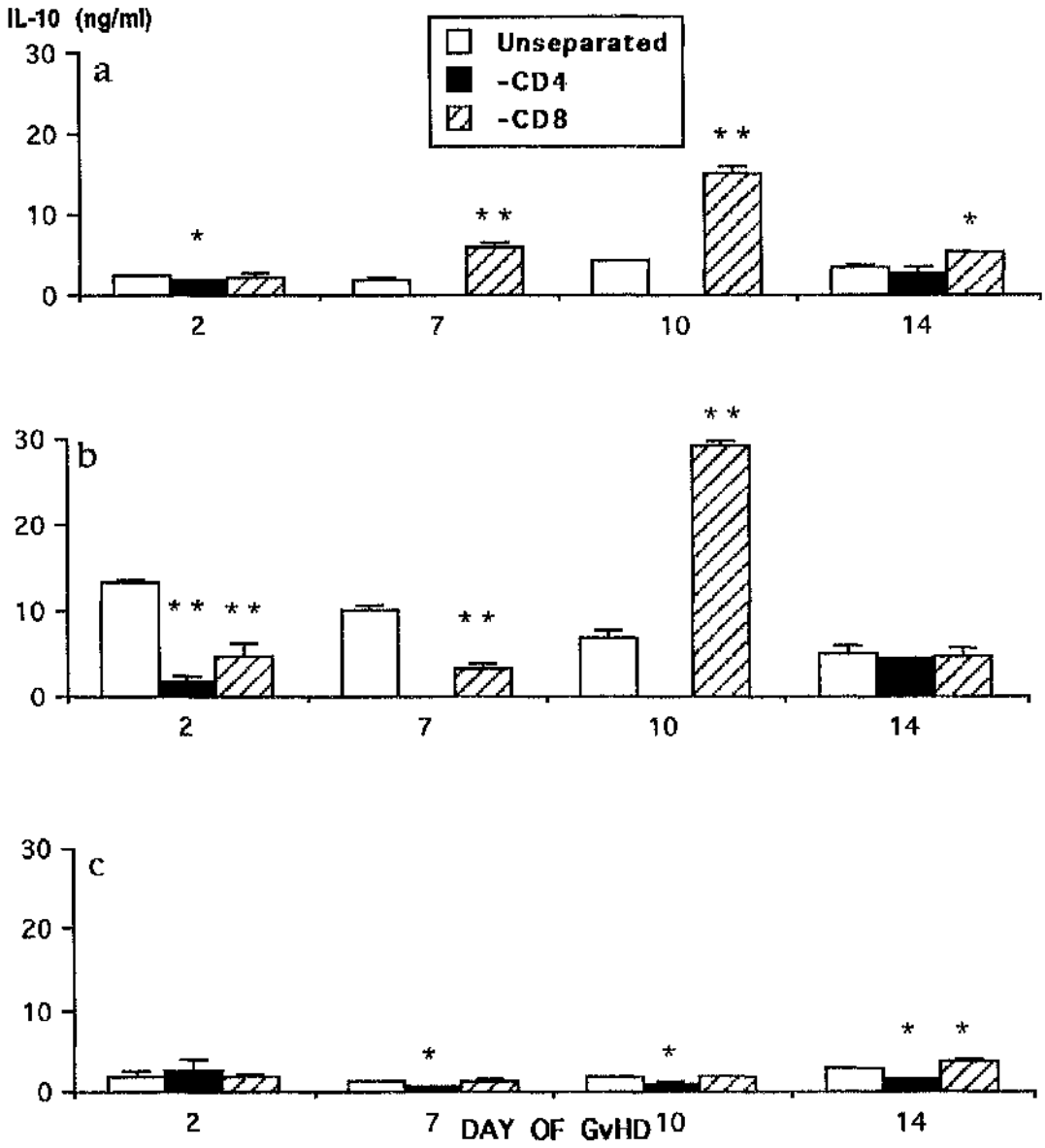


Fig. 9.6. Effects of depleting CD4+ and CD8+ cells *in vitro* on Con A induced IL-10 production during acute and chronic GvHD.

The results show the effect of depleting CD4+ or CD8+ lymphocytes on IL-10 levels in Con A stimulated spleen cell cultures from mice with acute GvHD (a), mice with chronic GvHD (b) and control mice (c). CD4+ or CD8+ cells were depleted *in vitro* using complement mediated lysis and the cells cultured with 10μg/ml Con A for 48 hours before the supernatants were assayed for the presence of IL-10 by ELISA. (* $p < 0.05$ ** $p < 0.001$ vs unseparated).

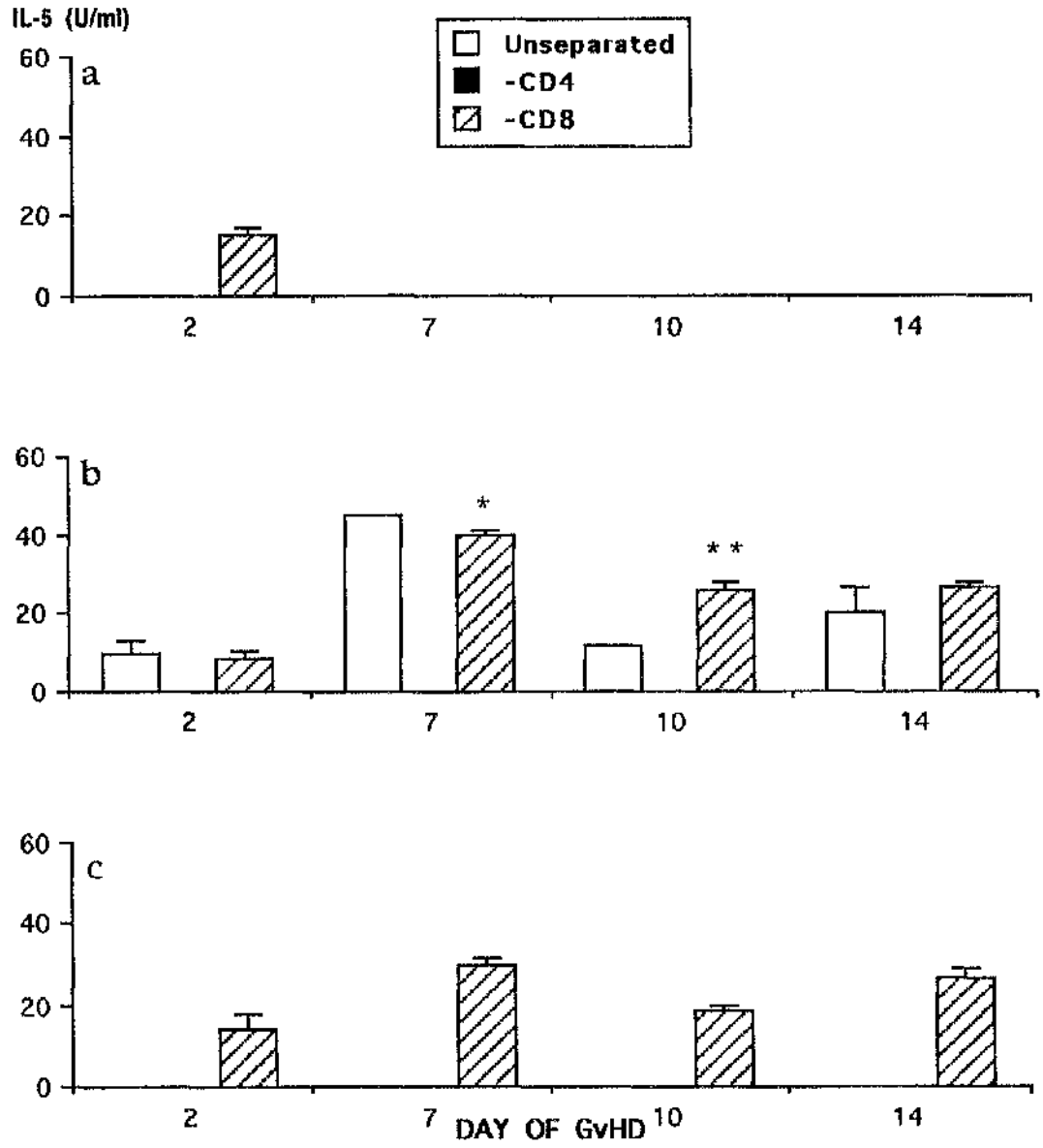


Fig. 9.7. Effects of depleting CD4+ and CD8+ cells *in vitro* on Con A induced IL-5 production during acute and chronic GvHD.

The results show the effect of depleting CD4+ or CD8+ lymphocytes on IL-5 levels in Con A stimulated spleen cell cultures from mice with acute GvHD (a), mice with chronic GvHD (b) and control mice (c). CD4+ or CD8+ cells were depleted *in vitro* using complement mediated lysis and the cells cultured with 10μg/ml Con A for 96 hours before the supernatants were assayed for the presence of IL-5 by ELISA. (* $p < 0.05$ ** $p < 0.005$ vs unseparated).

CHAPTER 10

THE ROLE OF NK CELLS IN GvHD

Introduction

I have shown in Chapters 4-8 that early production of IL-12 and IFN-γ was required for the development of acute GvHD. There is considerable evidence that NK cells may be a source of IFN-γ in other models of T cell-mediated immunity [112-114, 152, 153, 169] and I have shown that the increased IFN-γ in acute GvHD may be derived from a non-T cell source and coincides with activation of NK cells. I therefore decided to carry out a detailed study of the involvement of NK cells in polarising immune effector responses early in acute GvHD. To do this, I examined whether NK cell activation was exclusively a feature of acute GvHD at all time points, beginning a few hours after disease induction. Next, I focused on the role of NK cells in the initiation of B6 ⇒ BDF_1 acute GvHD by depleting NK cells immediately before induction of disease mice using anti-ASGM-1 antibody treatment *in vivo*.

Experimental protocol

The GvHR was induced by i.v. injection of 10^8 viable B6 or DBA/2 parental spleen cells into BDF_1 recipients as before. Levels of splenic NK cell-mediated cytotoxicity were measured throughout the first week of disease at effector : target (E : T) ratios from 100 : 1 to 12.5 : 1 using YAC-1 target cells. The results shown are for an E : T ratio of 100 : 1. NK cells were depleted from host mice *in vivo* by a single i.v. injection of anti-ASGM-1 rabbit antiserum. To avoid a contribution from donor-derived NK cells [103-105], I also treated donor mice with anti-ASGM-1 one day before cell harvest. Control mice received normal rabbit serum (NRS).

The intensity of the systemic GvHD *in vivo* was monitored by assessing splenomegaly, weight loss and mortality, while immune function was determined by measuring the proliferative capacity of spleen cells from GvHD mice either '*ex-vivo*', or in response to stimulation with T or B cell mitogens. IFN-γ, IL-2, IL-10, IL-5 and IL-4 production was measured by culturing spleen cells from GvHD mice either in medium alone or with Con A.

Results

Kinetics of NK cell activation during the early period of acute and chronic GvHD

B6 ⇒ BDF_1 mice displayed enhanced splenic NK cell activity from 4 hours after induction of the GvHR and this remained elevated throughout the period examined (up to day 7 of the GvHD) (Figs. 10.1). In contrast, enhanced splenic NK cell activity was not detectable in DBA/2 ⇒ BDF_1 mice before day 4 and this was markedly lower than that found in acute GvHD mice (Fig. 10.1). NK cell activity then remained enhanced to a similar extent on day 7 in chronic GvHD. Thus, early NK cell activation is mainly a feature of acute GvHD, although it subsequently does occur in chronic GvHD.

Effects of depleting NK cells on progression of GvHD

To determine whether the enhanced NK cell activity observed in B6 ⇒ BDF_1 mice is critical for disease progression, I examined the effect of NK cell depletion on acute GvHD.

Anti-ASGM-1 treatment *in vivo* ablated the levels of resting NK cell activity in normal BDF_1 mice and dramatically reduced, but did not ablate those observed on day 2 of acute GvHD in B6 ⇒ BDF_1 mice (Fig. 10.2).

i) Weight loss and mortality

In comparison to the previous experiments I have described in my thesis, the acute GvHD in this experiment was much less aggressive. Thus, weight loss was exhibited by only 1 of the 5 unmanipulated B6 ⇒ BDF_1 mice, was not apparent until day 20 and continued until day 24, when the mouse died. The remaining 4 mice showed no weight loss and did not die during the period of the experiment (12 weeks). However, 3 of 6 NK cell depleted B6 ⇒ BDF_1 mice lost weight from day 18 onwards. One of these mice died on day 21 and a further one on day 28. The third mouse which had lost weight recovered and there were no other deaths in this group.

ii) Splenomegaly

Despite the reduced morbidity and mortality observed in this experiment, unmodified B6 ⇒ BDF_1 mice showed splenomegaly on both days 2 (Fig. 10.3a) and 10 (Fig. 10.3b) which was comparable to that seen in previous experiments. Treatment with anti-ASGM-1 antiserum significantly reduced the splenomegaly on day 2 of the disease (Fig. 10.3a), but by day 10, treated mice showed significantly greater splenomegaly than untreated animals (Fig. 10.3b). Anti-ASGM-1 antiserum had no effect on the size of control spleens at either time point (Fig. 10.3a & b).

iii) Immune function

a) Spontaneous *'ex-vivo'* proliferation

In parallel with the splenomegaly observed in unmanipulated B6 ⇒ BDF_1 mice, spleen cells from these animals displayed an enhanced ability to proliferate spontaneously *'ex-vivo'* on both day 2 (Fig. 10.4a) and day 10 (Fig. 10.4b). Treatment with anti-ASGM-1 significantly reduced the spontaneous proliferation

observed on day 2 (Fig. 10.4a) and significantly increased that observed on day 10 (Fig. 10.4b).

On both days 2 and 10, spleen cells from control mice which had received anti-ASGM-1 anti-serum showed significantly increased levels of spontaneous proliferation compared with cells from unmodified control mice (Figs. 10.4a and 10.4b).

b) Proliferative responses to Con A stimulation

On day 10, spleen cells from unmanipulated B6 ⇒ BDF_1 mice showed similar proliferative responses to Con A stimulation to control cells (Fig. 10.5). This was in contrast to my findings in previous experiments, where the day 10 Con A responses of B6 ⇒ BDF_1 mice were suppressed and again highlighted the less aggressive GvHD in this experiment. Anti-ASGM-1 treatment significantly reduced the Con A responses of these mice, although it did not affect those of cells from control animals (Fig. 10.5).

c) Proliferative responses to LPS stimulation

As in previous experiments, on day 10, spleen cells from unmanipulated B6 ⇒ BDF_1 mice showed significantly reduced proliferative responses to LPS stimulation compared with control cells. This immunosuppression was enhanced further by treatment with anti-ASGM-1, but the antibody did not affect the LPS responses of cells from control animals (Fig. 10.6).

iv) Cytokine production

IFN-γ

a) Spontaneous

Cells from unmodified B6 ⇒ BDF_1 mice spontaneously produced high levels of IFN-γ on days 2 (Fig. 10.7a) and 10 (Fig. 10.7b). Treatment with anti-ASGM-1 did not significantly affect this at either time point (Figs. 10.7a & b). However, the results on day 2 were rather difficult to interpret, as spleen cells from both unmodified and anti-ASGM-1 treated control mice produced IFN-γ spontaneously at levels much higher than usually seen (Fig. 10.7a). The reasons for this are unclear and it did not occur on day 10 (Fig. 10.7b).

b) Con A stimulated

On day 2, cells from unmodified B6 ⇒ BDF_1 mice produced significantly enhanced levels of IFN-γ in response to Con A compared with control cells (Fig. 10.8a). By day 10, however, the levels produced by cells from B6 ⇒ BDF_1 mice were similar to those of controls (Fig. 10.8b). At both times, this IFN-γ production was unaffected by anti-ASGM-1 antiserum treatment (Figs. 10.8a & b). Furthermore, anti-ASGM-1 treatment did not affect the levels of IFN-γ produced by Con A stimulated control cells on either day 2 (Fig. 10.8a) or day 10 (Fig. 10.8b).

IL-2

IL-2 was not produced spontaneously by cells from any group (data not shown).

On both days 2 and 10, cells from untreated B6 ⇒ BDF_1 mice produced significantly lower levels of IL-2 in response to Con A stimulation than control cells (Fig. 10.9a & b). At both time points, treatment with anti-ASGM-1

antiserum significantly lowered IL-2 production by B6 ⇒ BDF_1 cells even further (Fig. 10.9a & b) and also reduced the levels of IL-2 produced by Con A stimulated control cells (Fig. 10.9a & b).

IL-10

IL-10 was not produced spontaneously by cells from any group (data not shown).

On both days 2 and 10, cells from untreated B6 ⇒ BDF_1 mice produced significantly higher levels of IL-10 in response to Con A stimulation compared with control cells (Fig. 10.10a & b). Once again, this was in contrast to my previous findings, since in other experiments B6 ⇒ BDF_1 cells and control cells produced similar levels of Con A stimulated IL-10. The reason for this disparity was unclear, but it should be noted that the levels of IL-10 produced by both groups in this study were very low. At both time points, treatment with anti-ASGM-1 antiserum did not affect IL-10 levels produced by either group (Fig. 10.10a & b).

IL-5

IL-5 was not produced spontaneously by cells from any group (data not shown).

On day 2, cells from untreated B6 ⇒ BDF_1 mice produced significantly higher levels of IL-5 in response to Con A stimulation compared with control cells (Fig. 10.11a). Anti-ASGM-1 treatment did not significantly affect this (Fig. 10.11a). On day 10, the level of IL-5 produced by cells from both untreated and anti-ASGM-1 treated B6 ⇒ BDF_1 mice were below the level of detection (Fig. 10.11b).

Anti-ASGM-1 treatment significantly increased the levels of IL-5 produced by Con A stimulated control cells on day 2 (Fig. 10.11a), but had no effect on the levels observed on day 10 (Fig. 10.11b).

v) CTL Activity

As before, untreated B6 ⇒ BDF_1 mice showed high levels of splenic CTL activity on day 10 of acute GvHD (Fig. 10.12). This was consistently reduced at every E:T ratio by anti-ASGM-1 treatment, but it should be noted that these treated mice still showed marked anti-host CTL activity (Fig. 10.12).

Summary and Conclusions

The results detailed in this chapter confirm that NK cell activation is a particular feature of the early stages of acute GvHD. Increased splenic NK cell activity was observed within 4 hours of the induction of acute GvHD in B6 ⇒ BDF_1 mice and this remained elevated throughout the first week of disease. In contrast, although increased NK cell activity was also observed in DBA/2 ⇒ BDF_1 mice with chronic GvHD, this was not evident until day 4 of the disease and was less marked than the levels observed in mice with acute GvHD. The exact role of NK cells in the development of acute GvHD was not straightforward and several effects were left unclear by my study, particularly since the GvHD was less aggressive than normal. Weight loss and mortality in B6 ⇒ BDF_1 GvHD were not dependent on increased NK cell activity early in the disease. Indeed, my results suggested that NK cell depletion may slightly increase the intensity of systemic GvHD since weight loss and mortality were higher in the treated group, as was the degree of T and B cell suppression. In contrast, anti-ASGM-1 reduced the early (day 2) lymphoid hyperplasia in B6 ⇒ BDF_1 mice, but this effect was transient. Anti-ASGM-1 treatment had a limited effect on immune function in control mice, although it increased the spontaneous proliferation of control cells on both days 2 and 10. The reason for this increase was not clear, but was consistent with reports suggesting ASGM-1 is expressed on a population of cells with 'suppressor-activity' [170]. However, anti-ASGM-1 treatment did not affect the Con A or LPS responses of control cells.

Anti-ASGM-1 treatment had very little effect on cytokine production by either B6 ⇒ BDF_1 or control cells. In particular, it did not affect the levels of IFN-γ produced by cells from B6 ⇒ BDF_1 mice either spontaneously, or in response to Con A. Anti-ASGM-1 did not affect IL-10 or IL-5 production by cells from B6 ⇒ BDF_1 mice at either time point and similarily, did not affect IL-10 production by control cells. It did however cause a striking increase in control IL-5 levels on day 2. The reason for this was unclear and by day 10 it was no longer evident. Finally, consistent with previous reports that anti-ASGM-1 antibody treatment prevents the full development of alloreactive CTL *in vitro* [171], I found that B6 ⇒ BDF_1 mice given anti-ASGM-1 *in vivo* showed somewhat reduced CTL activity compared with NRS treated B6 ⇒ BDF_1 mice. However, it should be noted that there was residual NK cell activity in the spleens of anti-ASGM-1 treated B6 ⇒ BDF_1 mice.

In conclusion, a single injection of anti-ASGM-1 antiserum on day -1 of acute GvHD had little consistent effect on either the progression of the disease or the cytokines produced during its course. Most importantly, my results suggested that NK cells did not seem to be a major source of IFN-γ during acute GvHD. Although there was residual NK cell activity in anti-ASGM-1 treated B6 ⇒ BDF_1 mice, these findings suggest that NK cells are not involved in the initial polarisation of acute vs chronic GvHD.

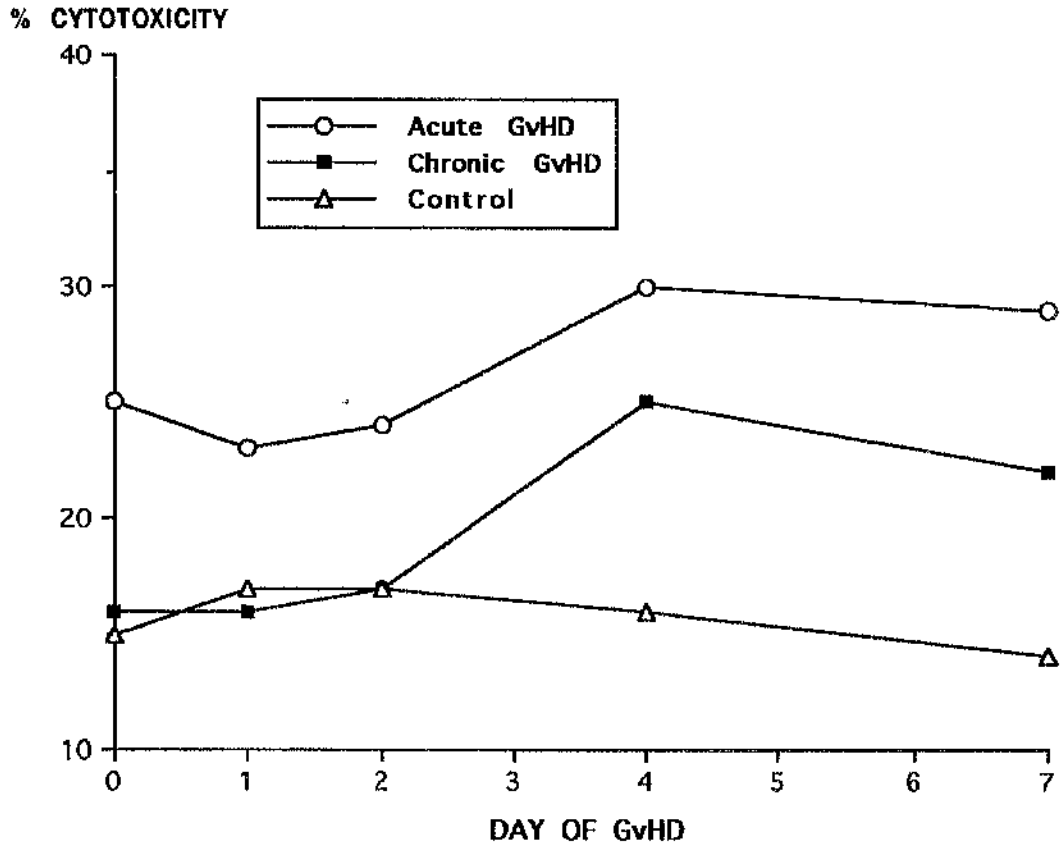


Fig. 10.1. Kinetics of NK cell activation in acute and chronic GvHD.

Splenic NK cell activity in BDF_1 mice with acute or chronic GvHD. The results are shown as the % cytotoxicity against YAC-1 target cells from quadruplicate assays measured at an E : T ratio of 100 : 1, using spleen cells pooled from 3 mice per group.

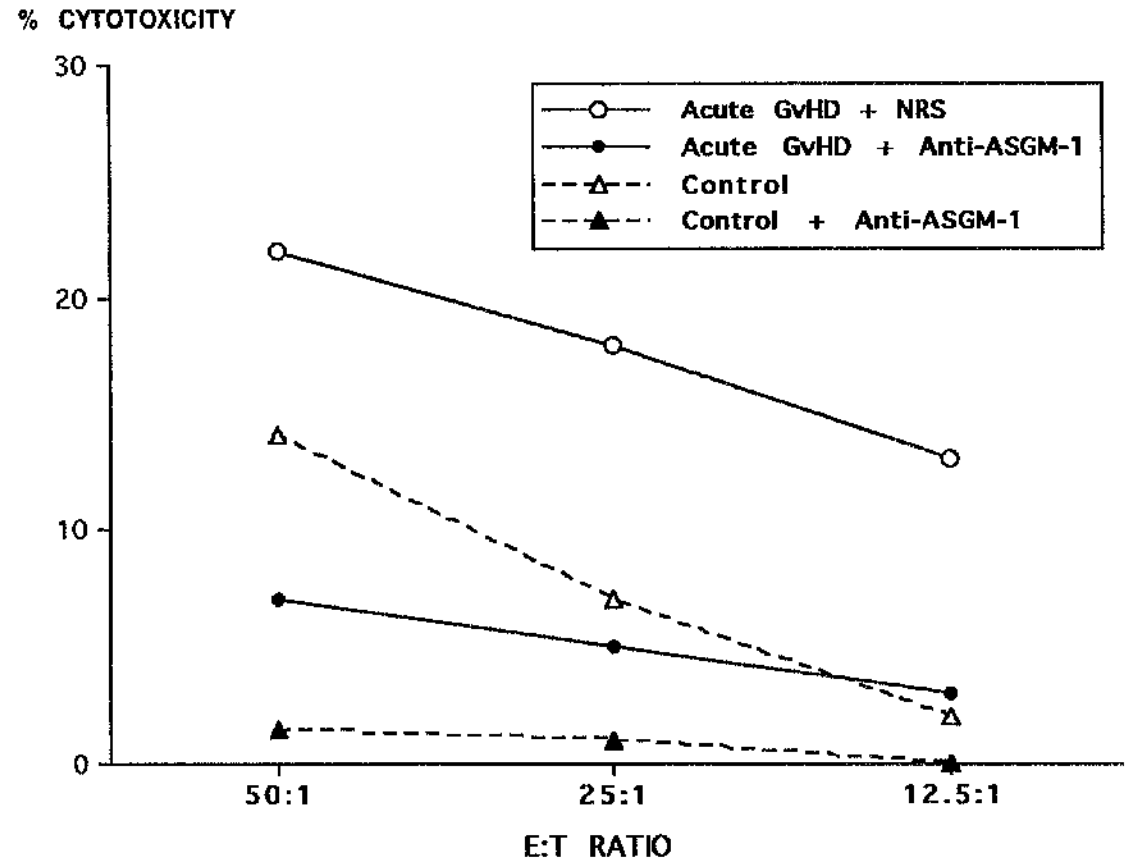


Fig. 10.2. Effects of anti-ASGM-1 treatment on splenic NK cell activity

The results show the effects of a single injection of anti-ASGM-1 antiserum on NK cell activity in control BDF_1 mice and BDF_1 mice given 10^8 B6 spleen cells i.v. The results are shown as the % cytotoxicity against YAC-1 target cells from quadruplicate assays measured at E : T ratios from 50 : 1 to 12.5 : 1, using spleen cells pooled from 3 mice per group on day 2 of GvHD.

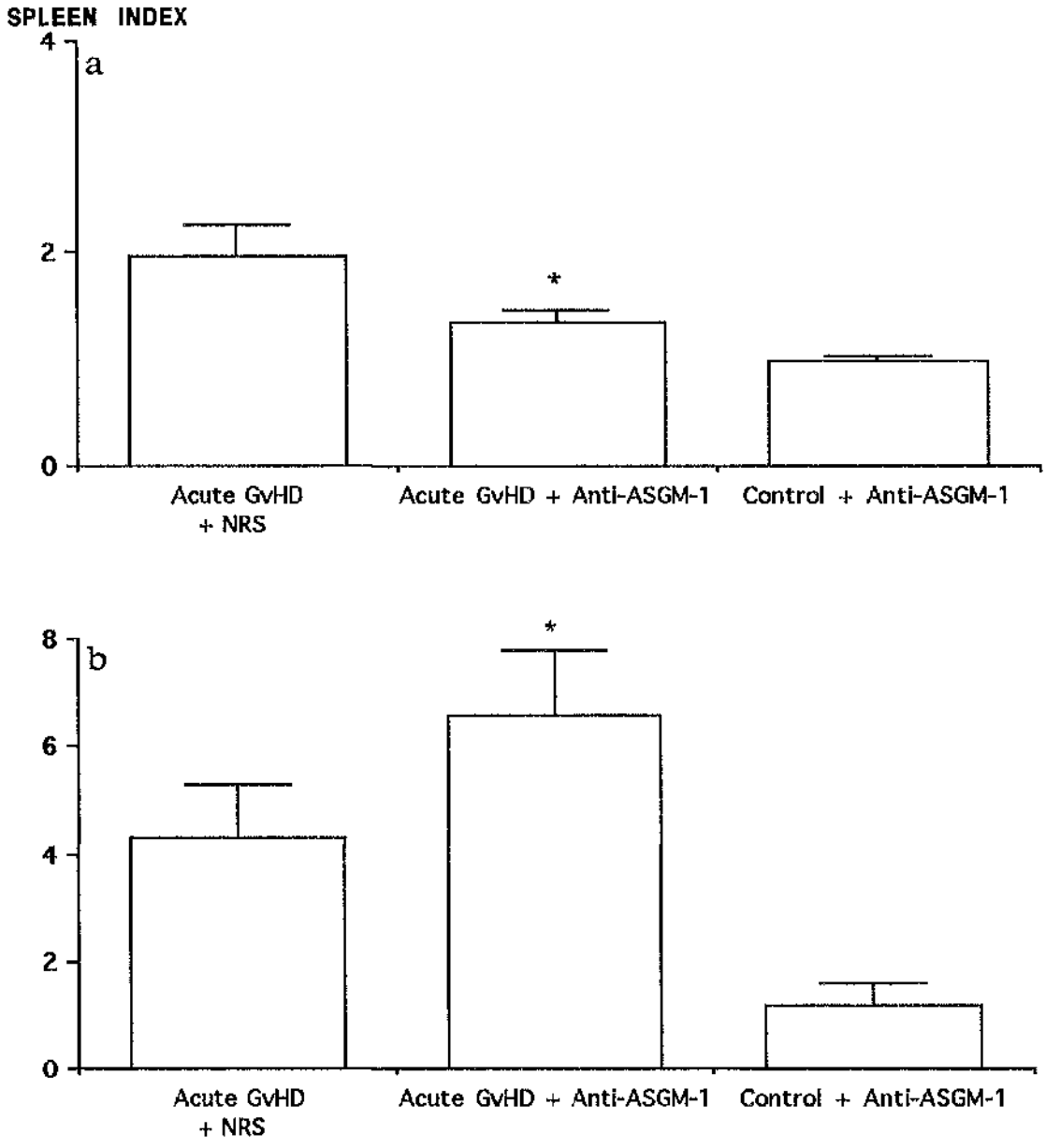


Fig. 10.3. Effects of anti-ASGM-1 treatment on splenomegaly in acute GvHD.

The results show the effects of a single injection of anti-ASGM-1 antiserum on splenomegaly in BDF_1 mice given 10^8 B6 spleen cells i.v. and are the mean spleen indices ± 1 SD for 3 mice per group on day 2 (Fig. 10.3a) and day 10 (Fig. 10.3b) of disease. (* $p < 0.05$ vs unmodified acute GvHD)

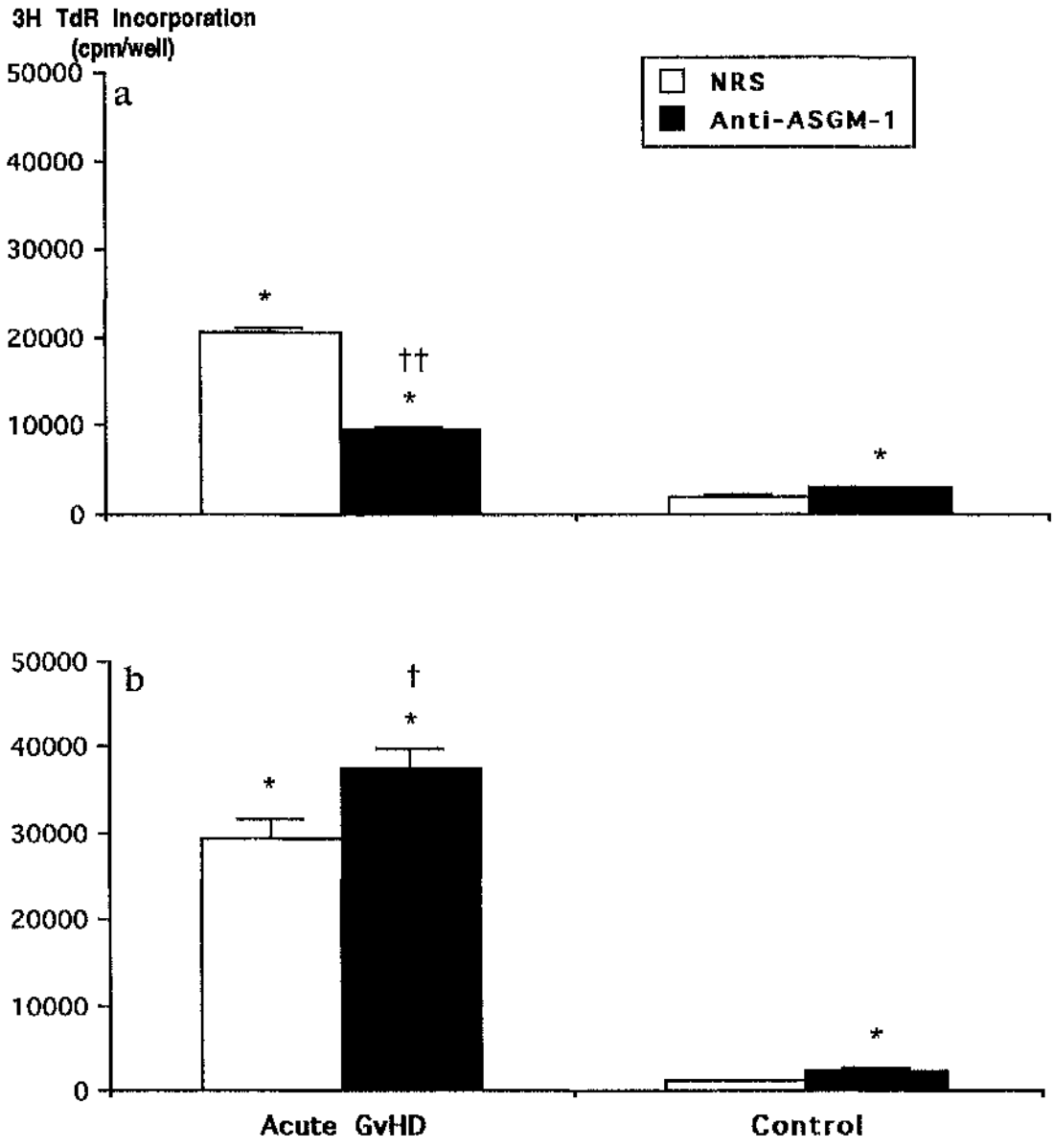


Fig. 10.4. Effects of anti-ASGM-1 treatment on spontaneous '*ex-vivo*' proliferation in acute GvHD.

The results show the effect of a single injection of anti-ASGM-1 antiserum on the spontaneous proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. and are the mean uptake of 3H-TdR ± 1 SD after 4 hours in quadruplicate cultures using spleen cells pooled from 3 mice per group on day 2 (Fig. 10.4a) and day 10 (Fig. 10.4b) of disease. (* $p < 0.001$ vs unmodified controls; † $p < 0.005$ †† $p < 0.001$ vs unmodified acute GvHD)

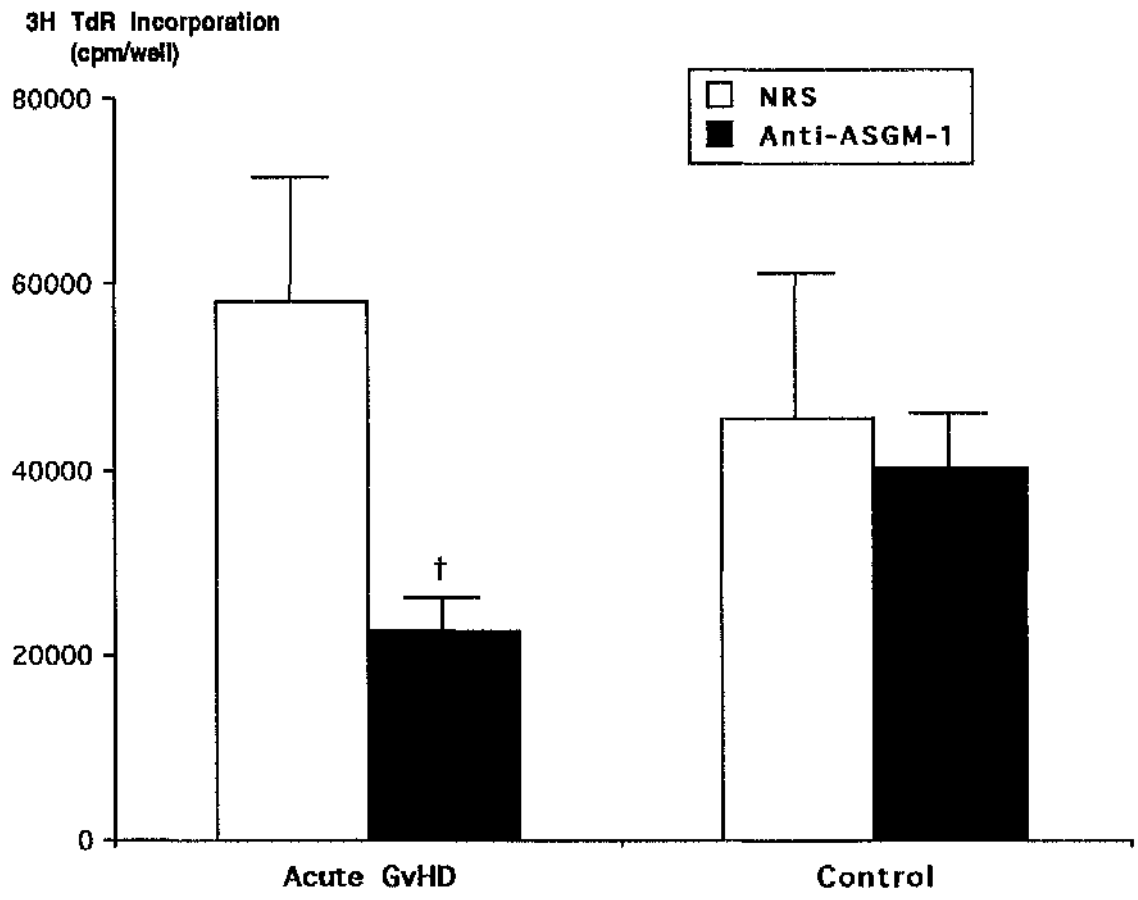


Fig. 10.5. Effects of anti-ASGM-1 treatment on Con A induced proliferation in acute GvHD.

The results show the effect of a single injection of anti-ASGM-1 antiserum on the proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. in response to stimulation with 10μg/ml Con A. The data are the mean uptake of 3H-TdR ± 1 SD after 48 hours in quadruplicate cultures, using spleen cells pooled from 3 mice per group on day 10 of acute GvHD. († $p < 0.005$ vs unmodified acute GvHD)

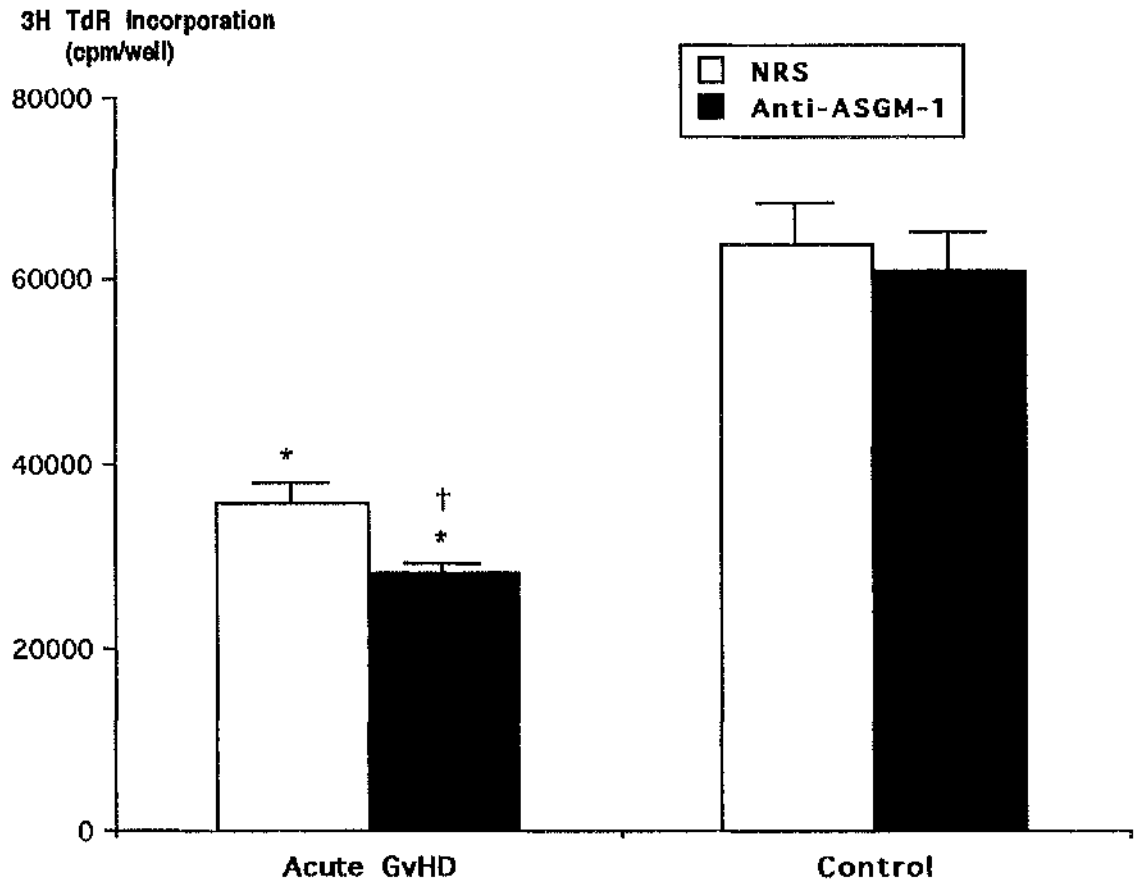


Fig. 10.6. Effect of anti-ASGM-1 treatment on LPS induced proliferation in acute GvHD.

The results show the effect of a single injection of anti-ASGM-1 antiserum on the proliferative capacity of splenocytes from BDF_1 mice given 10^8 B6 spleen cells i.v. in response to stimulation with 10μg/ml LPS. The data are the mean uptake of 3H-TdR ± 1 SD after 24 hours in quadruplicate cultures, using spleen cells pooled from 3 mice per group on day 10 of GvHD. (* $p < 0.001$ vs unmodified controls; † $p < 0.001$ vs unmodified acute GvHD)

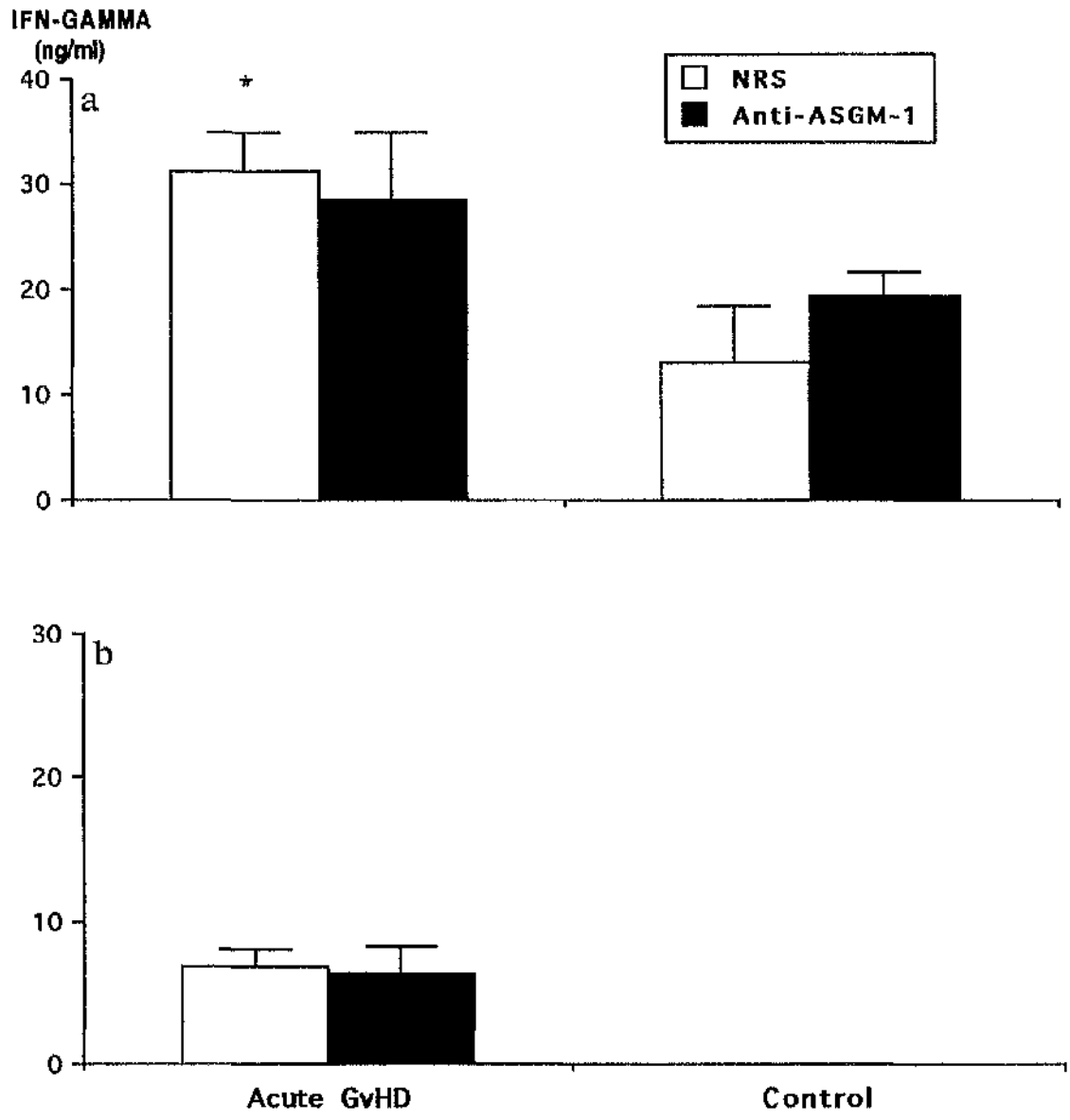


Fig. 10.7. Effects of anti-ASGM-1 treatment on spontaneous IFN-γ production in acute GvHD.

The results show the effect of a single injection of anti-ASGM-1 antiserum on spontaneous IFN-γ production in mice given 10^8 B6 spleen cells. Splenocytes were cultured in medium for 48 hours on day 2 (Fig. 10.7a) and day 10 (Fig. 10.7b) of acute GvHD before the supernatants were assayed for the presence of IFN-γ by ELISA. The results shown are means ± SD of triplicate samples. (* $p < 0.05$ vs unmodified controls).

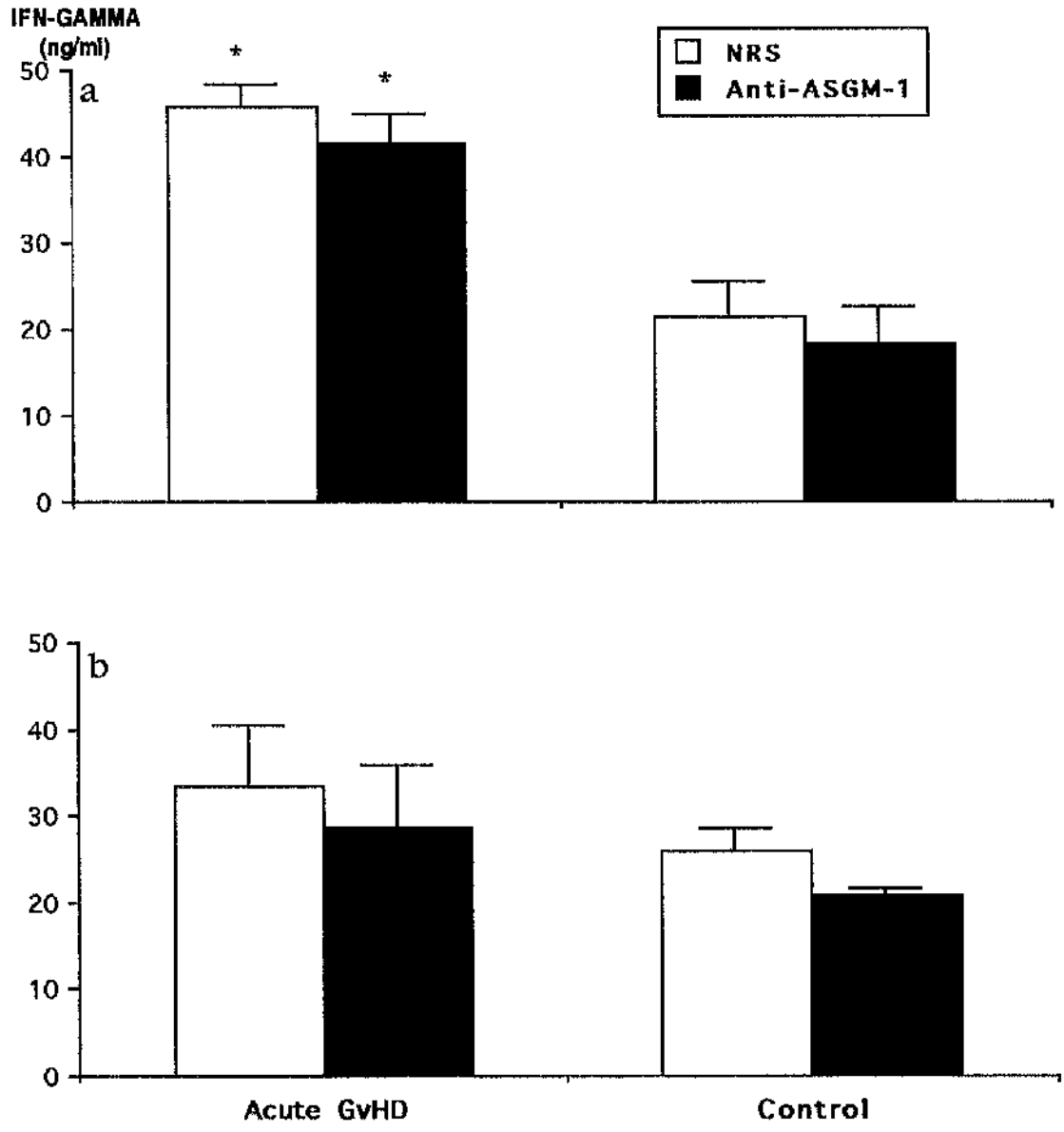


Fig. 10.8. Effects of anti-ASGM-1 treatment on Con A induced IFN-γ production in acute GvHD.

The results show the effect of a single injection of anti-ASGM-1 antiserum on IFN-γ production in BDF1 mice given 10^8 B6 spleen cells i.v. Splenocytes were cultured with 10μg/ml Con A for 48 hours on day 2 (Fig. 10.8a) and day 10 (Fig. 10.8b) of acute GvHD before the supernatants were assayed for the presence of IFN-γ by ELISA. The results shown are means ± SD of triplicate samples. (* $p < 0.001$ vs unmodified controls)

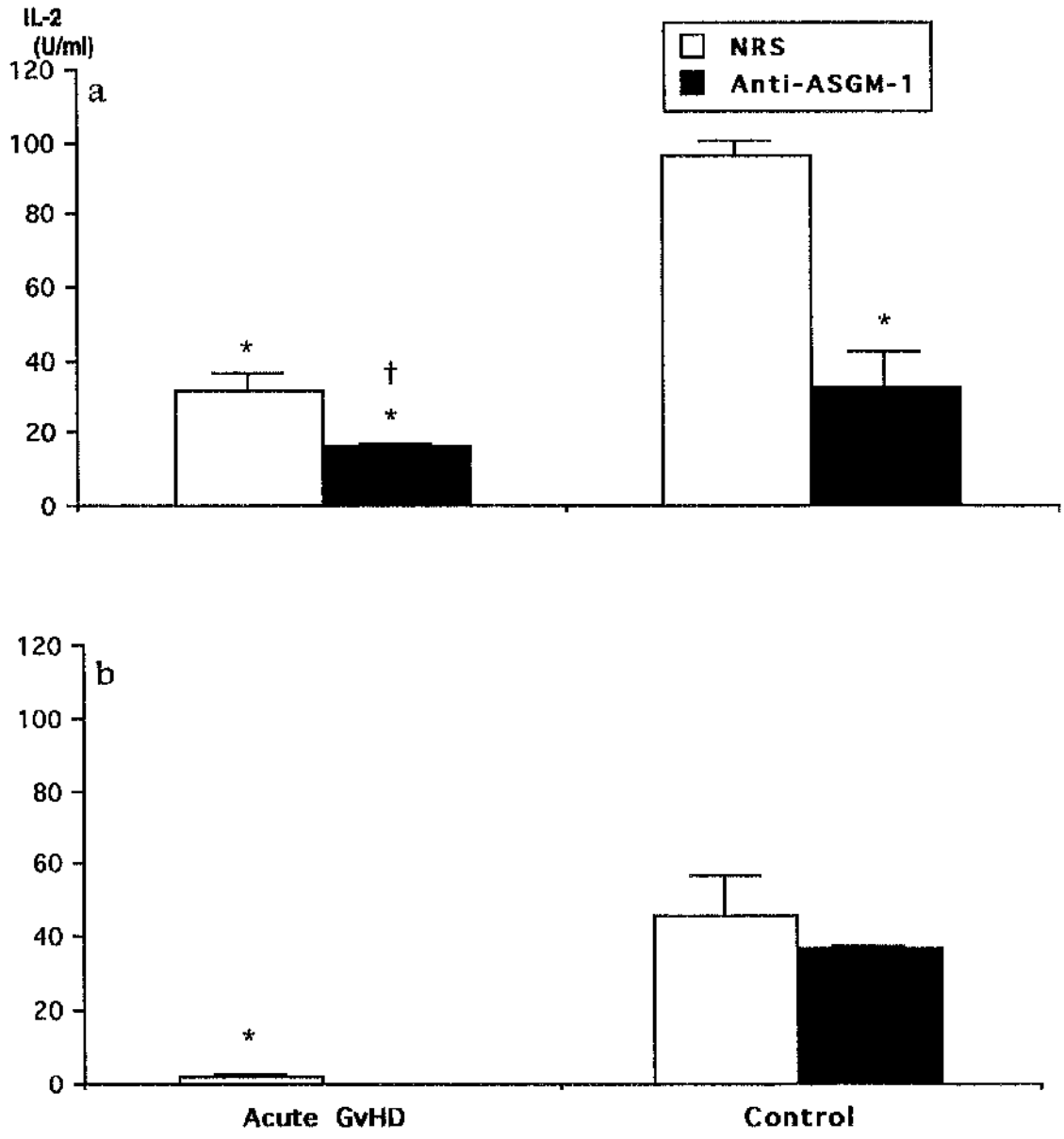


Fig. 10.9. Effects of anti-ASGM-1 treatment on IL-2 production in acute GvHD.

The results show the effect of a single dose of anti-ASGM-1 antiserum on IL-2 production in BDF_1 mice given 10^8 B6 spleen cells i.v. Splenocytes were cultured with of 10μg/ml Con A for 24 hours on day 2 (a) and day 10 (b) of acute GvHD before the supernatants were assayed for the presence of IL-2 by ELISA. The results shown are means ± SD of triplicate samples. (* $p < 0.001$ vs unmodified controls; † $p < 0.001$ vs unmodified acute GvHD)

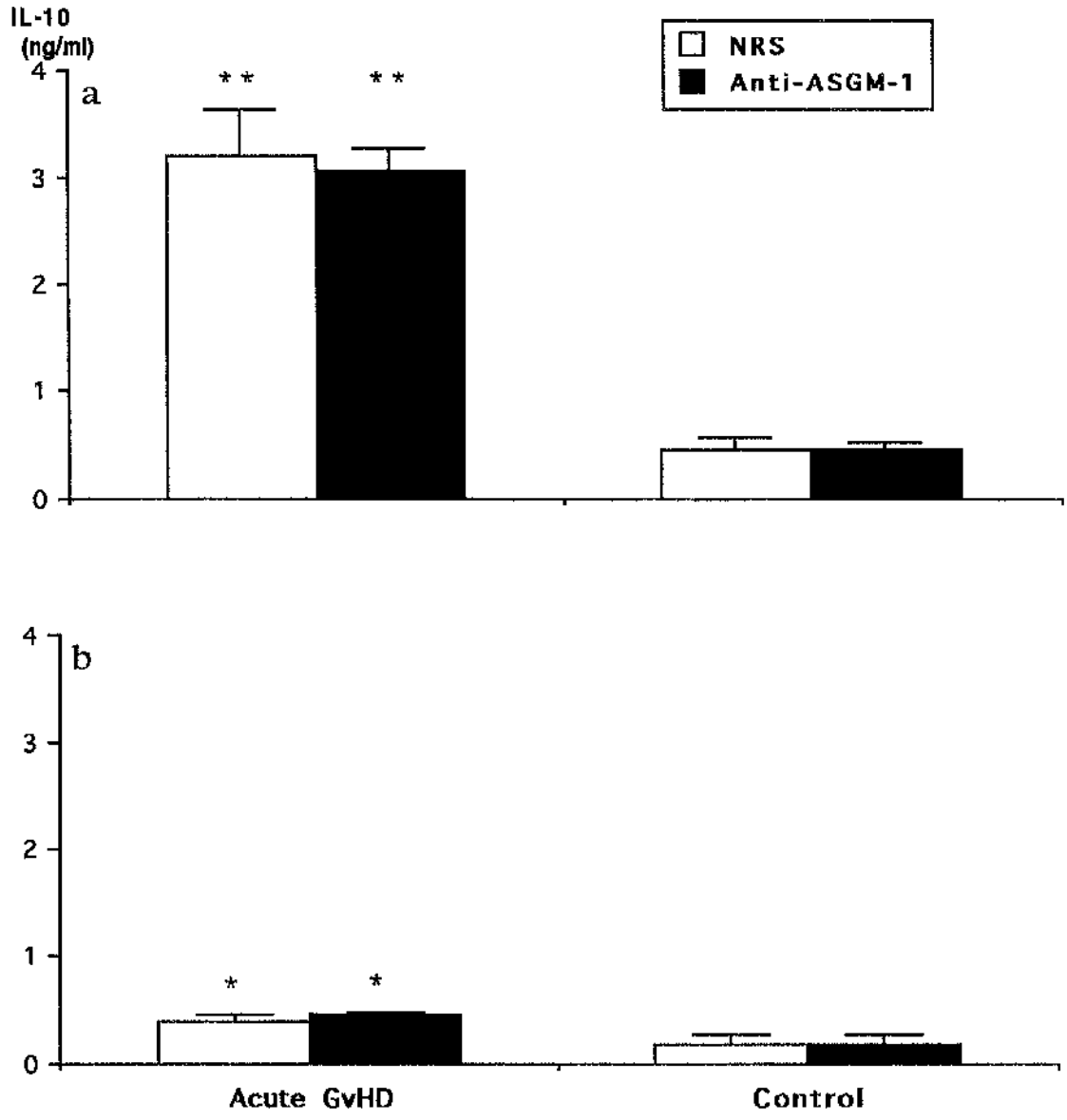


Fig. 10.10. Effects of anti-ASGM-1 treatment on IL-10 production in acute GvHD.

The results show the effect of a single dose of anti-ASGM-1 antiserum on IL-10 production in BDF_1 mice given 10^8 B6 spleen cells i.v. Splenocytes were cultured with 10μg/ml Con A for 48 hours on day 2 (Fig. 10.10a) and day 10 (Fig. 10.10b) of acute GvHD before the supernatants were assayed for the presence of IL-10 by ELISA. The results shown are means ± SD of triplicate samples. (* $p < 0.005$ ** $p < 0.001$ vs unmodified controls)

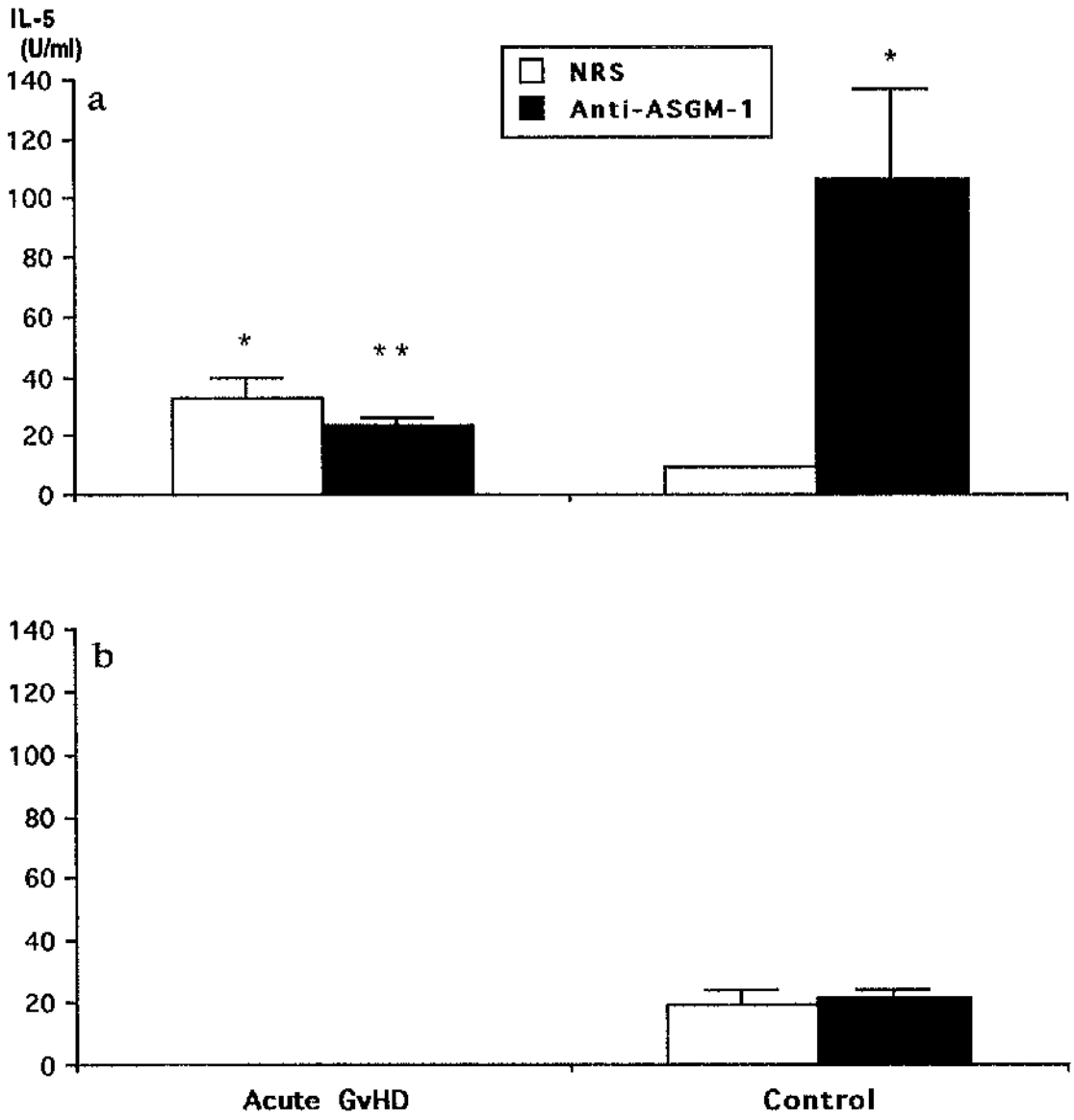


Fig. 10.11. Effects of anti-ASGM-1 treatment on IL-5 production in acute GvHD.

The results show the effect of a single dose of anti-ASGM-1 antiserum on IL-5 production in BDF_1 mice given 10^8 B6 spleen cells i.v. Splenocytes were cultured with 10μg/ml Con A for 96 hours on day 2 (Fig. 10.11a) and day 10 (Fig. 10.11b) of acute GvHD before the supernatants were assayed for the presence of IL-5 by ELISA. The results shown are means ± SD of triplicate samples. (* $p < 0.005$ ** $p < 0.001$ vs unmodified controls)

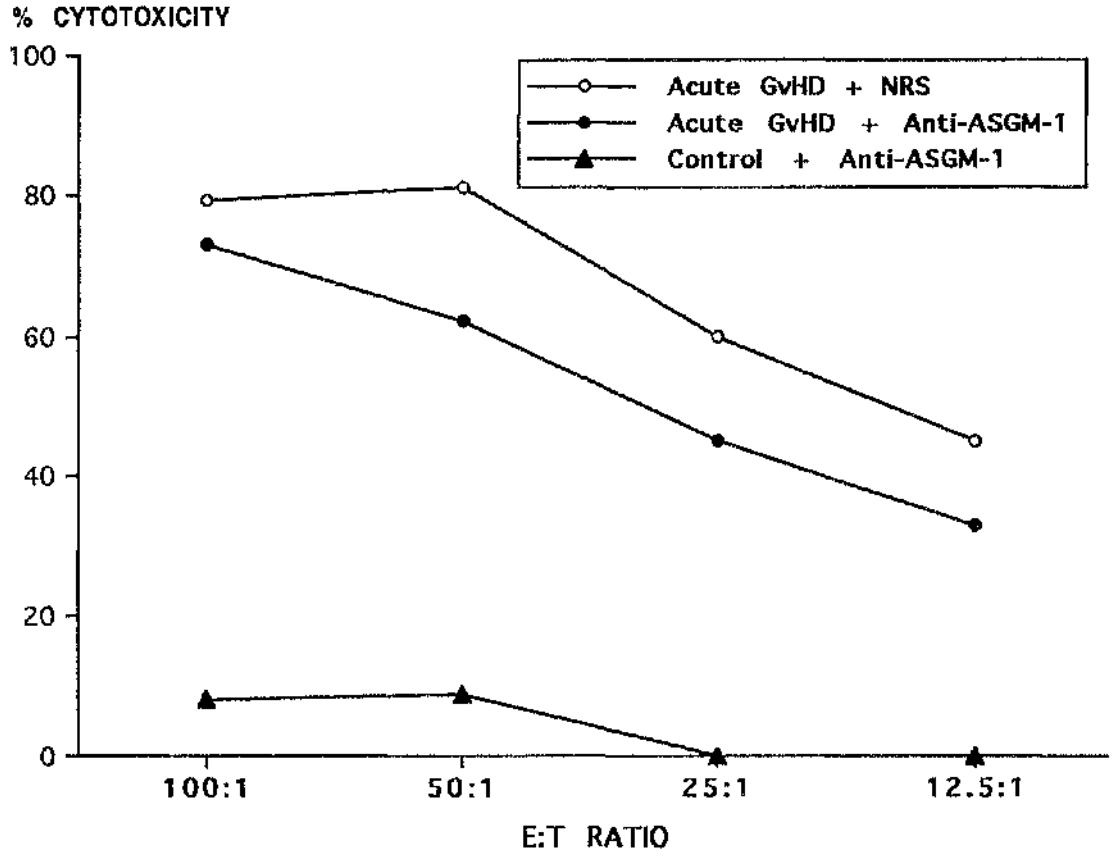


Fig. 10.12. Effects of anti-ASGM-1 treatment on CTL-activity in acute GvHD.

The results show the effect of a single injection of anti-ASGM-1 antiserum on splenic CTL activity in BDF_1 mice given 10^8 B6 i.v. and are the % cytotoxicity against P815 target cells from quadruplicate assays measured at effector : target (E:T) ratios from 100 : 1 to 12.5 : 1, using spleen cells pooled from 3 mice per group on day 10 of GvHD.

CHAPTER 11

DISCUSSION

The results presented in this thesis have confirmed and extended previous findings on the distinct forms of immunopathology which develop in BDF_1 mice with acute and chronic GvHD induced by B6 or DBA/2 parental cells respectively. In addition, my results have demonstrated the importance of individual cytokines in polarising the allogeneic immune response and determining its pathological outcome. These studies of the cellular and molecular interactions involved in murine GvHD have implications for understanding the pathogenesis of clinical GvHD and the development of specific therapy following BMT. Furthermore, they provide an important insight into the regulation of immune responses during other immunologically-mediated diseases.

KINETICS OF ACUTE AND CHRONIC GvHD

The results described in Chapter 3 showed that both acute and chronic forms of GvHD were characterised by an early period of immune activation. However, this stimulatory activity persisted throughout chronic GvHD, but in acute GvHD was replaced rapidly by a destructive, suppressive phase, culminating in weight loss and death in the majority of animals. My results also showed that the distinct outcomes of the GvHR were paralleled by different patterns of cytokine production, with high levels of IFN-γ produced spontaneously by cells from B6 $\Rightarrow$ BDF_1 mice, while enhanced IL-5 and IL-10 were produced by Con A stimulated DBA/2 $\Rightarrow$ BDF_1 cells. Although the different forms of pathology which occur in this model of GvHD have been described previously [21, 23, 24, 27], my study was the first to examine such a wide variety of *in vivo* and *in vitro* indices of disease over a detailed period of time. In

addition, this was the first study to correlate systemic features of acute and chronic GvHD with cytokine production.

Systemic Features of GvHD

The early proliferative period of both diseases was characterised by splenomegaly, increased numbers of splenic lymphocytes and enhanced spontaneous proliferative responses of GvHD cells '*ex-vivo*'. B6 cells induced a more aggressive hyperplastic phase than DBA/2 cells, particularly between days 7 to 10, when the splenomegaly and spontaneous proliferative response of acute GvHD mice reached a peak and was 2-3 fold higher than that of mice with chronic GvHD. Although I did not determine the phenotype and origin (donor vs host) of the proliferating cell populations, previous work implicates the involvement of a variety of cell types including donor $CD4^+$ and $CD8^+$ T cells [30], host B cells [124] and possibly haemopoietic stem cells. Of particular note was the fact that the most striking differences between the spontaneous proliferation of B6 $\Rightarrow$ BDF_1 and DBA/2 $\Rightarrow$ BDF_1 cells occurred on day 10, when there was a marked expansion of donor $CD8^+$ T cells in the former group. Thus at this time in B6 $\Rightarrow$ BDF_1 mice, the spontaneous response may be dominated by proliferating donor $CD8^+$ T cells.

This was also the time after which mice with acute GvHD exhibited lymphoid involution, dramatically reduced lymphocyte numbers and suppressed proliferative responses. This association between destructive GvHD and the preferential expansion of donor $CD8^+$ T cells during acute, but not chronic GvHD, has been described in numerous previous studies [24, 27-29, 31]. However, the role of $CD8^+$ T cells in the consequences of acute GvHD is controversial. Acute GvHD is critically dependent on the presence of donor $CD8^+$ T cells in the donor inoculum [30] and the differences between acute and chronic GvHD have been attributed to the fact that DBA/2 mice have low numbers of $CD8^+$ T cells and exhibit a particularly low anti-BDF_1 precursor CTL (pCTL) frequency compared

with mice of the B6 background [31]. Although my finding that CTL only appeared in acute GvHD was consistent with this idea, other studies suggest that this is unlikely to be the only factor accounting for the different capacities of DBA/2 and B6 $CD8^+$ T cells to expand and elicit anti-host CTL activity in BDF_1 mice [130, 131]. In addition, as I discuss below, acute GvHD-associated tissue destruction is not always associated with anti-host CTL activity [24, 27, 83].

When T cell function was assessed by examining the ability of GvHD spleen cells to proliferate to the T cell mitogen Con A, I found that chronic GvHD mice showed normal Con A responses throughout the course of disease and no loss of function was evident. In contrast, cells from acute GvHD mice showed slightly elevated proliferation until day 4 and then rapidly lost responsiveness. This indicated that while the spontaneous response of acute GvHD cells was reaching a peak on days 7-10, the Con A response was already suppressed below control levels. This may be explained by the fact that in other *in vitro* assays of T cell function, previously activated T cells have been shown to undergo activation-induced cell death (or 'apoptosis') when the TcR is crosslinked by polyclonal stimuli such as Con A [172]. Why a similar phenomenon was not observed when DBA/2 $\Rightarrow$ BDF_1 cells were stimulated with Con A *in vitro*, was not clear, but may be related to the lower level of spontaneous proliferation exhibited by these cells. Enumeration of GvHD cells following Con A stimulation *in vitro*, or examining GvHD cultures for evidence of apoptosis would help confirm this possibility.

The loss of Con A responsiveness exhibited exclusively by B6 $\Rightarrow$ BDF_1 mice has been described previously [51, 71, 73, 84, 165] and has been attributed to various mechanisms including deletion of host reactive T cells [50], defective lymphopoiesis [51, 52, 173-175] and active suppression mediated by anti-proliferative cytokines such as IFN-γ [71, 73]. Although defining the interactions underlying immune deficiency was beyond the scope of this inital study, it was interesting to note from my results that elevated IFN-γ production correlated with the abrogated Con A responses of B6 $\Rightarrow$ BDF_1 cells *in vitro* (see below).

However, the fact that during the latter stages of disease, reduced T cell numbers were recovered from B6 ⇒ BDF_1 spleens compared with either DBA/2 ⇒ BDF_1 or controls, may also indicate a role for T cell deletion *in vivo*. Recent studies in GvHD suggest that host-reactive T cells may be killed by apoptosis [50, 51] and that both Fas/Fas ligand and perforin-dependent cytotoxic pathways are involved in the disease [176]. However, TNF-α can also cause T cell apoptosis [177, 178] in synergy with IFN-γ [179]. Since both are produced preferentially during acute GvHD [41, 61], it is therefore also possible that a cytokine-mediated killing pathway may contribute to T cell deletion in B6 ⇒ BDF_1 mice.

Role of Cytokines in Acute and Chronic GvHD

When I started this work, several studies had been published which indicated that distinct patterns of T cell-derived cytokines were produced during acute and chronic GvHD in BDF_1 mice [42, 46]. These experiments showed that levels of Th1 cytokines were elevated during acute GvHD [41-43, 54], while Th2 cytokine production was preferentially associated with the chronic form of disease [42, 46, 47]. However, these studies did not determine when polarisation of the T cell response occurred and more importantly, did not attempt to correlate cytokine production with individual aspects of the diseases. A critical finding of Chapter 3 was therefore that the pattern of cytokines produced by acute and chronic GvHD mice had already started to diverge within the first 2 days of GvHD and by day 10, when the two diseases were distinct entities, they were also characterised by completely different cytokine profiles.

By day 2 and throughout the first 10 days of acute GvHD, B6 ⇒ BDF_1 cells produced elevated IFN-γ both spontaneously and in response to stimulation with Con A *in vitro*. Spontaneous and Con A induced cytokine levels *in vitro* provide different information regarding production *in vivo*. While spontaneous cytokine production indicates active secretion *in vivo*, Con A induced production provides a measure of lymphocyte priming [44]. It was therefore notable that in

my experiments, IFN-γ was the only cytokine which could be detected in unstimulated cultures, where it was produced exclusively by B6 ⇒ BDF_1 cells. This is consistent with the observation of Troutt et al. that up to 70% of lymph node cells from mice with acute GvHD contain IFN-γ mRNA [54].

The elevated levels of IFN-γ secretion were associated primarily with the early hyperplastic phase of systemic acute GvHD (days 0-10), an observation which has been confirmed in recent work by Rus et. al. [180]. Peak IFN-γ levels *in vitro* were detected on day 10, which was when the acute GvHD progressed into the later destructive period of the disease. Together with the results of Chapter 8, these findings indicate a potential role for IFN-γ in triggering the later pathological events characteristic of B6 ⇒ BDF_1 GvHD. Nevertheless, in common with production of other cytokines and the immune deficiency manifest in B6 ⇒ BDF_1 mice, IFN-γ was severely reduced late in acute GvHD.

Although IFN-γ was the predominant cytokine secreted by B6 ⇒ BDF_1 cells, enhanced levels of IL-2 and IL-5 were also produced by Con A stimulated cells on day 2 of acute GvHD. The priming of these cytokines was lost rapidly however and after day 4, levels were either similar to, or below, control levels. The early and transient increase in Con A stimulated IL-2 production has been described previously by others [23, 45, 181] and, consistent with the role of IL-2 as a T cell growth and survival factor [182], corresponded to the early activation and expansion of T cells in my study. In parallel, abrogated Con A induced IL-2 levels occurred on day 7, when these cells began to show reduced Con A driven proliferative responses. Although these observations may be attributed to classical acute GvHD-associated immunosuppression, they are difficult to interpret definitively since it was also possible that increased IL-2 consumption by highly activated cells *in vitro* led to reduced IL-2 levels. This would be consistent with the fact that B6 ⇒ BDF_1 cells continued to show high levels of spontaneous proliferation until day 10. Addition of soluble anti-IL-2 receptor (IL-2R) mAb to

GvHD cell cultures would help differentiate between reduced production vs increased production.

On days 2 and 4, B6 ⇒ BDF_1 cells produced enhanced levels of the Th2 cytokine, IL-5, as well as the Th1 cytokine, IFN-γ when stimulated with Con A *in vitro*. This was an important finding as it suggested that early in the disease, the immune response of B6 ⇒ BDF_1 mice was not completely polarised towards a Th1 phenotype. IL-5 priming was lost from day 7 onwards, at the time when IFN-γ levels were maximal, suggesting that the production of these cytokines is coordinately regulated in established acute GvHD. This is consistent with current views of Th1/Th2 cell development [132] and my later findings that neutralising IFN-γ *in vivo* increased IL-5 levels during the early period of B6 ⇒ BDF_1 GvHD supports the view that the high levels of IFN-γ actively suppress Th2 responses in these mice. Despite the increase in IL-5 in acute GvHD, I did not find priming of the other Th2-associated cytokines IL-10 and IL-4. Although studies published when I was carrying out my research reported elevated levels of IL-4 mRNA in acute GvHD [180], these workers were also unable to detect IL-4 cytokine product *in vitro*. This may reflect rapid *in vitro* consumption of transiently produced cytokine [183, 184], and/or the relative insensitivity of the assays employed.

In contrast to the Th1-dominated response found in acute GvHD, a more mixed pattern of cytokine production was seen during the initial phase of chronic GvHD. Although there was no spontaneous production of any cytokine by DBA/2 ⇒ BDF_1 cells at any time, from day 4 onwards, these cells were primed to produce high levels of IL-2, IL-5 and IL-10 when stimulated with Con A *in vitro*. In addition, there was some priming of IFN-γ production in the first 16 days of chronic GvHD, although the levels were markedly lower than those associated with the acute disease. Thus, the cytokine response of chronic GvHD mice was not completely polarised towards a Th2 phenotype at this time. However, it should be noted that the level of IFN-γ priming exhibited by DBA/2 ⇒ BDF_1 cells

was considerably lower than that shown by B6 ⇒ BDF_1 cells, and clearly insufficient to provoke the immunosuppression and destructive pathology associated with acute Th1-mediated GvHD.

In parallel with the persistent lymphoid stimulation in DBA/2 ⇒ BDF_1 mice, cells from these mice were primed to produce enhanced levels of IL-2 when stimulated with Con A *in vitro* at all time points. This contrasts with the suppressed IL-2 production in B6 ⇒ BDF_1 mice and correlated with my finding that the Con A induced proliferative response of chronic GvHD mice was normal at all time points examined. Furthermore, this is consistent with IL-2 being a product of precursor Th cells, rather than Th1 cells [132].

The chronically elevated levels of the Th2 cytokines IL-5 and IL-10 correlated with the persistent B cell activation and increased imunoglobulin levels which I observed in DBA/2 ⇒ BDF_1 mice. However, once again, I was unable to detect any IL-4. This was somewhat surprising given that Th2 responses are critically dependent on the presence of IL-4 [134, 135, 141] and appears to contrast with studies showing that the high levels of serum immunoglobulin, elevated proteinuria and death observed during chronic GvHD can be reduced by anti-IL-4 antibody *in vivo* [48, 128]. Although others have also been unable to detect IL-4 production in chronic GvHD cultures using ELISA [180], the upregulation of MHC class II on chronic GvHD spleen cells has been shown to be IL-4-dependent [46]. I decided to examine cytokine protein production rather than mRNA, since mRNA expression does not necessarily correlate with secretion. However, my results show that ELISA may not be the most appropriate means of assessing IL-4 production *in vivo,* and in future studies, I would utilise alternative detection systems such as ELISPOT, immunohistochemistry, or intracellular cytokine staining to detect IL-4.

B Cell Activation in GvHD

Between days 1-10 of GvHD, I found that spleen cells from both B6 ⇒ BDF_1 and DBA/2 ⇒ BDF_1 mice exhibited enhanced responsiveness to the B cell mitogen, LPS. At this time, both groups of mice also showed markedly increased levels of serum IgG and exhibited anti-ds DNA antibodies. From these results, B cell activation appeared to be an event common to the early period of both forms of disease, in agreement with studies showing that host B cell numbers initially increased in the lymphoid tissue of both acute and chronic GvHD mice [23]. After day 10, however, I found that B cell function in acute and chronic GvHD mice differed dramatically. The LPS response of B6 ⇒ BDF_1 cells was virtually absent by day 14, as were levels of serum IgG and anti-ds DNA antibodies. Furthermore, there was no evidence of oedema or antibody-mediated kidney pathology in the few long-term surviving B6 ⇒ BDF_1 mice, consistent with previous reports [33]. Thus the brief B cell hyperplasia in these mice was insufficient to provoke ICGN. In contrast, the hyperresponsiveness of DBA/2 ⇒ BDF_1 cells to LPS persisted for the duration of the study, their serum immunoglobulin levels continued to increase and several of these mice developed oedema and died from ICGN.

The reason why B cell activation and antibody production persisted during chronic GvHD, yet declined after day 10 of the acute disease, is the subject of much debate. Early studies showed that the B cell population of DBA/2 ⇒ BDF_1 mice continued to expand, whereas host B cell numbers were dramatically reduced during the latter stages of acute GvHD. This removes the source of pathogenic antibodies and risk of autoimmunity [23, 31]. Because anti-host CTL activity was readily detectable in B6 ⇒ BDF_1 mice, but not DBA/2 ⇒ BDF_1 mice at this time, it was assumed that CTL destroyed B cells during acute GvHD. In agreement with this, I found that the onset of B cell suppression coincided with expansion of $CD8^+$ T cells during acute GvHD. However, it is also possible that the loss of B cell function during acute GvHD reflected the polarised cytokine response. It is

now well established that while Th2 cytokines support the development of humoral immunity, Th1 cytokines actively inhibit antibody responses by reducing priming for Th2 cytokine production [132, 185]. Thus, the persistently high levels of IL-5 and IL-10 found in chronic GvHD correlated with B cell hyperplasia and immunoglobulin production. In contrast, although acute GvHD mice initially showed B cell hyperplasia and enhanced production of IL-5, B cell function and priming for IL-5 was lost when IFN-γ levels became maximal. The kinetics of these events suggests that IFN-γ plays a role in suppressing B cell responses during B6 ⇒ BDF_1 GvHD. Further support for IFN-γ as a regulator of B cell responses during GvHD is provided by the work of Umland et al which shows that elevated serum IgG and IgE, proteinuria and death from chronic GvHD can be reduced by exogenous IFN-γ [128]. Although my later results showed that neutralising either IL-12 or IFN-γ had little effect on the reduced LPS responses of B6 ⇒ BDF_1 mice, the possibility of incomplete cytokine neutralisation cannot be excluded. Therefore, whether CTL, or cytokines such as IFN-γ and TNF-α mediate B cell death in acute GvHD could be addressed directly by examining the effect of using either IFN-γ gene Knockout (KO) mice as a source of donor cells, or inducing GvHD in IFN-γ or TNF-α receptor KO recipients.

Therefore this initial study confirmed previous reports that differential activation of $CD4^+$ Th subsets may mediate the distinct systemic features of acute and chronic GvHD [42, 46]. However, I also found that the polarisation of the cytokine response occurred very rapidly after transfer of donor cells and I went on to examine how this might occur.

THE ROLE OF IL-12 IN GvHD

As IL-12 has been shown to be important in regulating Th1/Th2 cell development, I focused on its role in polarising GvHD. In my first series of experiments, I examined the effect of a single dose of neutralising anti-IL-12 polyclonal antibody given one day before induction of acute and chronic GvHD.

The rationale for this limited treatment period was that in a variety of systems, Th cell responses could be modified by manipulating the cytokine environment in which initial priming occurs [78, 82, 134, 135, 164]. My results indicated that although early depletion of IL-12 reduced the intensity of the initial period of acute GvHD, it did not affect subsequent disease progression.

Acute GvHD

A single injection of anti-IL-12 antibody reduced the splenomegaly, elevated NK cell activity and enhanced spontaneous and mitogenic responses observed on day 2 of acute GvHD. It also dramatically reduced the early priming of IFN-γ and IL-2 on day 2 of disease and increased priming of the Th2 cytokine IL-10. These effects were all consistent with the role of IL-12 as an enhancer of NK cell-mediated cytotoxicity [118, 120, 121, 150] and with its ability to induce IFN-γ production by T and NK cells [121, 151]. However, the reason why anti-IL-12 reduced the early splenomegaly in B6 ⇒ BDF_1 mice was less clear and may be related to the down-regulated IFN-γ levels observed in antibody treated mice. Since IFN-γ plays an important role in the recruitment and activation of inflammatory cells [58], reduced early priming for IFN-γ may be responsible for lowering the level of splenomegaly. This hypothesis was supported by my studies on the role of IFN-γ in acute GvHD (Chapter 8), which showed that the IL-12 induced increase in splenomegaly during GvHD in DBA/2 ⇒ BDF_1 mice, could be reduced by coadministration of neutralising anti-IFN-γ antibody. Furthermore, in the present study, when IFN-γ priming subsequently returned in anti-IL-12 treated B6 ⇒ BDF_1 mice on day 10, a simultaneous increase in spleen size was observed. Alternatively, since a noted biological effect of IL-12 *in vivo* is to provoke extramedullary haemopoiesis in the liver and spleen, resulting in splenomegaly [121], anti-IL-12 may inhibit this effect during acute GvHD.

Despite these findings early in disease, the effects of anti-IL-12 had disappeared by day 10. This was unexpected as IL-12 is usually held to be critical

for the permanent differentiation of Th1-type responses from uncommitted precursor $CD4^+$ T cells [78, 186]. The most likely explanation for my results is that the temporal window for Th cell phenotype comittment in acute GvHD exceeded the period of IL-12 neutralisation afforded by antibody treatment. I therefore went on to examine whether repeated administration of anti-IL-12 during the first week of acute GvHD had a more dramatic effect on disease progression. Administration of anti-IL-12 to B6 ⇒ BDF_1 mice until day 8 after donor cell transfer not only down-regulated several of the early proliferative features of acute GvHD, but also produced a marked reduction in the later pathological consequences of the disease. As with mice given a single injection of anti-IL-12, these mice had reduced levels of NK cell activity, IFN-γ production and splenomegaly compared with unmanipulated B6 ⇒ BDF_1 mice early in disease. However, anti-IL-12 had no effect on spontaneous proliferation and the degree of splenomegaly was unaltered after day 10, suggesting that anti-IL-12 may have prolonged the prolferative phase of disease. This was supported by the fact that anti-IL-12 treated mice did not show the lymphoid involution and reduced spontaneous and Con A proliferative responses evident in unmodified acute GvHD mice during the latter stages of disease.

Mice surviving after repeated neutralisation of IL-12 had consistently reduced IFN-γ production and increased IL-10 and IL-5 levels both at the early time points and later in the study. This contrasted with the temporary effects of a single injection of anti-IL-12 and indicates that the presence of IL-12 is required throughout the first week of disease for maximal production of IFN-γ. Some IFN-γ production remained in mice given anti-IL-12 repeatedly, although it was not clear if this reflected IL-12-independent IFN-γ, or the transience of the antibody effect. Although the residual levels of IFN-γ were insufficient to provoke lethal pathology, they may explain why these mice retained some of the proliferative alterations of acute GvHD.

Anti-IL-12 treatment prevented weight loss and mortality for up to 18 weeks and permitted full repopulation with donor B6 lymphocytes. However, engraftment of donor $CD4^+$ T cells appeared to occur more gradually in anti-IL-12 treated B6 $\Rightarrow$ BDF_1 mice than in unmodified GvHD mice. This was particularly evident on day 70 of the GvHD, when only 30% of the $CD4^+$ T cell population in anti-IL-12 treated GvHD mice was donor-derived, compared with 96% in unmodified B6 $\Rightarrow$ BDF_1 animals. Since IL-12 synergises with cytokines such as IL-3 and steel factor to stimulate the proliferation and differentiation of early lymphohaemopoietic progenitors [187, 188], blockade of IL-12 may have reduced the rate of donor stem cell haemopoiesis and therefore slowed down the repopulation process. However, anti-IL-12 did not adversely affect repopulation by donor $CD8^+$ T cells and both T cell compartments were fully replaced by donor-derived cells by day 130. Complete repopulation of the recipient immune system by donor cells in the absence of overt pathology would be consistent with the development of tolerance to host alloantigen, as previously described in survivors of acute GvHD [33, 52]. This explanation seems more likely than the alternative possibility that there was complete destruction of host MHC expressing tissues, as anti-IL-12 treated mice did not display major pathology at any time. However, it would have been interesting to determine directly whether the T cells which had repopulated anti-IL-12 treated B6 $\Rightarrow$ BDF_1 mice were tolerant to F_1 alloantigen in the long-term by examining the ability of T cells from these mice to respond to BDF_1 stimulator cells in an MLR *in vitro*. Nevertheless, the fact that chimeric mice had normal proliferative responses to Con A and did not succumb to opportunistic infections while housed in standard animal house conditions, shows that short-term IL-12 blockade allows full and long-term recovery of immune function.

Despite inhibiting the lethal outcome of the disease, anti-IL-12 was unable to significantly reverse the suppressed B cell responses or reduce the anti-host CTL activity normally found in B6 $\Rightarrow$ BDF_1 mice. These findings may be related,

as others have suggested that the profound B cell immune deficiency during acute GvHD is at least partially due to CTL-mediated destruction of host B cells [23, 31]. My results also indicate that the mechanisms of B and T cell suppression during acute GvHD may be distinct, since IL-12-dependent Th1 cytokines appear to be important in abrogating T cell responses, but are less involved in B cell suppression. Similar results have been obtained by others [71] and are consistent with the more dramatic effect of anti-IFN-γ mAb on T cell function, compared with B cell function, I found in Chapter 8. My observation that anti-IL-12 treated B6 ⇒ BDF_1 mice survived in the presence of high levels of specific cytotoxic activity also raises important questions about the mechanisms of tissue pathology and mortality in acute GvHD, as previous reports have suggested that mortality in B6 ⇒ BDF_1 mice is dependent on activation of $CD8^+$ anti-host CTL [31]. Instead, my results agree with studies in which there was no correlation between anti-host CTL activity and mortality or intestinal damage in acute GvHD [24, 27, 83] and suggest that anti-IL-12 may prevent immunopathology by down-regulating IFN-γ production and/or by promoting preservation of T lymphocyte function in these animals, hence reducing their susceptibility to secondary infections. Furthermore, although exogenous *IL-12* facilitates antigen-specific CTL responses in normal mice [121], the results of my study showed that IL-12 was not essential for anti-host cytotoxicity during acute GvHD. The fact that IL-12 KO mice have also recently been reported to mount normal allogeneic CTL responses [189], supports my finding that IL-12 is not critical for this aspect of cell-mediated immunity.

The reduced levels of IFN-γ and increased IL-10 and IL-5 production observed in anti-IL-12 treated B6 ⇒ BDF_1 mice, suggested that anti-IL-12 may be mediating its protective effect during acute GvHD by polarising the $CD4^+$ alloresponse towards a Th2 phenotype, similar to that observed during chronic, autoimmune GvHD. Furthermore, despite a limited capacity to reverse the suppressed LPS responses of B6 ⇒ BDF_1 mice early in acute GvHD, anti-IL-12

treated B6 ⇒ BDF1 mice then had enhanced LPS responses in the longer term. Previous reports showed that mice which recover from unmodified acute GvHD develop a mild stimulatory disorder termed "secondary chronic GvHD", which is characterised by a degree of lymphoid stimulation and autoantibody production [28, 58]. Taken together, these observations raised the possibility that anti-IL-12 treated long-term survivors might develop the B cell hyperreactivity and antibody-mediated kidney pathology associated with chronic GvHD. Although my anti-IL-12 treated mice had mildly elevated total serum IgG and IgE, these levels were severalfold lower than those associated with chronic GvHD in DBA/2 ⇒ BDF_1 mice. Anti-IL-12 treated animals also remained healthy and neither light nor electron microscopy revealed evidence of the antibody-mediated renal pathology apparent in mice with chronic GvHD. The heightened response of B cells from anti-IL-12 treated animals when stimulated *in vitro* with LPS may therefore simply reflect the fact that these cells were resident in a milieu rich in Th2 cytokines and were therefore primed to respond avidly to B cell mitogens. However, this was clearly insufficient to provoke the consequences of a classical chronic GvHD *in vivo*. This may be because the anti-IL-12 treated acute GvHD mice had full donor chimerism, whereas in DBA/2 ⇒ BDF_1 mice there is persistence of host B cells and other lymphohaemopoietic cells. Allorecognition of host B cells by donor $CD4^+$ T cells thus stimulates polyclonal B cell activation and the production of high levels of autoantibodies [39, 124, 126]. Although I did not confirm the absence of host B cells directly by assessing MHC expression on B cells in surviving anti-IL-12 treated B6 ⇒ BDF_1 mice, studies in unmodified B6 ⇒ BDF_1 mice show that newly generated donor-derived B cells appear in the spleens of recovering unmodified B6 ⇒ BDF_1 mice after 6-8 weeks and replace host cells [51].

In conclusion, the results from this study demonstrated that IL-12 blockade is an effective therapy for murine acute GvHD. Anti-IL-12 antibody prevented lethal immunopathology whilst allowing the survival of sufficient

donor-derived haemopoetic cells to permit full repopulation of the host by a functioning immune system. Although anti-IL-12 therapy prevented Th1-dependent lethality by permanently polarising the alloimmune response towards a Th2 phenotype, this did not result in the development of chronic GvHD-associated glomerulonephritis. In future studies, it would be important to directly assess the role of IL-12 in GvHD target organs such as the small intestine, liver and bone marrow by histological examination. In addition, since TNF-α causes cachexia during acute GvHD [65], IL-12 could indirectly enhance TNF-α release from activated mϕs by increasing IFN-γ levels. Whether increased serum TNF-α levels paralleled the increased weight loss and death in IL-12 treated B6 ⇒ BDF_1 mice would again be a subject for future studies.

Further support for the central role of IL-12 during acute GvHD was that administration of exogenous IL-12 exacerbated virtually all of the features of the systemic disease. Thus, IL-12 increased the level of splenomegaly and NK cell activity early in the disease and accelerated the onset of immunosuppression, weight loss and mortality found in established GvHD. One rather surprising effect of exogenous IL-12 was that it increased IL-10 levels in B6 ⇒ BDF_1 mice. Since IL-10 is a product of Th2 cells [53, 190, 191] and was initially defined as an inhibitor of Th1 responses [192, 193] this result was difficult to explain. However others have also shown that i.p. injection of IL-12 can cause increased IL-10 gene expression [163, 194, 195] and have suggested that this serves as a homeostatic function to control an excessive Th1 response. IL-12 did not increase either IFN-γ production or CTL activity in B6 ⇒ BDF_1 mice, possibly because these responses were already maximal and the peaks may already have passed.

Chronic GvHD

In contrast to my findings in acute GvHD, neutralising IL-12 had little effect on the early phase of chronic GvHD. However, it did slightly augment several of the Th2-mediated aspects of the disease in the long-term. Thus, anti-IL-

12 caused a further increase in the elevated serum IgE levels observed in treated DBA/2 ⇒ BDF_1 mice and increased the overall mortality rate associated with the chronic disease. In addition, the characteristic decline in serum IgG levels observed during the late stages of chronic GvHD, occurred more rapidly in anti-IL-12 treated DBA/2 ⇒ BDF_1 mice than in similar untreated animals. The ability of IL-12 to regulate Th2 responses has also been described in murine models of candidiasis, schistosomiasis, and toxoplasmosis [79, 152, 153, 194-196]. However, since others have shown that anti-IFN-γ antibody also decreased serum immunoglobulin levels during chronic GvHD [128], anti-IL-12 may act indirectly to reduce Th2 cytokines via IFN-γ down-regulation.

Administration of exogenous IL-12 induced an acute GvHD in DBA/2 ⇒ BDF_1 mice identical to that found normally in B6 ⇒ BDF_1 recipients, with a characteristic biphasic pattern of early hyperplasia progressing to activation of CTL, immunosuppression and death. Furthermore, IL-12 converted the Th2 cytokine profile associated with chronic GvHD into a Th1 dominated response. The systemic disease provoked in DBA/2 ⇒ BDF_1 mice given IL-12 was more aggressive than that observed in B6 ⇒ BDF_1 animals, with earlier and higher mortality rates. This may have reflected the effect of giving repeated high doses of exogenous IL-12, as opposed to the possibly lower, physiological levels induced in unmanipulated B6 ⇒ BDF_1 mice. However, it is important to note that the dose of IL-12 I used was not toxic per se in control mice. One aspect of acute GvHD not induced in DBA/2 ⇒ BDF_1 mice by IL-12 was enhanced NK cell activity on day 1. This was surprising in view of the reported stimulatory effect of IL-12 on NK cell-mediated cytotoxicity [121]. The dose of IL-12 used was also insufficient to induce more than a slight increase in NK cell activity in either B6 ⇒ BDF_1 or normal mice at this time, suggesting that the wrong time point was examined and more kinetic studies are indicated. The ability of exogenous IL-12 to elicit high levels of anti-host CTL activity in DBA/2 ⇒ BDF_1 mice contrasted with my own and other findings [189] that endogenous IL-12 is not required for allogeneic CTL

responses. Again, this may reflect differences between administration of pharmacological doses of IL-12 vs the physiological function of the cytokine.

The acute GvHD induced in IL-12 treated DBA/2 ⇒ BDF_1 mice was, as expected, accompanied by a shift in the cytokine profile from a Th2 to a Th1 phenotype, with extremely high levels of IFN-γ production. A similar switch in cytokine pattern has been observed in other models of Th2 immunity modified by exogenous IL-12 [81, 194-196]. In DBA/2 ⇒ BDF_1 GvHD, this could reflect the stimulatory effect of IL-12 on $CD4^+$, $CD8^+$ and/or NK cells, since each of these cell types produce IFN-γ in response to IL-12 [121, 151]. As discussed above, I did not observe an increase in NK cell mediated cytotoxicity in IL-12 treated DBA/2 ⇒ BDF_1 mice, suggesting that, at least on day 1 of GvHD, NK cells were not the source of the IL-12-induced IFN-γ. However, it was important to note that on day 1, while IL-12 had a pronounced effect on spontaneous IFN-γ levels in DBA/2 ⇒ BDF_1 mice, Con A induced IFN-γ production was not significantly increased. A possible explanation for this was that T cells were not yet primed to produce increased IFN-γ on day 1 of disease and a non-T cell was the main source of the cytokine at this time.

The differential role of IL-12 in acute and chronic GvHD was supported by the finding that although spleen cells from both groups produced enhanced levels of IL-12 p75 heterodimer when stimulated *in vitro* with LPS, this continued to increase for up to 10 days only in B6 ⇒ BDF_1 mice. The kinetics of IL-12 priming in GvHD thus closely resembled those of IFN-γ production. Although I did not determine the source of IL-12 during the course of these studies, the main IL-12-producing cells *in vivo* are believed to be mϕs [76, 122, 152], although other APC types are emerging as alternative IL-12 producers e.g. B cells and DCs [118, 158, 197]. It will therefore be very important for future studies to explore the source of IL-12 in acute GvHD using either immunohistochemistry, or by enriching different cell populations and examining their IL-12-producing potential. Furthermore, the relative contribution of host and

donor-derived cells to IL-12 production could be assessed in vivo by utilising appropriate combinations of IL-12 deficient and wild-type host and donor animals.

Since both forms of GvHD are initiated by recognition of alloantigen, presumably expressed on the same host APC, the similar levels of IL-12 produced initially during acute and chronic GvHD were not surprising. The difference in the persistence of IL-12 production may therefore be secondary to differences in T cell IL-12 responsiveness between the two donor strains, as has been suggested in studies of genetic susceptibility to *L. major* infection in BALB/c and B10.D2 mice [139, 140, 198]. Here, T cells from the two strains are equally capable of initiating antigen specific Th1 responses, but BALB/c T cells become unresponsive to IL-12 and acquire a Th2 phenotype, whereas B10.D2 T cells maintain IL-12 responsiveness and produce the IFN-γ required for eliminating the parasite. The Th2 phenotype default which BALB/c mice exhibit can also be reversed by early administration of rm IL-12 [80, 82, 139]. Whether T cells from DBA/2 and B6 mice also have intrinsic differences in their ability to sustain IL-12 responsiveness to BDF_1 alloantigen has not been investigated directly. There are, however, a number of pieces of circumstantial evidence which suggest that a similar genetic bias could be operative during BDF_1 GvHD. Firstly, it is known that DBA/2 mice preferentially elicit Th2-type responses [79, 140, 199], while B6 mice are biased towards making Th1 responses to a variety of stimuli [200, 201]. In addition, an inability to retain IL-12 responses would explain why DBA/2 ⇒ BDF_1 mice did not produce high levels of IFN-γ, despite exhibiting some early IL-12 production. A disparity at the level of the donor T cells is also supported by studies which have attributed the dichotomy between acute and chronic GvHD in BDF_1 mice to differences in the ability of the donor $CD8^+$ T cells to engraft and generate CTL responses [23, 31]. Although my results show that CTL activity itself does not appear to be responsible for the lethal consequences of acute GvHD, other aspects of $CD8^+$ T cell function, such as IFN-γ production, may also differ between B6 and DBA/2 mice and could be a manifestation of inherent

differences in the ability of these T cells to respond to IL-12. A final similarity between the BDF_1 model of GvHD and susceptibility to *L. major* is that the putative default Th2 response exhibited by BALB/c mice (in the case of L. major) and DBA/2 donor cells (in GvHD) could be overcome when rm IL-12 is given at the time of antigen contact, but not if IL-12 treatment was delayed [81, 82, 139, 166].

If time had allowed, it would have been interesting to further examine the genetic basis for Th subset development during BDF_1 GvHD. T cells from B6 and DBA/2 mice could be analysed directly to determine whether they are genetically predisposed to mount preferential Th1 and Th2 responses to *in vitro* stimulation with BDF_1 alloantigen respectively. In addition, the hypothesis that B6 and DBA/2 donor cells show differential responsiveness to IL-12 could be tested using a similar system as that utilised by Guler et al [139], in which T cells were stimulated with antigen for several days under neutral conditions *in vitro* and then restimulated in the presence or absence of IL-12. T cells which retained IL-12 responsiveness during primary culture were thus primed to produce high levels of IFN-γ when restimulated, whereas cells which lost IL-12 responsiveness did not show such priming.

My finding that IL-12 plays a pathological role in acute GvHD contrasts with recent work which shows that anti-IL-12 treatment can actually reverse the protective effect of combined CD80 and CD86 blockade in irradiated mice with acute GvHD [202]. However, this study used irradiated mice and the requirement for IL-12 in haemopoiesis [188, 203] may conflict with its enhancing effect on Th1-mediated immunopathology. Furthermore, the blockade of costimulatory pathways may exaggerate any immunosuppressive effect of anti-IL-12 in these mice. Others have also suggested that IL-12 was protective during certain models of GvHD [204]. Thus, a single, high dose injection of IL-12 was found to inhibit GvHD in lethally irradiated mice receiving allogeneic bone marrow. Although the mechanism of protection was not defined, it was suggested that this dose of IL-12

may exhaust host-reactive $CD8^+$ T cells and induce their deletion. A similar IL-12-induced phenomenon has also been described in murine viral models [205]. Why IL-12 did not similarly ameliorate acute GvHD in B6 ⇒ BDF_1 mice is unclear, although one possible critical difference may be the different dosing protocols utilised in my experiments compared to those of Sykes et al [204]. Thus, repeated, lower doses of IL-12, as used in my studies may not lead to clonal deletion and its associated protection. Dose-response studies of IL-12 effects in B6 ⇒ BDF_1 mice would help address this possibility. However, most reports support my demonstration that Th1 cytokines, such as IFN-γ, play a pathogenic role in B6 ⇒ BDF_1 mice [55, 57, 180]. Furthermore, as in the studies by Saito et al described above [202], in irradiated models, the requirement for IL-12 in haemopoiesis [188, 203] may be greater than in unirradiated mice. An important concept for the potential therapeutic use of IL-12 blockade would therefore be to prevent Th1-mediated pathology, without preventing donor BM engraftment.

ROLE OF IFN-γ IN IL-12-DEPENDENT ACUTE GvHD

The principal role of IL-12 is to regulate IFN-γ production [123, 147] and the effects of anti-IL-12/rm IL-12 were paralleled by changes in IFN-γ production. However, it is currently controversial as to how much of the effects of IL-12 in immune responses are secondary to IFN-γ. I therefore examined the effects of neutralising IFN-γ both during IL-12-dependent acute GvHD in B6 ⇒ BDF_1 mice and in IL-12 treated DBA/2 ⇒ BDF_1 mice with acute GvHD. My results showed that IFN-γ played a significant role in many of the characteristic features of acute GvHD, including splenomegaly, immunosuppression, lymphoid involution, weight loss and mortality. Furthermore, IFN-γ appeared to be involved in the regulation of the cytokine response during acute GvHD.

Overall, anti-IFN-γ mAb treatment had similar beneficial effects on both forms of GvHD and the results supported a role for IFN-γ both during the early proliferative phase and also later, when tissue destruction, weight loss and

mortality were evident. In both models, anti-IFN-γ mAb ameliorated the acute GvHD without reducing anti-host CTL activity, further indicating that CTL activity does not necessarily correlate with mortality. One difference between the effects of anti-IFN-γ on the two models of acute GvHD, was that anti-IFN-γ reduced the level of T cell suppression in unmodified B6 ⇒ BDF_1 mice, but not in IL-12 treated DBA/2 ⇒ BDF_1 mice. This may be because IL-12 treated DBA/2 ⇒ BDF_1 mice have a more aggressive GvHD, with higher mortality and IFN-γ production. In addition, others have reported that immunosuppression in B6 ⇒ BDF_1 mice with acute GvHD may not be solely due to IFN-γ and may also involve other factors such as TGF-β [71, 73], or deletion of host-reactive lymphocytes [50] and deficient lymphopoiesis [173-175]. These additional factors may therefore be more important in IL-12 treated DBA/2 ⇒ BDF_1 mice.

An interesting finding was that neutralisation of IFN-γ *in vivo* did not reduce the stimulation of IFN-γ production by GvHD. Indeed, IFN-γ levels may even have been increased. This may reflect a compensatory mechanism activated in the absence of biologically active IFN-γ, since similar over-production of IFN-γ is observed in mice which are defective in the gene coding for the IFN-γ cytokine receptor [206]. In addition, these findings suggest that the IL-12 induced IFN-γ production during acute GvHD does not require endogenous IFN-γ. This is in agreement with other *in vitro* [78] and *in vivo* [163, 164] studies of Th phenotype development, which have demonstrated that IL-12 exerts its effects directly on T cells. However, this contrasts with the findings of other recent *in vitro* and *in vivo* studies suggesting that significant IL-12 production requires priming of mϕs by IFN-γ [148, 160]. The possibility that the antibody did not competely neutralise IFN-γ *in vivo* cannot be excluded, however, and studies using either IFN-γ receptor or gene knockout mice would help to rule this out.

Despite exerting little influence over Th1 cytokine production, anti-IFN-γ mAb treatment dramatically increased priming for IL-5 production by acute GvHD cells, suggesting that IFN-γ directly regulates IL-5 production during the disease.

This finding is consistent with recent work showing that IFN-γ directly inhibits Th2 cytokine production [162, 163, 194, 196]. Whether IFN-γ exerted a similar regulatory influence over production of the other Th2 cytokine, IL-10, was less clear from my studies. IL-10 production by B6 ⇒ BDF_1 cells was below the level of detection and although anti-IFN-γ increased IL-10 production by IL-12 treated DBA/2 ⇒ BDF_1 cells on day 2, a similar effect was not evident on day 10, when biologically active IFN-γ would presumably be present.

Overall, I found that the effects of neutralising IFN-γ and IL-12 were very similar. Both treatments prevented the weight loss and mortality observed during the first 3 weeks of acute GvHD and prolonged the early proliferative features of disease. In addition, both anti-IFN-γ and anti-IL-12 treatment reduced the T cell immunosuppression observed late in the acute disease, although there was a less marked effect on the abrogated B cell responses in GvHD mice. Despite these effects, both antibodies failed to reduce the high levels of anti-host CTL activity. Anti-IL-12 and anti-IFN-γ also increased the levels of IL-5 and IL-10 production. A final critical finding was that although DBA/2 ⇒ BDF_1 mice given IL-12 and anti-IFN-γ showed increased levels of Th2 cytokine production, they did not exhibit any of the symptoms of classical chronic GvHD. Thus, I found IL-12-mediated inhibition of chronic GvHD-associated autoimmunity to be IFN-γ-independent, in agreement with other recent studies [166]. As in anti-IL-12 treated B6 ⇒ BDF_1 mice with acute GvHD, this may be because anti-IFN-γ treated DBA/2 ⇒ BDF_1 mice given IL-12 retained anti-host CTL activity which could destroy host B cells. In support of this, others recently showed that DBA/2 ⇒ BDF1 mice given IL-12 and anti-IFN-γ mAb had profoundly reduced B cell numbers [166].

Taken together, my results show that many of the effects of IL-12 in GvHD are IFN-γ dependent including; lymphoid involution, T cell immune deficiency, regulation of Th2 cytokine production, weight loss and mortality,

whereas others, such as CTL activity, induction of IFN-γ production and possibly B cell destruction/suppression are IFN-γ-independent.

CELLULAR SOURCE OF CYTOKINES DURING GvHD

An important finding of my T cell subset depletion studies was that since depletion of $CD4^+$ or $CD8^+$ T cells did not affect the early spontaneous IFN-γ production by B6 ⇒ BDF_1 cells, it appeared to be non-T cell-derived. NK cells are an important source of IFN-γ early during other T cell-dependent immune responses [112-114, 152, 153]. However, when I examined whether NK cells were involved in IFN-γ production during acute GvHD, I found that NK cell depletion did not affect either spontaneous or Con A induced IFN-γ production at any time during the disease. However, this work needs to be repeated as control mice given either NRS or anti-ASGM-1 produced uncharacteristically high levels of spontaneous IFN-γ on day 2 of this study. Furthermore, the dose of anti-ASGM-1 used to deplete NK cells did not completely inhibit NK cell activity in B6 ⇒ BDF_1 mice, making it impossible to exclude NK cells as IFN-γ producers in acute GvHD.

A more complex picture was found at the later time points in the acute disease, when I could not identify any single population that was responsible for IFN-γ production. Both $CD4^+$ and $CD8^+$ T cells and possibly non-T cell populations all appeared to contribute to the high levels of spontaneous and Con A induced IFN-γ at different times, in agreement with studies which highlight the potential of each of these cell types to produce IFN-γ [53, 86, 87, 180, 207]. $CD4^+$ and $CD8^+$ T cells also contributed to the lower levels of Con A induced IFN-γ which were found in chronic GvHD.

$CD4^+$ T cells appeared to be the main source of IL-2, IL-10 and IL-5 during both acute and chronic GvHD, although there were a few exceptions. On day 2 of chronic GvHD, for example, CD8 depletion also reduced IL-2 levels, presumably because the highly activated $CD4^+$ T cells in these cultures were

utilising IL-2. Furthermore, on day 14 of both acute and chronic forms of disease, neither CD4+ nor CD8+ T cells appeared to be responsible for IL-10 production. An alternative source of IL-10 are activated mϕs [190, 191, 208]. IL-10 was only induced in Con A stimulated cultures and mϕs would not be expected to respond directly to a T cell mitogen. However it is possible that mϕs were activated secondary to T cell stimulation with Con A. Some IL-10 production during chronic GvHD also appeared to be CD8+ T cell-derived. IL-10-producing CD8+ T cells have been described in other systems [94] and since IL-10 inhibits Th1 differentiation [192, 193], this may be important to the preferential Th2 response in DBA/2 ⇒ BDF_1 mice. Paradoxically, there was also evidence that CD8+ T cells were responsible for down-regulating IL-10 levels during chronic GvHD, since on day 10, CD8+ T cell depletion caused a severalfold increase in IL-10. Depleting CD8+ cells also increased IL-10 levels in B6 ⇒ BDF_1 cultures at all times during the disease. Similarly, CD8 depletion revealed some IL-5 production on day 2 of acute GvHD. These observations raised the possibility that CD8+ T cells down-regulated Th2 cytokine production during acute GvHD. A similar phenomenon has been observed in other experimental systems in which CD8+ T cells inhibited Th2 development [87, 209, 210], at least in part through their production of IFN-γ [87, 210]. This was also consistent with my own and other [180, 207] findings that CD8+ T cells were primed to produce IFN-γ production during acute GvHD. The absence of IFN-γ-mediated inhibition of Th2 cytokine production by CD8+ T cells could explain the uncontrolled Th2 response in chronic GvHD. Also, recent studies have demonstrated that BDF_1 mice given DBA/2 donor cells which had been enriched with either total T lymphocytes [211] or purified CD8+ T cells [180] developed features typical of acute GvHD, together with a small increase in IFN-γ levels [180].

The results detailed above indicated that cytokines produced by both CD4+ and CD8+ T cells may be important in determining the outcome of GvHD. However, the pattern was very complex and negative depletion may not be the

ideal wasy to assess the source of cytokines. If time had allowed, I would therefore have used alternative strategies to determine the cell types involved. Positively selecting individual cell populations using magnetic beads may have provided more information than depletion, since in the absence of one population, other cell type(s) may compensate. In order to study the possible interactions between cells, however, it may be advantageous to adopt an *in vivo* approach such as immunohistochemistry or the recently developed intracellular cytokine staining [183, 184, 186].

Finally, my studies did not attempt to assess whether the T cell populations involved in cytokine production during acute and chronic GvHD were of host or donor origin. Because the GvHR in parent $\Rightarrow$ F_1 mice is unidirectional, alloreactive donor T cells should be the main cytokine producing cells during the disease. However, the local production of high levels of T cell growth factors such as IL-2 may also lead to bystander host T cell activation and cytokine production. If time had allowed, I would have utilised Thy-1 congenic mouse strains in order to examine the contribution of host cells. Thus, by using donor cells and host cells which expressed different forms of the T cell-specific Thy-1 marker, it would be possible to specifically purify or deplete cells of donor/host origin before culture and examine the effect on cytokine levels.

THE ROLE OF NK CELLS IN GvHD

A notable feature of my studies was that, during the first week of GvHD, B6 donor cells induced earlier and higher levels of NK cell activity in BDF_1 mice than DBA/2 cells. The greater enhancement of NK cell activity evident after injection of B6 cells correlated with the high levels of IFN-γ in these mice, implicating NK cells as a source of IFN-γ production during acute GvHD. Although I did not examine the reason for differential NK cell activation during acute versus chronic GvHD, the augmented NK activity observed in B6 $\Rightarrow$ BDF_1 mice, correlated with the known ability of H-2^b parental cells to provoke F_1

resistance to donor haemopoietic cells [49, 110, 111, 212, 213]. This "hybrid resistance" is elicited when NK cells of appropriate F_1 mice recognise haemopoicitic histoincompatibility antigens (Hh) on the homozygous parental cells and is induced by H-2^b, but not H-2^d strains such as DBA/2 [110, 111, 212]. However, previous work in our laboratory found no evidence that the activation of NK cells during acute GvHD was associated with *in vivo* host resistance to parental cells and instead suggested that both donor and host NK cells are non-specific effector cells recruited into lymphoid tissues by cytokines such as IL-2 and IFN-γ produced during the anti-host inflammatory response [95]. Thus the higher levels of NK cell activity observed in B6 ⇒ BDF_1 vs DBA/2 ⇒ BDF_1 mice probably reflect the superior ability of B6 cells to induce IL-12-dependent IFN-γ production and recruit and activate inflammatory cells. Unfortunately, I was unable to directly examine whether neutralising IFN-γ reduced NK cell activity.

When NK cells were depleted by injection of anti-ASGM-1 antiserum, there was a transient reduction in the degree of lymphoid hyperplasia early in the disease and a slight reduction in CTL levels. However, I found little effect on other aspects of systemic GvHD. If anything, NK cell depletion slightly exacerbated acute GvHD, as higher numbers of anti-ASGM-1 treated mice lost weight and died than equivalent NRS treated GvHD controls. Furthermore, on day 10, anti-ASGM-1 treated GvHD mice showed lower T and B cell proliferative responses than GvHD mice given NRS. Although most studies suggest a pathogenic role for NK cells during GvHD by showing that NK cell depletion prevents tissue pathology and death [102, 104-106], some evidence also exists which supports a protective role for NK cells in GvHD [214, 215]. However, my results were rather inconclusive, perhaps because of the unusually mild acute GvHD that occurred in NRS treated B6 ⇒ BDF_1 animals. It would be important to repeat these studies using both untreated and NRS treated recipients to exclude the possibility that giving foreign immunoglobulin to GvHD mice affects disease progression. There should also be similar reservations about the effect of depleting

NK cells on IFN-γ production, as control mice given either NRS or anti-ASGM-1 antiserum produced uncharacteristically high levels of spontaneous IFN-γ on day 2 of this study. In addition, as noted above, the dose of anti-ASGM-1 used in this study did not completely inhibit NK cell activity in B6 ⇒ BDF_1 mice and it was not possible to rule out NK cells as a potential source of IFN-γ during the disease. Anti-NK1.1 treatment *in vivo* may provide an alternative, more efficient method of depleting NK cells for future studies. In addition, if time had allowed, I would have repeated this study and also examined IFN-γ production by NK cells purified from the spleens of B6 ⇒ BDF_1 mice using magnetic beads.

THE ROLE OF CYTOKINES IN THE PATHOGENESIS OF MURINE GvHD

The results which I have presented in this thesis have extended previous work on the distinct forms of GvHD which develop in BDF_1 mice given different parental donor splenocytes. I have shown that cells and cytokines of the early non-specific immune response may be important in determining the outcome of the alloresponse and in particular, my studies have highlighted the importance of the proinflammatory cytokine, IL-12, in polarising the specific T cell response. Furthermore on the basis of my findings, it is tempting to hypothesise that targetting IL-12 may be an effective and specific therapy for GvHD following BMT.

Murine models of GvHD are useful tools for examining the complex cellular interactions which lead to GvHD following clinical BMT and other forms of tissue-specific immunopathology [7, 8, 21-23, 37, 83]. An advantage of the parent ⇒ F_1 model employed in this thesis is that a predominantly cell-mediated immune response or a predominantly humoral response can be elicited in the same F_1 hybrid mouse depending on the genotype of the injected parental cells [24, 27]. This allowed me to trace the development of these distinct diseases from the common starting point of parental donor cell transfer, in order to determine when

polarisation of the alloimmune response occurred and what caused its divergence. Taken together, the findings detailed in this thesis agreed with previous reports suggesting that differential activation of $CD4^+$ T helper cell subsets may underlie the distinct forms of GvHD in BDF_1 mice [42, 46]. However, other cell types, particularly APC, $CD8^+$ and possibly NK cells are inextricably involved in the development and regulation of the anti-host response.

The responses elicited by B6 and DBA/2 parental cells diverged rapidly, since as early as 4 hours after donor cell transfer, enhanced NK cell activity was observed only in B6 $\Rightarrow$ BDF_1 mice and within two days, increased spontaneous and primed IFN-γ production were evident in acute, but not chronic GvHD mice. These distinguishing features of early acute GvHD were IL-12 dependent, which strongly suggested that the first critical events in B6 $\Rightarrow$ BDF_1 GvHD involved interactions between APC, NK cells and $CD4^+$ T cells. The possible events occurring in acute and chronic GvHD in BDF_1 mice are illustrated in Figs. 11.1A & B. These clearly proceed more efficiently in acute GvHD. After injection of parental donor cells, donor $CD4^+$ T cells recognise alloantigen expressed on the surface of host dendritic cells (DC). As with responses to nominal antigen [216-218], engagement of the TcR by host alloantigen results in T cell activation and CD40 ligand (CD40L) expression on the T cell surface. Expression of CD40L allows donor T cells to bind to and activate host mϕs via CD40 : CD40L interactions [216, 217, 219, 220], provoking IL-12 release by the mϕ population [148, 149]. IL-12 then exerts a number of effects on a variety of different cell types to induce the symptoms of acute GvHD. Because DBA/2 cells exhibit defective IL-12 responses, the manifestations of acute GvHD are not induced. In the absence of IL-12-dependent effects, a default Th2 response and autoimmune pathology are elicited as oulined below.

IL-12 stimulates IFN-γ production by $CD4^+$ and $CD8^+$ T cells and possibly NK cells during acute GvHD. The initial IFN-γ production then induces more IL-12 production from mϕs, followed by even greater levels of IFN-γ,

which will eventually provoke many of the pathological alterations associated with acute GvHD, including immune deficiency and pathology in target tissues like the gut, weight loss and mortality. In addition, as my results suggested, IFN-γ may also directly inhibit Th2 cytokine production, thus preventing the B cell hyperplasia and autoimmunity which accompany chronic GvHD. The high levels of anti-host CTL activity found in B6 ⇒ BDF_1 mice may also prevent autoimmunity by directly destroying host B cells [23, 31], although this was not examined directly in these studies.

Several additional elements may contribute to this basic pathway and warrant future study. Firstly, although I showed that IFN-γ was not essential for IL-12 to appear, I did not examine directly if IFN-γ was required for optimal IL-12 production, as suggested by other work [148, 152, 160]. This could be determined by examining if IL-12 production occurs normally during GvHD in B6 ⇒ BDF_1 host mice which are deficient in the gene coding for the IFN-γ receptor. It will also be important to determine the kinetics of IFN-γ and IL-12 production during acute GvHD in unmanipulated B6 ⇒ BDF_1 mice using a more sensitive method such as PCR. In addition, the source of IFN-γ and in particular, whether NK cells are involved, needs to be explored directly. One conflicting aspect of my studies was the inability of anti-IL-12 to prevent CTL activation despite induction of CTL by exogenous IL-12. This was possibly due to incomplete neutralisation of IL-12 by the antibody and could be better examined in appropriate combinations of IL-12 deficient donor and host mice.

A further focus for future studies is the source of IL-12. In the model above I cite host mϕs as the major IL-12-producing population. Although mϕs purified from mice undergoing acute GvHD produce high levels of mRNA for the inducible p40 subunit of IL-12 [221], activated mϕs are not the only cell type capable of IL-12 production *in vivo*. In particular, DCs have also been described as potential IL-12-producers [123], suggesting that the initial host DC-donor $CD4^+$ T cell interaction could potentially elicit some IL-12 production. Future

studies should explore the role of mϕs in IL-12 production by enriching them from spleen cell cultures and measuring IL-12 production. Furthermore, given the potent cytokine environment which acute GvHD elicits, it is likely that donor-derived mϕs may also be stimulated to produce IL-12. The relative contribution of host and donor-derived cells to IL-12 levels could again be assessed using appropriate combinations of IL-12 gene knockout and replete host and donor mice.

The simplest explanation for the fact that chronic GvHD in DBA/2 ⇒ BDF_1 mice was largely unaffected by IL-12 blockade, but could be converted into an acute Th1-mediated GvHD by the administration of exogenous IL-12, would be that DBA/2 donor cells cannot elicit IL-12 production in BDF_1 recipients. Since IL-12 is essential for Th1 differentiation [76-78], the Th2 response which develops in DBA/2 ⇒ BDF_1 mice may therefore be a default which occurs in the absence of a Th1 polarising signal. However, despite the absence of early IL-12 dependent effects such as NK cell activation and enhanced IFN-γ production, I found that DBA/2 ⇒ BDF_1 mice had equivalent initial priming of IL-12 production as B6 ⇒ BDF_1 animals. It would be useful to study the full kinetics of IL-12 p40 mRNA expression *in vivo* using a senstive method such as PCR. Time did not permit me to do this.

An alternative explanation is that there are genetically controlled differences in the IL-12 responsiveness of B6 and DBA/2 donor cells, as reported in other models [139, 140]. According to this idea, T cells from both strains induce similar initial levels of IL-12, but only the B6 cells can respond by producing IFN-γ and then inducing more IL-12. This is consistent with studies of *L. major* and with my finding that initial IL-12 priming was similar in both acute and chronic GvHD. In addition, DBA/2 mice preferentially elicit Th2 dominated responses in other systems [79, 140, 199], while B6 mice tend to make strong Th1 responses to a variety of stimuli [200, 201]. It would therefore be important to assess directly whether donor T cells from B6 and DBA/2 mice show an inherent predilection to

produce Th1 and Th2 cytokines in response to allostimulation and to assess whether regulation of the IL-12 signalling pathway differed between the two strains of mice. The inability of DBA/2 donor T cells to respond to IL-12 could explain why the high levels of IFN-γ released by IL-12 stimulated $CD4^+$ and $CD8^+$ T cells during acute GvHD, are not observed. As a result, the feed-back loop which operates between IL-12 and IFN-γ production in B6 ⇒ BDF_1 GvHD is eliminated and IFN-γ–dependent immunopathology will not occur. Furthermore, since IFN-γ regulates Th2 cytokine production during GvHD, a preferential Th2 response is allowed to develop. The high levels of Th2 cytokines then provide help for host B cells, already activated due to their recognition by donor T cells [124], provoking autoantibody production and ICGN. The defective IL-12 response of DBA/2 $CD8^+$ T cells could also explain why $CD8^+$ T cell engraftment is not observed during chronic GvHD and why potentially autoreactive B cells are not eliminated by anti-host CTL.

Therapeutic Potential of IL-12 Neutralisation in GvHD

Acute GvHD remains a major obstacle for the use of BMT to treat leukaemia and many congenital immunodeficiencies and the results presented in this thesis have practical implications for the development of specific therapy during GvHD. Traditional approaches to GvHD prophylaxis depend on immunosuppressive drugs such as methotrexate and cyclosporine which often have to be used in combination, resulting in severe side effects [10]. Recent attempts to use the more selective approach of depleting T cells from the donor BM have been largely unsuccessful, since clinical trials showed that T cell depletion led to an increased incidence of leukaemic relapse and failure of engraftment [20]. An ideal strategy for GvHD prophylaxis would therefore be to prevent anti-host immunopathology, while allowing the survival of sufficient donor-derived haemopoetic cells to permit full repopulation of the host. Recent approaches have therefore included selective depletion of individual donor T cell

subsets from the donor inoculum [18, 19], or blocking T cell activation *in vivo* by inhibiting the CD28/CTLA4 : B7/BB1 or CD40 : CD40L T cell costimulatory pathways [202, 222-225]. This latter approach has been moderately effective in reducing GvHD lethality, but in many cases the disease was not completely prevented and recurred when treatment was ceased [222, 223]. Furthermore, in one study [225] the use of high doses of CTLA4-Ig (a chimeric fusion protein with high affinity for B7 molecules on APC) largely prevented donor cell engraftment. Although combined blockade of several costimulatory pathways was more effective in alleviating lethality [226], this type of therapy may also be accompanied by deficits in T cell function. Neutralising individual effector cytokines *in vivo* has therefore become an attractive alternative to inhibiting lymphoid responses during GvHD [55, 57, 64, 65, 227]. My results show that IL-12 blockade was particularly useful in treating murine GvHD and may be of therapeutic use following clinical BMT. Most importantly, neutralising endogenous IL-12 for a brief period during the initiation of acute GvHD conferred long-term protection from the pathological outcome of the disease. Because IL-12 was critical for the afferent phase of GvHD, selective targetting of this cytokine prevented the cascade of events which culminates in lethal immunopathology. Importantly, IL-12 blockade did not prevent engraftment of donor cells, but permitted full repopulation with donor B6 lymphocytes and long-term survivors showed normal T cell function and remained visibly healthy for over four months after withdrawal of antibody treatment. Finally, although anti-IL-12 therapy prevented Th1-dependent lethality in B6 $\Rightarrow$ BDF_1 mice by permanently polarising the alloimmune response towards a Th2 phenotype, this did not result in the development of autoimmune pathology in the kidney, which might otherwise limit its application. In order to fully capitalise on the beneficial effects of IL-12 blockade, future studies will have to compare different strategies for neutralising IL-12 in vivo. Possible candidates are polyclonal and monoclonal anti-IL-12 antibodies, soluble IL-12 receptor-Fc fusion protein or recombinant IL-12 p40

protein. These latter agents may be particularly useful since they can block IL-12 binding to its receptor at very low concentrations [123] and may thus avoid hypersensitivity reactions provoked by heterologous antibodies.

Fig. 11.1A. Model of the Cellular and Molecular Interactions Involved in the Pathogenesis of Acute GvHD

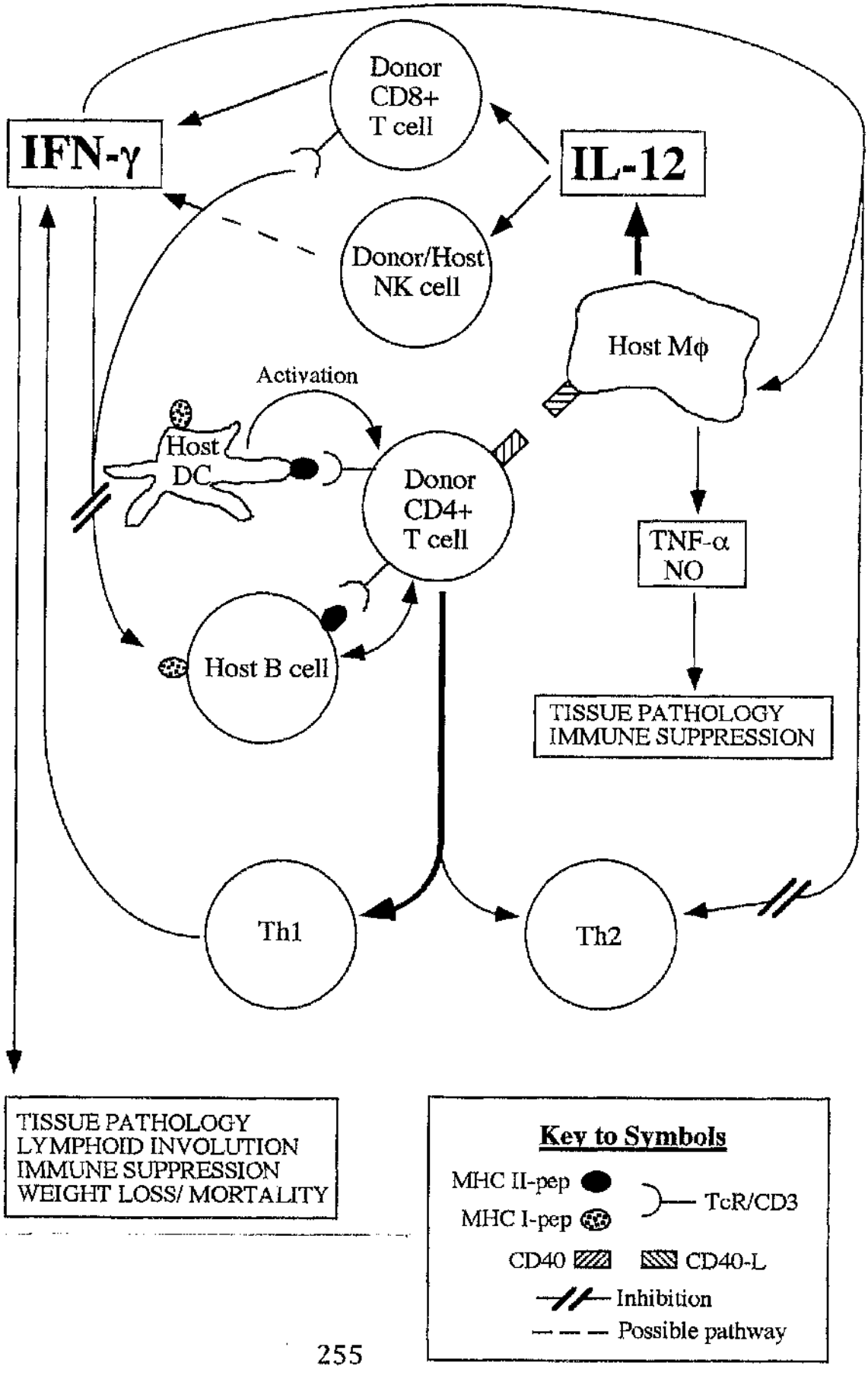
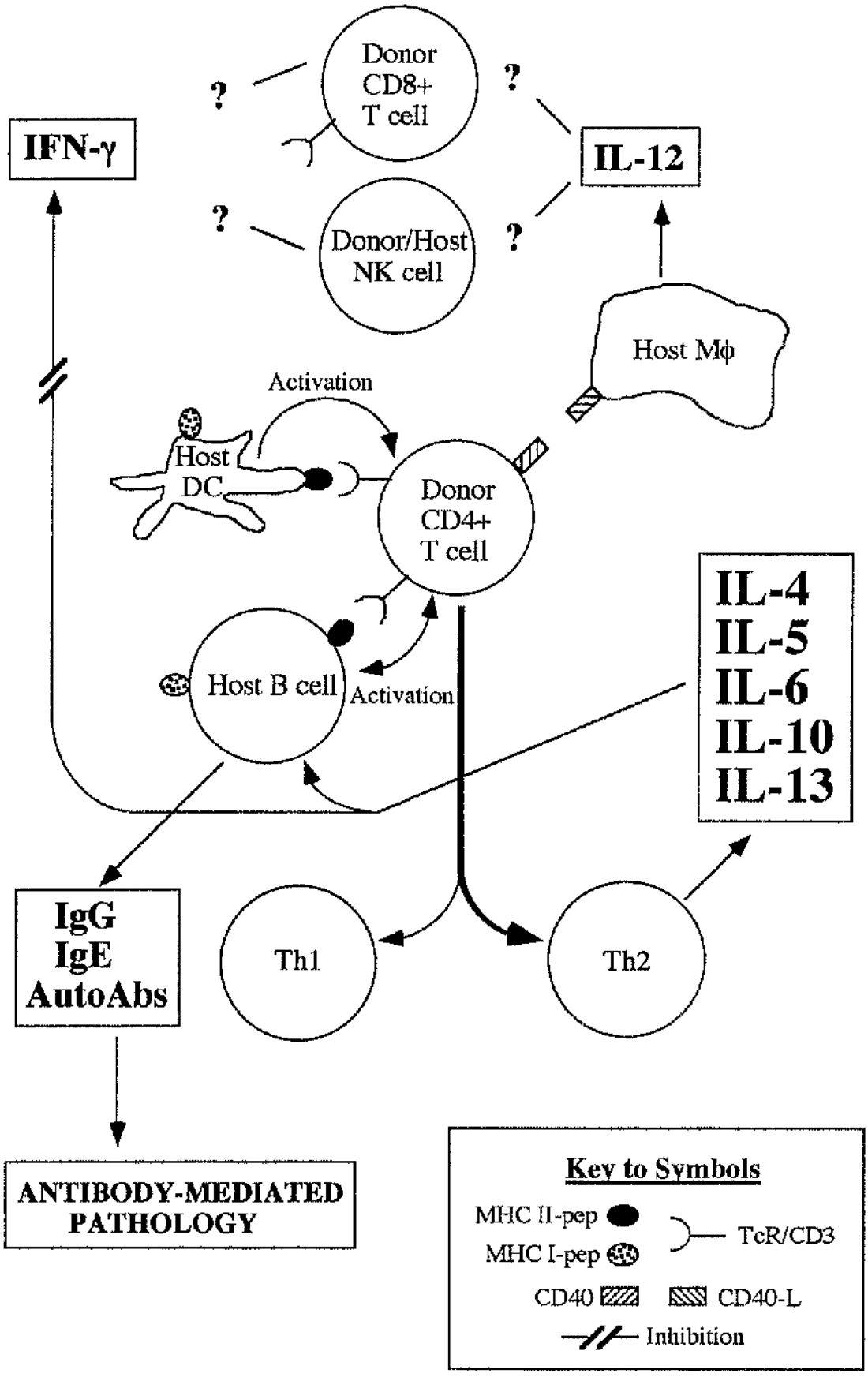


Fig. 11.B. Model of the Cellular and Molecular Interactions Involved in the Pathogenesis of Chronic GvHD



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